Profiling of autoantibodies to tumor antigens from cancer of the gingivo-buccal complex using immunoproteomics

by

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A thesis submitted to the
Board of Studies in Life Sciences

In partial fulfillment of requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Of

HOMI BHABHA NATIONAL INSTITUTE

August, 2013

Homi Bhabha National Institute

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CERTIFICATE

I certify that the thesis titled "**Profiling of autoantibodies to tumor antigens from cancer of the gingivo-buccal complex using immunoproteomics**" submitted for the degree of Doctor of Philosophy by Mr. Atul Pranay is a record of the research carried out by him during the period 2006 to 2012 under my supervision. This work has not formed the basis for the award of any degree, diploma, associateship or fellowship at this or any other institute or university.

Navi Mumbai,

August 2013

S. M. Zingde

Dedicated to

My dearest Parents

Acknowledgments

I am highly delighted to take this opportunity to say my thanks to all those who have contributed in making this journey of Ph.D work a wonderful and learning experience which would indeed remain with me for my entire life.

First and foremost, I am highly thankful to my mentor Dr. S.M Zingde and I express my deepest gratitude and regards to her for providing me the opportunity to work on this project and for her valuable supervision and guidance throughout my research tenure at ACTREC. She taught me to work in an organised and meticulous manner which tremendously helped to move my work with better pace with less errors and best results. I really appreciate her punctuality and devotion to her work. I am thankful to her for being so patient with me. I would like to thank her for always encouraging me to think and work independently.

I wish to express to my sincere thanks to Dr. Rajiv Sarin, Director, ACTREC and my mentor Dr. Surekha M. Zingde as Deputy Director, ACTREC for providing me the opportunity to work at this institute and for creating excellent infrastructure to carry out research work. I would also like to thank them for providing me financial support during my research work. I acknowledge the financial support received from CSIR for the project through a grant sanctioned to my guide.

I wish to express my sincere gratitude to all my DC members Dr. Malini Krishna, Dr. Padma Shastry, Dr. Robin Mukhopadhayay and Dr. Milind Vaidya for their valuable and critical suggestions during all my DC meets which helped me in taking my project in the right direction and making progress in my work. Discussions with them about the progress of my work filled me with sense of new enthusiasm and encouragement for my

work and helped me in deciding the right kind of experiments for the better progress of my work.

I would like to extend my thanks to Dr. Asawari Patil for all clinical analysis. I am thankful to Mrs. Sadhana Kannan for helping me in all the statistical analysis.

I express my warmest thanks to all my lab members Siddhi, Sanjeev, Rukmini mam, Parag, Poonam, Peter, Dait and Amit fulzele. I am thankful to Sanjeev who taught me all the techniques and gave me all initial training to work in the lab. There was always friendly and helping atmosphere in the lab because of all my lab members. Siddhi was always there to help me whenever I was stuck in any technicalities of my experiments. I am extremely thankful to her for all help. Parag was also a great help to me in my work and also otherwise. I am thankful to Rukmini Mam for her invaluable advises about work.

I am thankful to all CIR faculty members especially Dandeker Sir for rescuing me from any technical difficulties.

I would also like to thank all my seniors and juniors for keeping my spirit high by creating positive and lively environment in the Institute.

I owe my warm thanks to all my batch mates cum friends Amit Fulzele, Sapna, Lalit, Amit Gautam, Manoj Ramteke, Poulami, Amit Ranjan, Ajit Chande, Tabish and Pallavi. All my batchmates were very caring and helping. I would like to say special thanks to Amit Fulzele and Sapna for bearing with me and my nonsense talks all through my PhD without any complaint.

Last but not the least I am highly thankful to all the patients for providing me with the samples for my study otherwise this thesis would not have been possible. Now comes my dearest family without whose support and love nothing would have been possible. I

thank them all from the bottom of my heart for being so supportive, caring, loving and patient all through these years of my work.

CONTENTS:

Publications

Synopsis

Homi Bhabha National Institute

Ph. D. PROGRAMME

1. Name of the Student: Atul Pranay

2. Name of the Constituent Institution: Tata Memorial Centre

3. Enrolment No.: LIFE 09200604004

4. Title of Thesis: Profiling of autoantibodies to tumor antigens from cancer of the gingivo-buccal complex using immunoproteomics

5. Board of Studies: Life Sciences

SYNOPSIS

Introduction

Oral cancer is a major cause of cancer morbidity and mortality in males in India [1]. Squamous cell carcinoma of the oral cavity ranks as the15th most common cancer in the world and 10th most frequent in males [1]. In India, the gingivo-buccal complex of the oral cavity is the common site of cancer in males due to the prevalent habit of chewing tobacco [2]. A major hurdle in the management of oral cancer is high rate of loco regional recurrence [3]. Currently, diagnosis, treatment and prognosis of the disease are entirely based on clinical and radiologic and standard histopathology findings. Such techniques do not allow for recognition of genotypically different tumors with identical morphology and different prognosis. Therefore identification of biological markers is necessary to complement clinicopathological findings for a more accurate prediction of individual patient's prognosis and to help clinicians in planning more effective therapeutic strategies.

A large number of biomarkers are being reported for cancer of the head and neck region [4, 5] and their diagnostic and prognostic utility [6-9] are being assessed. Autoantibody response is also receiving increasing attention as a molecular sieve for identifying specific biomarkers for cancer for detection, prognosis, progression and response to therapy [10-19]. It is apparent that autoantibodies to tumor antigens have potential for clinical evaluation as their levels can be determined in blood using less invasive techniques without the need for biopsies.

Various approaches have been used for identification of autoantibody response in head and neck cancer. These include ELISA immunoproteomics, phage display library and AMIDA. In most studies, wherein biomarkers are being identified, attention has not

been given to sub-site specificity of the oral cavity/Head and Neck region.

Using immunoproteomics, many tumor antigens and their cognate antibodies have been identified and reported as biomarkers in different cancers. Our laboratory has used an immunoproteomics approach to identify tumor associated antigens for cancer of gingivo-buccal complex (GBC). The antigens identified are α enolase, Annexin II , HSP 70, Peroxiredoxin-6, ATP-synthase, α-tubulin, ß tubulin, Pyruvate kinase, Triose phosphate isomerase, Phosphoglycerate mutase, Aldose reductase, and Cyclophilin A, which elicit an autoantibody response in cancer of the GBC. Autoantibody response to some of these antigens is also observed in sera of individuals with leukoplakia of GBC suggesting that they may have utility as early markers. These results needed further investigation in order to determine whether the antibody response to the identified tumor antigens can be used as an early biomarker or as a prognostic marker and to generate a multiplex array for their evaluation. Towards this end the following objectives were undertaken:

Objectives:

1) To evaluate autoantibody response to the identified tumor antigens in sera of healthy individuals with and without chewing /smoking habit, individuals with leukoplakia and in patients with T1/T2, T3/T4 tumors of cancer of GBC, using an immunoproteomics approach.
2) To evaluate the prognostic utility of autoantibody response to tumor antigens.
3) To express and purify recombinant proteins of identified tumor antigens
4) To assess autoantibody response on a multiplex array of the recombinant antigens

Materials and Methods:

Samples used for the study:

This study was approved by the Hospital ethics committee of the Tata Memorial Centre, Mumbai, India. Blood samples were collected from patients with cancer of GBC (n=60), individuals with leukoplakia (n=30), from healthy individuals with (n=30) and without chewing/smoking habit (n=30). Blood samples were also collected from six individuals with gingivitis and periodontitis. Serum was isolated and stored at -80°C.

Antigen source: KB cell line was used as the source of antigens. Other oral cancer cell lines AW85073, and AW13516 were also used to evaluate the specificity of the autoantibody response. Cell lysates were prepared in urea lysis buffer. Protein was estimated using TCA precipitation modification of Petersons method [20] .

Two Dimensional separation of KB cell lysate proteins: KB cell lysate proteins were separated in two dimensions essentially according to Lamelli [21]. Forty µg of lysate protein was resolved by isoelectric focussing using precast Immobilized pH Gradient (IPG) gel strips (pH 3-10, 7cm) from Bio-Rad**,** followed by 1DE-SDS-PAGE. Multiple gels were run in a Bio-Rad PROTEAN 3 Dodeca Cell. One gel was used for silver staining to ensure resolution and condition of the proteins. The resolved proteins from the rest of the gels were transferred on to PVDF membranes and the blots probed with sera IgG (5µg/ml) from healthy individuals and those with cancer**.**

Purification of IgG from sera: IgGs were purified from the collected sera with Melon Gel IgG Spin Purification Kit (Cat. No: 45206) from Pierce Biotechnology. Purified IgGs were kept at 4°C and used within one week.

Immunostaining of KB 2D blots: KB 2D blots were blocked with 5 % milk powder and then probed with IgG purified from the collected sera. After washing, blots were

incubated with secondary antihuman antibody. Immunodetection was done using the enhanced chemiluminescence's ECL plus and the signals captured on X-ray film.

Analysis of 2D autographs: To identify the proteins reactive with the sera, autographs were superimposed over their corresponding blots stained with Amido Black / Colloidal gold and only those signals which completely overlapped with the protein spots on blots in shape and size were considered positive. The signals obtained were tabulated and percentage positivity calculated.

Statistical analysis: Statistical analysis was performed using the SPSS 16.0 software package. Clinical information such as age, gender, node, differentiation status and tumor stage was obtained from the clinical and histopathological reports of the patients. Clinico-pathological parameters were correlated with autoantibody response detected in sera against each tumor antigen using Chi square test. The association of follow up information with autoantibody response observed in the patients was evaluated by Kaplan-Meier analysis and multivariate analysis by Cox-regression method.

Cloning, expression and purification of recombinant proteins of tumor antigens: Gateway cloning was used for expression of the recombinant proteins for the identified tumor antigens. Entry clones in pDonr 221 carrying genes coding for the identified tumor antigens were obtained from Harvard Institute of Proteomics, Boston. They are α tubulin, ß-tubulin, Aldose reductase, Phosphoglycerate mutase, Pyruvate kinase, Cyclophilin A, Peroxiredoxin 6 and ATP synthase clones. Aldose reductase gene was sub cloned in pGEX4T1 while rest of the clones were sub cloned in pDEST15 gateway vector. Conditions such as (IPTG conc., temperature. for induction) for the optimum expression of each of proteins were determined and used for their expression. All recombinant proteins were expressed in Escherichia coli BL21 (DE3) cells and purified as GST fusion proteins using affinity chromatography. Proteins were also purified

without the GST tag by thrombin cleavage. Protein was estimated using Bradford assay. Purified proteins were evaluated by 1DE SDS-PAGE.

Autoantibody assay by Dot Blot analysis: An outline of the template for the protein microarray was designed. For the optimization of assay conditions, purified recombinant proteins were manually spotted on to the nitrocellulose membrane in an array in different concentrations and dilutions and probed with different concentrations of serum IgG from cancer patients and healthy controls for determining the optimum concentration of IgG for dot blot experiments. The blots were developed using the enhanced chemiluminescence's ECL plus kit and the signals captured on X-ray film.

Evaluation of autoantibody response to recombinant proteins, α enolase and HSP70 in patients and healthy individuals by ELISA: 96-well microtiter plates were coated with the recombinant proteins and kept overnight at 4°C. The wells were then washed and the unused sites blocked with BSA. Diluted patient sera were added to each well of the coated ELISA plate and the same incubated for 1 h at 37°C. The bound autoantibodies to α enolase/Hsp 70 were detected using horse radish peroxidase (HRP) conjugated sheep anti human IgG secondary antibody. The bound secondary HRP-antibody was measured using the tetramethylbenzidine substrate and stopping the reaction with H_2SO_4. The optical density was measured at 450 nm. Each sera sample was evaluated in duplicate on each of the protein coated and non coated wells and the readings normalised.

Evaluation of autoantibody response to recombinant α enolase and HSP70 in sera of tumor patients by Multiwestern analysis: Five µg of purified recombinant α enolase and HSP70 each were loaded in a single large well of a preparative SDS-polyacrylamide gel. After electrophoresis, the protein was transferred on to PVDF membrane. The blots were placed in a Mini-PROTEAN II Multiscreen Apparatus

(BIO-RAD) and probed for the presence of autoantibody to α enolase and HSP70 as described for the 2DE blots above.

Results:

Evaluation of autoantibody response against the tumor antigens by immunoproteomics: Autoantibody response in 30 individuals with leukoplakia, 30 patients with T1/T2 tumors of GBC cancer, 30 patients with T3/T4 tumors of GBC cancer, 30 healthy individuals each with and without chewing/smoking habit was evaluated using immunoproteomics. The highest autoantibody response observed in sera of cancer patients was against α enolase c (50%) and the lowest (1%) for Triose phosphate isomerase and Aldose reductase 1. Autoantibody response to many tumor antigens was also detected in individuals with leukoplakia and healthy individuals with chewing habit. However percentage of autoantibody response was less compared to cancer patients. Autoantibody response to α enolase b and c, ATP synthase and Peroxiredoxin 6 showed a trend of increasing antibody response from healthy individuals and leukoplakia to patients with cancer of the GBC with tumor size T1/T2 and T3/T4.

The pattern of autoantibody response was similar with the two different oral cancer cell lines used as antigen source. The autoantibody response to identified tumor antigens was absent in individuals with gingivitis and periodontitis.

Combined Analysis: To validate the observations obtained for these 60 patients and 30 healthy individuals without chewing habits, a pooled analysis was undertaken with data reported earlier from the laboratory. Autoantibody response patterns from healthy

individuals (n=49), individuals with leukoplakia (n=12), and 50 patients with T1/T2 (n=12) and T3/T4 (n=38) from the earlier studies [16, 19, 22] were included to increase the total patient sample size to 110 for calculating the final percentage of autoantibody response to each antigen.

Autoantibody response to the antigens varied between 5% for Triose phosphate isomerase to 62% for α enolase c and the combination of antigens eliciting an autoantibody response was different among patients. The intensity of the signal for each protein spot was distinct for each patient which reflects the specific autoantibody response of the individual to each antigen. Autoantibody response against α Enolase isoforms a, b and c was detected in 27%, 51% and 62% of the 110 patients respectively. The autoantibody response to Hsp 70 was seen in 23% of the patients. Autoantibody response to three isoforms of α Enolase and HSP70 was much lower and was seen in 6%, 10%, 20% and 2% respectively of normal healthy individuals.

Correlation of autoantibody response between healthy individuals and those with leukoplakia showed significant increase in the response to α enolase b and c, Annexin II and PKM2. This increase was also clearly seen between the healthy individuals and those with T1/T2 tumors wherein correlation was significant for the response to α enolase, b and c, HSP70, Peroxiredoxin 6, PKM and ATP synthase. Between healthy individuals and patients with T3/T4 tumors, autoantibody response was increased and significant for all the antigens being investigated.

Correlation of the autoantibody profile for each of the tumor antigens with clinicopathological parameters

Clinical significance of the autoantibody response to tumor antigens and clinicopathologic parameters were assessed using Chi-square test. Significant correlations were seen with HSP70 and α ENO (α enolase - the three isoforms taken together). Autoantibody response to HSP70 showed significant correlation with stage, tumor size, nodal status, and recurrence while autoantibody to α ENO correlates only with differentiation at p=0.055. The autoantibody response for both the antigens did not correlate with age and sex of the patients. There was no significant correlation to clinico- pathological parameters for the other antigens.

Evaluation of prognostic utility of autoantibody response to the tumor antigens: Kaplan Meier survival analysis was performed for autoantibody response against each tumor antigen to assess their independent association with disease free survival (DFS) of patients. Of the 60 patients with cancer of GBC investigated for this thesis, follow up information was available for 42 patients. Patients were grouped in those who do not show antibody response to tumor antigens and those who show antibody response and survival curves were plotted for both the groups and compared with respect to their disease-free survival (DFS) using log rank test. Among the three isoforms, autoantibody response to the isoform α enolase-b showed significant correlation with DFS of patients (p= 0.039). A similar trend was seen with autoantibody response to α ENO (three isoforms taken together) at (p=0.103). HSP70 also showed significant correlation with (DFS) of patients (p=0.021). Autoantibody response to the other antigens did not show significant correlation with DFS.

To validate the results, a ***combined analysis,*** was done wherein antibody response to the tumor antigens reported in the earlier investigation were included. Of the 50 GBC samples that were included from earlier investigations, follow up information was available for 36 samples which together with 42 patients from present study provided data for autoantibody response from 78 GBC samples. Autoantibody profile of these 78 samples was used for correlation studies with DFS and clinical parameters. Patients positive for autoantibody response to α enolase and HSP70 individually and in combination, showed significantly reduced disease free survival (DFS) compared to those who do not show autoantibody response to either of them. Further the patients, who exhibit autoantibody response to α ENO and HSP70 in combination with nodal involvement and/or differentiation status, have significantly lowered DFS. The relative risk of recurrence is 3.41 for patients who exhibit autoantibody response to both the antigens. Autoantibody response to the other antigens did not exhibit any prognostic significance.

Cloning, expression and purification of recombinant proteins of tumor antigens: Recombinant clones for eight identified tumor antigens were successfully generated in pDEST15 expression vector. Recombinant proteins for all tumor antigens except (ATP synthase, ß tubulin and α tubulin) were expressed and purified in sufficient quantity. Three recombinant proteins from the clones for ATP synthase, ß tubulin and α tubulin were expressed mostly as inclusion bodies from which only low amounts of the soluble proteins could be recovered. Recombinant proteins for tumor antigens α enolase, Cyclophilin A, Annexin II, HSP70, Aldose reductase, Phosphoglycerate mutase and Peroxiredoxin 6 with the GST tag and Annexin II, HSP70, α Enolase, Aldose reductase,

Phosphoglycerate mutase and Peroxiredoxin 6 without the GST tag were finally prepared and used for further analysis.

Assessment of autoantibody response to the recombinant tumor antigens by dot blot assay: Analysis of sera using the optimized conditions gave results which did not match with 2DE immunoblotting results for the same sample. Some of the tumor sera as well as sera from healthy samples which did not react with tumor antigens in 2D western analysis showed reactivity with recombinant proteins on dot blot.

Assessment of autoantibody response to α Enolase and HSP70 by ELISA: To investigate whether uniform results could be obtained with another technique, ELISA was undertaken. Autoantibody response to α Enolase and HSP70 which showed prognostic significance in the Kaplan Meier analysis were used for analysis by ELISA. Autoantibody response was evaluated in sera from 86 patients with cancer of GBC in which 68 samples were also used for immunoproteomics analysis and 18 were new. Autoantibody response was also evaluated in sera from 63 normal healthy individuals of which 33 samples were used for the immunoproteomics analysis in an earlier study from the lab. The median OD value from healthy individual sera 0.29 was set as cut off value for ENO autoantibodies which provided sensitivity and specificity of 51% and 52% respectively. There was no difference in mean serum levels of autoantibodies to α enolase in patients and healthy individuals (0.29 ± 0.64 vs. 0.28 ± 0.7 resp.). The median OD value from healthy individual sera 0.18 was set as cut off value for HSP70 autoantibodies which provided sensitivity and specificity of 36% and 55% respectively. There was no difference in mean serum levels of autoantibodies to Hsp 70 in between patients sera and healthy individual sera (0.15 ± 0.14 vs. 0.18 ± 0.14). Kaplan Meier

analysis with autoantibody response to α enolase evaluated with ELISA show similar trend of correlation with disease free survival but it is not significant. This correlation is better in patients with T3/T4 size tumors.

Comparison of the ELISA and 2DE data showed that for α enolase, 57% of the tumor and 57% healthy individual samples showed similar result, while for Hsp 70, 62% of the tumor samples and 48% of the healthy individual samples showed similar results between the two techniques. The differences observed may be attributed to the difference in epitope presentation due to different state of proteins in the two techniques.

Evaluation of autoantibody response to α Enolase and HSP70 in sera of tumor patients by Multiwestern analysis: In order to minimise the difference of epitope presentation and also use another option for multiplexing, autoantibody to recombinant α enolase and HSP70 was evaluated in sera by Multiwestern analysis. In this analysis 72% of the tumor samples and 86 % of the healthy individual samples showed similar result for α enolase as was obtained in 2DE analysis. Further the intensity of signals obtained in the two techniques was similar for greater percentage of the samples thereby validating our 2DE autoantibody results for α enolase to a large extent. For HSP70, (52%) tumor samples and (75%) healthy individual samples show similar results to that obtained by 2DE analysis.

Discussion:

Autoantibody response against tumor antigens has been receiving attention for its utility in early detection and prognosis of cancer. There are only a few reports which evaluate

the autoantibody response in oral / Head and Neck cancers. The work in this thesis investigates the utility of autoantibody response to the identified tumor antigens for early detection and prognosis of cancer in a subsite of the oral cavity i.e. GBC. Significant correlation of the autoantibody response to Eno b, Eno c and Anx II was seen between healthy individuals and those with leukoplakia indicating that these antigens have promising potential in the early detection of cancer. Significant correlation was also seen for autoantibody response to Eno b, Eno c, HSP70, Peroxiredoxin 6, PKM and ATP synthase between healthy individuals and patients with T1/T2 tumors. The autoantibody response to all the antigens correlated significantly between healthy individuals and patients with T3/T4 tumors showing that autoantibody response increases as the disease progresses.

Evaluation of prognostic utility shows that the autoantibody response against α ENO (α Enolase a, b and c taken together) and HSP70 provides an additional parameter for predicting disease free survival and recurrence and may be utilized along with nodal involvement and differentiation status for better prognosis of cancer of GBC.

α Enolase is reported to be multifunctional and to elicit an autoantibody response in several cancers [23-25]. The exact mechanism involved in the production of autoantibodies to α enolase in oral cancer is presently unclear. We have earlier reported that expression of α-enolase is increased in cancer of GBC [26]. Tsai *et.al* [27] have shown *ENO1* overexpression as a prognostic marker in head and neck cancer. Increased expression of the antigen in cancer may be a reason for the generation of autoantibody response.

HSP70 is another multifunctional protein which plays a role in regulating protein quality and turnover in normal conditions as well under stress. Autoantibodies to HSP70 have been reported in several cancers including the head and neck [16, 28, 29]. HSP70 is reported to be overexpressed in the cancer of head and neck and oral cavity [30]. There are divergent reports of its prognostic relevance in oral cancer [31]. In this study the autoantibody response to HSP70 detected in sera of patients with cancer of GBC correlated significantly both with the size and stage of tumor and was more in patients with advanced stage of tumor. Further there is a decrease in DFS with increase in autoantibody response.

In a recent review Murphy et al [32] have observed that due to the changing repertoire of the antigens as the tumor progresses, there is probably a constant qualitative and quantitative change in the auto antibody response to an antigen. In addition the autoantibody response will depend on the technique used to assess it since the immune response could be to a sequence in the linear protein or to an epitope presented as a consequence of the folding of the native protein [33]. These factors along with differential expression of an antigen, its post translational modifications and its relocation would provide a complex pattern which has to be deciphered to explain the autoantibody response profiles obtained for the different antigens.

Autoantibodies have been either reported to improve the prognosis of cancer patients, to worsen the clinical outcome, or even to be irrelevant for the course of the disease [34-36]. It is apparent that the scenario is very complex and as described by Tan and Coussens in their review [37] enhanced humoral immunity (HI) and inflammation in combination with suppressed cell mediated immunity (CMI) are responsible for the

pathogenenesis of several human cancers. Further studies are therefore required to understand the observations in this study.

It is now well accepted that a single antigen has low sensitivity for detection of cancer and there is a need of panel of biomarkers for better diagnosis and prognosis of cancer. Multiplex protein array provides the suitable platform to screen autoantibody response against large number of tumor antigens simultaneously. Towards the generation of multiplex antigen array, recombinant proteins of the identified tumor antigens were prepared and used in dot blot analysis for the evaluation of autoantibody in sera to the purified recombinant proteins. In dot blot analysis some of the sera from healthy individuals showed strong reactivity with some of the proteins which was not observed in the 2D immunoproteomics. On further investigation it was found that the free GST protein present in many of the recombinant protein preparations was reacting non specifically with some of the sera.

To overcome challenges related to manual spotting, presence of free GST protein, and degradation of proteins ELISA was done for quantitative evaluation of autoantibody response against α enolase and HSP70 in serum samples of patients. It is seen that the autoantibody response to enolase as evaluated by ELISA also shows a similar trend of correlation with prognosis of disease though it is not as significant as was found with 2DE western based autoantibody profile. Evaluation of autoantibody response to HSP70 by ELISA showed a trend of positive correlation with DFS. Therefore it is apparent that each antigen has an unique behaviour when its reactivity with sera in native state on an ELISA plate and linear denatured form as on 2DE resolved membrane blotted protein are compared.

In 2DE immunoproteomics, autoantibody positivity was assigned on the basis of presence and absence of signal on the autograph while in ELISA, autoantibody seropositivity was assigned on the basis of the cut off value which in this case was the median value of OD of healthy individual samples. Also the two techniques differ largely in respect to the state of protein used for autoantibody evaluation. In 2DE analysis proteins were linear and well resolved. In this state the epitopes may be more exposed while in ELISA, recombinant fusion proteins were used and they were in their native 3D conformation and same epitopes may not be equally exposed. Similar kind of observation was reported by Murphy et. al [33] in which they showed that the antigen presentation is an important determinant for detection of antigen by antibody. This may be one of the causes for the difference in detection in addition to absence of post translational modification (PTM) on the protein.

Multiwestern immunoblot analysis was used to minimize issues related to the structural difference of the proteins used for antibody evaluation. Autoantibody response to α enolase was close to the result obtained by 2DE analysis. Also there was a similarity in the intensity of signal obtained in the two techniques for most of samples thereby validating our 2DE autoantibody results for α enolase to a large extent. These observations strongly indicate that epitope presentation may be an important parameter for autoantibody evaluation when different techniques are used.

The autoantibody response is seen to be dependent on the size of the tumor, the differentiation and nodal status. All these factors would create complex patterns which have to be deciphered to explain the differences in the patterns obtained by the different techniques. The observations in this thesis provide some clues to the reason for failure

of validation of many tumor markers predicted as strong candidates on the basis of a single technique.

In summary, the work in the thesis has identified antigens which elicit autoantibody response with potential for early detection and prognosis in oral cancer. It has also identified the parameters which need attention while generating multiplex arrays for validation of data in large sample sizes for translation into the clinics.

References:

1. *Ferlay, J., et al., GLOBOCAN 2008,Cancer Incidence and Mortality Worldwide : IARC Cancerbase No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer; 2010 Available from: http://globocan.iarc.fr, accessed on 27/06/2012.*
2. *Dinshaw, K. and B. Ganesh, Hospital Based Cancer Registry, Annual report 2002-2005,Tata Memorial Hospital, 2005. **2005**.*
3. *Pathak, K.A., et al., Advanced squamous cell carcinoma of lower gingivobuccal complex: patterns of spread and failure. Head Neck, 2005. **27**(7): p. 597-602.*
4. *Schaaij-Visser, T.B., et al., Protein biomarker discovery for head and neck cancer. J Proteomics, 2010. **73**(10): p. 1790-803.*
5. *Leemans, C.R., B.J. Braakhuis, and R.H. Brakenhoff, The molecular biology of head and neck cancer. Nat Rev Cancer, 2011. **11**(1): p. 9-22.*
6. *Wang, Z., et al., Comparative proteomics approach to screening of potential diagnostic and therapeutic targets for oral squamous cell carcinoma. Mol Cell Proteomics, 2008. **7**(9): p. 1639-50.*
7. *Meyer, F., et al., Serum prognostic markers in head and neck cancer. Clin Cancer Res, 2010. **16**(3): p. 1008-15.*
8. *Schaaij-Visser, T.B., et al., Differential Proteomics Identifies Protein Biomarkers That Predict Local Relapse of Head and Neck Squamous Cell Carcinomas. Clin Cancer Res, 2009. **15**(24): p. 7666-7675.*
9. *Bleijerveld, O.B., et al., Protein signatures associated with tumor cell dissemination in head and neck cancer. J Proteomics, 2011. **74**(4): p. 558-66.*
10. *Mudenda, B., et al., The relationship between serum p53 autoantibodies and characteristics of human breast cancer. Br J Cancer, 1994. **69**(6): p. 1115-9.*
11. *Trivers, G.E., et al., Anti-p53 antibodies in sera from patients with chronic obstructive pulmonary disease can predate a diagnosis of cancer. Clin Cancer Res, 1996. **2**(10): p. 1767-75.*
12. *Disis, M.L., et al., High-titer HER-2/neu protein-specific antibody can be detected in patients with early-stage breast cancer. J Clin Oncol, 1997. **15**(11): p. 3363-7.*
13. *Mitsudomi, T., et al., Clinical implications of p53 autoantibodies in the sera of patients with non-small-cell lung cancer. J Natl Cancer Inst, 1998. **90**(20): p. 1563-8.*

14. *Chang, J.T., et al., Enzyme immunoassay for serum autoantibody to survivin and its findings in head-and-neck cancer patients. Clin Chem, 2004.* ***50****(7): p. 1261-4.*
15. *Lin, H.S., et al., Autoantibody approach for serum-based detection of head and neck cancer. Cancer Epidemiol Biomarkers Prev, 2007.* ***16****(11): p. 2396-405.*
16. *Shukla, S., et al., Tumor antigens eliciting autoantibody response in cancer of gingivo-buccal complex. Proteomics Clin Appl, 2007.* ***1****(12): p. 1592-604.*
17. *Tjalsma, H., R.M. Schaeps, and D.W. Swinkels, Immunoproteomics: From biomarker discovery to diagnostic applications. Proteomics Clin Appl, 2008.* ***2****(2): p. 167-80.*
18. *Qiu, J. and S. Hanash, Autoantibody profiling for cancer detection. Clin Lab Med, 2009.* ***29****(1): p. 31-46.*
19. *Shukla, S., et al., Immunoproteomics reveals that cancer of the tongue and the gingivobuccal complex exhibit differential autoantibody response. Cancer Biomark, 2009.* ***5****(3): p. 127-35.*
20. *Peterson, G.L., A simplification of the protein assay method of Lowry et al. which is more generally applicable. Anal Biochem, 1977.* ***83****(2): p. 346-56.*
21. *Laemmli, U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. NATURE, 1970.* ***227****(5259): p. 680-5.*
22. *Shukla, S., Study of tumor antigens eliciting humoral response in Oral Cancer. Ph.D. Thesis, University of Mumbai, 2008.*
23. *Capello, M., et al., a enolase: a promising therapeutic and diagnostic tumor target. FEBS J, 2011.* ***278****(7): p. 1064-74.*
24. *Cappello, P., et al., An integrated humoral and cellular response is elicited in pancreatic cancer by a enolase, a novel pancreatic ductal adenocarcinoma-associated antigen. Int J Cancer, 2009.* ***125****(3): p. 639-48.*
25. *He, P., et al., Proteomics-based identification of a enolase as a tumor antigen in non-small lung cancer. Cancer Sci, 2007.* ***98****(8): p. 1234-40.*
26. *Govekar, R.B., et al., Proteomic profiling of cancer of the gingivo-buccal complex: Identification of new differentially expressed markers. Proteomics Clin Appl, 2009.* ***3****(12): p. 1451-62.*
27. *Tsai, S.T., et al., ENO1, a potential prognostic head and neck cancer marker, promotes transformation partly via chemokine CCL20 induction. Eur J Cancer, 2010.* ***46****(9): p. 1712-23.*
28. *Fujita, Y., et al., Proteomics-based identification of autoantibody against heat shock protein 70 as a diagnostic marker in esophageal squamous cell carcinoma. Cancer Lett, 2008.* ***263****(2): p. 280-90.*
29. *Looi, K.S., et al., Using proteomic approach to identify tumor-associated antigens as markers in hepatocellular carcinoma. J Proteome Res, 2008.* ***7****(9): p. 4004-12.*
30. *Lin, H.P., Y.P. Wang, and C.P. Chiang, Expression of p53, MDM2, p21, heat shock protein 70, and HPV 16/18 E6 proteins in oral verrucous carcinoma and oral verrucous hyperplasia. Head Neck, 2011.* ***33****(3): p. 334-40.*
31. *Tavassol, F., et al., Prognostic significance of heat shock protein 70 (HSP70) in patients with oral cancer. Head Neck Oncol, 2011.* ***3****: p. 10.*
32. *Murphy, M.A., J.J. O'Leary, and D.J. Cahill, Assessment of the humoral immune response to cancer. J Proteomics, 2012.*
33. *Murphy, M.A., et al., Epitope presentation is an important determinant of the utility of antigens identified from protein arrays in the development of autoantibody diagnostic assays. J Proteomics, 2012.*

34. *Saffroy, R., et al., Clinical significance of circulating anti-p53 antibodies in European patients with hepatocellular carcinoma. Br J Cancer, 1999.* ***79****(3-4): p. 604-10.*
35. *Tang, R., et al., Humoral response to p53 in human colorectal tumors: a prospective study of 1,209 patients. Int J Cancer, 2001.* ***94****(6): p. 859-63.*
36. *Hermsen, B., et al., Humoral immune responses to MUC1 in women with a< i> BRCA1</i> or< i> BRCA2</i> mutation. European Journal of Cancer, 2007.* ***43****(10): p. 1556-1563.*
37. *Tan, T.T. and L.M. Coussens, Humoral immunity, inflammation and cancer. Curr Opin Immunol, 2007.* ***19****(2): p. 209-16.*

Publications:

a. Published

1. Sanjeev Shukla, **Atul Pranay**, Anil K. D'Cruz, Pankaj Chaturvedi, Shubhada V. Kane, Surekha M. Zingde. Immunoproteomics reveals that cancer of the tongue and the gingivo-buccal complex exhibit differential autoantibody response. Cancer Biomarkers, 5, 127-135, 2009.

2. **Pranay A**, Shukla S, Kannan S, Malgundkar SA, Govekar RB, Patil A, Kane SV, Chaturvedi P, D'Cruz AK, Zingde SM. Prognostic utility of autoantibodies to α-enolase and HSP70 for cancer of the gingivo-buccal complex using immunoproteomics. Proteomics Clin Appl. 2012 Nov 17. doi: .1002/prca.201200081. [Epub ahead of print] PMID:23161576

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LIST OF ABBREVIATIONS

APS	Ammonium per sulphate
AMIDA	Autoantibody mediated identification of antigens
CTC	Copper tartarate carbonate
DTT	Dithiothretol
DMSO	Dimethyl sulfoxide
DFS	Disease free survival
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethedium bromide
GBC	Gingivo buccal complex
GST	Glutathione S transferase
HRP	Horseradish peroxidase
IEF	Isoelectric focusing
IPTG	Isopropyl beta thiogalactopyranoside
IPG	Immobilized pH gradient
LB	Luria broth
MD	Moderately differentiated
PD	Poorly differentiated
PGM	Phosphoglycerate mutase
PBS	Phosphate buffered saline
PMSF	Phenyl methyl sulfonyl fluoride
2-DE	Two dimensional electrophoresis

SDS	Sodium dodecyl sulphate
SCC	Squamous cell carcinoma
TBS	Tris buffered saline
Tris	2-Amino-2-hydroxymethyl-propane-1,3-diol
TPI	Triose phosphate isomerase
TEMED	N, N, N’, N’-Tetra methylethylenediamine
WD	Well differentiated

List of Figures

List of Tables

Preamble

Squamous cell carcinoma of the oral cavity ranks as the15th most common cancer in the world and 10th most frequent in males. In India, the gingivo-buccal complex of the oral cavity is the common site of cancer in males due to the prevalent habit of chewing tobacco. It is a major cause of cancer morbidity and mortality. A major hurdle in the management of oral cancer is high rate of loco regional recurrence. Currently, diagnosis, treatment and prognosis of the disease are entirely based on clinical and radiologic and standard histopathological findings. Such techniques do not allow for recognition of genotypically different tumors with identical morphology and different prognosis. Therefore identification of biological markers is necessary to complement clinico pathological findings for a more accurate prediction of individual patient's prognosis and to help clinicians in planning more effective therapeutic strategies.

A large number of biomarkers are being reported for cancer of the head and neck region and their diagnostic and prognostic utility are being assessed. Autoantibody response is also receiving increasing attention as a molecular sieve for identifying specific biomarkers for cancer for detection, prognosis, progression and response to therapy. It is apparent that autoantibodies to tumor antigens have potential for clinical evaluation as their levels can be determined in blood using less invasive techniques without the need for biopsies.

Using immunoproteomics, many tumor antigens and their cognate antibodies have been identified and reported as biomarkers in different cancers. Our laboratory has also reported a set of tumor antigens that elicit autoantibody response in patients with cancer of gingivo-buccal complex (GBC), a subsite of the oral cavity. This thesis evaluates the

utility of the identified tumor antigens for early detection, prognosis and evaluates the markers with different techniques for their validation.

The **Introduction** provides recent information relevant for the investigations undertaken and in turn the aim and objectives of this thesis. In **Chapter 2**, the **Materials and Methods** used for the study are described. **Chapter 3, Results** presents the data on the autoantibody response in healthy individuals with and without chewing/smoking habits, patients with leukoplakia, and cancer of the gingivo buccal complex (GBC). The significance of the autoantibody responses and their utility for early detection and prognosis is evaluated. The chapter also presents the results for purification of recombinant proteins for the tumor associated antigens and assesses the evaluation of autoantibody responses using different techniques. In **Discussion** the data obtained in the thesis is summarized and discussed in the light of present literature.

Chapter 1

Introduction

1.1 Introduction:

With the advent of the OMICs technologies, pathogenesis of disease is now being characterised by genomic and proteomic signatures comprising of several molecules referred to as Biomarkers. These molecules are directly or indirectly associated with the disease process and can be utilized for early detection, diagnosis, prognosis and monitoring treatment response. Biomarkers can be measured in the blood, other body fluids, or tissues. An ideal biomarker is a molecule which is highly specific and has high sensitivity for the disease and most importantly can be detected easily in a noninvasive manner at a stage when disease is not clinically detectable.

1.2 Tumor biomarkers:

Cancer is the leading cause of death in developed countries and the second leading cause of death in developing countries [1]. One of the major factors behind the cancer related deaths is failure of its detection at an early stage. Many of the cancers are diagnosed at a late stage when any kind of therapy is less/not effective for its treatment. Biomarkers can assist in detection of cancer at an early stage.

Tumor biomarkers are molecules often produced by the tumor itself or the host system in response to the tumor and provide the biological material to determine the risk of getting cancer, to detect cancer, to classify cancer or to provide insight into prognosis and modalities for therapy. Apart from this they can also help in tracing the pathway involved in tumorigensis.

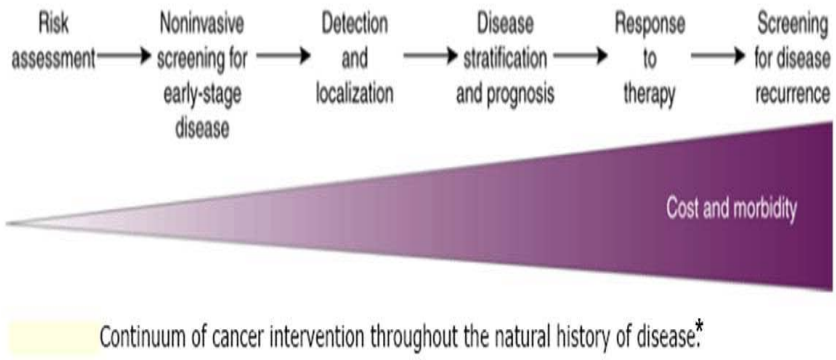


Figure 1.1**:Use of cancer biomarkers in different clinical settings** [2]

OMICS technologies have provided immense information and as per the Cancer Gene Census, so far 488 cancer genes have been reported which represent more than 1% of all the genes in the human genome [3]. Genetic alterations encompass mutation, change in copy number, change in expression, translocation of a gene, modification in the promoter region and epigenetic changes. Now changes at the proteome level are receiving attention as they are more representative of the disease as effector molecules for any phenotype. Using proteomic profiling a large repertoire of protein biomarkers have been identified from biological fluids and tissues of different cancers. Biological fluids are a better choice for the assessment of protein biomarkers as they can be obtained in a less invasive manner.

Currently available serum biomarkers are based on the measurement of cancer antigens. For example, the prostate specific antigen (PSA) is measured for prostate cancer, the carcinoembryonic antigen (CEA) for colorectal cancer, the cancer antigen CA15-3 for breast cancer, the cancer antigen CA19-9 for gastrointestinal cancer and the cancer antigen CA125 for ovarian cancer. However these biomarkers are not suitable for early screening of cancers owing to their low sensitivity and low

specificity in early stages. Thus there is great need to discover novel biomarkers and translate them into routine clinical use.

1.3 Oral cancer:

Oral cancer is a major cause of cancer morbidity and mortality in males in India [4]. Squamous cell carcinoma of the oral cavity ranks as the15th most common cancer in the world and 10th most frequent in males [4]. In India, the gingivo-buccal complex of the oral cavity is the common site of cancer in males due to the prevalent habit of chewing tobacco [5].

Oral cancers are relatively uncommon in the west, accounting for only 10% of oral cavity cancers, while they account for 40% of oral SCC among the Indian communities in Africa and in Southeast Asia [6]. The role of tobacco in the epidemiology of these cancers has been well established in numerous studies. Approximately 90% of people with mouth cancer are tobacco users. In India and Southeast Asia, the chronic use of betel quid (*paan*) in the mouth has been strongly associated with an increased risk for oral cancer [7-9].

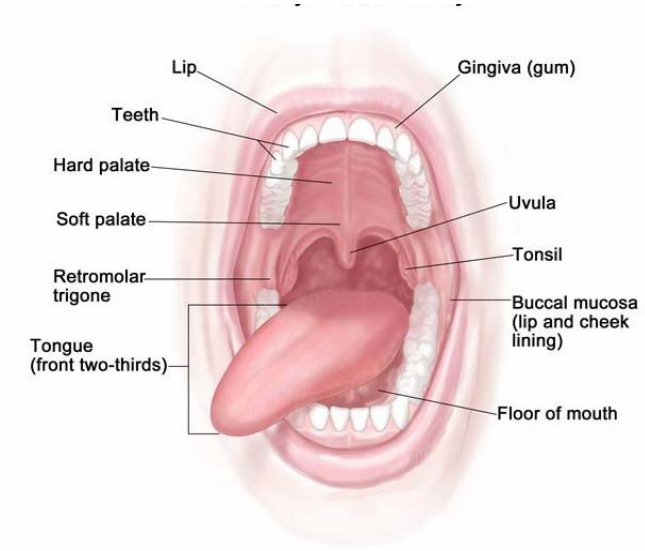


Figure 1.2: Anatomy of Oral cavity [10]

Of all the cancers of oral cavity, cancer of Gingivo-buccal complex (GBC) shows the highest incidence according to the TMH annual report [5]. The GBC constitutes the buccal mucosa, alveolus, retro molar trigone, and gingiva.

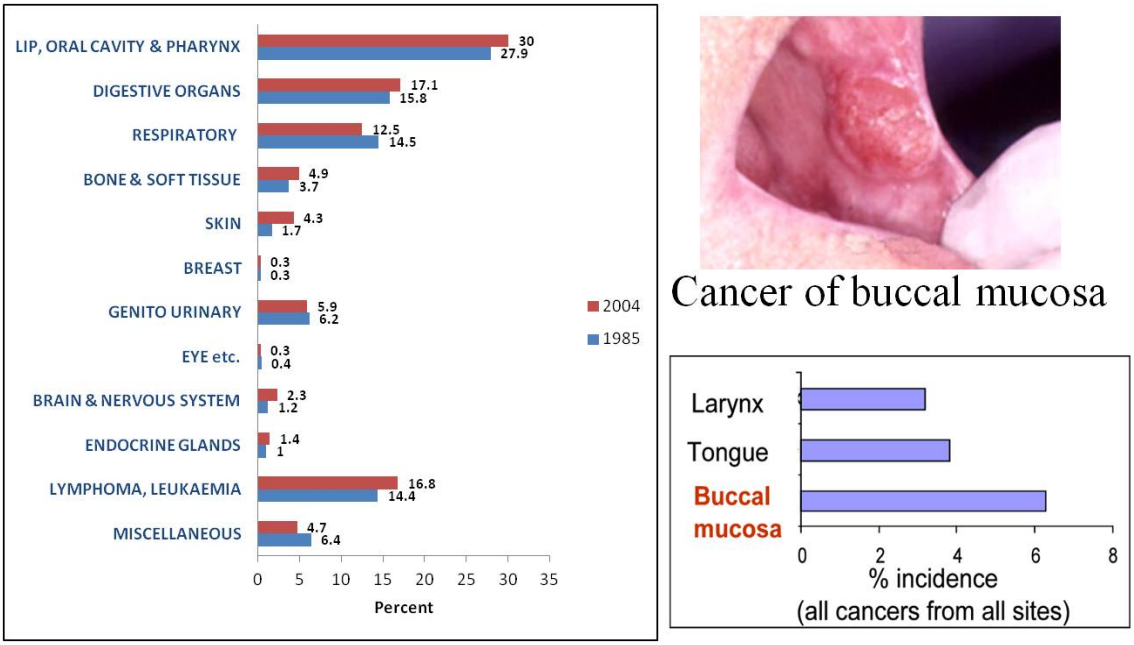


Figure 1.3: **Incidence of different cancers at the Tata Memorial Centre.** Graphs generated from data in *Hospital based cancer registry, Annual report 2005, TMH 2008* [5]

More than two thirds of patients with HNSCC are diagnosed at an advanced stage when the 5-year survival is < 40% [11]. In contrast to this, patients diagnosed at an early stage have an excellent 5-year survival rate of >80% [11] and experience significantly less effect on their quality of life after treatment with single modality therapy [11]. A large proportion of oral cancer patients present at Tata Memorial Hospital for treatment with T3/T4 size tumors when the chances of their survival become very low. During the last decades, progress has been made in the development of novel treatment modalities for HNSCC [12-14] still survival rate has not improved substantially [15].

Invasive oral Squamous cell carcinomas are often preceded by the presence of clinically identifiable premalignant changes of the oral mucosa. These lesions often present as either white or red patches, known as leukoplakia and erythroplakia. Currently the diagnosis, treatment, and prognosis of the disease are entirely based on clinical and radiologic and standard histopathological findings which may fail to detect early cancerous lesions or precancerous changes. Such techniques do not allow for recognition of genotypically different tumors with identical phenotypes, which could affect prognosis, because certain genetic mutations may be predictive of behaviour or prognosis. Therefore identification of a biological marker is of utmost importance, to complement clinicopathological findings for a more accurate prediction of individual patients prognoses and to help clinicians in planning more effective therapeutic strategies. Unfortunately, there has been little improvement in the early detection of oral cancer because many patients do not present for diagnosis and treatment until they have Stage III or Stage IV disease. Therefore it becomes imperative to identify biomarkers for:

(1) Screening for risk of cancer;

(2) Early detection of premalignant and malignant changes resulting in earlier treatment;

(3) Evaluation of margins of resection for residual disease.

(4) Development of new treatment approaches and

(5) Monitoring treatment response

Oral cancer exhibits inter tumor and intra tumor heterogeneity. Therefore a single biomarker cannot be very useful in the diagnosis and prognosis of the disease.

Identification of multiple markers is necessary and can be identified using global genomic and proteomic profiling of cancer samples.

1.4 Biomarkers for head and neck cancers:

Like other cancers, head and neck carcinogenesis is also a multistep process and is a result of accumulation of several genetic and epigenetic changes. Apart from environmental risk factors, genetic factors also play a major role in head and neck tumorigenesis. This is well demonstrated by the observation that patients with Fanconi anaemia (FA) are highly prone to develop oral Squamous cell carcinoma. Based on information reported, a genetic model for progression has been presented by Califano *et al* [16] and later on modified by Leemans et. al [17] wherein the generic alterations from the precancerous to cancer are well presented. It is now revealed that head and neck cancers are heterogeneous both at the molecular level and clinical level [18, 19]. There are HPV positive and HPV negative head and neck tumors which show different prognoses and alteration of different set of genes / signalling pathways [20, 21]. *TP53*, *CCND1* and *CDKN2A* are established cancer genes in HPV-negative HNSCC and *TP53* and the genes encoding the Rb family (comprising *RB1*, *RBL1* (which encodes p107) and *RBL2* (which encodes p130) are established cancer genes in HPV-positive HNSCC [22]. Several studies have shown that HPV positive HNSCC patients respond better to radiotherapy, chemotherapy and have higher overall survival [23]. *CCND1*, which encodes cyclin D1, is located on chromosome 11q13, is amplified or gained in more than 80% of cases of HPV-negative HNSCC [24]. Another gene *CDKN2A*, which encodes p16INK4A, is located on chromosome 9p21 and is frequently inactivated in HNSCC by mutation or methylation in combination with chromosome loss or, by homozygous deletion [25]. Alterations in signalling

pathways involving EGFR , TFG ß and nuclear factor-κB are also reported in many head and neck cancer cases. Both gene amplification and activating mutations of EGFR have been reported in tumors. Downregulation of TGFβ receptors is often found in tumours. Apart from EGFR another growth factor receptor tyrosine kinase coded by *MET* gene has recently been implicated in head and neck carcinogenesis. Mutations and gene amplifications of *MET* have been reported in HNSCC [26]. Another crucial pathway that is reported to be altered in HNSCC is the PI3K-PTEN-AKT pathway. Besides activating *PIK3CA* mutations, inactivating mutations or homozygous deletions of *PTEN* have also been identified in approximately 10% of HNSCCs.

With the advancement of proteomic technologies, protein biomarkers have gained more attention as these techniques have made possible differential protein expression profiling of the entire tissue, cell line or any biological fluid in a quantitative manner. With these robust techniques even very minute changes in the level of proteins can be detected. In addition, there are many alterations involved in tumourigenesis which are confined only to post transcriptional and post translational level which can be detected only at the protein level. Importantly, low-abundance proteins are being identified and characterized, not only from the tumor tissues, but also from body fluids (plasma, saliva, and urine) in a high-throughput and unbiased manner. A large number of protein biomarkers are being reported for cancer of the head and neck and their diagnostic and prognostic utility are being assessed.

Comparison of the data emerging from proteomic studies reveals that very few of the identified markers overlap between studies. This is due to use of microdissected /

whole tissue, technique used for analysis (2D gel electrophoresis / 2D liquid chromatography), quality of tissue sample (tissue rich in stroma), collection of tissues, no attention to subsites of the HNSCC and various other confounding factors which may vary between two studies. Some common protein markers identified in more than one study are 14-3-3 sigma [27, 28] , 14-3-3 zeta/delta and S100-A7 which are all upregulated in HNSCC, while keratin 4 [29-31] and keratin 13 [29, 31] are downregulated in HNSCC.

Some of the recent studies have shown the potential of protein biomarkers in the prognosis of cancer prediction of relapse or metastasis. Increased level of EGFR has been correlated to poor survival of patients [32]. Schaaij-Visser *et al.* [29] have identified forty proteins differentially expressed in head and neck tumor tissue out of which low expression of keratin 4 and cornulin showed significant correlation with local relapse of tumor .

Ralhan *et al*. [27] have identified stratifin (SFN), YWHAZ, and hnRNPKs as biomarkers for discriminating dysplasias from normal tissues using iTRAQ-LC-MS/MS analysis which they further verified by immunohistochemistry. In another study Matta *et al.* [33] showed the prognostic utility of stratifin (SFN), YWHAZ for head and neck cancer. They found significant decrease in median disease-free survival (13 months) in HNOSCC patients showing overexpression of both stratifin and YWHAZ proteins, as compared to patients that did not. In a recent study Chang *et al.* [34] have used iTRAQ-based quantitative proteomic approach and shown that higher levels of peroxiredoxin 4 and P4HA2 are associated with lymph node metastasis in oral squamous cell carcinoma. PRDX4 overexpression proved as a

significant prognostic factor for disease-specific survival in both univariate and multivariate analyses.

Ye *et al.* [35] have used immunohistochemistry to show that elevated SOD2 levels are associated with lymph node metastasis in OSCC. Bleijerveld *et al.* [36] have used 2D-DIGE and identified a signature of 51 differentially expressed proteins which could classify between patients with presence or absence of disseminated tumor cells (DTC) in the bone marrow i.e. DTC positive and DTC negative patients. The most prominent feature within this signature was the down-regulation of CK19 in DTC-positive tumors. The presence of disseminated tumor cells (DTC) in the bone marrow predicts development of distant metastasis. MMPs are another class of molecules which are over expressed in OSCC tissues and their overexpression has been associated with poor prognosis. [37-39].

1.5 Autoantibodies as potential biomarkers:

Circulating serum autoantibodies to tumor associated antigens (TAA) are group of potential biomarkers. In the 1960s, it was demonstrated for the first time that the immune system could react to a developing tumor. The immune response to tumor is both cell mediated and a humoral response. There has been tremendous increase in the number of reports of TAAs and their cognate autoantibodies in patients with cancer.

1.5.1 Biological role of autoantibody response in cancer:

The biological role of the autoantibodies generated against TAAs and their immunological role is, not completely understood. Do the autologous antibodies

directed against tumor-specific or tumor-associated antigens play a cancer-promoting role, whether they take part in a concerted immune attack against the malignancy, or whether they have no effect on tumor growth at all and simply represent an epiphenomenon of tumor persistence and/or progression are questions that are receiving attention [40]. It has been demonstrated that in cancer, activation of humoral response leads to suppression of anti tumor cell mediated response thereby promoting tumor growth and progression [41, 42]. This hypothesis is further supported by studies where high titre of autoantibodies correlated with poor prognosis and decreased survival for several human cancer types [43] and presence of infiltrating T lymphocytes correlate with improved prognosis in colon cancer [44]. Activation of B cells and humoral immune responses promote tumor by chronic activation of innate immune cells in neoplastic tissues [40]. By contrast, activation of adaptive immunity elicits antitumour responses through T-cell-mediated toxicity (by induction of FAS, perforin and/or cytokine pathways) in addition to antibody-dependent cell-mediated cytotoxicity and antibody-induced complement-mediated lysis [40]. Tan and Coussens in their review [45] have discussed that the situation is complex wherein enhanced humoral immunity (HI) and inflammation in combination with suppressed cell mediated immunity (CMI) are responsible for the pathogenesis of several human cancers. In some studies protective role of autoantibodies in cancer have also been observed [46, 47]. Lu *et al.* investigated the role of autoanti-dsDNA autoantibodies on growth of tumor in vitro and in vivo. tumors were inhibited in mice bearing anti-dsDNA autoantibodies. In the invitro study, anti-dsDNA autoantibodies induced apoptosis of SP 2/0 and Wehi 164 tumor cells [48].

1.5.2 Autoantibody response to cellular proteins in cancer:

Some of the immune responses in cancer patients recognize neo-antigens that are found only in tumors, but most tumor-associated autoantibodies are directed against self-antigens. The mechanism by which these autoantibodies are generated to normal cellular proteins is still not understood.

Cordes *et al.* [49] investigated the overexpression of p53 in head and neck tumor tissue and the presence of p53 protein and their autoantibodies in sera. No correlation was seen between p53-overexpression in tissue and p53-protein levels in sera or between p53-autoantibody levels in sera and in mutation frequency of the p53-gene or p53-overexpression in tissue. These results suggest that there is no single factor which determines the eliciting of autoantibody response in the sera of cancer patients.

Different studies indicate that some of the autologous proteins in cancer cells are altered in such a manner that they start behaving as tumor antigens and elicit an antibody response. Both cell surface proteins and intracellular proteins elicit an immune response. Production of autoantibodies to self proteins is a distinct phenomenon also found in autoimmune diseases. Autoantibody response to a single antigen may not be seen in all patients even with same type of cancer which may be due to heterogeneous nature of cancer, differences in epitope presentation and also due to the fact that the immune response differs between individuals. The alterations that provide immunogenicity to a self protein include posttranslational modification (eg. calreticulin and annexin I [50, 51]), mutations (p53) [52, 53], aberrant expression (e.g HER2/neu, p53 and ras) [54-57], altered localisation (cAMP-dependent protein kinase (PKA), [58, 59], cyclin B1[60-63].

1.5.3 Tumor antigens:

The tumor antigens identified so far belong to any of these three groups:

(1) Proteins associated with tumorigenesis (eg, L-myc, c-myb, p21 ras, HER2/neu, cyclin B1, survivin, livin, p53) [54, 55, 64, 65]

(2) Cancer-testis antigens (eg. MAGE, SSX2, NY-ESO-1) which are expressed only in germ cells [66].

(3) oncofetal proteins aberrantly expressed in tumors (e.g. p62) [67]

1.5.4 Strategies to detect tumor associated antigens and their cognate antibodies:

Various approaches have been used for the identification and validation of tumor antigens eliciting an autoantibody response in different cancers. They are shown in Fig. 1.4.

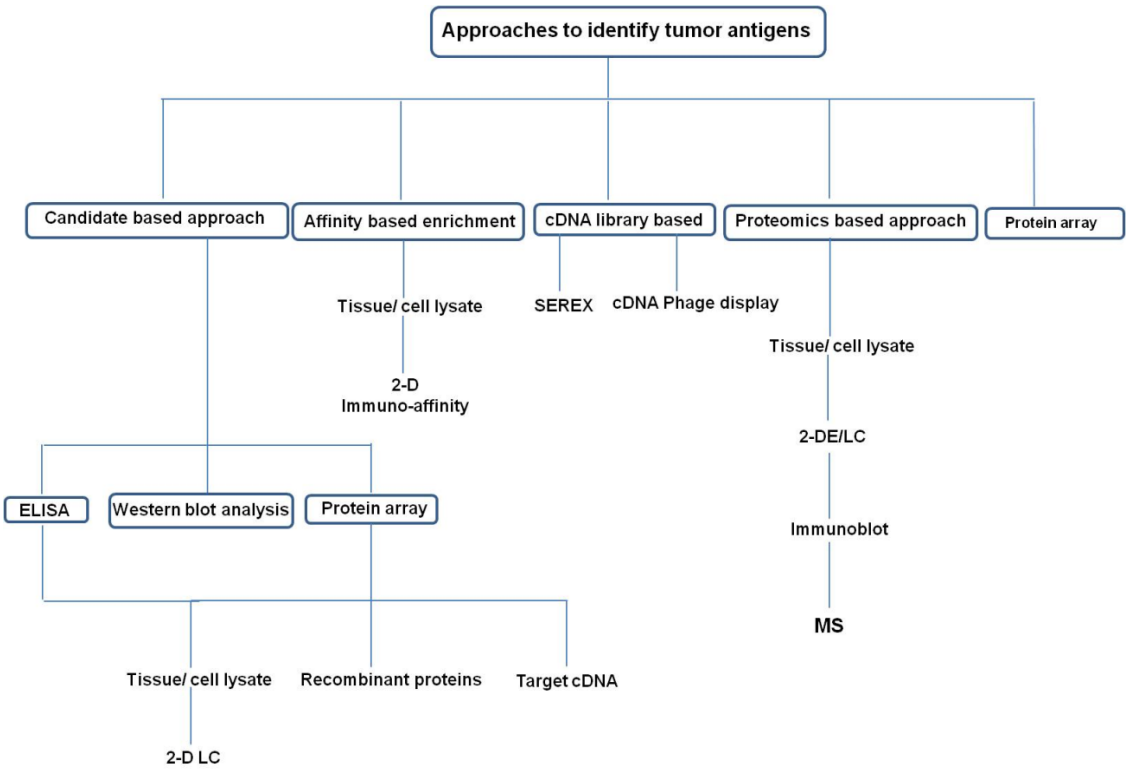


Figure 1.4: Overview of different techniques used for identification of tumor antigens and for detection of autoantibodies against them.

The major techniques used for discovery of tumor antigens are serological screening of cDNA expression libraries (SEREX), phage-display libraries, protein microarrays, 2D immunoblotting and 2D immunoaffinity chromatography. A number of tumor antigens have been identified by *SEREX* wherein a cDNA recombinant expression library constructed from tumor tissues/cancer cell lines is screened with patient sera and healthy control sera. Clones that exhibit differential reactivity between the two sera groups are isolated and identified. Tumor antigens identified using this approach include CTAs (e.g. NY-ESO-1, SSX2, MAGE), [68, 69] mutational antigens (e.g. p53), differentiation antigens (e.g. tyrosinase, SOX2, ZIC2) and embryonic proteins [69, 70]. Based on a similar approach but slightly modified is the *phage-display cDNA expression library* generated from cancer cell lines or tumor tissue. Only after several rounds of biopanning final phage clones reacting only with cancer sera are selected for their identification. TAAs for prostate and ovarian cancers, amongst others, have been identified using this approach. *Random peptide library phage display* is also used to select for peptides recognised by tumor sera. Using this approach Chinnaiyans group [71] identified a panel of 22 antigenic peptides that discriminated the prostate cancer patients and healthy individuals with 81.6% sensitivity and 88.2% specificity. For high-throughput serological screening of a large cohort of cancer patients phage-based protein/peptide micro-arrays, containing thousands of phages, have been generated. For example, Wang *et al.* [71] analysed sera from 119 prostate cancer patients and 138 healthy individuals using an array of a phage-display library. However one of the major limitation of these approaches is that antibodies to posttranslational modifications of the tumor antigens are not recognised. Another approach most frequently used is Immunoproteomics (Fig 1.5). Proteins from cancer cell line/ tumor tissue are separated by two dimensional gel electrophoresis and

the separated proteins are transferred on to blots by western blotting. The blots are then screened with tumor sera and normal sera. Proteins showing reactivity with cancer sera are identified by mass spectrometry. This approach allows identification of antigens with epitopes resulting from any post translational modification. However with this approach only antigens with linear epitopes can be identified.

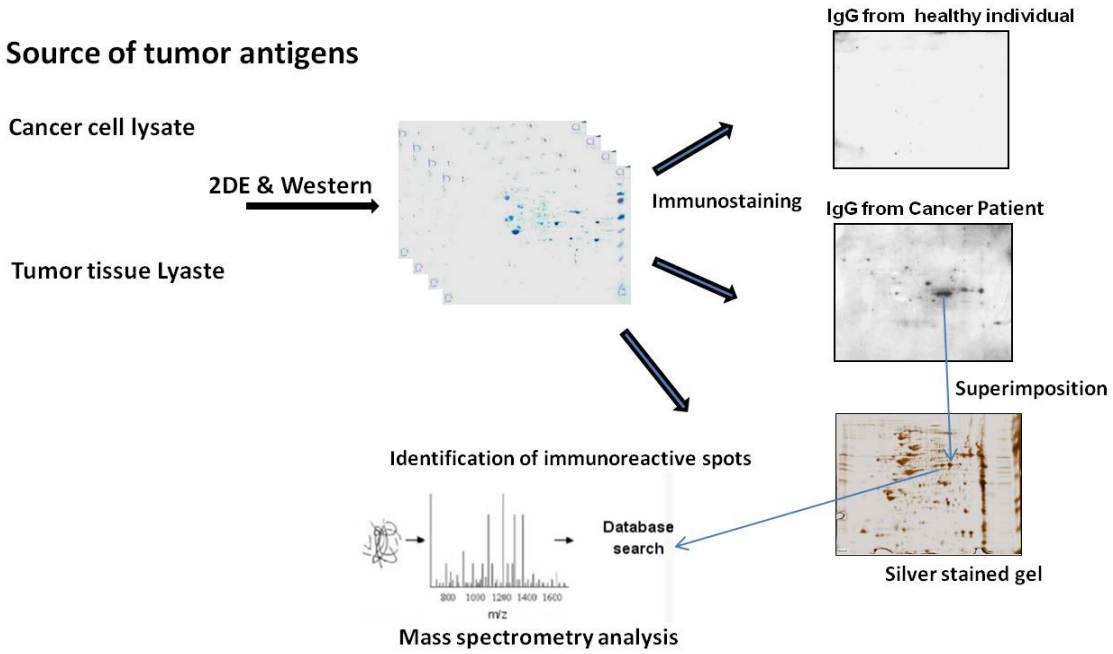


Figure 1.5: Overview of methodology used for identification of tumor antigens using Immunoproteomics

As an improvement of this approach Hanash's team [72] used 2D liquid chromatography to separate proteins in different fractions from cancer cell line / tumor tissue and developed protein arrays of these fractions to screen them with tumor sera and control sera. Proteins from fractions reactive with tumor sera were identified by mass spectrometry. This was called as natural protein microarray. Recently, similar natural protein microarrays have been generated to identify autoantibodies in lung and prostate cancer [73, 74]. Antigens with conformational epitope/s as well as with

epitope/s arising out of post translational modifications have been identified using these techniques.

Another approach for identifying TAAs eliciting autoantibodies is an immunoaffinity based technique called multiple affinity protein profiling *MAPPING* developed by Caron *et al*. [75]. In this technique, cell lysate is first passed through a column coupled with IgG isolated from a healthy individual in order to adsorb out tumor antigens which react with healthy sera. The cleared lysate is then passed through another column which is coupled with IgG isolated from tumor sera. Proteins that bind the tumor sera IgG are those that are specific for cancer and these are then identified by mass spectrometry [76, 77]. Hardouin *et al.* [78] have used this approach to screen sera for autoantibodies from patients with colo rectal cancer.

Immunoproteomics: Several terms have been proposed for this approach, such as SERPA (serological proteome analysis) [79], PROTEOMEX (a combination of proteomics and SEREX) [80], AMIDA (autoantibody-mediated identification of antigens) [81], or immunoproteomics approach [82]. With this approach it is possible to identify tumor-associated antigens eliciting immune response and detect isoforms and post-translational modifications (PTMs) of these antigens. Using immunoproteomics approach several tumor antigens have been identified in different cancers and their potential as biomarkers assessed. Table 1.1 below summarises the antigens identified in different cancers using this approach.

Cancer	Tumor antigens eliciting autoantibodies	Occurrence % (no of patients)	Reference
Neuroblastoma	β-tubulin I and III	48(n=23)	[83]
Breast	RS/DJ-1	13(n=23)	[84]
	Hsp60	47(n=40)	[85]
	Prdx2	37(n=40)	
	β-tubulin	42(n=40)	
Lung	Annexin I and II	60(n=54)	[51]
	PGP 9.5	14(n=64)	[86]
	Peroxiredoxin-I	47(n=53)	[87]
	Alpha enolase	27(n=94)	[88]
	Triose Phosphate Isomerase	28(n=40)	[89]
	Superoxide dismutase	20(n=40)	
Liver(HCC)	Calreticulin	27(n=37)	[90]
	β-tubulin	24(n=37)	
	Hsp-60	14(n=37)	
	Cytokeratin-18	14(n=37)	
	Cytokeratin-8	11(n=37)	
	Creatine kinase-B	14(n=37)	
	F1-ATP synthase b subunit	11(n=37)	
	NDPKA	14(n=37)	
	eEF2	39(n=28)	[91]
	AIF	42(n=28)	
	Prostatic binding protein	46(n=28)	
	DEAD box polypeptide 3	53(n=28)	
Pancreatic cancer	Calreticulin	58(n=36)	[92]
	DEAD-box protein 48	63(n=55)	[93]
Renal (RCC)	SM22-α	45(n=11)	[79]
	Carbonic anhydrase I (CAI)	27(n=11)	
Ovarian Cancer	Mesothelin	47(n=24)	[94]
Esophageal Cancer	Peroxiredoxin-6	50(n=30)	[95]
Adenocarcinoma	Alpha Enolase	60(n=5)	[96]
	Triose Phosphate Isomerase	40(n=5)	
CML	Beta tubulin	27(n=30)	[97]
	HSP 70	17(n=30)	
	Alpha Enolase	33(n=30)	
	Aldolase A	27(n=30)	
	Tropomyosin isoforms	13(n=30)	

Table 1.1: Antigens identified in different cancers by Immunoproteomics approach:

Protein microarray has emerged as a most promising technique for identification of multiple tumor antigens in parallel and also for high throughput screening of thousands of sera from different cancer patients. With the use of a panel of biomarkers there is great improvement in terms of specificity and sensitivity of

detection of cancer. Protein microarrays are based on immobilization of multiple candidate antigens on a support in an array format followed by differential analysis of antigen–antibody interactions. Protein arrays can be generated from either protein fractions obtained by 2D liquid chromatography of tissue or cell lysates or recombinant proteins [98] or by in-situ transcription and translation of proteins from cDNA arrayed on slides. The latter technique is called *NAPPA* (Nucleic Acid Protein Programmable Array). NAPPA has been used to identify tumor antigens for detection of early breast cancer patients [99]. Protein microarrays that enable large-scale testing on more than 80,000 recombinant antigens are now commercially available from invitrogen. Protein arrays have great potential as biomarker tools for clinics as they provide a modality for multiplexing of signature antigens in a single assay.

1.6 Clinical Implications of autoantibodies:

1.6.1 Autoantibodies as early biomarkers:

The immune response to tumor associated antigens occurs at an early stage of tumorigenesis, as our immune system is capable of sensing even those alterations which happen during early tumorigenesis. In addition, autoantibodies possess characteristics that enable them to be valuable as early cancer biomarkers. They are much more stable compared to other serum proteins and are present in easily detectable amount in the sera for a relatively long period and can be detected much before the clinical onset of disease. This is clearly apparent with the detection of p53 antibodies in heavy smokers who were diagnosed as lung cancer patients few months later. Hence autoantibodies serve as reporters for any aberrant processes happening during tumorigenesis

Cancer screening in high-risk populations, such as smokers, patients with chronic obstructive pulmonary disease and asbestosis has shown that autoantibodies are detectable before radiographic detection in lung cancer patients.

Trivers *et al.* [100] were able to detect p53 autoantibodies in serum in a subset of smokers with chronic obstructive pulmonary disease. p53 autoantibodies (Abs) were detected in 23% of the cancer patients, 80% of whom had detectable p53-Abs before diagnosis: 2 lung cancers (7 and 6 months before), 1 prostate cancer (11 months), and 1 breast cancer (5 months). Li *et al.* [101] have demonstrated significant relationship between presence of p53 autoantibodies in sera of an asbestosis cohort at high risk of cancer with their subsequent development of malignancy with a positive predictive value of 0.76 and an average lead time to diagnosis of 3.5 years. Zhong *et al.* [102] also showed the potential of autoantibody profiling in detection of early stage cancer and occult NSCLC. They used 102 samples from the Mayo Clinic CT Screening Trial (six prevalence cancer samples, 40 drawn 1 to 5 years before diagnosis, and 56 risk-matched controls) for this study. By measuring five most predictive antibody markers they correctly predicted six of six prevalence cancers, 32 of 40 cancers from samples drawn 1 to 5 years before radiographic detection on incidence screening, and 49 of 56 risk-matched controls.

Desmetz *et al.* [103] investigated autoantibodies to Hsp60 antigen in sera from early stage breast cancer and ductal carcinoma in situ using ELISA. Autoantibodies were present in 16/49 (31%) early stage breast cancer and 18/58 (32.6%) DCIS patients, compared to 4/93 (4.3%) healthy subjects. They also investigated the expression of Hsp60 in tissue samples by IHC staining and found that Hsp60 expression gradually

increases from normal through DCIS to invasive tissues. In a very recent blinded case-control study of p53 autoantibodies in colorectal cancer Pederson *et al.* [104] identified 25.8 % of colorectal cancer patients 1.4 years before clinical diagnosis.

1.6.2 Combination of Autoantibodies for better diagnosis:

It is well acknowledged that cancers are heterogeneous in nature and immune response is not same in every individual. The chance of detecting immune response to an individual antigen in all the patients is therefore not high. As a result, single markers lack sensitivity and specificity in assays for early detection, diagnosis and prognosis of cancer. There are many studies which have shown that markers in combination show improved sensitivity and specificity for the assay. Chen *et al.* [105] have shown that combined detection of autoantibodies to survivin and CEA provided better sensitivity (51.3 %) and specificity (89.9 %) compared to the sensitivity of CEA (40.9 %) and the specificities of the individual markers (64.1 % and 86.6 %, respectively). Similarly Zhang *et al.* [106] used miniarrays of recombinant proteins of a panel of seven already reported tumor antigens for detection of autoantibodies in six different cancers. They found an increase of positive antibody reactions from 15–20 % for single TAAs to 44–68 % for seven TAAs. Further patients with breast, lung, and prostate cancer showed separate and distinct profiles of reactivity, suggesting that there are unique autoantibody signatures for each type of cancer.

In a protein microarray based blinded validation study Qiu *et al.* [107], were able to detect antibodies to Annexin I, PGP9.5, 14-3-3 theta antigens and LAMR1 in the prediagnostic lung cancer sera collected from 85 patients from the Carotene and Retinol Efficacy Trial (CARET) cohort within 1 year before a diagnosis of lung

cancer. Lu *et al.* [108] investigated presence of antibodies to HER2/neu, p53, carcinoembryonic antigen (CEA) and cyclin B1 in sera collected 150 days before the diagnosis of breast cancer. They found 15 % of cases (n=33) were positive for HER2 autoantibodies, and 6 % were positive for p53 autoantibodies whereas no controls (45) were positive for autoantibodies to either protein.

Desmetz *et al.* [109] tested 5 tumor antigens PPIA, PRDX2, FKBP52, Hsp 60 and MUC1 for their ability to detect early stage breast cancer using ELISA. This test was done on 235 samples (60 from primary breast cancer patients, 82 from CIS patients, and 93 from healthy controls). Three of five autoantibodies, FKBP52, PPIA, and PRDX2, showed significantly increased reactivity in primary breast cancer and CIS compared with healthy controls.

Chang *et al.* [110] identified five- phage peptide clones which did not react with healthy sera but reacted with sera from colorectal patients. They validated these clones on an independent set of patients, 60 with colorectal cancer (CRC), 60 healthy controls, 52 with polyps and 30 with autoimmune diseases. The sero reactivities were significantly upregulated in the patients with colorectal polyps than in the healthy controls ($P < 0.005$ for each). Sero reactivities of the 4– phage peptide were significantly lower in the patients with colorectal polyps than in the CRC patients ($P < 0.005$ for each). These results suggested that the five-phage peptide clones can be useful as early biomarkers for colorectal cancer.

1.6.3 Autoantibodies and disease progression:

Autoantibody levels change during the progression of the cancer. The pattern of change is not uniform as observed in many different studies in different cancers. Li. *et. al* [111] have shown that serum IGFBP-2 autoantibody levels were significantly elevated in tumors and higher in early stage tumors than advanced stage tumors. IGFBP-2 autoantibody effectively discriminated between controls and grade II and III gliomas **(***P* < 0.0001). IGFBP-2 autoantibody levels were also high in patients with advanced polyps who were at high risk of colorectal cancer (p<0.0001).

Mudenda *et al.* [112] found the presence of p53 antibodies in sera from all stages (carcinoma in situ, primary invasive breast cancer and in metastatic disease) of breast cancer patients. The presence of antibodies to p53 correlated positively with high histological grade (P = 0.0012) and a history of second primary cancer (six positive out of eight cases). Results indicated that titre of autoantibodies varied with the progression of the disease.

Mange *et al.* [113] examined autoantibody targets in 20 DCIS and 20 IBC patients using protein microarrays and identified a signature (RBP-Jk, HMGN1, PSRC1, CIRBP, and ECHDC1) that was able to distinguish the two groups. The identified antibody signature was further tested on 120 independent samples 61 ductal in situ carcinoma and 59 Invasive breast cancers. This signature significantly discriminated between DCIS and IBC patients on the basis of levels of antibodies. Antibody levels also correlated significantly with the histological grade of DC.

1.6.4 Autoantibodies and disease prognosis:

Autoantibodies have been shown to be implicated in poor prognosis of disease, better outcome of the disease or to be irrelevant for the course of disease. Two proteins p53 and NY-ESO-1 have been extensively studied for their prognostic significance as tumor antigens in different cancers.

Anderson *et al.* [114] evaluated the diagnostic and prognostic value of p53 antibodies for ovarian cancer using ELISA. They analysed preoperative sera from women which included those subsequently diagnosed with invasive serous ovarian cancer, serous ovarian cancer and benign cancer. Detectable p53 antibodies in pretreatment sera correlated with improved overall survival in serous ovarian cancer.

Houbiers *et al.* [115] measured p53 autoantibodies in preoperative sera from 255 patients with colorectal cancer by enzyme-linked immunosorbent assay (ELISA) and showed significant association with poor prognosis of patients.

Kulic *et al.* [116] used ELISA to analyse sera from 61 patients with breast cancer and 20 individuals without malignancies. Concentration of anti-p53 antibodies was significantly related to the tumor size (p=0.028). Concentration of anti-p53 antibodies was significantly associated with histological grade of tumors (p=0.013). A statistically significant relationship was found between the presence of anti-p53 antibodies and the number of involved axillary lymph nodes (P = 0.0242), patients with higher levels of anti-p53 antibodies had shorter 5-year survival than patients with lower levels of antibodies (P = 0.0022).

Atta *et al.* [117] found significant association of serum anti-p53 antibodies with overall survival of patients with HCC (p=0.019) with a shorter survival time in patients positive for autoantibodies to p53. Blixt *et al.* [118] have reported that autoantibodies to specific cancer associated glycoforms of MUC1 are found more frequently and at higher levels in early stage breast cancer patients than in women with benign breast disease or healthy women. Further, presence of autoantibodies showed strong correlation with reduced rate and delay in metastasis thereby proving their utility in prognosis of disease.

Budiu. *et al.* [119] have shown that increased serum MUC1 and high anti-MUC1 antibody levels correlate with poor clinical response and reduced overall survival in platinum-resistant or platinum-refractory ovarian cancer. Table 1.2: shows information from different studies in which autoantibody response has been of prognostic utility.

Autoantibody to	No. of patients	Tumor Type	Disease outcome	Ref.
Laminin	71	Breast cancer	Decreased OS	[120]
HSP90	327	Breast cancer	Decreased OS	[121]
p53	9489	Breast, Gastric, Colon, NSCLC, Oral cancer	Increased OS	[43]
p53	60	Serous Ovarian cancer	Increased OS	[114]
p53	255	Colon cancer	Decreased OS	[115]
p53	61	Breast cancer	Decreased OS	[116]
NY-ESO-1	207	Prostate cancer	Decreased OS	[122]
Nucleophosmin	100	Breast cancer	Decreased RFS	[123]
CTSP-1	147	Prostate cancer	Increased RFS	[124]
p53	130	HCC	Increased RFS	[125]
p53	41	HCC	Decreased OS	[117]
Laminin-Receptor	67	CLL	Increased RFS	[126]
CML66	15	CML	Increased RFS	[127]
GLEA 3 and PHF 3	62	Glioblastoma	Increased OS	[128]
MUC1	30	NSCLC	Increased RFS	[46]
MUC1	100	Ovarian cancer	Increased OS	[129]
MUC1	28	Platinum resistant and platinum refractory Ovarian cancer	Decreased OS	[119]
MUC5AC	30	Colon cancer	Increased OS	[130]
SOX1	90	SCLC	Increased OS	[131]
CEA	52	Breast cancer	Increased RFS	[132]
SCP1	100	Pancreatic cancer	None	[133]
Cyclin B1	42	AML	None	[63]
p53	120	Oral cancer	None	[134]
Hsp90	116	Ovarian cancer	None	[135]
ALK	21	ALL	None	[136]
SEREX CLONES	25	SCC	None	[137]
MUC-1	125	Breast cancer	None	[138]
Glycosylated forms MUC1	395	Breast cancer	decreased RM & delay in metastasis	[118]
Survivin	76	NSCLC	None	[139]
NY-ESO-1	12	Different Cancers	None	[140]
NY-ESO-1	69	Esophageal	None	[141]
Phage display clones	176	Breast cancer	None	[142]
Braf	372	Melanoma	None	[143]
Panal of 29 antigens	60/59	Ovarian cancer	Increased OS	[144]

Table 1.2: Studies showing prognostic correlation of autoantibodies in different cancers, OS-overall survival; RFS-recurrence free survival; RM-Rate of metastatis

All these findings strongly suggest that the autoantibodies have immense potential for early detection, diagnosis and prognosis of cancers. However clinical utility of individual antigens is very limited because of their low sensitivity and specificity or low predictive value. Panel of antigens have shown better potential. Using protein microarrays, an autoantibody signature (RBP-Jk, HMGN1, PSRC1, CIRBP, and ECHDC1) was identified which could be used to divide the DCIS patients into a poor-prognosis group (local recurrence) and a good-prognosis group [113].

However there is an immense need to discover combination of different antigens. Several recent studies have identified a combination of antigens which show high specificity for a particular cancer and allow better discrimination between the test group and the control group. For selecting such a combination of tumor antigens elegant statistical analysis and algorithms are being used along with proteomic techniques.

There are many challenges which need to be addressed to ensure that data from the laboratory can be transferred to the clinic. In particular the identified set of tumor antigens will have to be validated on larger and different cohorts of patients with appropriate controls.

1.7 Autoantibodies and head and neck cancer:

Autoantibody response is being widely considered as a molecular sieve for identifying specific biomarkers for cancer, which can be used for early detection, prognosis and cancer management. The search for autoantibodies in oral cancer was first reported in 1969 by Gangal and Vernekar [145]. Using an indirect antiglobulin consumption test,

they showed presence of autoantibodies in five patients with oral cancer before and after treatment. In their analysis there was no significant autoantibody response after therapy and the autoantibody response was not evaluated in healthy individuals. Their study did not identify the antigens, which elicit autoantibody response. This study evaluated autoantibody response in only five patients before and after therapy and autoantibody response was not compared with healthy individuals. Thereafter, Ralhan et al and Castelli et al have reported autoantibodies against a single antigen p53 in oral cancer [134, 146]. These studies have assessed autoantibody response against a single antigen resulting in low sensitivity of cancer detection (19 % and 34 % respectively) and have also interpreted their results considering all head and neck sites together.

Marcos *et. al* [147] have shown that the predictive value of MMP-13 for cervical lymph node metastasis in oral cancer improved when it is combined with the presence of p53 autoantibodies. Lin. *et. al* [148] have used microarray-based serologic profiling for identification of a panel of antigens for early diagnosis of head and neck cancer. From the entire cDNA phage display library derived from three different HNSCC tissues a panel of 130 markers were identified which showed sensitivity of 79.8 % and specificity of 90.1 % for the diagnosis of cancer.

Eto M *et. al* [149] have reported the detection of autoantibodies to survivin in 71 % of head and neck cancer patients. High levels of anti-survivin were detected in patients with advanced stage. Murase *et. al* [150] have reported autoantibodies to sideroflexin 3 as a novel tumor marker for oral squamous cell carcinoma.

Our laboratory has used an immunoproteomics approach to identify tumor associated antigens for cancer of gingivo buccal complex (GBC). The antigens identified are Alpha enolase, Annexin II, HSP 70, Peroxiredoxin VI, ATP synthase, Alpha tubulin, Beta tubulin, Pyruvate kinase, Triose phosphate isomerase, Phosphoglycerate mutase, Aldose reductase, and Cyclophilin A, which elicit an autoantibody response in cancer of the gingivo-buccal complex [151] . Their site specificity is evident, as there is negligible autoantibody response to these antigens in patients with cancer of tongue[152]. Autoantibody response to some of these antigens is also observed in sera of individuals with leukoplakia of gingivo-buccal complex suggesting that they may have utility as early markers. These results needed further investigation in order to determine whether the antibody response to the identified tumor antigens can be used as an early biomarker or as a prognostic marker and to generate a multiplex array for their evaluation. Towards this end the following objectives were undertaken:

1.8 Objectives:

1) To evaluate autoantibody response to the identified tumor antigens in sera of healthy individuals with and without chewing/smoking habit, individuals with leukoplakia and in patients with T1/T2, T3/T4 tumors of cancer of GBC, using an immunoproteomics approach.

2) To evaluate the prognostic utility of autoantibody response to tumor antigens.

3) To express and purify recombinant proteins of identified tumor antigens

4) To assess autoantibody response on a multiplex array of the recombinant antigens.

Chapter 2

Materials and Methods

2.1 Materials for Immunoproteomics analysis:

2.1.1 Blood samples: This study was approved by the Institutional scientific review committee and the Hospital Ethics Committee of the Tata Memorial Centre. Blood samples were collected from patients undergoing surgery at the Tata Memorial hospital. These patients had not undergone any prior treatment. Blood was collected after obtaining informed consent of all the patients and healthy individuals with and without chewing/smoking habit and individuals with leukoplakia. Clinical information for all samples used in study is given in Appendix Tables A1 to A5.

2.1.1.1 Materials Used for Blood collection:

- BD vacutainer tubes from BD vacutainer Systems, UK. (Cat # 288231)
- 5 ml disposable syringe from Becton Dickinson India Ltd. India.
-22 gauge disposable needle with infusion set from Meditop Corporation, Malaysia.
-10 ml Screw cap tubes from Axygen Scientific, USA. (Cat# SCT-150-C)

2.1.2 Kit for IgG purification from serum: Melon Gel IgG purification kit (Pierce, Catalogue # 45206)

2.1.3 Cell lines: The **KB cell line**, originally derived from epidermal carcinoma of the mouth, has been used in this study as a source of tumor antigens. Other oral cancer cell lines **AW 13516** and **AW 8507** (derived from poorly differentiated SCC and epidermoid carcinoma of the tongue) were also used for some of the experiments.

2.1.4 Reagents for maintenance of KB Cell Line:

2.1.4.1 Dulbecco's modified Eagle's medium (DMEM) (Gibco-12100-046). The powdered medium (16.2 gm) was dissolved in 950 ml of deionized water and 3.7 gm of Na_2HCO_3 was added. The pH of the solution was adjusted to 7.5 with HCl and the final volume was made up to 1 litre. The solution was then filtered through 0.22 micron filter inside a Bio-safety hood.

2.1.4.2 10% Fetal Bovine Serum (FBS) (JRH Biosciences, USA)

50 ml of heat inactivated FBS was added to 450 ml filtered DMEM.

2.1.4.3 Antibiotic mixture: An antibiotic mixture consisting of 1 g streptomycin (Nicholas Piramal, India), 80 mg gentamicin (Nicholas Piramal India), and 2.5 mg amphotericin B (Himedia RM 468) was added to 1 liter of filtered DMEM.

2.1.5 Reagents used in the preparation of KB cell line lysate:

Lysis buffer: 8 M Urea (Sigma U-5378), 2 M Thiourea (Sigma T-7875), 10 % CHAPS (USB-13361), 10 % Dithiothrietol (DTT) (USB-15397)

480 mg Urea and 152 mg Thiourea were mixed and 200 µl of 10% CHAPS and 100 µl of 10% DTT were added. The total volume of the solution was adjusted to 1 ml.

2.1.6 Reagents for protein estimation of KB cell lysate by TCA Precipitation modification of Peterson's method

2.1.6.1 Protein standard: 1 mg/ml Bovine Serum Albumin (Sigma A7906)

2.1.6.2 Solutions for precipitation of proteins:

1.5 % Sodium deoxycholic acid, sodium salt (Sigma D-6750)

72 % Trichloro acetic acid (Sisco Research Laboratories 20482)

2.1.6.3 Copper Tartarate Carbonate (CTC) solution :

0.1 % copper sulphate, 0.2 % potassium tartarate, 10 % Sodium carbonate:

20 % Sodium carbonate was prepared by dissolving 20 gm of sodium carbonate in deionized water to a final volume of 100 ml.

0.2 gm copper sulphate dissolved in 40 ml deionized water and 0.4 gm of potassium tartarate dissolved in 40 ml deionized water was mixed together and the volume was made up to 100 ml to a final concentration of 0.2 % and 0.4 % respectively. Equal volume of 20 % sodium carbonate solution was then added to the mixture to obtain the final CTC solution.

2.1.6.4 10 % Sodium Dodecyl Sulphate (SDS) (Sigma L3771): 10 gm SDS was dissolved in deionized water to a final volume of 100 ml..

2.1.6.5 0.8N NaOH: 3.2 gm of NaOH solubilized in deionized water to a final volume of 100 ml.

2.1.6.6 Working solution A: Equal volumes of CTC solution, 10 % SDS, 0.8 N NaOH and deionized water were mixed together and the mixture was diluted 1:1 (v/v) with deionized water.

2.1.6.7 Working solution B:

2 N Folin and Ciocalteau's Phenol Reagent (FC reagent) (Sisco Research Laboratories 62015) diluted 1:5 with deionized water.

2.1.7 Reagents for Two Dimensional separation of KB cell lysate proteins

2.1.7.1 Precast Immobilized pH Gradient (IPG) gel strips

Ready Strip IPG strips 3-10, 7cm (Bio-Rad 163-2000)

Ready Strip IPG strips 3-10, 17cm (Bio-Rad 163-2009)

2.1.7.2 Rehydration buffer

8 M Urea, 2 M Thiourea, 2 % CHAPS, 1 % DTT, 0.2 % Ampholyte (Bio-Rad) (163-1113), 0.0002 % Bromophenol blue (Sigma B 5525):

480 mg Urea and 152 mg thiourea were mixed and 200 µl of 10 % CHAPS and 100 µl of 10 % DTT were added. Then 2 µl of 0.2 % ampholytes (pH 3-10) and 2 µl of 0.1 % bromophenol blue were added. The final volume was adjusted to 1ml .

2.1.7.3 Mineral oil (Bio-Rad 163-2129)

2.1.7.4 Equilibration buffer-1

6 M Urea, 0.375 M Tris (pH 8.8), 2 % SDS, 20 % Glycerol, 2 % DTT:

7.2 gm Urea, 0.4 gm SDS, and 0.4 gm DTT were mixed and 7.5 ml of 1 M Tris (pH 8.8) and 4 ml glycerol were added. The total volume of the solution was adjusted to 20 ml.

2.1.7.5 Equilibration buffer-2

6 M Urea, 0.375 M Tris (pH 8.8), 2 % SDS, 20 % Glycerol, 2.5 % Iodoacetamide:

7.2 gm Urea, 0.4 gm SDS, and 0.5 gm iodoacetamide were mixed and 7.5 ml of 1 M Tris (pH 8.8) and 4 ml glycerol were added. The total volume of the solution was adjusted to 20 ml.

2.1.8 Reagents for SDS-PAGE and Western blotting

Acrylamide (USB-75820), Bisacrylamide (USB- 75821), SDS (Sigma L-4509), Tris (USB-75825), Ammonium persulphate (S.D. Fine Chem. Pvt. Ltd- 20032), TEMED (USB-76320)

2.1.8.1 Stock acryl amide solution

30 % acrylamide solution: 29.2 gm acrylamide and 0.8 gm bisacrylamide were dissolved to a final volume of 100 ml in deionized water.

2.1.8.2 Separating gel for SDS-PAGE:

12 % Acrylamide, 375 mM Tris-Cl (pH 8.8), 0.1 % SDS:

For two 7 X 7 cm mini gels, 4.8 ml of 30 % acrylamide solution, 4.44 ml of 1 M Tris (pH 8.8), 120 µl of 10 % SDS, and 2.54 ml of deionized water were mixed and 100 µl of 10 % ammonium per sulphate and 10 µl of TEMED were added just before pouring gel in between the glass plates.

2.1.8.3 Stacking gel for SDS-PAGE:

5 % Acrylamide, 0.175 M Tris-Cl (pH 6.8), 0.2% SDS:

For two 7 X 7 cm mini gels, 600 µl of 30 % acrylamide, 672 µl of 1 M Tris (pH 6.8), 18 µl of 10 % SDS and 1.71 ml deionized water were mixed and 60 µl of 10 % APS and 3 µl TEMED were added just before pouring the gel.

2.1.8.4 10% N,N,N',N'-Tetramethylethylenediamine (TEMED)

2.1.8.5 10% Ammonium persulphate (APS)

2.1.8.6 SDS Sample buffer

0.189 M Tris-Cl (pH 6.8), 30 % Glycerol, 6 % SDS:

2.4 ml 1 M Tris-Cl (pH 6.8), 3 ml 20 % SDS, 3 ml 100 % glycerol, and 1.6 ml β-mercaptoethanol were mixed and the final volume made up to 10 ml with deionized water.

2.1.8.7 Electrode buffer

0.025 M Tris, 0.19 M Glycine and 0.2 % SDS:

3 gm Tris, 14.3 gm glycine and 2 gm SDS were dissolved in deionized water to make final volume up to 1 liter.

2.1.9 Reagents for Silver and Coomassie blue staining of gels:

2.1.9.1 Destainer

40 % Methanol and 10 % Acetic acid.

2.1.9.2 Silver staining-Solution 1

0.02 % Sodium thiosulphate (Sisco Laboratories- 14455):

0.02 gm Sodium thiosulphate was dissolved in deionized water to a final volume of 100 ml.

2.1.9.3 Silver staining-Solution 2

0.2 % Silver nitrate (Qualigens-15804), 0.075 % formaldehyde (Merck-1/17837):

0.2 gm silver nitrate and 75 µl of formaldehyde were dissolved in deionized water to a final volume of 100 ml.

Note: 75 µl of formaldehyde was added at the time of staining.

2.1.9.4 Silver Staining- Solution 3

2 % Sodium carbonate (Qualigens: 15955), 0.05 % formaldehyde, 2 % silver staining solution 1:

2 gm sodium carbonate, 50 µl of formaldehyde and 2 ml silver staining solution 1 were mixed in deionized water to a final volume of 100 ml. Note: 50 µl of formaldehyde was added just prior to staining.

2.1.9.5 Silver Staining-Stop solution

10 % Acetic acid.

2.1.9.6 Commassie Blue stain

0.3 % Commassie brilliant blue R250 (Sigma B0149) in 40 % Methanol and 10 % Acetic acid.

2.1.10 Reagents for immuno detection on western blots:

2.1.10.1 Blocking buffer - Tris Buffered Saline with Tween 20 (TBST):

20 mM Tris-Cl (pH 7.6), 150 mM NaCl containing 0.1 % Tween 20 (USB- 20605).

2.1.10.2 Blocking solution

5 % skimmed milk powder (Nestle Carnation non fat dry milk) in blocking buffer.

2.1.10.3 Washing buffer- TBST :

20 mM Tris-Cl (pH 7.6), 150 mM NaCl containing 0.05 % Tween 20.

8.77 gm of NaCl, 20 ml of 1M Tris(pH 7.6) and 1 ml of Tween 20 was added in 980 ml of deionized water to make 1 lt. of buffer.

2.1.10.4 Primary antibodies used in this study:

IgGs purified from sera of patients and healthy individuals.

Rabbit anti-α-enolase antibody (Santacruz sc-15343)

Rabbit anti HSP-70 antibody (Abcam ab31010)

2.1.10.5 Secondary antibodies

Horseradish peroxidase-conjugated (HRP) sheep anti-human IgGs (GE Healthcare NA-933)

HRP conjugated donkey anti-rabbit IgGs (GE healthcare NA-934)

2.1.10.6 ECL plus western blotting detection reagents:

ECL plus detection reagents A & B supplied by GE Healthcare in the ECL plus detection kit (RPN 2132).

2.1.10.7 X-ray films:

X-ray films supplied by Kodak India Pvt Ltd (MXB 4908703)

2.1.11 Staining of blots:

2.1.11.1 Bio-Rad's Colloidal Gold total protein stain (Cat No:170-6527)

2.1.11.2 AMIDO BLACK stain (Naphthalene Black 10B GURR'S 21044)

2.2 Methods for immunoproteomics analysis

2.2.1 Maintenance of cell lines: Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10 % FBS and the antibiotic mixture at 37°C in a humidified atmosphere of 95 % air and 5 % CO_2.

2.2.2 Preparation of KB cell lysate: For 2DE analysis, (1 x 10^8) cells were lysed in 500 µl of lysis buffer (2.1.5) and the mixture centrifuged at 55,000 rpm for 1 h at 4^0C in the Beckman TLD ultracentrifuge. The supernatant was collected and stored in aliquots at -80^0 C. Concentration of the protein in the lysate was estimated in one of the aliquots by the Peterson's modified Lowry method given below.

2.2.3 Protein estimation of KB cell lysate by modified TCA precipitation method [153] **:** KB cells were lysed in in lysis buffer (2.1.5). As urea was found to interfere in the protein estimation, the TCA Precipitation method was used. BSA was used as protein standard. A stock solution of BSA (1mg/ml) was prepared in 2DE buffer. Different dilutions of BSA were prepared from this stock by diluting in 2DE buffer so as to obtain the required concentration in 100 µl and the final volume was then made to 1ml with deionized water. Similarly 10 µl of KB cell lysate and 90 µl of 2DE buffer were mixed and final volume was made to 1ml with deionized water. Hundred µl of 1.5 % sodium deoxycholate was added to each tube. The tubes were vortexed and allowed to stand at RT for 10 min. Hundred µl of 72 % TCA was then added to each tube and the mixtures were gently shaken. Addition of 72 % TCA causes precipitation of proteins, which is seen as a white fluffy precipitate. The precipitates were spun out by centrifuging the suspension at 3000 rpm for 60 min in

rotor 13 of the Plasto craft Rota 4 centrifuge at RT. The supernatants were decanted completely. One ml of 1:10 diluted 1.5 % sodium deoxycholate was added to each tube, the mixture was vortexed and allowed to stand at RT for 10 min. Hundred µl of 72 % TCA was added to the precipitate and the mixtures were gently shaken. The suspensions were then spun at 3000 rpm for 60 min. The supernatants were decanted completely. 2 ml of solution A (2.1.6.6) was added to each tube and the precipitates were solubilized by vortexing. The mixtures were allowed to stand at RT for 10 min, 500 µl of 1:5 diluted FC reagent was added to each tube and the solutions were vortexed. The mixtures were incubated for 30 min at RT in dark. At the end of the incubation period the contents were vortexed again and their O.D (optical density) read at 750 nm using a spectrophotometer. The readings obtained with the BSA standards were used to plot the standard curve and the protein content of the cell lysate was determined by extrapolation from the standard curve.

2.2.4 Two Dimensional separation of KB cell lysate

2.2.4.1 Rehydration and sample application: KB lysate proteins were resolved by 2DE essentially according to Laemmli [154]. Precast Immobilized pH Gradient (IPG) gel strips (pH 3-10, 7cm) from Bio-Rad were used for isoelectric focussing. Each single IPG strip was rehydrated with 125 µl of rehydration buffer (2.1.7.2) containing 40 µg of KB cell lysate protein. The rehydration buffer containing the protein was dispensed as a line along the edge of a channel of the rehydration tray with a pipette. The plastic cover of the the IPG strips, was removed and the strips were gently placed gel side down, on the sample in the tray. After 20 minutes of incubation at room temperature, 1-2 ml. of mineral oil was overlaid over each strip to avoid evaporation of the sample during rehydration. Rehydration of strips was done for overnight at RT.

During rehydration, the protein sample dissolved in the rehydration buffer gets absorbed into the precast Immobilized pH Gradient (IPG) gel strip**.**

2.2.4.2 Isoelectric focussing: After overnight rehydration (14-16 h), small paper wicks were cut from Whatman filter paper (Cat-1441150) and placed at both ends of the electrodes in the Protean IEF focusing tray (Bio-Rad). The paper wicks were moistened with 8 µl of deionized water. The rehydrated strips were then removed from the rehydration tray and excess mineral oil was absorbed out by placing them on a tissue paper with the gel surface facing up. The rehydrated strips were then placed in the IEF focussing tray, with gel side down and 1-1.5 ml of mineral oil was overlaid over each strip to avoid evaporation of protein sample. The IEF tray was then placed inside the IEF protean cell for isoelectric focussing, with the positive mark on the strips towards the positive electrode of the cell. The proteins were focused using the following programme.

Table 2.1: IEF programme for 7 cm IPG strip

	Start voltage	End voltage	Time	Final Volt-Hours	
Step 1	0 V	250 V	20 min	--	Linear
Step 2	250 V	4000 V	2 h	--	Linear
Step 3	4000 V	4000 V	--	10000 V-h	Rapid

Table 2.2: IEF programme for 17 cm IPG strip

	Start voltage	End voltage	Time	Final Volt-Hours	
Step 1	0 V	250 V	20 min	--	Linear
Step 2	250 V	10000 V	2 h, 30 min	--	Linear
Step 3	10000 V	10000 V	--	40000 V-h	Rapid

After the completion of IEF, IPG strips were transferred from the IEF tray in to a washed rehydration tray (gel side facing up) after draining the mineral oil on the strips on to a tissue paper. The strips were equilibrated with equilibration buffer-1 and 2 one after another for 10 min. each.

2.2.4.3 SDS-PAGE followed by Western Blotting:

After the completion of IEF, proteins on each IEF strip were incubated in equilibration buffer-1 for 10 min to reduce the disulphide bonds and then in equilibration buffer 2 to alkylate the reduced amino acids. After equilibration, IPG strips were dipped in electrode buffer and each strip placed on the top edge of a 12 % SDS-PAGE gel and fixed into position with 1 % agarose solution. The proteins so processed were resolved further in the second dimension by 12 % SDS-PAGE. Multiple gels (from 3 to a maximum of 9) were run in a Bio-Rad PROTEAN 3 Dodeca Cell. Second dimension separation was carried out at a constant voltage of 200 V.

Six or more gels were run, one gel was used for silver staining to ensure resolution and condition of the proteins and the separated proteins from the rest of the gels were transferred on to PVDF membranes (Millipore) by Western Blotting in a Mini transfer Transblot Cell from (Bio-Rad) at 55 volts for 55 min. The blots were then probed with sera IgG (5 µg/ml) from individuals with leukoplakia, chewing habitués, cancer patients and healthy individuals.

2.2.5 Immunostaining of KB 2D blots:

2.2.5.1 Purification of IgG from sera: IgGs were purified from the collected sera with Melon Gel IgG Spin Purification Kit and the protein estimated by the Lowry method

[155]. The quality and concentration of IgG was further assessed on Commassie stained 10 % SDS PAGE gel. Purified IgGs were kept at 4°C and used within one week

2.2.5.2 Immunostaining of KB 2D blots with sera IgG: KB 2DE blots were marked at three different asymmetric locations on the corners of the blot. The blots were then blocked in 5 % milk powder in TBST (20 mM Tris-Cl, pH 7.6, 150 mM NaCl containing 0.1 % Tween 20 in a poly bag for 1 h at RT. After blocking, blots were incubated overnight at 4°C with serum IgG (5µg/ml in 2.5 % milk powder in TBST) from healthy individuals and patients.

After three washes with TBST buffer, blots were incubated with secondary antihuman antibody (1:10,000 dilution in 2.5 % milk powder in TBST) for 1h at RT. Immuno-detection was done using the Enhanced Chemiluminescence ECL plus kit and the signals captured on X-ray film. Four of six blots processed in a single run were arranged in a single cassette and multiple exposures ranging from (5 min to 10 min) were taken on the X-ray film so as to get the best signals with least background. In each run atleast two cancer samples, one control and a sample for which the pattern was known were taken. To confirm weak signals the experiments were repeated.

2.2.5.3 Selection of proteins that elicit autoantibody response: All the blots used in the experiment were first stained with Amido black and finally with Colloidal Gold to visualize the proteins below 30 kD. To identify the signal corresponding to the protein on the blot, autographs were superimposed over its respective amido/colloidal stained blot so as to overlap the marks made on the edges. The signals on the X-ray film which completely overlay in size and shape with the Amido stained protein spots on the blot

were considered as positive. Signals on protein spots already reported in our earlier studies were only considered. Other signals which did not fall on an amido stained protein were not taken into consideration. This analysis was done for every blot. The blot was evaluated by the student and guide. As there was no indication about the signals which would be obtained for any sample, there was no blinding. The signals obtained were tabulated and percentage positivity calculated.

2.2.6 Statistical analysis for correlation studies:

Clinical information such as age, gender, node, differentiation status and tumor stage was obtained from the clinical and histopathological reports of the patients.

In order to determine the association of autoantibody response to tumor antigens with disease free survival of patients Kaplan Meier survival analysis was performed for autoantibody response against each tumor antigen. SPSS 16.0 software package was used for all the statistical analysis.

Survival analysis was first performed with the samples evaluated for autoantibody response in the work done for this thesis and then samples from the earlier investigation were also included in a pooled analysis. For survival analysis with each of tumor antigens, patients were grouped into those who are positive for antibody response against that protein and those negative for antibody response. The survival curves were plotted for both the groups and rate of survival of patients in two groups was compared. The significance of this analysis was estimated by log rank test. A p value of 0.05 was considered significant.

2.3 Materials for cloning, expression and purification of recombinant proteins:

2.3.1 Bacterial Strains:

DH5α E.Coli Ultra competent cells, BL21(DE3) Competent Cells , ccdB Survival™ chemically competent E. Coli cells (Invitrogen),

2.3.2 Bacterial cell culture medium:

2.3.2.1 2 % Luria Broth (LB) Medium:

Twenty gm Luria Broth powder (HIMEDIA M575) was dissolved in deionized water and the final volume made to 1000 ml. The medium was sterilized by autoclaving at 15 lbs for 20 min.

2.3.2.2 2 % Luria Broth agar medium:

2.0 % Luria Broth medium, 1.5 % Agar (Himedia RM 301):

15 gm of agar was added to the LB medium and the mixture autoclaved at 15 lbs for 20 min.

2.3.2.3 Antibiotics:

Ampicillin sodium salt (Sigma A-9518):

Stock: A solution of 100 mg/ml was made in deionized water and filtered through a 0.45 µ filter. LB media containing 100 µg/ml ampicillin was used for growing cultures.

Kanamycin sulfate (Sigma K4378) :

Stock: A solution of 100 mg/ml was made in deionized water and filtered through a 0.45 µ filter. 100 µg/ml ampicillin was used in LB media.

Spectinomycin dihydrochoride pentahydrate (Sigma S4014): A solution of 100 mg/ml was made in deionized water and filtered through a 0.45 µ filter. LB media containing 100 µg/ml spectinomycin was used for growing cultures .

2.3.2.4 Sterile disposable 85 mm petri plates (Laxbro Cat No: PD-85):

2.3.3 Reagents for preparation of E. coli DH5-α ultra competent cells:

2.3.3.1 SOB medium

2 % bacto-tryptone (Himedia RM 014), 0.5 % yeast extract (Himedia RM 027), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, pH 6.7 to 7.0,

2 M Mg solution

1 M $MgSO_4.7H_2O$ (6.15 gm in 25 ml deionized water),

1 M $MgCl_2$. $6H_2O$ (5.075 gm in 25 ml deionized water)

25 ml 1 M $MgSO_4.7H_2O$ and 25 ml $MgCl_2.6H_2O$ were mixed to make 2 M Mg solution and the solution was filtered through 0.22 micron filter .

To make SOB medium, 6 gm bacto-tryptone, 1.5 gm yeast extract, 175.32 mg NaCl, 55.91 mg KCl were dissolved in 297 ml deionized water and autoclaved. After autoclaving, 3 ml of 2 M Mg solution was added prior to use.

2.3.3.2 TB buffer:

10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$:

393 mg PIPES, 286.65 mg $CaCl_2$, and 2.42 gm KCl were dissolved in 120 ml deionized water and pH was adjusted to 6.7 with 5 N NaOH. After adjusting pH, 1.414 gm $MnCl_2$ was added to the solution and volume was made up to 130 ml with deionized water. The solution was filtered through 0.22 micron filter.

2.3.3.3 Dimethyl Sulfoxide (DMSO) (Sigma D-8779):

2.3.4 Reagents for preparation of one Shot® ccdB Survival™ chemically competent E. Coli cells :

2.3.4.1 Transformation buffer 1 (TFB-1)

100 mM KCl, 30 mM CH_3COOK, 7 mM $CaCl_2.2H_2O$, 15 % glycerol, 30 mM $MnCl_2,4H_2O$:

0.75 gm KCl, 0.3 gm CH_3COOK, 0.11 gm $CaCl_2$, $2H_2O$, 15 ml glycerol, 0.635 gm $MnCl_2,4H_2O$ were dissolved in water to a final volume of 100 ml. The buffer was filtered through a 0.22 µ filter and kept at 4^0C.

2.3.4.2 Transformation buffer 2 (TFB-2)

0.04 mM Na-MOPS, 10 mM KCl, 57 mM $CaCl_2$, $2H_2O$, 15 % glycerol:

1mg sodium-MOPS, 0.075 gm KCl, 0.84 gm $CaCl_2$, $2H_2O$, 15 ml glycerol were dissolved in water to a final volume of 100 ml. The buffer was filtered through a 0.22 µ filter and kept at 4^0C.

2.3.5 Reagents for gateway Cloning:

2.3.5.1 pDEST 15 Vector (Invitrogen 11802-014)

2.3.5.2 Gateway LR Clonase II Enzyme mix (Invitrogen 11791-020)

2.3.5.3 2 µg/µl Proteinase K solution (Invitrogen 11791-020)

2.3.5.4 50 µg/µl pENTR-gus Positive Control (Invitrogen 11791-020)

2.3.6 Entry clones: cDNA entry clones carrying genes for the identified eight tumor antigens were obtained from Harvard Institute of Proteomics.

Table 2.3 : List of entry clones for the identified tumor antigens obtained from Harvard Institute of Proteomics

S.N0.	Gene name	Clone ID	Description	Selection Property
1.	Aldo-Keto Reductase family1[AKR1B1]	HsCD00042775	Gateway type cloning Donor vector pDONR221	Kanamycin resistant
2.	Pyruvate Kinase Muscle [PKM2]	HsCD00044791	Gateway type cloning Donor vector pDONR221	Kanamycin resistant
3	ATP Synthase, H Transporting,Mitochondrial F1 [ATP5B]	HsCD00043668	Gateway type cloning Donor vector pDONR221	Kanamycin resistant
4	Tubulin, Alpha 2 [TUBA1B]	HsCD00044921	Gateway type cloning Donor vector pDONR221	Kanamycin resistant
5	Tubulin, Beta [TUBB]	HsCD00043825	Gateway type cloning Donor vector pDONR221	Kanamycin resistant
6	Phospho Glycerate Mutase1(Brain) [PGAM1]	HsCD00043580	Gateway type cloning Donor vector pDONR221	Kanamycin resistant
7	Peroxiredoxin 6 [PRDX6]	HsCD000288707	Gateway type cloning entry vector pENTR223	Spectinomycin resistant
8	Peptidyl Prolyl Isomerase A (Cyclophilin A) [PPIA]	HsCD00044677	Gateway type cloning Donor vector pDONR221	Kanamycin resistant

2.3.7 Plasmids :

pGEX4T1 (GE Healthcare Code No. 28-9545-49)

pTZ57R/T (Thermo Scientific Fermentas K1213)

2.3.8 Polymerase Chain Reaction (PCR)

Recombinant Taq DNA Polymerase **(** GIBCO-BRL 10342-020), 10 X PCR buffer supplied with Taq DNA polymerase, 25 mM $MgCl_2$, dNTPs mix (2 mM each,GIBCO-BRL R0241)

TE buffer: 10 mM Tris-Cl (pH-8.0), 1 mM EDTA

2.3.9 PCR Primers from Sigma Genosys

PCR primers were designed using the DNA Star software.

Table 2.4 : Primers for confirmation of the presence of genes of identified tumor antigens in the clones obtained from Harvard Institute of Proteomics,

AKR	FP	5'CCGTCTCCTGCTCAACAAC3'
	RP	5'GTGGTCATATCCTGGCTGCT3'
PKM2	FP	5'GTCGAAGCCCCATAGTGAAG3'
	RP	5'GAAGATGCCACGGTACAGGT3'
ATP SYNTHASE	FP	5'TCTAAATGCCCTGGAAGTGC3'
	RP	5'GCTGGAATCCTTTGATGGTC3'
ALPHA TUBULIN	FP	5'AATGCCTGCTGGGAACTGTA3'
	RP	5'CACGCCCACCTCTTCATAAT3'
BETA TUBULIN	FP	5'CTGGACCGCATCTCTGTGTA3'
	RP	5'CTCTCAGCCTCGGTGAACTC3'
PGM	FP	5CGCTACGAGATGCTGGCTA3'
	RP	5'AACTGCATGGGCTTGATAGG3'
CYCLOPHILIN A	FP	5'CACCGTGTTCTTCGACATTG3'
	RP	5'GCCATCCAACCACTCAGTCT3'

FP: forward primer; RP: reverse primer

Table 2.5 : Gene specific primers with restriction sites for PCR/TA cloning:

AKR	FP	5'GAATTCATGGCAAGCCGTCTCCTG3'
	RP	5'GTCGACTCAAAACTCTTCATGGAAGG3'
PKM2	FP	5'GAATTCATGTCGAAGCCCCATAGTGAAGC3'
	RP	5'GTCGACTCACGGCACAGGAACAACACG3'
ATP SYNTHASE	FP	5'GAATTCATGTTGGGGTTTGTGGGTCG3'
	RP	5'GTCGACTCACGATGAATGCTCTTCAGCC3'
ALPHA TUBULIN	FP	5'CTCGAGATGCGTGAGTGTATCTCTATCCACG3'
	RP	5'GCGGCCGCTCAGTATTCTTCACCTTCTTCAGCCTCG'3
BETA TUBULIN	FP	5'GAATTCATGAGGGAAATCGTGCACATCC3'
	RP	5'GTCGACTTAGGCCTCCTCTTCGGCCTC'3
PGM	FP	5'GAATTCATGGCCGCCTACAAACTGGTG3'
	RP	5'GTCGACTCACTTCTTGGCCTTGCCCTG3'
PEROXIREDOXIN6	FP	5'GAATTCATGCCCGGAGGTCTGCTTCTCG3'
	RP	5'GTCGACGTAAGGCTGGGGTGTGTAGCG3'
CYCLOPHILIN A	FP	5'GAATTCATGGTCAACCCCACCGTGTTCTTC3'
	RP	5'GTCGACTTATTCGAGTTGTCCACAGTCAGC3'

FP: forward primer; RP: reverse primer

Table 2.6: Primers used in sequencing for confirmation of cloned genes in pDEST15:

pDEST 15	FP	5'ATATAGCATGGCCTTTGCAG3'
	RP	5'CTTTCGGGCTTTGTTAGCAG3'

FP: forward primer; RP: reverse primer

All the primers were constituted in TE buffer to give final concentration of 100 picomole/µl. This was further diluted in deionized water to give final concentration of 10 picomole/µl.

2.3.10 Reagents for plasmid DNA extraction by alkaline lysis method

2.3.10.1 Solution 1

50 mM glucose, 25 mM Tris (pH 8.0), 10 mM EDTA:

0.9 gm glucose, 2.5 ml of 1 M Tris (pH 8.0), and 2 ml of 0.5 M EDTA (pH 8.0) were dissolved in deionized water to a final volume of 100 ml.

2.3.10.2 Solution 2

0.2 M NaOH, 1 % SDS:

1 ml of 2 M NaOH, 1 ml of 10 % SDS was dissolved in deionized water to a final volume of 10 ml.

2.3.10.3 Solution 3

3 M potassium acetate, 11.5 % glacial acetic acid:

49.07 gm of potassium acetate was dissolved in deionized water to make 5M potassium acetate in 100 ml. 60 ml of 5 M potassium acetate and 11.5 ml of glacial acetic acid were dissolved in deionized water to a final volume of 100 ml.

2.3.10.4 Equilibrated phenol (Sigma P-4557)

2.3.10.5 Chloroform and Isoamyl alcohol in 24:1 ratio

2.3.10.6 Ethanol (Changshu yangyuan chemicals, China)

2.3.10.7 TE buffer: 10 mM Tris-Cl (pH-8.0), 1 mM EDTA

2.3.10.8 3 M Sodium acetate, pH 5.2 :

40.8 gm sodium acetate, $3H_2O$ was dissolved in 80 ml deionized water and the pH adjusted to 5.2 with glacial acetic acid. The total volume of the solution was made to 100 ml.

2.3.11 Reagents for restriction Digestion

2.3.11.1 Restriction enzymes

10 u/µl *Bam HI* (Fermentas ER0051)

10 u/µl *Sal I* (Fermentas ER0641)

10 u/µl *Eco RI* (Fermentas ER0271)

10 u/µl *Bsrg1*(Fermentas ER0931)

2.3.11.2 Digestion buffer

10 X Tango buffer supplied with restriction enzymes.

2.3.12 Reagents for agarose Gel Electrophoresis:

2.3.12.1 10 X TBE buffer

0.89 M Tris base, 0.89 M Boric acid, 0.02 M EDTA, Na_22H_20:

108 gm Tris base, 55 gm boric acid and 7.44 gm EDTA, $Na_{2,}2H_2O$ were dissolved in deionized water to a final volume of 1 liter.

2.3.12.2 Ethidium bromide (Sigma E-8751); 10 mg/ml in deionized water, stored at 4^0C.

2.3.12.3 Agarose (Sigma A-6013); 1 % Agarose in 1 X TBE buffer.

2.3.12.4 1kb DNA ladder; 0.5 µg/µl (Fermentas SM0311)

2.3.12.5 Loading dye (6X); 0.25 % Bromophenol blue, 0.25 % Xylene cyanol, 30 % Glycerol

2.3.13 Reagents for expression and purification of recombinant GST fusion proteins:

2.3.13.1 1M Isopropyl-beta-D-thiogalactopyranoside (Sigma I-5502)

1.19 g Isopropyl-beta-D-thiogalactopyranoside (IPTG) was dissolved in 5 ml deionized water, filter sterilized and stored at -20^{0}C.

2.3.13.2 Lysis buffer for bacterial cells

2.3.13.2.1 Protease inhibitor mix

10 µg/ml Aprotinin (USB-11388), 10 µg/ml Leupeptin (Sigma L-2884), 1 mM EDTA, 1 mM Sodium ortho vanadate (Sigma S-6508), 0.1 mM PMSF (Sigma P-7626), 1 mM Pefabloc SC (Roche-1429876)], Triton-X-100:

110 µl of 1 mg/ml Aprotinin, 110 µl of 1 mg/ml Leupeptin, 22 µl of 0.5 M EDTA, 55 µl of 0.2 M sodium ortho vanadate, 11 µl of 100 mM PMSF, 110 µl of 100 mM Pefabloc SC and 110 µl Triton-X-100 were dissolved in PBS to make a final volume of 11 ml.

2.3.13.3 50% Glutathione Sepharose 4-B (GE Healthcare 17-0756-01)

2.3.13.4 Elution buffer

10 mM reduced glutathione, 50 mM Tris-Cl (pH-8.0):

0.015 gm reduced glutathione and 250 µl 1M Tris (pH 8.8) were dissolved in deionized water to make a final volume of 5 ml.

2.3.13.5 Thrombin: GE Healthcare 500 units (27-0846-01)

2.3.13.6 Thrombin cleavage buffer: PBS, pH 7.3

Thrombin was dissolved in cold filtered 500 µl of PBS, pH 7.3 (1U/µl). Fifty µl aliquots were made and stored at -80°C.

2.3.14 Molecular weight markers: Fermentas page ruler prestained protein ladder SM 0671

2.4 Reagents for dot Blot Analysis :

2.4.1 Nitrocellulose membrane type SCN (mdi Membrane technologies)

2.4.2 Blocking buffer - Tris Buffered Saline with Tween 20 (TBST):

20 mM Tris-Cl (pH 7.6), 150 mM NaCl containing 0.1 % Tween 20 (USB 20605).

2.4.3 Blocking solution

5 % skimmed milk powder (Nestle Carnation non fat dry milk) in blocking buffer.

2.4. 4 Washing buffer-TBST :

20 mM Tris-Cl (pH 7.6), 150 mM NaCl containing 0.05 % Tween 20.

2.5 Reagents for ELISA:

2.5.1 Maxisorp plates (Nunc Fischer scientific Cat No: 442404)

2.5.2 Anti-GST antibody coated plates (Pierce Thermo Scientific No. 15144)

2.5.3 Coating buffer: 50 mM carbonate/bicarbonate buffer, pH 9.6

1.59 gm of Na_2CO_3 and 2.93 gm NaHCO3 were dissolved in deionized water and the volume made up to 1 liter.

2.5.4 Phosphate buffered saline, pH 7.4, containing 0.05 % Tween 20 (PBS-T):

8.00g NaCl, 0.20g KH_2PO_4, 1.15g Na_2HPO_4, 0.20g KCl, 0.5ml Tween 20 in 1 liter. of deionized water.

2.5.5 Polyvinyl alcohol (Sigma-cat. No. 341584)

2.5.6 2.5% Bovine serum albumin: 2.5 gm of BSA (Sigma A7906) was dissolved in PBS to make a 100 ml solution.

2.5.7 Dilution buffer: 0.2 % BSA-PBS

2.5.8 TMB sustrate: tetramethylbenzidine substrate (BD Bioscience, Cat No. 555214)

2.5.9 0.5 M H_2SO_4: 5.5ml H_2SO_4 was added in 44.5 ml deionized water to make 50 ml. of solution.

2.6 Methods for cloning, expression and purification of recombinant proteins

2.6.1 Preparation of *E.coli* DH5-α ultra competent cells:

The procedure followed was essentially according to Inoue et al [156]. *E.coli* DH-5α cells were streaked on LB agar and the plate kept overnight at 37^0C. A single colony of DH-5α cells was picked up and grown in 250 ml of SOB medium at 18^0C until OD_{600} reached to 0.6. The cells were then kept on ice for 10 min and pelleted by centrifugation at 5000 rpm for 10 min at 4^0C using a SS-34 rotor in a Sorvall RC5C centrifuge. After centrifugation, cells were resuspended in 80 ml of ice-cold TB buffer **(2.3.3.2)** and kept on ice for 10 min. The cell suspension was then centrifuged at 5000 rpm for 10 min at 4^0C using a SS-34 rotor in a Sorvall RC5C centrifuge. After centrifugation, cells were resuspended gently in 20 ml of ice-cold TB buffer and 1.4 ml DMSO was added to make the final concentration to 7%. Cells were kept on ice for 10 min and aliquots of 100 µl were made and snap frozen in liquid nitrogen. The ultra competent cells were then stored at -80^0C.

2.6.2 Preparation of one Shot® ccdB Survival™ chemically competent E. coli cells:

ccdB Survival™ *E.coli* cells were streaked on a LB agar plate and the same incubated overnight at 37^0C. A single colony of ccdB Survival™ *E.coli* cells from the plate was picked up and grown in 5 ml of LB medium at 37^0C ON to make a starter culture. Twenty five ml of LB was inoculated with 250 µl of starter culture and the suspension grown at 37^0C for 2.5 to 3 h until OD_{600} reached 0.5-0.6. The cell suspension was then kept on ice for 15 min and the cells pelleted by centrifugation at 4000 rpm for 5 min at

4^0C using a SS-34 rotor in an Sorvall RC5C centrifuge. The pelleted cells were resuspended in 30 ml of ice-cold TFB-1 and kept on ice for 90 min. The cells were then centrifuged at 4000 rpm for 5 min at 4^0C using a SS-34 rotor in a Sorvall RC5C centrifuge. After centrifugation, cells were resuspended gently in 1 ml of ice-cold TFB-2 . Aliquots of 50 µl were made and snap frozen in liquid nitrogen. The competent cells were then stored at -80^0C.

2.6.3 Strategy for cloning of genes for the identified tumor antigens:

The strategy used for cloning of identified tumor antigens in expression vectors is shown below. Of the 12 tumor antigens identified, three antigens, alpha enolase, annexin and Hsp70 were subcloned in pGEX4T1 vector by PCR cloning. These clones were obtained as gifts from Dr. Peter J Hornsby, University of Texas, USA, Dr. Volker Gerke, Institute of Medical Biochemistry, Munster and Dr Marja Jaattela, Danish Cancer Institute, Denmark through Dr Santosh Kumar, RGCB, India. cDNA entry clones carrying genes for the remaining eight tumor antigens, Aldose reductase, Pyruvate kinase, ATP synthase, Alpha tubulin, Beta tubulin, Phosphoglycerate mutase, Peroxiredoxin 6 and Cyclophilin A were obtained from Harvard Institute of Proteomics, USA. The details of the clones are given above in Table 2.3 . The clones were subcloned in to pDEST15 vector using gateway cloning. Aldose keto reductase was also subcloned in pGEX4T1 vector via TA PCR cloning as it did not express well in pDEST15 vector. The cloning strategy for the different proteins is summarized in Fig. 2.1.

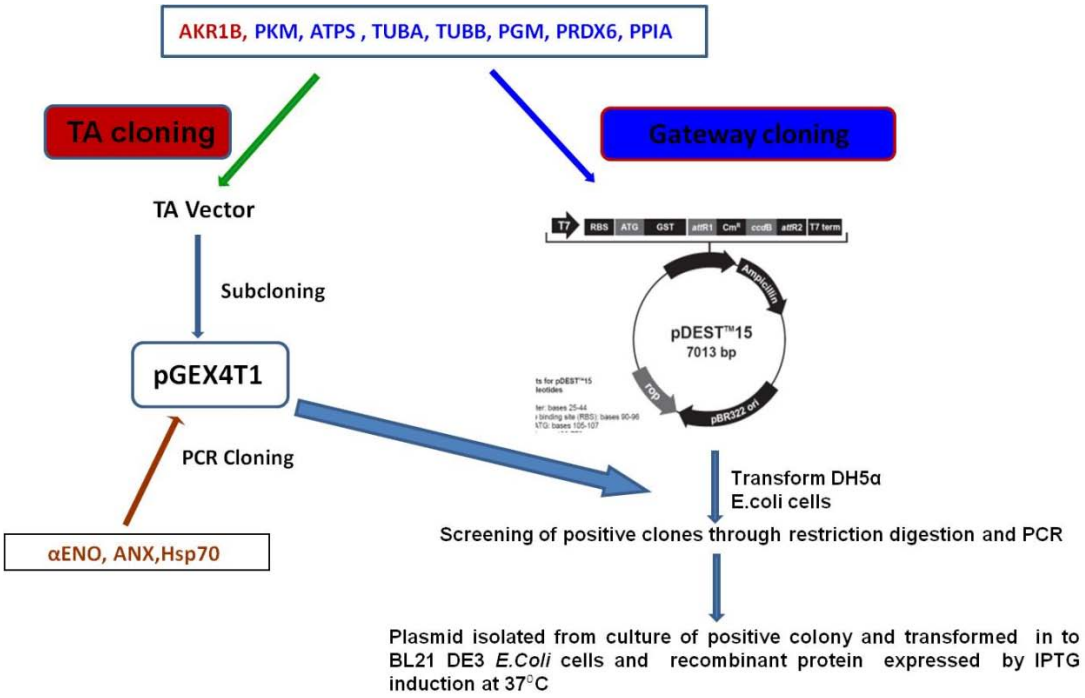


Fig. 2.1: Overview of strategy used for cloning and expression of recombinant GST proteins for the identified tumor antigens.

2.6.4 Gateway cloning:

2.6.4.1 Propagation of entry clones for the tumor antigens and the destination vector:

The entry clones for the identified tumor antigens were transformed in DH5α competent cells and colonies were obtained for each clone. Single colony for each clone was inoculated in 10 ml of LB kanamycin media and grown overnight at 37°C in an incubator shaker. Five ml of overnight culture was used for preparing glycerol stocks for each clone and rest 5 ml of culture was used for plasmid isolation. Plasmids were isolated for each clone from their respective culture using Qiagen Miniprep kit. Plasmid DNA was quantitated by recording absorbance at 280 nm. pDEST15 vector was used as the destination vector for gateway cloning. As this vector contained a lethal gene

*ccd*B gene it was propagated in one Shot® *ccd*B Survival™ chemically competent *E. coli* cells which was resistant to CcdB effects. The identity of pDEST15 vector was confirmed by digestion with SalI and EcoRI. A 1784bp fragment was obtained confirming that the plasmid is pDEST15.

2.6.4.2 Confirmation of presence of genes for the tumor antigens in the entry clones obtained from Harvard Institute of Proteomics:

This was done by PCR analysis of all the clones using gene specific primers. PCR reactions were set up for each of entry clone by mixing 1X PCR buffer, 1 mM dNTPs mixture, 1.5 mM $MgCl_2$, 10 *p*M forward primer, 10 *p*M reverse primer, 1 unit Taq DNA polymerase, deionized water and 0.5 µl (10 pg-1µg) of purified plasmid DNA. All the reactions were carried out in a final volume of 50 µl. The conditions used for PCR were: initial denaturation at 94^0C for 5 min, followed by 35 cycles of melting at 94^0C for 1 min, annealing at 60°C for 1 min and extension at 72^0C for 2 min, followed final extension for 10 min at 72^0C. Five µl of the amplified reaction was mixed in 1 µl of 6 X gel loading buffer {}, and run on 1 % agarose gel (pre-mixed with 10 µg EtBr) in 1 X TBE at 80 V.

2.6.4.3 Generating the expression clones through Gateway cloning: The genes for the identified tumor antigens obtained in pDONR221 entry clone were subcloned in pDEST15 destination vector through gateway cloning reactions where in the LR recombination reaction between the entry clone and the destination vector led to the transfer of the gene from the entry clone to the destination vector generating the expression clone. The LR recombination reaction is mediated by LR clonase II enzyme Mix which is a mixture of bacteriophage Integrase (Int) and Excisionase (Xis) proteins.

LR recombination reactions for each of the clones were as follows: Each 10 µl reaction mixture consisted of an entry clone (100 ng), destination vector (150 ng), LR Clonase II Enzyme mix (2µl) and the TE buffer (pH 8.0). The reaction mixture was mixed by vortexing and incubated at 25°C for 1 h. The reaction was stopped by adding 1 µl of Proteinase K solution. As a positive control the gateway LR recombination reaction was set up with pENTR-gus in place of entry clone.

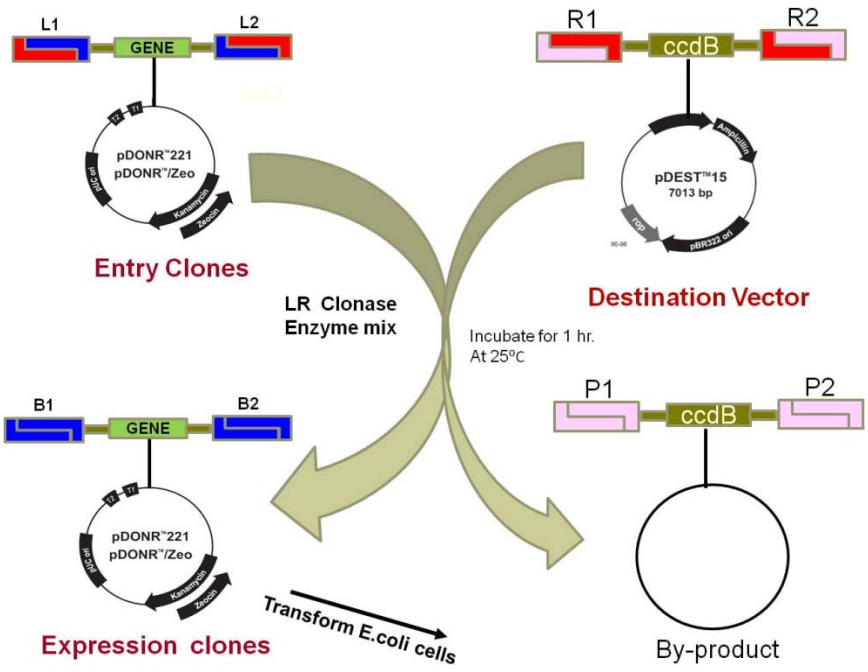


Fig: 2.2 Schematic representation of Gateway LR reaction between an entry clone and destination vector to yield expression clone.

2.6.4.4 Transformation of *E.Coli* DH5α cells with LR recombination reaction mixture:

For transformation, ultracompetent *E.Coli* DH5α cells were taken out from -80^{0}C and kept at 4^{0}C for 5 min and then thawed at RT. Five µl of LR recombination reaction mixture was added to the cells and the suspension was incubated on ice for 30 min. Cells were given a heat shock at 42^{0}C for 90 sec and immediately kept on ice for 2-5

min. After heat shock, 800 µl of LB media was added and the cell suspension was incubated at 37^0C for 30 min on a shaker. After incubation, cells were separated by centrifugation at 10,000 rpm for 1 min at 4^0C. The supernatant was discarded leaving 100 µl of LB media in the tube. Cells were resuspended in 100 µl of LB media and spread on LB-Agar with ampicillin (100 µg/ml) plate. The plate was incubated at 37^0C for 16-18 h.

2.6.4.5 Selection of expression clones positive for the gene of interest

The colonies obtained for each clone were screened for the selection of clones carrying the gene of interest. Colonies for each clone were picked and inoculated in 5 ml of LB-Amp medium (100 µg/ml). They were incubated at 37^0C for 14-16 h on a shaker at 220 rpm. After incubation, glycerol stocks of cells were made by adding 400 µl of 50 % glycerol in 600 µl of culture. The glycerol stocks were stored at -80^0C. The remaining culture was used to purify plasmid DNA. Plasmids were isolated by QIAprep Spin Miniprep kit. The plasmids were analyzed by PCR amplification using gene specific primers for some of genes as well as primers for the destination vector for each gene. The presence of the gene and the correct sequence of the gene cloned was confirmed for AKR, PKM, TUBB, TUBA, ATPS and PGM by DNA sequencing on the 3500 Genetic analyser (Applied Biosystems) using gene specific primers and also by digestion with Bsrg1 restriction enzyme for TUBB, ATPS, PKM and PRDX clones. The expression clones positive for the genes were then transformed in BL21DE3 *E.Coli* cells for expression of the recombinant protein.

2.6.4.6 Expression of recombinant GST fusion proteins

A single colony was picked from the transformants obtained on a LB (amp) plate and used for preparation of a 5ml of overnight starter bacterial culture. The starter culture was used to make a master culture by adding it to LB broth (1:100 v/v) followed by incubation at 37°C for 2-2.5 h till OD reached to 0.6. IPTG (0.1mM) was then added to the master culture which was then kept at 18°C for 16 h for induction of protein expression. The cells were then harvested by centrifugation at 7000 rpm for 10 min. The cell pellet obtained was kept at -80°C.

2.6.4.7 Purification of recombinant GST fusion proteins:

Bacterial cell pellet containing the expressed protein was taken out from -80°C, thawed on ice and then suspended in lysis buffer for 10 min . The cells were sonicated using a 9.5 mm probe, pulse set at 15-20 (10 pulse each time) and power set at 60. The sonication was repeated 5-10 times on ice depending upon the extent of lysis of the cells. After sonication, the mixture was centrifuged at 17000 rpm in a Sorvall Centrifuge in a SS-34 rotor for 30 min at 4^0C. The supernatant was then collected in a fresh 15 ml conical tube and 500 µl of 50 % Glutathione-Sepharose beads were added. The mixture was kept at 4^0C for 1 h on a rocker. The beads were then separated by centrifugation at 1000 rpm for 5 min at 4^0C in the Plastocraft Rota 4, rotor 13. The supernatant was discarded leaving 500 µl in the tube. The beads were resuspended in 1ml of PBS buffer and transferred in to a fresh T15 tube. The beads were washed three times with PBS buffer, with 5 min for each wash. After three washes with PBS, the bound recombinant protein was eluted from the beads with the elution buffer containing reduced glutathione. Elution buffer (500 µl) was added to the beads and the same mixed gently in the buffer for 10 min on a rocker. The eluted protein was recovered by

centrifugation at 1000 rpm for 5 min at 4^0C. The supernatant was collected as the first eluent in a fresh eppendorf tube. The entire elution procedure was repeated three more times in order to obtain all the protein bound to the beads. Ten µl of the eluted fractions of purified protein were subjected to I DE and the gel was stained with Coomassie blue to verify the size of the purified protein. The identity of recombinant proteins expressed and purified was also confirmed by mass spectrometry.

2.6.4.8 Purification of GST-fusion proteins using BPER reagent:

Bacterial cell pellet obtained from 250 ml of culture was suspended in 10 ml of BPER reagent and left for 20 min. The cell suspension was subjected to centrifugation for 45 min at 17000 rpm. Supernatant was collected and 500 ml of 50% glutathione sepharose beads slurry was added to it for the GST fusion protein to bind to the beads. The mixture was kept at 37°C for 10 min, the beads were then collected and washed with PBS buffer 3 times and the bound protein was eluted with elution buffer.

2.6.5 Sub Cloning of Aldose keto reductase in pGEX4T1 vector via TA cloning:

2.6.5.1 Cloning and expression of Aldose keto reductase: Clone for aldose keto reductase was obtained in a gateway donor vector pDONR221. It was subcloned in a gateway expression vector pDEST15 via gateway cloning. The protein did not express well in this vector. It was then subcloned in pGEX4T1 vector via TA cloning for the expression of the protein.

2.6.5.1.1 Cloning of AKR gene in TA cloning vector pTZ57R/T: AKR gene in pDONR221 vector was PCR amplified using a gene specific forward primer carrying an

EcoRI restriction site and reverse primer carrying a Sal I restriction site. The PCR product was run on a 1% agarose gel and was purified from the gel using the kit from Qiagen. A 20µl TA cloning reaction was set with the purified PCR product using thermo Scientific InsTAclone PCR cloning kit (Cat. No. #K1213).

Components	Volume
Vector pTZ57R/T, (0.17 pmol ends)	0.5µl
5X Ligation Buffer	2µl
PCR product (0.52 pmol ends)	5µl
Water, nuclease-free	9.5µl
T4 DNA Ligase	1µl
PEG	2µl

Table 2.7: Ligation reaction for TA cloning

The ligation mixture was incubated at 22°C, ON. The reaction mixture was transformed in ultra competent E.coli DH5α cells.

2.5.5.1.2 Screening of positive clones for AKR gene in TA vector: The colonies obtained next day were picked up and inoculated in 5ml of LB broth with 100 µg/ml ampicillin in it. The plasmid DNA was isolated from cultures obtained from each colony. The presence of AKR gene in the plasmid was confirmed by restriction digestion with EcoRI and Sal I enzymes. The 20µl reaction mixture was set up for each of the plasmids and incubated at 37°C. On next day, the whole reaction mixture was run on 1% agarose gel. The cleaved AKR gene (950bp) was seen on the gel. Six of the seven clones screened showed an insert for the AKR gene.

2.5.5.1.3 Subcloning of AKR gene from TA vector to pGEX4T1 vector: The plasmid was double digested with EcoRI and SalI restriction enzymes to release the

insert and the mixture run on an agarose gel. The insert with EcoRI and SalI overhangs was purified from the gel using the kit from Qiagen. Simultaneously the pGEX4T1 vector was also digested with the same restriction enzymes. The digested product was run on the gel and the pGEX4T1 vector with EcoRI and SalI overhangs were purified from the gel with a gel extraction kit. The insert and vector DNA were quantitated by spectrophotometry. A ligation reaction was set up with digested vector and digested insert at 22°C, O/N as follows.

Digested pGEX4T1 vector	5µl (50ng)
Insert (AKR)	1µl (30ng)
Ligation buffer	2µl
T4 DNA ligase	0.5µl
D/W	11.5µl
Total	20µl

Table 2.8: Ligation reaction for AKR insert in to pGEX4T1

2.5.5.1.4 Selection of positive clones for AKR gene in pGEX4t1 vector: The ligation mixture was transformed in ultra competent E.coli DH5α cells. The two colonies seen on the plate on the next day were picked up and inoculated in 5ml of LB broth containing 100 µg/ml ampicillin. Plasmid DNA was isolated from the cultures obtained from each colony. The presence of AKR gene in the plasmid was confirmed by restriction digestion with EcoRI and SalI enzymes. The 20 µl reaction mixture was set up for each of the plasmids and incubated at 37^0C ON. Next day the whole reaction mixture was run on 1% agarose gel. A 950 bp band was seen on the gel confirming the presence of AKR gene in the pGEX4t1 vector. The positive clone was used for expression of recombinant aldose keto reductase protein in BL21 DE3 E.coli cells.

2.6.5.2 Expression and purification of recombinant GST fusion Aldose keto reductase protein:

Aldose keto reductase was expressed and purified using the same protocol used for expression and purification of other GST fusion recombinant proteins which is mentioned above in detail. The purified protein was resolved by SDS PAGE and the identity of the protein confirmed by western blot analysis using the commercial antibody to the protein. The identity of expressed protein was further confirmed by mass spectrometry.

2.6.6 Optimization of expression of recombinant proteins expressed mostly as insoluble protein:

Conditions for expression of ATP synthase, alpha and beta tubulin were optimized by varying the induction temperatures and IPTG concentrations as shown in the Table 2.9 below. Induction was done at different temperatures 37°C, 30°C, 24°C and 18°C and also at different IPTG concentrations 0.1mM and 0.4 mM in separate cultures.

Five hundred µl of cell suspensions from cultures induced at different conditions were collected. Cell pellets obtained from each cell suspension was suspended in 25 µl of B-PER reagent and the mixture centrifuged to collect the supernatants. An uninduced culture was used as a negative control. The supernatants obtained from induced and uninduced cultures were run on the SDS-PAGE gel and the protein visualised by staining with Coomassie blue. The cell pellet was also analyzed for the presence of insoluble protein, by solubilization in 3X Lamelli buffer followed by 1DE. The induced protein was seen in the pellet indicating that it was expressed as an insoluble protein.

	INDUCTION		
Protein	**Temps.**	**IPTG concs.**	**Time (Hrs.)**
ATP synthase	37°C, 30°C, 24°C, 18°C	0.1mM, 0.4 mM	3h.
ATP synthase	18°C	0.1mM, 0.4mM	O/N
	28°C	0.05 mM	4hrs.
	37°C, 30°C, 24°C, 20°C	0.1mM, 0.05mM	2h, 3h, 4h

Table 2.9: Induction conditions used for expression of ATP synthase protein

Alpha and Beta tubulin were induced at two temperatures 37°C and 20°C with 0.1mM IPTG concentration. The expression of Beta tubulin improved at 20°C though it was still not sufficient for the experiments.

Different strains of BL21 DE3 strains such as C41(DE3), C43(DE3) and pLYSE were also used for improving the expression of soluble ATP synthase , alpha tubulin and beta tubulin. However the solubility of the proteins did not improve.

2.6.7 Solubilization of proteins expressed as insoluble proteins

2.6.7.1 Solubilization of insoluble recombinant ATP synthase from the inclusion bodies:

The insoluble protein was solubilised using urea gradient. Cell pellet was suspended in the lysis buffer and sonicated. After sonication, the mixture was centrifuged at 17000 rpm for 45 min and the supernatant was removed into a separate tube so as to remove away all the soluble proteins from the bacterial pellet. The remaining pellet was washed three times in PBS buffer containing 1% Triton X100. This pellet was solublized in different concentrations of urea. A major fraction of the protein was soluble in 4M and 8M urea. 4M urea was finally used for solubilisation of insoluble protein from the inclusion bodies. Urea was removed by gradient dialysis to permit renaturation of the

protein. After gradient dialysis, glutathione Sepharose beads were added to the protein for binding. The bound protein was eluted using reduced glutathione.

2.6.7.2 Solubilization of insoluble recombinant alpha tubulin from the inclusion bodies:

The insoluble protein was solubilised using 2% Empigen in PBS buffer. Cell pellet was suspended in lysis buffer and was sonicated. After centrifugation for 45 min at 17000 rpm, supernatant containing the solubilized proteins was collected. The remaining pellet containing insoluble proteins was suspended in 2% Empigen in PBS buffer and left for 30 min at RT. The suspension was centrifuged to collect the supernatant. The proteins in fifty µl of supernatant as well as the pellet were resolved by 1DE and the gel was stained with Coomassie blue. More than 50% of the protein was seen in the supernatant fraction on the gel. To recover the protein, glutathione sepharose beads were added to the supernatant and the mixture kept on a rocker for 1 h at 4°C for binding the protein. The protein bound to the beads was pelleted at 2000 rpm. Beads were washed 4 times with PBS buffer and the bound protein eluted with elution buffer.

2.6.8 Purification of recombinant GST-Fusion proteins without the GST tag using thrombin cleavage:

The glutathione sepharose beads with the bound GST tagged protein were washed three times with 10 volumes of PBS, pH 7.3. Twenty µl (20 units) of thrombin enzyme was mixed in 500 µl of PBS pH 7.3 and added to 200 µl of beads with ~2mg bound protein and incubated on a rocker, at 25°C, ON. Supernatant which contained the cleaved protein was collected by centrifugation at 2000 rpm for 2 min. and the protein content in

the supernatant estimated using the Bradford protein assay kit. The size and purity of the protein was determined by 1DE using 5µg protein.

2.6.9. Expression and purification of recombinant α enolase, Hsp70 and annexin 2:

pCMV-SPORT6 α-enolase, pCDNA3 Annexin A2 construct and pDS-HSP70 were subcloned into pGEX4T1 vector. The recombinant protein was expressed in BL21 (DE3) *Escherichia coli.* Recombinant GST-alpha-enolase and GST-HSP70, were released from glutathione Sepharose 4B beads by thrombin cleavage as described above. To determine if the recombinant protein was detected by the antibody to the protein, the resolved proteins were transferred to PVDF membrane and probed with their respective antibodies essentially as described in section using rabbit polyclonal anti-Eno antibody (SantaCruz Biotechnology, Cat No: sc15343) and rabbit polyclonal anti-Hsp70 antibody (Abcam, Cat No: ab 31010) at 1:2000 and 1:1000 dilution respectively. Annexin 2 was also subcloned in pGEX4T1 vector .

2.6.10 Purification of proteins by FPLC:

Several of the GST-fusion proteins were accompanied by a 26 kD protein. To remove this low molecular weight contaminant, the proteins eluted from the GST-Sepharose beads were subjected to FPLC on AKTA Superdex^{TM} 200 10/300GL, (Amersham-Pharmacia Biotech). The coloumn was prepacked to 600 mm bed heights in 16mm diameter. The bed volume of the column was 120 ml. Before loading the sample on the column, the eluate was centrifuged for 10 min to remove any precipitated protein or beads. The clear supernatant containing 5 mg protein in 2 ml was injected on to the column which was pre equilibrated in PBS buffer pH 7.4. The proteins were eluted

from the column with PBS pH 7.4 at 0.5ml/ min. Buffer used for equilibration of column was autoclaved and filtered through a 0.4 micron filter. The effluent was monitored at 280 nm and the peak fractions collected as 1 ml aliquots in microcentrifuge tubes. The protein in alternate fractions was evaluated by SDS-PAGE. Fractions were pooled depending on the pattern obtained.

2.7 Dot blot Analysis with recombinant proteins:

Protein was estimated in each of the purified protein preparations using the Bradford protein assay kit. Aliquots of the protein were manually spotted on the nitrocellulose membrane and allowed to dry for 5 min. Rest of the procedure was similar to the immunostaining protocol given above in section 2.2.5.2.

2.7.1 Optimization of conditions for Dot blot experiments:

An outline of the template for the protein microarray was designed where in the order of spotting of proteins was arranged as per the order of increasing percentage of autoantibody response. Recombinant GST-Fusion proteins for all the tumor antigens were spotted manually in three volumes 0.20µl, 0.35µl and 0.5µl on a small strip of nitrocellulose membrane in the order of the template and the blot was probed with commercial anti-GST antibody to ascertain uniform spotting. To standardise conditions for blotting the purified recombinant proteins were manually spotted on to the nitrocellulose membrane in different dilutions, or at different concentrations of protein i.e 50 ng, 100 ng and 200 ng according to the template designed. The blots with the spotted proteins were probed with serum IgG (5µg/ml) from few of cancer patients and healthy controls. The autographs were developed and analyzed to find the immunoreactivity of each sera with the recombinant proteins. To determine the IgG

concentration optimum for immunostaining of dot blots, three different IgG concentrations (3µg/ml, 4µg/ml and 5µg/ml) were used. Two dilutions (1:100 and 1:200) of the sera were also used for immunostaining of the dot blots.

2.8 ELISA with recombinant proteins

2.8.1 Standardization of ELISA: Two types of ELISA plates, Maxisorb plate and Anti-GST antibody coated plate, were initially used for coating recombinant GST-fusion proteins diluted in carbonate buffer. Different concentrations of the proteins in 100 µl were incubated in the wells for overnight at 4°C. The unbound protein was removed and the wells washed with PBS. To decide on a blocking reagent which would give minimum background, three different reagents 2.5 % BSA, 0.5 % polyvinyl alcohol (PVA) and 5 % milk powder in PBS were used. Different blocking times and temperatures and serum dilutions were also tried. Table 2.8 given below summarizes the standardization conditions followed.

Protein coated per well	ELISA plate	Blocking buffer	Blocking time	Serum
100 ng, 125 ng 150 ng, 200 ng	Maxisorp, Anti-GST antibody coated plate	BSA, PVA, Milk Powder	1h. at 37°C, 2 h. at 37°C, 1h. at 37°C followed by ON at 4° C	1:100, 1:200

Table 2.10: Conditions used for standardisation of ELISA experiments

2.8.2 ELISA with thrombin cleaved recombinant α Enolase and HSP70:

Purified recombinant protein was diluted to a final concentration of 1µg/ml in 100 mM bicarbonate buffer, (pH-9.6) . Hundred µl (100 ng/well) was added to each well of a 96 well microtiter plate and the same kept overnight at 4°C. After washing three times

(300 µl /wash) with PBS, free binding sites in the wells were blocked with 2.5 % BSA and the plates incubated for 1 h at 37°C followed by overnight incubation at 4°C. The plates were washed 2 times with PBS. Patient sera were diluted 1:100 in 0.2 % BSA in PBS and 100 µl added to each well of the coated ELISA plate and the same incubated for 1 h at 37°C. The wells were then washed 6 times (300 µl /wash) with PBS 0.05% Tween 20. The bound alpha enolase autoantibodies were detected using sheep anti human IgG secondary antibody conjugated with horse radish peroxidase (HRP) at 1: 5000 dilution for 1 h at 37°C. For measuring the bound secondary HRP-antibody, 100 µl of tetramethylbenzidine substrate was added to each well and incubated for 15 min at RT. The reaction was stopped with 0.5 M H_2SO_4 and optical density was measured at 450 nm in an ELISA reader.

2.8.3 Normalization of ELISA readings: The data obtained from different experiments was normalized. Each sera sample was evaluated in duplicate on protein coated and non coated wells. The optical density (OD) of each test sera was calculated as the average OD of protein coated wells minus the average OD of non coated wells. An ELISA was performed for 63 control sera samples in case of α enolase and 67 control sera in case of Hsp70. Ten patient sera samples with OD above and below the mean OD value were identified. The average OD (a) of these 10 samples was used to normalize the ELISA readings in all the other plates. In each ELISA plate the chosen 10 control human patient sera were evaluated along with the test sera. The average OD (a) was divided by the average OD of the chosen 10 control samples (b) assayed on each plate for each experiment and the factor so obtained was used to multiply the other OD readings for the test sera under evaluation. In case of HSP70 instead of ten, eight control samples were used in each experiment for normalization of all the readings.

2.9 Multiwestern analysis with recombinant proteins:

Five µg of purified recombinant alpha enolase and Hsp70 each were loaded in a single large well of a 12% preparative SDS-polyacrylamide gel so that the total protein runs uniformly across the entire gel as a single horizontal band. After electrophoresis, the protein was transferred on to PVDF membrane by electroblotting. The blot was stained with Ponceau to ascertain uniform transfer of the protein and after washing off the stain the blot was placed in a Mini-PROTEAN II Multiscreen Apparatus (BIO-RAD). The blot was blocked in 5% milk powder in TBST for 1 h at RT. Eighteen different sera samples diluted 1:200, were used to probe the blots for the presence of autoantibody to alpha enolase and Hsp70.

Chapter 4

Results

3.0 Results:

3.1 Evaluation of autoantibody response to identified tumor antigens in sera of normal individuals with and without chewing/smoking habit, individuals with leukoplakia, and patients with T1/T2 and T3/T4 tumors of GBC, using immunoproteomics

The autoantibody response against the antigens identified in our earlier study [151], namely α Enolase (isoforms a, b and c), Peroxiredoxin VI (Prdx 6), Annexin II (Anx2 2), Hsp70, Pyruvate kinase (PKM), alpha Tubulin (Tuba), beta Tubulin (Tubb), ATP synthase (ATPS), Phosphoglycerate mutase (PGM), Aldose reductase (AR1) and Triose phosphate isomerase (TPI) was evaluated using the 2DE immunoproteomics approach. Autoantibody response was evaluated in sera from healthy individuals with and without chewing /smoking habit (n=30 each), individuals with leukoplakia (n=30) and patients with T1/T2 and T3/T4 tumors (n=30 each) from cancer of GBC so as to determine if the pattern of autoantibodies in circulation has any correlation with the habit of chewing tobacco, presence of early lesions and tumor size. Figures 3.1, 3.2, 3.3 and 3.4 each show the pattern of the signals obtained on six representative autographs from individuals with and without chewing habit, individuals with leukoplakia, patients with T1/T2 and T3/T4 cancers.

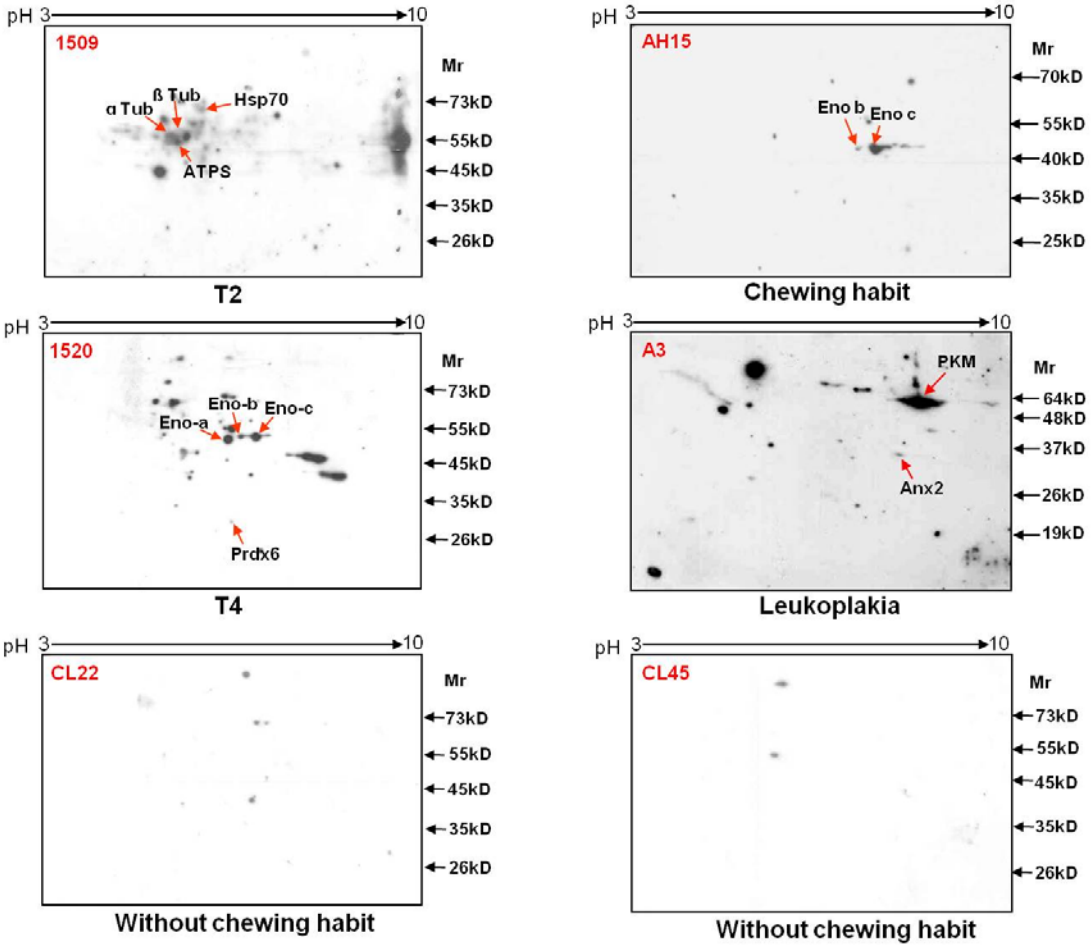


Fig. 3.1: Representative autographs of KB 2D blots immunostained with IgG purified from sera of patients with T2 and T4 tumor of cancer of the GBC, individuals with leukoplakia and healthy individuals with and without chewing habit. The pH range and position of the molecular weight standards is shown. Sample number is given on the top left hand corner of each autograph. Arrows show position of spots which elicit autoantibody response in patients with cancer of GBC

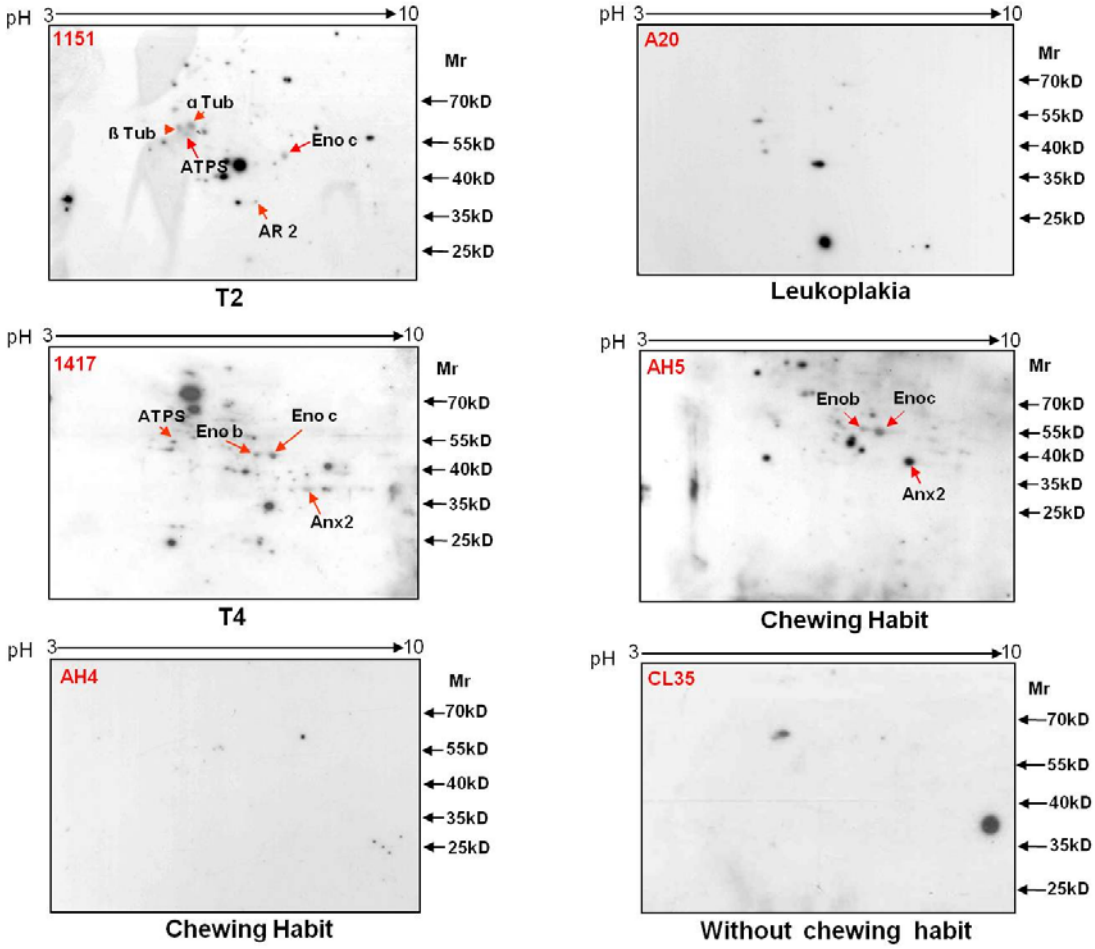


Fig. 3.2: Representative autographs of KB 2D blots immunostained with IgG purified from sera of patients with T2 and T4 tumor of cancer of GBC, individuals with leukoplakia, healthy individuals with and without chewing habit. The pH range and position of the molecular weight standards is shown. Sample number is given on the top left hand corner of each autograph. Arrows show position of spots which elicit autoantibody response in patients with cancer of GBC.

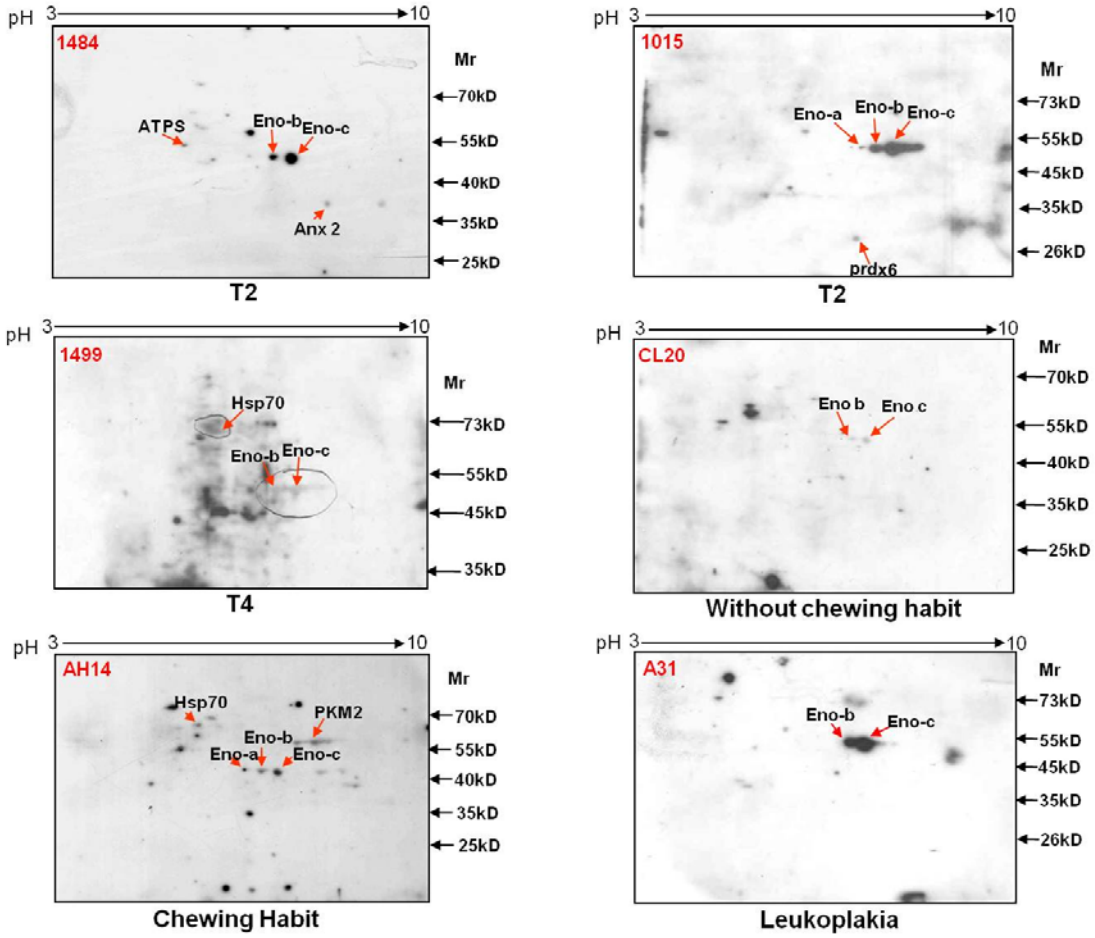


Fig. 3.3: Representative autographs of KB 2D blots immunostained with IgG purified from sera of patients with T2 and T4 tumor of cancer of GBC, individuals with leukoplakia, healthy individuals with and without chewing habit. The pH range and position of the molecular weight standards is shown. Sample number is given on the top left hand corner of each autograph. Arrows show position of spots which elicit autoantibody response in patients with cancer of GBC.

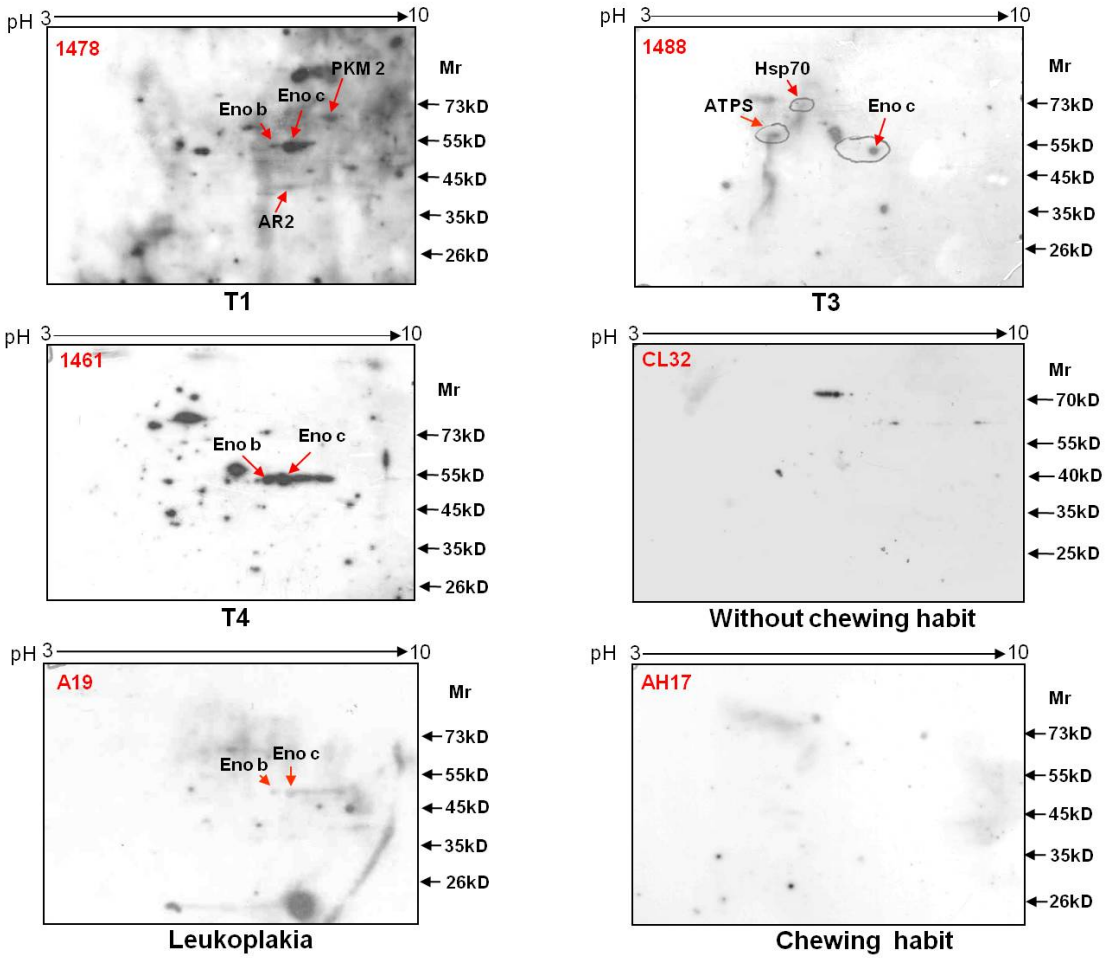


Fig. 3.4: Representative autographs of KB 2D blots immuonostained with IgG purified from sera of patients with T1 and, T3/T4 tumor of cancer of GBC, healthy individuals with and without chewing habit and individuals with leukoplakia. The pH range and position of the molecular weight standards is shown. Sample number is given on the top left hand corner of each autograph. Arrows show position of spots which elicit autoantibody response in patients with cancer of GBC.

Table 3.1 summarises the autoantibody response for each of the tumor antigens for the different groups investigated. The details of the response against each of the antigens by each of the individuals in the study group are given in the Appendix in Tables A6, A7, A8, A9 and A10. The highest autoantibody response observed in sera of cancer patients was against alpha enolase c (50%) and the lowest (1%) for aldose reductase 1 and triose phosphate isomerase. Autoantibody response to many tumor antigens was also detected in individuals with leukoplakia and healthy individuals with chewing

/smoking habit. However percentage of autoantibody response was less compared to cancer patients. Autoantibody response to tumor antigens alpha enolase b and c, ATP synthase, phosphoglycerate mutase and peroxiredoxin 6 show trend of increasing antibody response from individuals with chewing/smoking habit and leukoplakia to patients with GBC cancer with tumor size T1/T2 and T3/T4. Some of the individuals with chewing/smoking habit and leukoplakia show autoantibody response against several of the tumor antigens while some other individuals with leukoplakia and chewing habit do not show any autoantibody response. The follow up information for these individuals which have shown strong autoantibody response is crucial in order to assess its utility in early detection.

Tumor Antigens	Percentage of autoantibody response against each antigen					
	Healthy individuals (n=30)	Healthy individuals with C/S habit (n=30)	Leukoplakia (n=30)	GBC Cancer T1/T2 (n=30)	GBC cancer T3/T4 (n=30)	GBC Cancer (n=60)
Alpha enolase a	3	17	10	10	13	12
Alpha enolase b	7	27	27	27	47	37
Alpha enolase c	23	37	40	43	57	50
Aldose reductase 1	0	0	0	3	0	1
Aldose redutase 2	0	0	3	13	3	8
Annexin 2	10	13	13	7	7	7
Pyruvate kinase	0	10	7	17	7	12
HSP70	3	13	7	3	13	8
ATP synthase	10	3	13	20	23	21
Alpha tubulin	0	3	0	7	3	5
Beta tubulin	0	7	7	7	7	7
Phosphoglycerate mutase	3	7	3	10	7	8
Peroxiredoxin 6	7	7	10	30	23	26
Triosephosphate isomerase	0	3	0	0	3	1

Table 3.1: Percentage of autantibody response to identified tumor antigens detected by immunoproteomics in the sera of healthy individuals with and without chewing/smoking (CS) habit, individuals with leukoplakia, patients with T1/T2 tumor, T3/T4 tumor of cancer of GBC.

Figure 3.5 shows the number of antigens in different individuals in each of the groups. The median number of antigens is shown in Fig. 3.6. It is seen that patients with T3/T4 tumors elicit autoantibody response against more antigens than those with T1/T2 tumors. The median number of antigens seen in individuals with leukoplakia is higher than those with and without chewing/smoking habits. Analysis of the Tables A6, A7, A8 A9 and A10 in Appendix shows that there is no specific pattern of autoantibody response against any of the antigens.

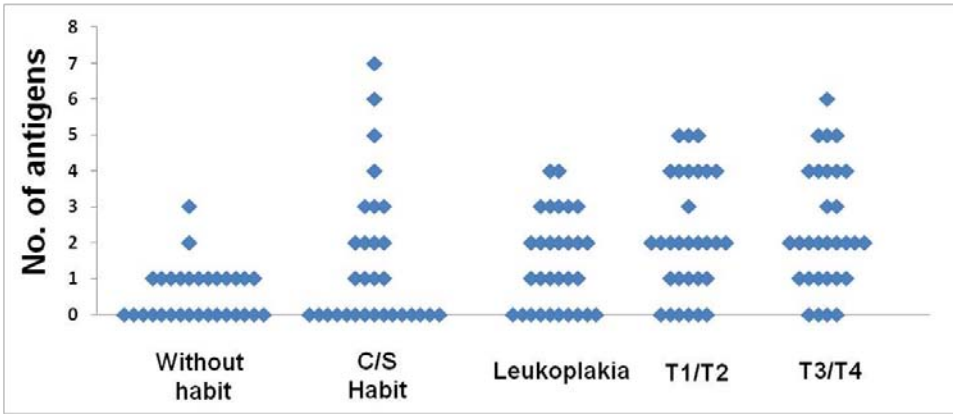


Fig. 3.5: Scatter plot for number (No.) of antigens eliciting an autoantibody response in the different groups. Each dot represents number. of antigens immunoreactive with sera for each sample in each group. C/S-chewing/smoking

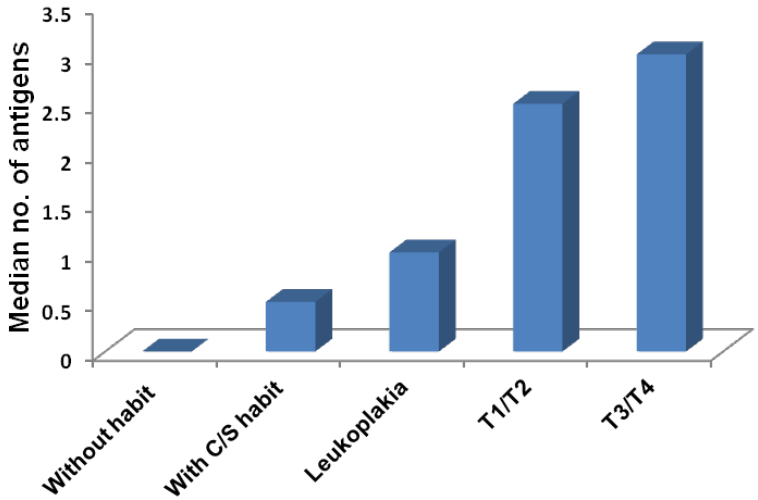


Fig. 3.6: Median number of antigens eliciting an autoantibody response in different groups. C/S-chewing/smoking habit

3.1.1 Evaluation of autoantibody response using other oral cancer cell lines as the source of tumor antigens: In order to determine whether the source of protein affects the pattern of antibody response, 1D and 2DE blots were prepared with lysates from different oral cancer cell lines and were probed with same IgG. Fig. 3.7 shows the patterns of autoantibody response obtained for three different sera with three different cell lines in 1D analysis. It is seen that the patterns are similar although the intensity of the bands differ.

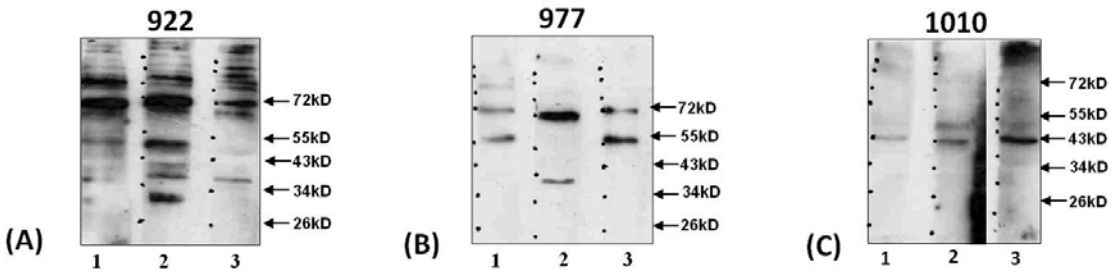


Fig. 3.7: Autographs of 1DE blots prepared from lysates of three different oral cancer cell lines and probed with IgG from samples 922, 977 and 1010. In each of the panels, lane 1: KB cell lysate, lane 2: AW 85073 cell lysate and lane 3: AW13516 cell lysate, The position of the molecular weight standards is shown on the right of each panel in kD

Figure 3.8 shows the pattern of signals obtained when 2DE blots of lysates from KB, AW85073, and AW13516 cell lines are probed with IgG from sample 922 and 1010. The amido black stained pattern on the corresponding blots is shown. The patterns seen on the different cell lysates is similar for the IgG from the same sample.

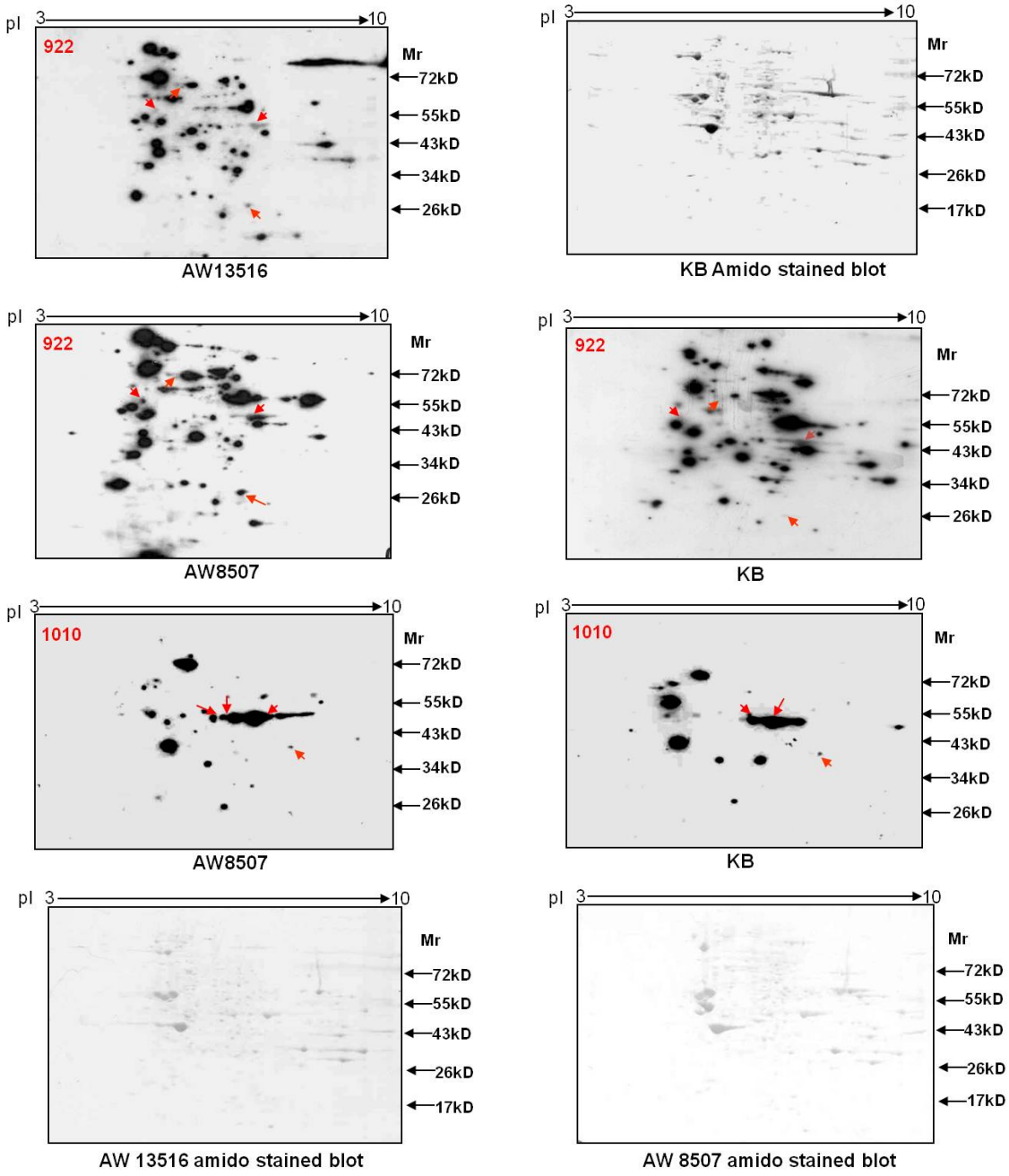


Fig. 3.8: Autographs of 2DE blots of different lysates from KB, AW8507 and AW13516 cell lines probed with IgG from sample 922 and 1010. The amido black stained blots of the different cell lines are also shown in the lower most panel.

3.1.2 Evaluation of autoantibody response in normal individuals with other inflammatory conditions of the mouth: Autoantibody response to the antigens under

study was evaluated in individuals with gingivitis, periodontitis and multiple abscesses to determine if they show any response and to check the specificity of autoantibody response detected in sera of patients with cancer of GBC. Six samples were analysed. Figure 3.9 shows that autoantibody response to the identified tumor antigens was absent in these individuals.

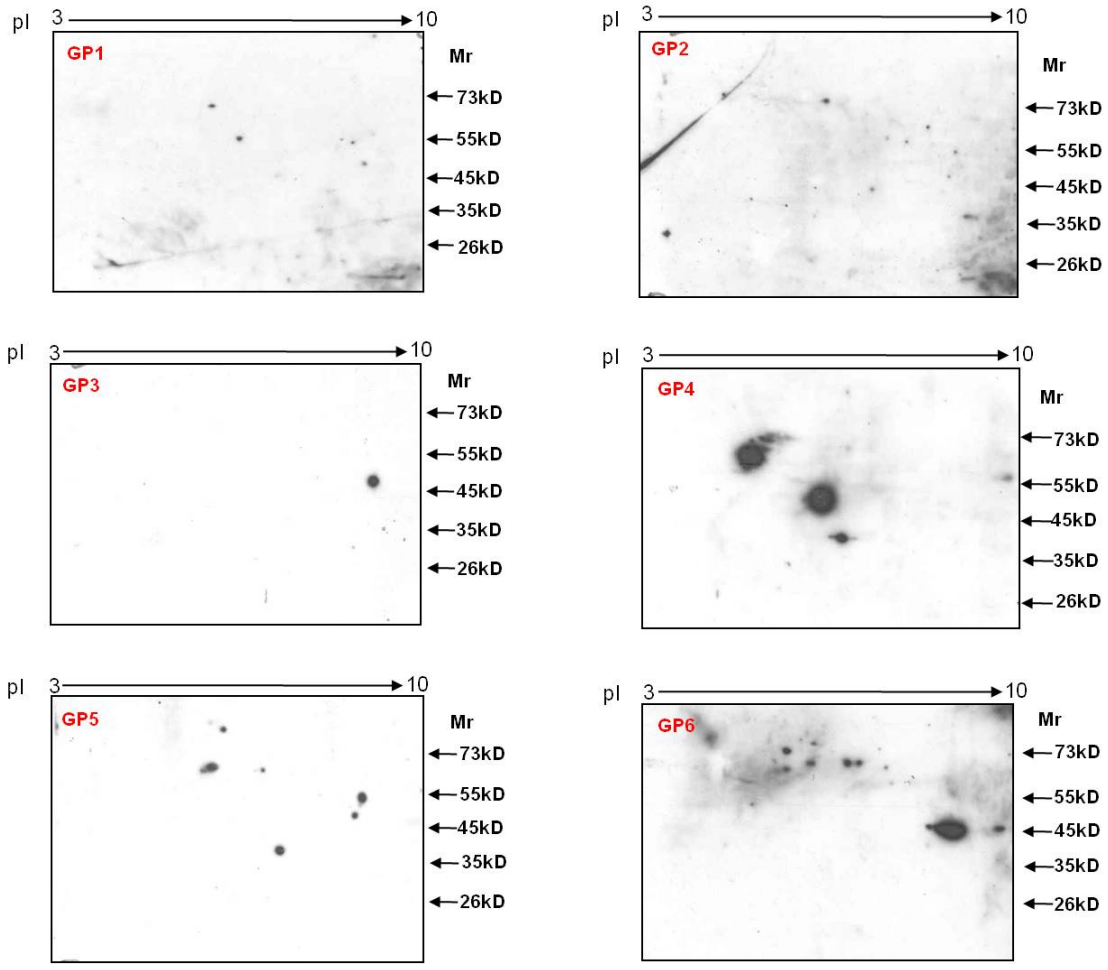


Fig. 3.9: Autographs of KB 2D blots probed with IgG from individuals with other inflammatory conditions of mouth 1) GP1-Gingivitis 2) GP2-Multiple abscesses 3) GP3 Periodontitis 4) GP4-Periodontitis 5) GP5-Gingivitis 6) GP6-Periodontitis

3.2 Evaluation of prognostic utility of autoantibody response to tumor antigens:

3.2.1 Association of autoantibody response with disease free survival (DFS): Of the 60 patients analysed follow up information was available for only 42 patients. Kaplan Meier survival analysis was performed for autoantibody response against each tumor antigen, to assess their independent association with disease free survival of patients. For this, the patients were grouped into those, who exhibited an autoantibody response against the antigen and those who did not. The survival curves obtained for each of these groups were compared using log rank test.

Fig. 3.10 A to E: shows the results for alpha Enolase and Hsp70 taken individually. Autoantibody response to only α Enolase b (Fig 3.10 B) and Hsp70 (Fig. 3.10 E) showed significant correlation with the disease free survival (DFS) of the patients. Autoantibody response to α ENO (α Enolase a, b and c together) showed similar correlation with DFS though it was not significant, Fig.3.10 D. Patients were also grouped in those, who do not show antibody response to either Hsp70 or α ENO, those who exhibit autoantibody response to either Hsp70 or α ENO, those who show antibody response to both Hsp70 and α ENO. Autoantibody response seen in these groups was correlated with DFS of patients. It is seen that patients with autoantibody response to both antigens recur faster than those without an autoantibody response, p=0.008, Fig.3.10F.

The survival analysis for the other tumor antigens is shown in Fig. 3.11. It is seen that there is no significant correlation between autoantibody response and DFS for these antigens.

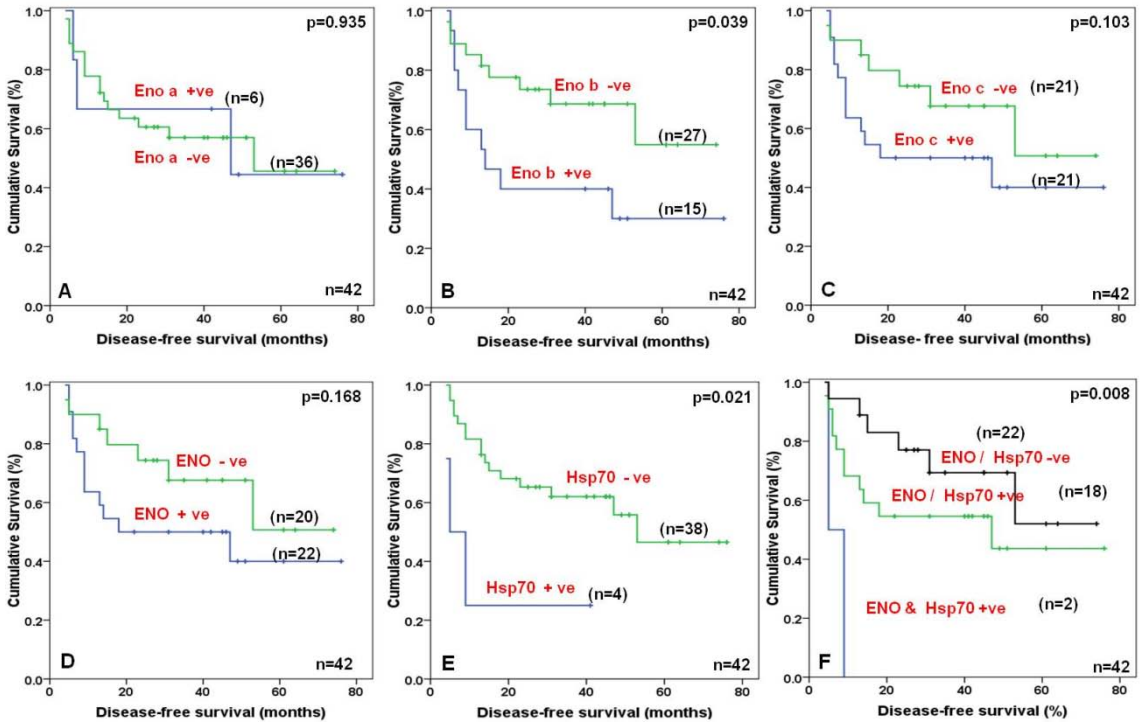


Figure. 3.10: Kaplan-Meier curves with univariate analysis for correlation of cumulative survival and DFS (in months) with autoantibody response in groups of patients: negative or positive for autoantibody response to each of the antigens shown A) α Enolase- a; **B)** α Enolase-b; **C)** α Enolase-c; **D)** α ENO (α Enolase a, b, c taken together) ; **E)** Hsp70; **F)** Either α ENO or Hsp70 or both . The number of patients in each group is given as (n) against each curve.

3.2.2 Nodal and differentiation status of the tumor and DFS: The nodal and differentiation status of the tumor are well established prognostic clinical parameters. Fig. 3.12 shows the survival curves for the patients in this study with respect to these parameters. It is seen that there is a trend for decrease in DFS with nodal involvement and decrease in differentiation status of the tumors.

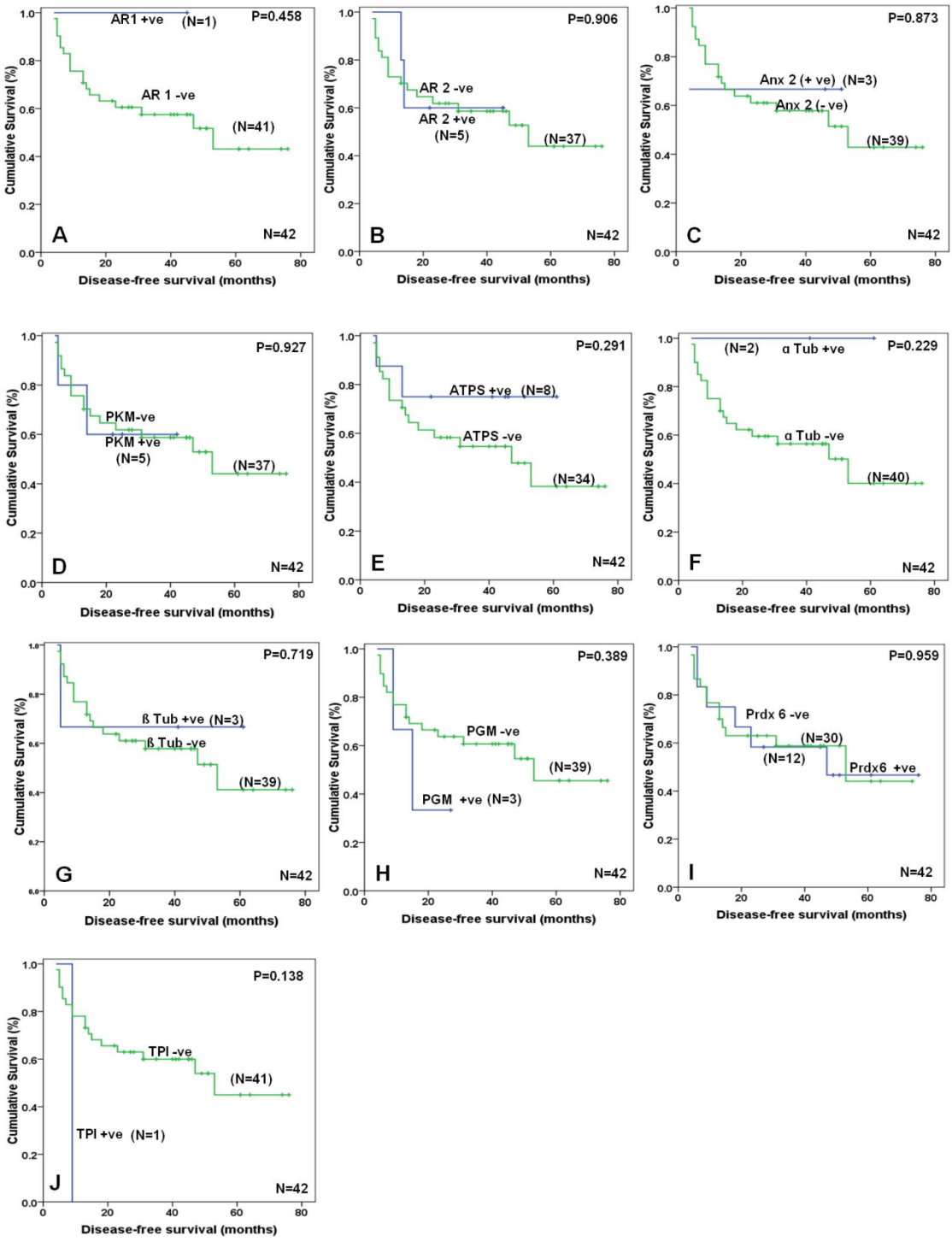


Figure 3.11: Kaplan-Meier curves with univariate analysis for correlation of cumulative survival and DFS (in months) with autoantibody response in groups of patients negative and positive for the response: **A)** Aldose keto reductase 1 (AR 1); **B)** Aldose keto reductase 2 (AR 2); **C)** Annexin 2 (Anx 2); **D)** Pyruvate kinase (PKM); **E)** ATP synthase (ATPS); **F)** alpha Tubulin (α Tub); **G)** beta Tubulin (ß Tub); **H)** Phosphoglycerate mutase (PGM); **I)** Peroxiredoxin 6 (Prdx 6); **J)** Triose phosphate isomerase (TPI). The number of patients in each group is given as (N) against each curve.

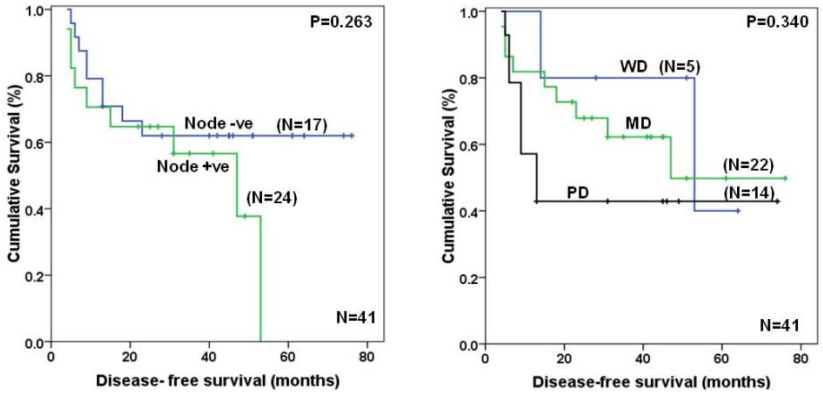
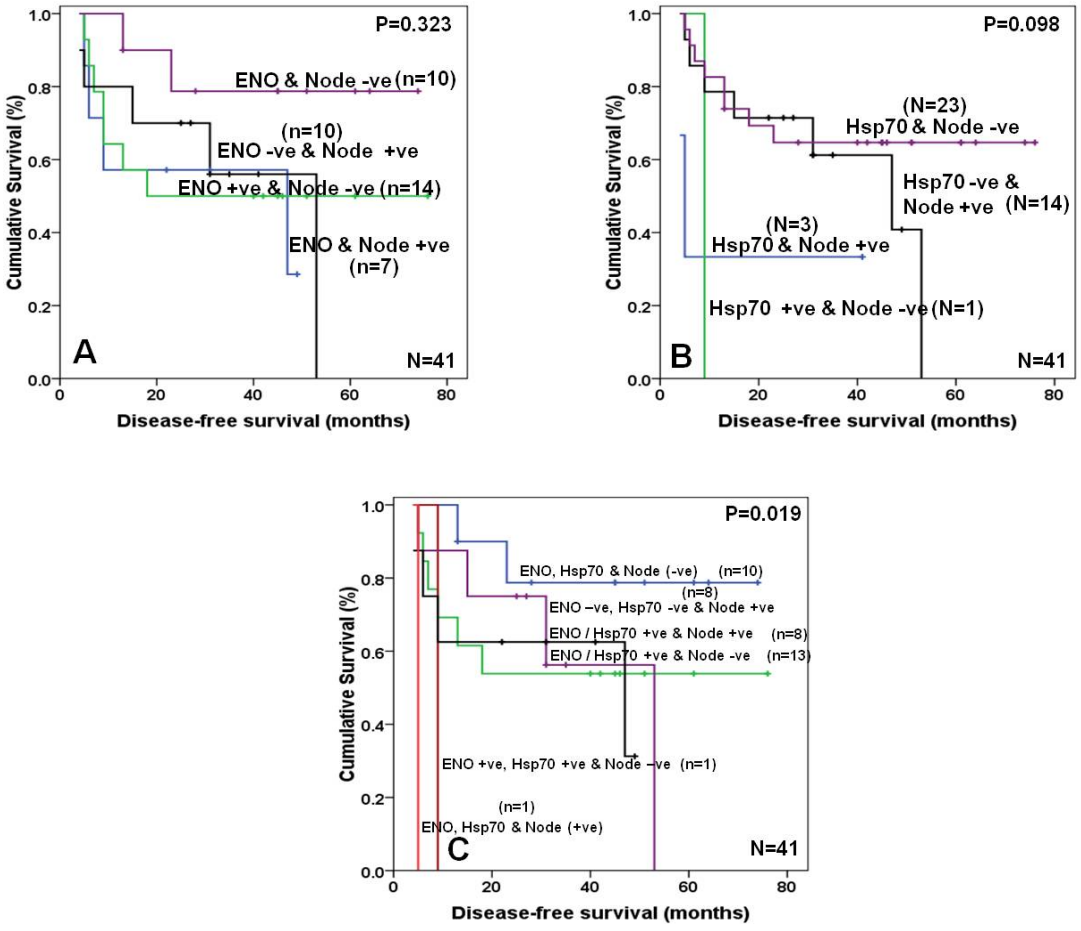


Fig. 3.12: Kaplan-Meier curves for correlation of cumulative survival and DFS (in months) in A) patients with and without nodal involvement, (node +ve and node –ve; **B)** patients with well differentiated (WD), moderately differentiated (MD) and poorly differentiated (PD) tumors. The number of patients in each group is given as (N) against each curve.

3.2.3 Autoantibody response to α ENO, Hsp70, nodal status and DFS: Autoantibody response to α ENO (α enolase, a, b and c taken together) and Hsp70 detected in the patients was correlated with their disease free survival along with their nodal involvement status, Fig.3.13. Data from 41 patients was divided into four groups based on the presence of antibody response to α Enolase and nodal involvement. The survival curve obtained for each group is shown in Fig. 3.1.3A. There is a clear decrease in DFS in patients who have node negative tumors and exhibit an autoantibody response to α ENO and those who do not. Similarly among node positive patients those who exhibit autoantibody response to α ENO recur faster than those who do not. However the overall trend is not statistically significant at p=0.323.

Data from 41 patients was divided in to four groups based on the presence of antibody response to Hsp70 and nodal involvement. There is a clear decrease in DFS in patients who have node negative tumors and exhibit an autoantibody response to Hsp70 and those who do not. The survival curves obtained for each group is shown in Fig. 3.13 B.

Presence of autoantibody response to either Hsp70 or α ENO or both in sera correlates independently to the disease free survival of patients. To determine their potential as an additional prognostic marker in combination, survival analysis was performed with nodal involvement. Data from 41 patients was divided in to six groups based on presence of antibody response to either α ENO or Hsp70 or both and nodal status. Fig. 3.13 C shows the survival curves obtained for each group. It is seen that patients who are negative for both autoantibody response to Hsp70 and α ENO and node fare better than those who are positive for antibody response to either Hsp70 or α ENO or both and negative for node. Overall trend is significant at p=0.019. However sample size is small in group positive for both Hsp70 and α ENO with node negative (n=1) as well as with node positive (n=1).



Legend on next page

Figure 3.13: **Kaplan-Meier curves with univariate analysis for correlation of cumulative survival and DFS (in months) with autoantibody response in: (A) Four groups of patients viz** those negative for autoantibody response to both α ENO and node; those positive for autoantibody response to α ENO but negative for node; those negative for autoantibody response to α ENO but positive for node; those positive for both autoantibody response to α ENO and node; **(B) Four groups of patients viz those** negative for both autoantibody response to Hsp70 and node; those negative for autoantibody response to Hsp70 but positive for node; those positive for autoantibody response to Hsp70 but negative for node; those positive for both autoantibody response to Hsp70 and node.(C) **) Six group of patients viz those** negative for autoantibody response to Hsp70 and α ENO and node; those negative for autoantibody response to Hsp70 / α ENO and positive for node; those negative for autoantibody response to both Hsp70 and α ENO and positive for node; those positive for autoantibody response to either Hsp70 and α ENO and negative for node; those positive for autoantibody response to both Hsp70 and α ENO and negative for node; those positive for autoantibody response to Hsp70 and α ENO and node. The number of patients in each group is given as (N) against each curve.

3.2.4 Autoantibody response to α ENO, differentiation status and DFS: Autoantibody response to α Enolase and Hsp70 detected in the patients was correlated with their disease free survival along with their differentiation status. Data from 41 patients was divided into four groups based on the presence of antibody response to α ENO and differentiation status of their tumors. Patients with well differentiated tumors and moderately differentiated tumors were clubbed together in one group. The survival curve obtained for each group is shown in Fig. 3.14A. Patients with WD/MD tumors and positive for antibody response to α enolase recur faster compared to patients with WD/MD tumors but negative for antibody response to α enolase. Similar trend was observed for patients with poorly differentiated (PD) tumors.

3.2.5 Autoantibody response to Hsp70, differentiation status and DFS: Data from 41 patients was divided into four groups based on the presence of antibody response to Hsp70 and differentiation status of their tumors. Survival curves for each of the groups

are shown in Fig. 3.14 B. Patients with WD/MD tumors and positive for antibody response to Hsp70 have reduced disease free survival compared to patients with WD/MD tumors but negative for antibody response to Hsp70. Similar was the trend observed for patients with poorly differentiated (PD) tumors. However sample size in both these groups was very small. Overall trend was significant at p=0.028.

3.2.6 Autoantibody response to α ENO, Hsp70, differentiation status and DFS: The data from 41 patients was divided into six groups based on autoantibody positivity and differentiation status of their tumors. The survival curves in Fig 3.14 C show that those patients with PD tumors who exhibit autoantibody to α ENO and Hsp 70 recur faster than those who are negative for an autoantibody response to both or either antigens. However sample size is very small in both the groups. Similarly patients with MD/WD tumors who exhibit antibody response to either of antigens recur faster than those who are negative for autoantibody response to both the antigens. The overall trend is significant at p=0.040.

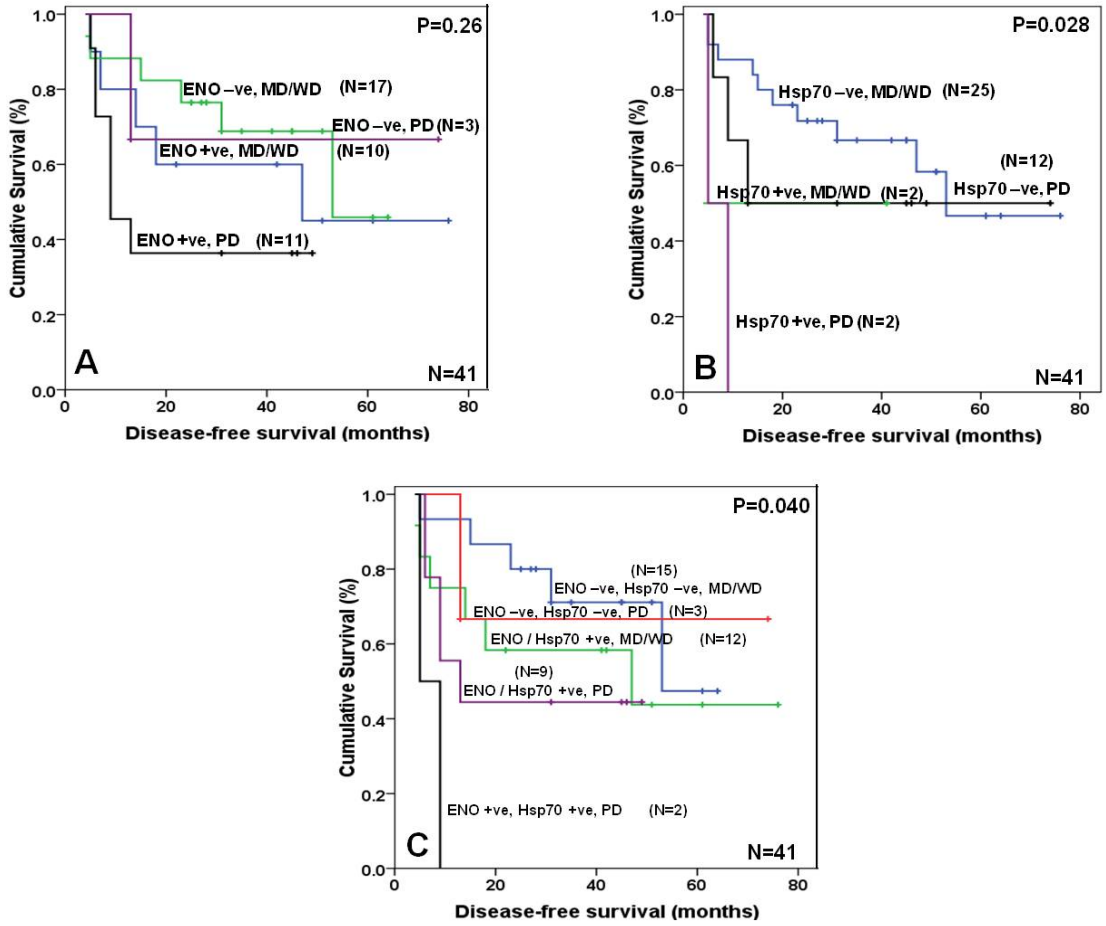


Fig. 3.14: Kaplan-Meier curves with univariate analysis (log rank) for correlation of cumulative survival and DFS (in months) in:(A) Four groups of patients viz those with WD / MD tumor and negative for autoantibody response to α ENO; those with WD / MD tumor and positive for autoantibody response to α ENO antibody; those with PD tumor and negative for autoantibody response to α ENO; those with PD tumor and positive for autoantibody response to α ENO; **(B) Four group of patients viz** those with WD / MD tumor and negative for autoantibody response to Hsp70; those with WD /MD tumor and positive for autoantibody response to Hsp70; those with PD and negative for autoantibody response to Hsp70 antibody; those with PD tumor and positive for autoantibody response to Hsp70; **C) Five groups of patients viz** those negative for autoantibody response to α ENO and Hsp70 and with WD / MD tumor; those positive for autoantibody response to α ENO / Hsp70 and with WD / MD tumor; those negative for autoantibody response to both α ENO and Hsp70 and with PD tumor; those positive for autoantibody response to α ENO / Hsp70 and with PD tumor; those positive for autoantibody response to both α ENO and Hsp70 and with PD tumor. There were no patients positive for autoantibody response to both α ENO and Hsp70 and with WD / MD tumor. The number of patients in each group is given as (N) against each curve.

3.2.7 Combined Analysis

It is apparent from the results presented above, that there is a definite trend in autoantibody response to α ENO and Hsp70 and DFS. However in some of the subgroups the numbers were very less. To validate the observations obtained for these 60 patients and 30 healthy individuals, a combined analysis was undertaken with data reported earlier from the laboratory. Autoantibody response patterns from healthy individuals (n=49), individuals with leukoplakia (n=12), and patients with T1/T2 (n=12) and T3/T4 (n=38) from the earlier studies [151, 152, 157] were included to increase the total patient sample size to 110 for calculating the final percentage of autoantibody response to each antigen. Table 3.2 shows the autoantibody response for the combined data n=110 for GBC, n=79 for healthy individuals. Autoantibody response to the antigens varied between 5% for Triose phosphate isomerase to 62% for alpha Enolase c and the combination of antigens eliciting an autoantibody response was different among patients. The intensity of the signal for each protein spot was distinct for each patient which reflects the specific autoantibody response of the individual to each antigen. Autoantibody response against α Enolase isoforms a, b and c was detected in 27%, 51% and 62% of the 110 patients respectively. The autoantibody response to Hsp 70 was seen in 23% of the patients. Autoantibody response to three isoforms of α Enolase a, b and c and Hsp70 was much lower and was seen in 6%, 10%, 20% and 2% respectively of healthy individuals. Detailed information about antibody response to each antigen by each patient considered in this study is given in Appendix in Table A11a to A11c.

Tumor Antigens	Percentage of autoantibody response against each antigen					
	Healthy individuals (n=49+30)	Healthy individuals with C/S habit (n=1+30)	Leukoplakia (n=12+30)	T1/T2 (n=12+30)	T3/T4 (n=38+30)	GBC (n=50+60)
Alpha enolase a	6	16	14	19	32	27
Alpha enolase b	10	26	24	31	63	51
Alpha enolase c	20	35	50	52	68	62
Aldose reductase1	0	0	5	7	7	7
Aldose redutase 2	0	0	3	12	15	14
Annexin 2	4	13	17	14	28	23
Pyruvate kinase	2	10	12	14	21	18
HSP70	2	16	9	12	29	23
ATP synthase	4	3	12	17	26	23
Alpha tubulin	1	3	2	7	19	14
Beta tubulin	0	6	9	12	16	14
Phosphoglycerate mutase	1	6	5	7	13	11
Peroxiredoxin 6	2	6	9	31	35	34
Triose phosphate isomerase	0	3	2	0	9	5

Table 3.2: Final percentage of autoantibody response to each of the tumor antigens in patients and healthy individuals. The numbers underlined in the headings are from the earlier studies. C/S-chewing/smoking habit

Out of the 50 GBC samples from the earlier studies that were included for making combined table of percentage of autoantibody response, follow up information was available for 36 samples which together with 42 patients from the present study add to 78 samples. Autoantibody profile of these 78 samples was used for correlation studies with DFS and clinical parameters. Detailed information about autoantibody response to each tumor antigen in each patient and their Follow up information is given in Appendix in Table A 12.

3.2.7.1: Correlation of autoantibody response to tumor antigens between different groups of samples :

The autoantibody response to identified tumor antigens evaluated by immunoproteomics approach was correlated between different groups of samples by Chi square test using SPSS analysis. Table 3.3 summarizes the correlation analysis for autoantibody response to each of the antigens between the different groups given in Table 3.2. The details of the correlation analysis are given in Appendix Tables A13a , A13b and A13c.

It is seen in Table 3.2 and 3.3 that there is increasing autoantibody response for α Eno b, α Eno c ,Anx 2 and Tubb in the sera of individuals who are at high risk of cancer, such as *chewing/smoking habitues and individuals with leukoplakia* indicating that these markers have promising potential in the early detection of cancer. Autoantibody response to α Enolase b and c, PKM 2 and Tubb showed a trend of increasing response from *healthy individuals and leukoplakia* to patients with cancer of the GBC with tumor size *T1/T2 and T3/T4.*

Autoantibody response to α Enolase b, α Enolase c, Anx2 PKM2 and Tubb was significantly higher in *individuals with leukoplakia* as compared to *healthy individuals.* The increase was also clearly seen between the *healthy individuals* and those with *T1/T2 tumors* wherein correlation was significant for the response to α Enolase, b and c, Hsp70, Peroxiredoxin 6, PKM2, ATP synthase, Tubb and AR2. Between *healthy individuals* and patients with *T3/T4 tumors,* autoantibody response was further increased and difference became significant for all the antigens being investigated. Further autoantibody response to α Enolase b and Hsp70 is significantly higher in

patients with *T3/T4* tumors as compared to those with *T1/T2* tumors showing correlation with the tumor size.

			Tumor antigens eliciting autoantibody response									
	Sample type	**Eno a**	**Eno b**	**Eno c**	**Hsp70**	**Anx 2**	**Prdx 6**	**PKM2**	**ATPS**	**Tubb**	**Tuba**	**AR2**
Healthy individuals (n=79)		N=5 (6.3%)	N=8 (10.1%)	N=16 (20.3%)	N=2 (2.5%)	N=3 (3.8%)	N=2 (2.5%)	N=2 (2.5%)	N=3 (3.8%)	N=0 (0%)	N=1 (1.2%)	N=0 (0%)
	C/S habit (N=31)	N=5 (16.1%)	N=8 (25.8%)	N=11 (35.5%)	N=5 (16.1%)	N=4 (12.9%)	N=2 (6.5%)	N=3 (9.7%)	N=1 (3.2%)	N=2 (6.5%)	N=1 (3.2%)	N=1 (3.3%)
	P value	0.141	0.067	0.095	**0.018**	0.097	0.315	0.135	1	0.078	0.486	0.347
	Leukoplakia (N=42)	N=6 (14.3%)	N=10 (23.8%)	N=21 (50%)	N=4 (9.5%)	N=7 (16.7%)	N=4 (9.5%)	N=5 (11.9%)	N=5 (11.9%)	N=4 (9.5%)	N=1 (2.4%)	N=1 (3.3%)
	P value	0.187	**0.044**	**0.001**	0.181	**0.031**	0.181	**0.048**	0.124	0.013	1	0.347
	T1/T2 (N=42)	N=8 (19%)	N=13 (31%)	N=22 (52.4%)	N=5 (11.9%)	N=6 (14.3%)	N=13 (31%)	N=6 (14.3%)	N=7 (16.7%)	N=5 (11.9%)	N=3 (7.1%)	N=5 (11.9%)
	P value	0.060	**0.004**	**0.00029**	**0.048**	0.063	**0.00001**	**0.020**	**0.031**	0.004	0.120	0.004
	T3/T4 (N=68)	N=22 (32.4%)	N=43 (63.2%)	N=46 (67.6%)	N=20 (29.4%)	N=19 (27.9%)	N=24 (35.3%)	N=14 (20.6%)	N=18 (26.5%)	N=11 (16.2%)	N=13 (19.1%)	N=10 (14.7%)
	P value	**0.00005**	**0.00000**	**0.00000**	**0.00001**	**0.00004**	**0.00000**	**0.00046**	**0.00009**	**0.00020**	**0.00024**	**0.000**
C/S habit	Leukoplakia	P=0.540	P=0.845	P=0.217	P=0.481	P=0.750	P=1	P=1	P=0.230	P=1	P=1	P=1
	T1/T2	P=0.747	P=0.631	P=0.152	P=0.734	P=1	P=0.010	P=0.724	P=0.127	P=0.691	P=0.632	P=0.068
	T3/T4	P=0.093	P=0.001	P=0.003	P=0.158	P=0.1	P=0.002	P=0.182	P=0.006	P=0.220	P=0.058	P=0.028
Leukoplakia	T1/T2	P=0.558	P=0.463	P=0.827	P=1	P=0.763	P= 0.015	P=0.746	P=0.533	P=1	P=0.616	P=0.090
	T3/T4	P=0.035	**P=0.00006**	P=0.065	**P=0.014**	P=0.176	**P=0.003**	P=0.242	P=0.068	P=0.323	P=0.011	P=0.049
T1/T2	T3/T4	P=0.128	**P=.001**	P=0.109	**P=0.033**	P=0.097	P=0.640	P=0.405	P=0.233	P=0.537	P=0.084	P=0.677

Table 3.3: Correlation of autoantibody response to tumor antigens between different groups of samples, C/S-chewing/smoking habit:

3.2.7.2: Correlation of the autoantibody profile for each of the tumor antigens with clinicopathological parameters

Of the 50 GBC samples that were included from earlier investigations, follow up information was available for 36 samples which together with 42 patients from present study provided data for autoantibody response from 78 GBC samples. Autoantibody profile of these 78 samples was used for correlation studies with DFS and clinical parameters.

Clinical significance of the autoantibody response to tumor antigens and clinicopathologic parameters were assessed using Chi-square test. Significant correlations were seen with Hsp70 and α ENO (α enolase a, b and c taken together). Table 3.4 shows the data for these two antigens and those for the other antigens is given in the Appendix, Tables A14a to A14e. Autoantibody response to Hsp70 shows significant correlation with stage, tumor size, nodal status, and recurrence while autoantibody to α ENO correlates only with differentiation at p=0.055. The autoantibody response for both the antigens did not correlate with age and sex of the patients. However autoantibody response to other antigens does not show any correlation with stage, tumor size, nodal status, differentiation status and recurrence of the tumor.

Clinico-pathological parameter		Antibody response against different tumor antigens in serum of patients with cancer of GBC						
		N=78 (100%)	Hsp70		p- value	Alpha enolase		p-value
			+ve	-ve		+ve	-ve	
Age (Median, 50yrs) (29-80 Yrs)	≤50	N=43(55%)	N=11(26%)	N=32(74%)	0.989	N=28(65%)	N=15(35%)	0.956
	>50	N=35(45%)	N=9(26%)	N=26(74%)		N=23(66%)	N=12(34%)	
Sex	Male	N=52(67%)	N=12(23%)	N=40(77%)	0.463	N=33(63%)	N=19(37%)	0.614
	Female	N=26(33%)	N=8(31%)	N=18(69%)		N=18(69%)	N=8(31%)	
Stages	I/II	N=23(29%)	N=43(55%)	N=11(26%)	0.027	N=15(65%)	N=8(35%)	0.984
	III/IV	N=55(71%)	N=35(45%)	N=9(26%)		N=36(65%)	N=19(35%)	
Tumor Size	T1/T2	N=37(47%)	N=5(14%)	N=32(86%)	0.020	N=23(62%)	N=14(38%)	0.57
	T3/T4	N=41(53%)	N=15(37%)	N=26(63%)		N=28(68%)	N=13(32%)	
Nodal Status	+ve	N=34(44%)	N=14(41%)	N=19(58%)	0.006	N=21(62%)	N=12(35%)	0.62
	-ve	N=42(55%)	N=6(14%)	N=36(86%)		N=29(69%)	N=13(31%)	
Differentiation	WD/ MD	N=52(67%)	N=12(23%)	N=32(77%)	0.403	N=30(57%)	N=22(42)	0.055
	PD	N=25(32%)	N=8(32%)	N=17(68%)		N=20(80%)	N=5(20%)	
Recurrence	Yes	N=38(48%)	N=15(39%)	N=23(61%)	0.006	N=28(74%)	N=10(26%)	0.133
	No	N=40(52%)	N=5(12%)	N=35(88%)		N=23(58%)	N=17(42%)	

Table 3.4: Correlation of antibody response to Hsp70 and alpha enolase with clinical parameters

3.2.7.3: Association of autoantibody response with disease free survival (DFS)

Kaplan Meier survival analysis was performed for autoantibody response against each tumor antigen reported earlier to assess their independent association with disease free survival of patients. Patients were grouped into those, who exhibited an autoantibody response against the antigen and those, who exhibited no autoantibody response to the same antigen. For each antigen the two survival curves obtained for each of these groups were compared using log rank test.

3.2.7.4: Association of autoantibody response to α Enolase isoforms and Hsp 70 individually with DFS

Correlation of autoantibody response to each of the three isoforms of α Enolase individually, with disease free survival of the patients is shown in Figure 3.15 (A-D). Among the three isoforms, autoantibody response to the isoform α enolase-c showed significant correlation with disease free survival of patients (p= 0.0187), Fig 3.15 C. A

similar trend was seen with autoantibody response to α ENO at p=0.053, Fig 3.15 D and for Hsp 70 at p=0.0002, Fig. 3.16 A. It is seen that patients who exhibit autoantibody response to each of the antigens recur significantly faster than those who are negative for autoantibody response.

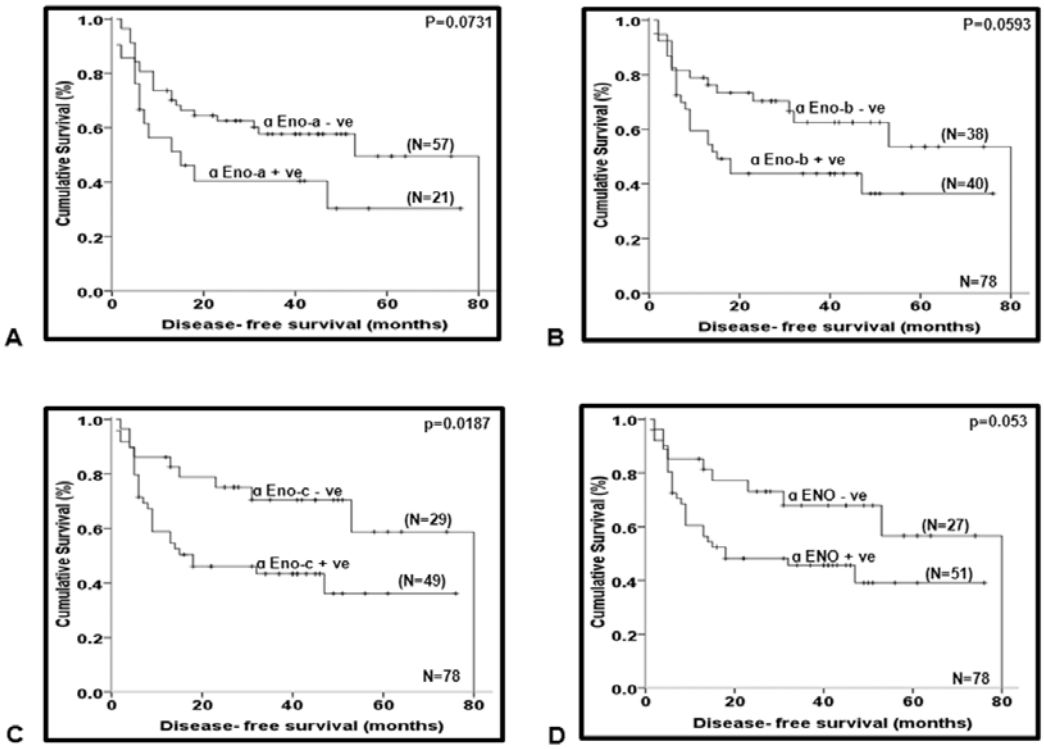


Figure 3.15: Kaplan-Meier curves with univariate analysis for correlation of cumulative survival and DFS (in months) with autoantibody response in groups of patients: negative or positive for autoantibody response to each of the antigens shown **(A)** α -Enolase a; **(B)** α Enolase-b; **(C)** α Enolase-c; **(D)** α ENO (α Enolase a, b, c taken together). The number of patients in each group is given as (N) against each curve.

3.2.7.5: Association of autoantibody response to Hsp70 and α ENO in combination with DFS

Patients were grouped in those, who do not show antibody response to either of the antigens, those who exhibit autoantibody response to either Hsp70 or α ENO, those who show antibody response to both Hsp70 and α ENO. Autoantibody response seen in these groups was correlated with DFS of patients. The survival curves are shown in Figure 3.16 B. It is seen that patients with autoantibody response to both antigens recur

faster than those without an autoantibody response, p=0.0013. Similarly presence of autoantibody response to Hsp70 and alpha enolase isoform c in combination significantly correlates with disease free survival of patients (p=0.0017), Fig. 3.16 C.

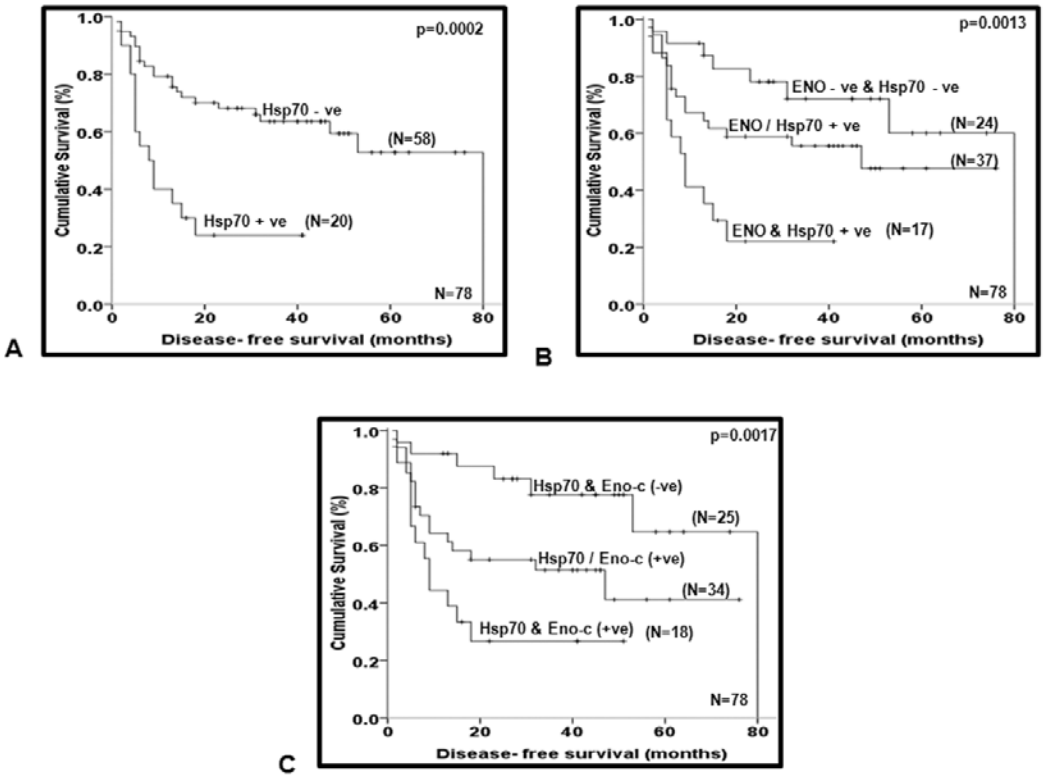


Fig. 3.16: Kaplan-Meier curves with univariate analysis for correlation of cumulative survival and DFS (in months) with autoantibody response in: **(A)** group of patients positive and negative for auto antibody response to-Hsp70; **(B)** group of patients positive and negative for either auto antibody response to α ENO (α Enolase- a, b, c taken together) or Hsp70 or both; **(C)** group of patients positive and negative for either auto antibody response α Enolase c or anti Hsp70 antibody or both. The number of patients in each group is given as (N) against each curve.

From the above it is clear that presence of an autoantibody response to the two antigens has correlation with DFS and this can be an added parameter for prognosis of cancer of the GBC. Autoantibody response to *other tumor antigens* also shows a trend towards their negative correlation with DFS of patients although not statistically significant except for triose phosphate isomerase, Fig.3.17 A-J

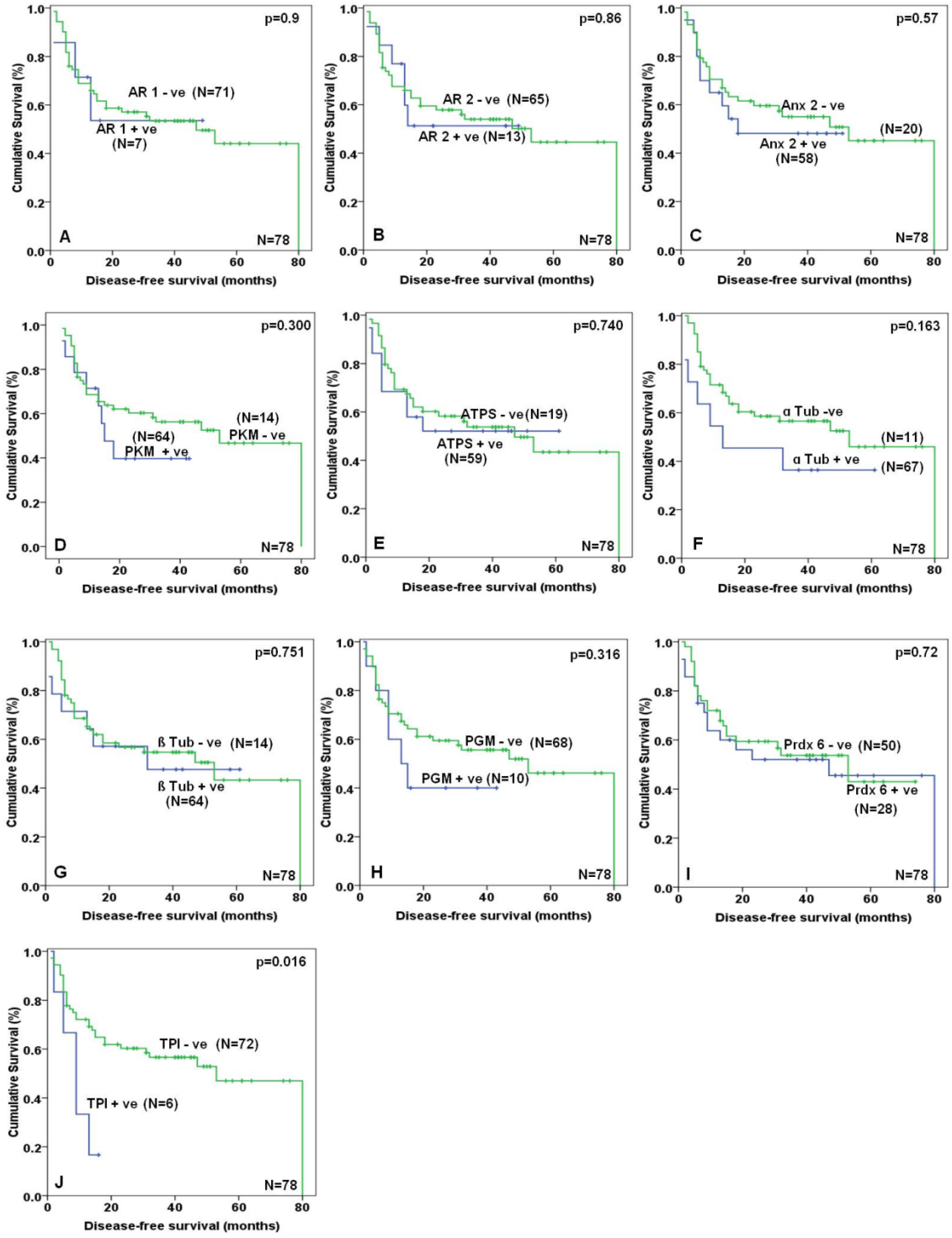


Figure 3.17: Kaplan-Meier curves with univariate analysis for correlation of cumulative survival and DFS (in months) with autoantibody response in groups of patients negative and positive for the response: **A)** Aldose keto reductase 1 (AR 1); **B)** Aldose keto reductase 2 (AR 2); **C)** Annexin 2 (Anx 2); **D)** Pyruvate kinase (PKM); **E)** ATP synthase (ATPS); **F)** alpha Tubulin (α Tub); **G)** beta Tubulin (ß Tub); **H)** Phosphoglycerate mutase (PGM); **I)** Peroxiredoxin 6 (Prdx 6); **J)** Triose phosphate isomerase (TPI). The number of patients in each group is given as (N) against each curve

3.2.7.6: Nodal and differentiation status of the tumor and DFS

The nodal and differentiation status of the tumor are well established prognostic clinical parameters. Fig. 3.18 (A-B) shows the survival curves for the patients in this study with respect to these parameters.

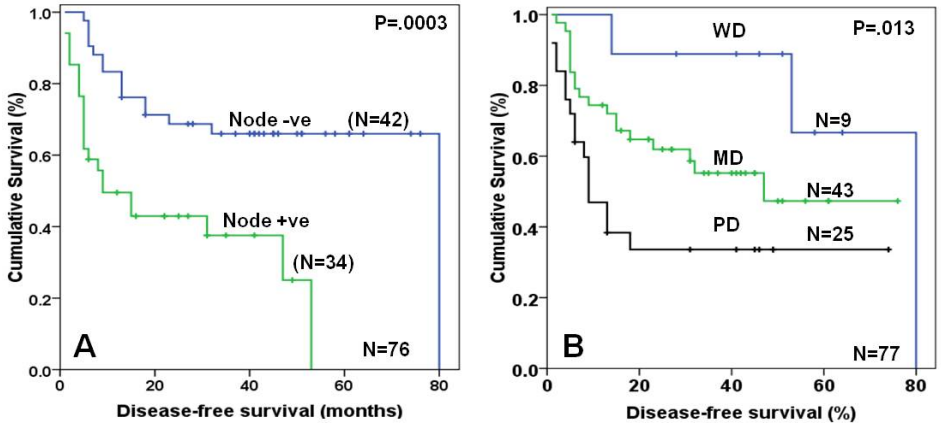


Figure 3.18: Kaplan-Meier curves with univariate analysis for correlation of cumulative survival and DFS (in months) in groups of patients: **A)** negative or positive for node **B)** with well differentiated tumors, moderately differentiated tumors and poorly differentiated tumors.

3.2.7.7: Autoantibody response to α ENO, nodal status and DFS

The autoantibody response to Hsp70 and α Enolase independently correlate with disease free survival. The association of each of these parameters in combination with nodal status was evaluated with DFS.

Data from 76 patients (for two patients pathological information was not available to assess nodal status) was divided into four groups based on the presence of antibody response to α enolase and nodal involvement. The survival curve obtained for each group is shown in Fig. 3.19 A. The overall trend is statistically significant at p=0.0002. Importantly, there is a clear decrease in DFS in patients who have node negative tumors and exhibit an autoantibody response to α ENO (α Enolase a, b, and c taken together)

and those who do not. Similarly among node positive patients, individuals who exhibit autoantibody to α ENO recur faster. A similar significant trend was observed when autoantibody response to only α Enolase c isoform was correlated with disease free survival of patients along with the nodal involvement (p=0.0003), Fig. 3.19 B.

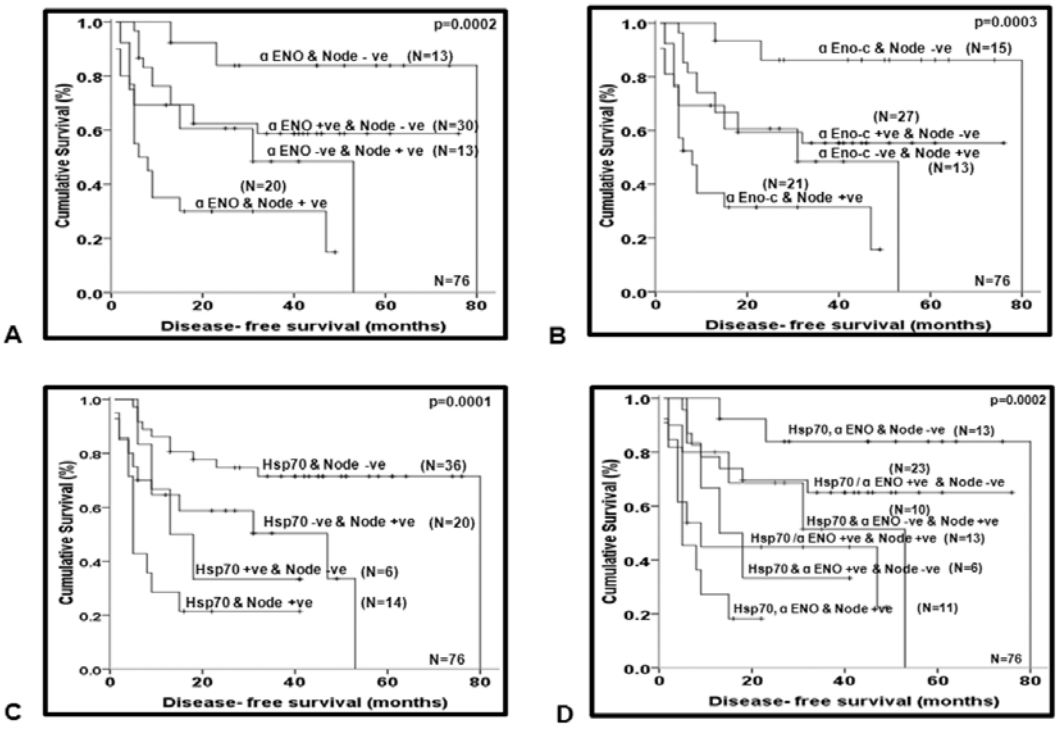


Fig. 3.19. **Kaplan-Meier curves with univariate analysis for correlation of cumulative survival and DFS (in months) with autoantibody response in:** (A) Four groups of patients viz those negative for autoantibody response to both α ENO and node;those positive for autoantibody response to α ENO but negative for node; those negative for autoantibody response to α ENO but positive for node; those positive for both autoantibody response to α ENO and node; **(B) Four group of patients viz** those negative for both autoantibody response to α enolase-c antibody and node;those positive for autoantibody response to α enolase-c but negative for node; those negative for autoantibody response to α enolase-c but positive for node; those positive for both autoantibody response to α enolase-c and node; **(C) Four groups of patients viz those** negative for both autoantibody response to Hsp70 and node; those negative for autoantibody response to Hsp70 but positive for node; those positive for autoantibody response to Hsp70 but negative for node; those positive for both autoantibody response to Hsp70 and node **(D) Six group of patients viz those** negative for autoantibody response to Hsp70 and α ENO and node; those positive for autoantibody response to Hsp70 / α ENO and negative for node; those positive for autoantibody response to both Hsp70 and α ENO and negative for node; those negative for autoantibody response to both Hsp70 and α ENO and positive for node; those positive for autoantibody response to Hsp70 / α ENO and positive for node; those positive for autoantibody response to Hsp70 and α ENO and node. The number of patients in each group is given as (N) against each curve.

3.2.7.8: Autoantibody response to Hsp70, nodal status and DFS

Data from 76 patients was divided in to four groups based on presence of antibody response to Hsp70 and nodal involvement. The survival curve obtained for each group is shown in Fig. 3.19 C . The overall trend is significant at p= 0.0001. It is noted that there is a significant decrease in DFS (p=0.049) in patients positive for autoantibody response to Hsp70 and nodal involvement as compared to patients positive only for node. In addition, there is also a significant difference in disease free survival of patients (p=0.050) between the group negative for both node and antibody response to Hsp70 and the group positive for antibody response to Hsp70 but negative for node.

3.2.7.9: Autoantibody response to both α ENO and Hsp70, nodal status and DFS

Presence of autoantibody response to either Hsp70 or α ENO or both in sera correlates independently to the disease free survival of patients. To determine their potential as an additional prognostic marker in combination, survival analysis was performed with nodal involvement. Data from 76 patients was divided in to six groups based on presence of antibody response to either α ENO or Hsp70 or both and nodal status. Fig. 3.19 D shows the survival curves obtained for each group. It is seen that patients who are negative for both autoantibody response to Hsp70 and α ENO and node fare better than those who are positive for antibody response to either Hsp70 or α ENO or both and negative for node. Similarly, the patients negative for antibody response to both Hsp70 and α ENO and positive for node fare better than patients positive for antibody response to either Hsp70 or α ENO or both as well as node.

In summary, autoantibody response to both antigens is an added parameter to node positivity for prognosis of cancer of GBC.

3.2.7.10: Autoantibody response to α ENO and Hsp70 individually, differentiation status and DFS

The parameters, differentiation and autoantibody response to α ENO alone or Hsp70 alone independently correlated with disease free survival as shown above. To determine whether the two parameters have any additive effect on disease free survival of the patients, data from 77 patients (for one patient differentiation status was not available) were divided in to six groups based on the presence of autoantibody response to α ENO and differentiation status of the tumors. The survival curves obtained are shown in Fig. 3.20 A. The group with well differentiated or moderately differentiated (WD/MD) tumors and positive for antibody response to α ENO has reduced DFS relative to the group with WD/MD tumors and negative for antibody response to α ENO. The group with poorly differentiated (PD) tumors and positive for antibody response to α ENO has significantly reduced disease free survival from group with PD tumor and negative for autoantibody to α ENO. The overall trend is significant at ($p=0.012$). Similarly presence of antibody response to Hsp70 showed significant correlation with DFS along with the differentiation status of tumor ($p=0.0002$), Fig. 3.20 B.

3.2.7.11: Autoantibody response to α ENO and Hsp70, differentiation status and DFS

The data from 77 patients was divided into six groups based on autoantibody positivity and differentiation status. The survival curves in Fig 3.20 C show that those patients with PD tumors who exhibit autoantibody to α ENO and Hsp 70 recur faster than those who are negative for an autoantibody response to both or either antigens. Similarly patients with MD/WD tumors who exhibit antibody response to both antigens

recur faster than those who are negative for autoantibody response. The overall trend is significant at p=0.001.

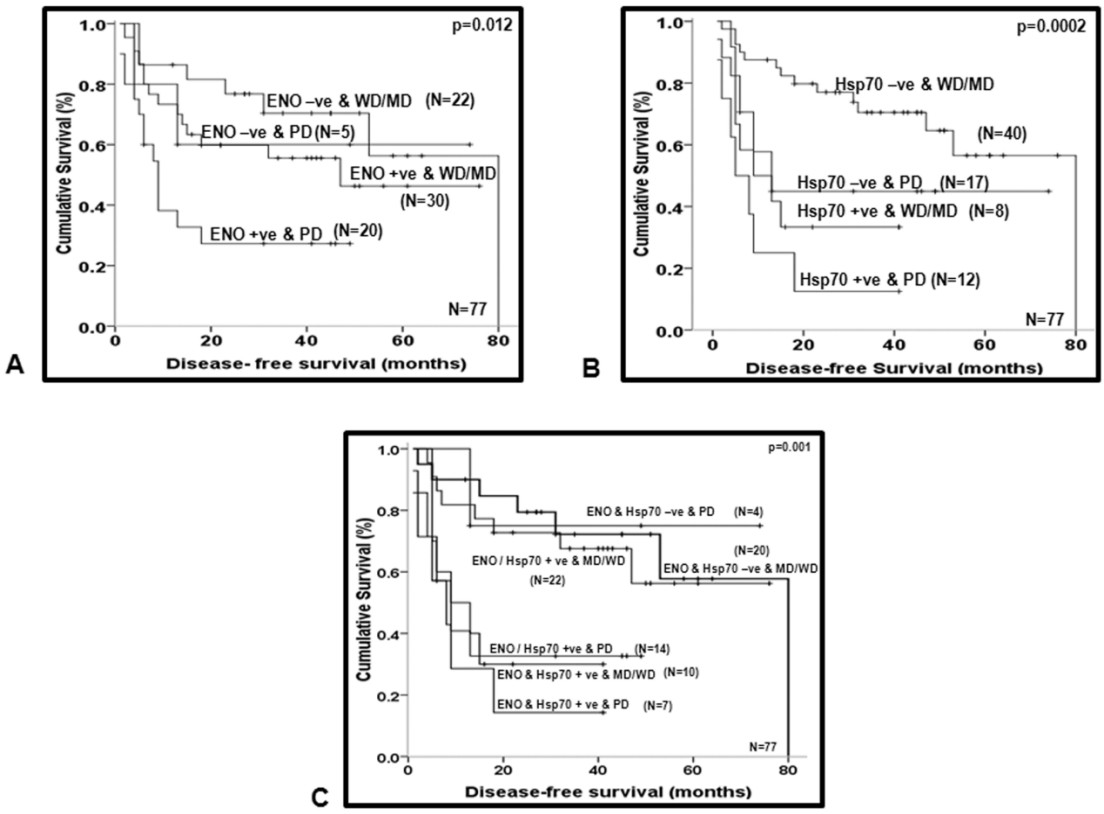


Fig. 3.20: Kaplan-Meier curves with univariate analysis (log rank) for correlation of cumulative survival and DFS(in months) in: (A) Four groups of patients viz those with WD (well differentiated) tumor / MD (moderately differentiated) tumor and negative for autoantibody response to α ENO; those with WD tumor / MD tumor and positive for autoantibody response to α ENO antibody; those with PD (poorly differentiated) tumor and negative for autoantibody response to α ENO; those with PD tumor and positive for autoantibody response to α ENO; **(B) Four group of patients viz** those with WD tumor / MD tumor and negative for autoantibody response to Hsp70; those with WD tumor /MD tumor and positive for autoantibody response to Hsp70; those with PD and negative for autoantibody response to Hsp70 antibody; those with PD tumor and positive for autoantibody response to Hsp70; **(C) Six group of patients viz** those negative for autoantibody response to α ENO and Hsp70 and with WD / MD tumor; those positive for autoantibody response to α ENO / Hsp70 and with WD / MD tumor; those positive for autoantibody response to both α ENO and Hsp70 and with WD / MD tumor; those negative for autoantibody response to both α ENO and Hsp70 and with PD tumor; those positive for autoantibody response to α ENO / Hsp70 and with PD tumor; those positive for autoantibody response to both α ENO and Hsp70 and with PD tumor. The number of patients in each group is given as (N) against each curve.

The emerging data shows that autoantibody response to α ENO and Hsp 70 alone and in combination is an added parameter which with differentiation status of the tumor can improve the prognosis of cancer of GBC.

3.2.7.12: Multivariate analysis

Cox regression analysis was performed to determine the prognostic potential of autoantibody response to α ENO and Hsp70 for cancer of GBC in combination with the clinical parameters, differentiation and nodal status. Table 3.5 shows that the relative risk of recurrence is 3.41 when the patients exhibit autoantibody response to both the antigens supporting their potential as added parameters which influence the prognosis of the disease.

		No. of patients	Relative risk	95% CI for relative risk		Significance *(p)*
				lower	upper	
Differentiation status	WD/MD	51	2.29	1.14	4.6	0.020
	PD	24				
Nodal involvement	-ve	41	2.98	1.49	5.96	0.002
	+ve	34				
Autoantibody response to Hsp70 and α Enolase	(-ve/-ve)	23	1.6	0.63	4.1	0.318
	(-ve/+ve)	35				
	(+ve/+ve)	17	3.41	1.28	9.0	0.014

Table 3.5: Multivariate analysis evaluating the relative risk of recurrence vis a vis nodal status, differentiation status of the tumor and autoantibody response using Cox regression analysis

3.3 To express and purify recombinant proteins of the tumor antigens for generation of a multiplex array.

3.3.1 Generating the expression clones for the identified tumor antigens by Gateway Cloning: Clones for Aldose reductase (AKR1B), Pyruvate kinase (PKM2), ATP synthase (ATPS), Alpha tubulin (TUBA), Beta tubulin (TUBB), Phosphoglycerate mutase (PGM), Peroxiredoxin 6 (PRDX6) and Cyclophilin A (PPIA) were obtained from Harvard Institute of Proteomics as entry clones in the pDONR221 vector except for Peroxiredoxin 6. The genes for tumor antigens in pDONR221 entry clones were subcloned in pDEST15 destination vector for their expression. The cloned genes except for Cyclophilin A were confirmed by DNA sequencing using Sanger's method (sequence given in Appendix in Fig. A1). The cloning of ATPS, TUBB and PGM in pDEST15 was also confirmed by PCR analysis using gene specific primers. The cloning of PKM2, ATPS, TUBB and PRDX6 in pDEST15 vector was also confirmed by restriction digestion with EcoR1 and Bsrg1. Fig. 3.21 A to C shows the inserts obtained after restriction enzyme cleavage and Fig. 3.22 A shows the amplified PCR products for the different clones.

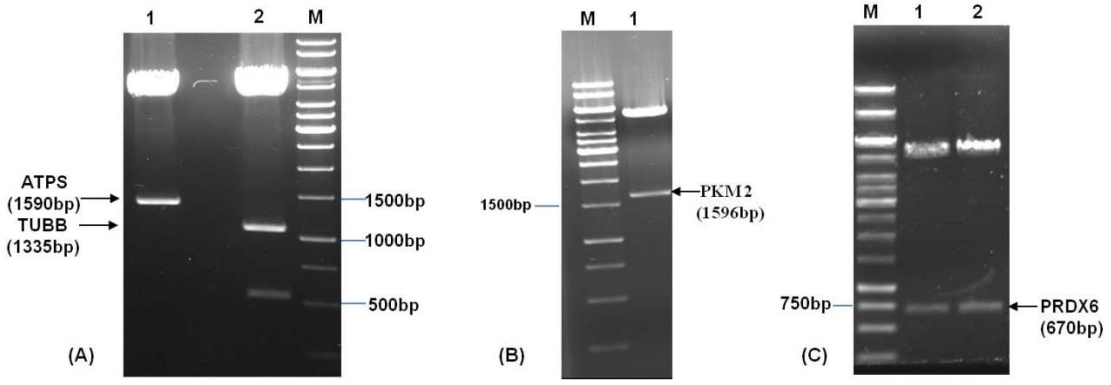


Fig. 3.21: Screening of pDEST15 clones positive for different genes by restriction digestion with ECoR1 and Bsrg 1 A) lane 1: TUBB, lane 2: ATPS; B) lane 1: PKM2; C) lane 1 and lane 2 : PRDX 6 M: 1Kb DNA ladder

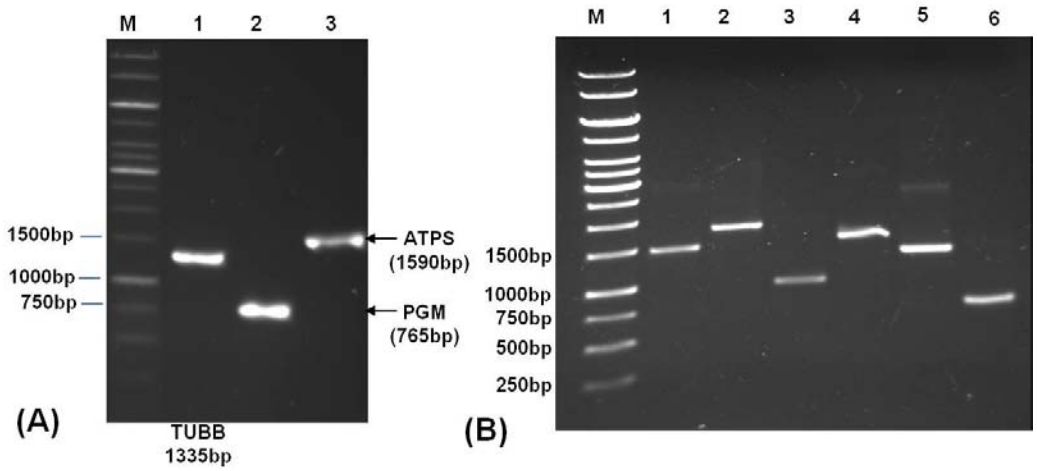


Fig. 3.22: Confirmation of pDEST15 clones positive for different genes by PCR analysis: (A) with gene specific primers, lane 1: TUBB, lane 2: PGM, and lane 3: ATPS, M: 100 bp DNA ladder, (B) with primers for the pDEST15 vector, lane 1: TUBA, lane 2: PKM, lane 3: PGM, lane 4: ATPS, lane 5: TUBB, lane 6: PPIA and M: 1Kb DNA ladder.

3.3.2 Expression of recombinant GST-fusion proteins for the identified tumor antigens cloned in pDEST 15 vector:

All recombinant proteins were expressed in Escherichia coli BL21 (DE3) strain and purified as GST fusion proteins using affinity chromatography. Three proteins (ATPS, Tuba and Tubb) were obtained in very less amount after purification as they were expressed mostly as inclusion bodies. Fig. 3.23 shows the pattern of the proteins obtained. Purified protein preparations were assessed by 1DE for their molecular size. Most of the protein preparations showed presence of a 26 kD protein on the SDS-PAGE gel.

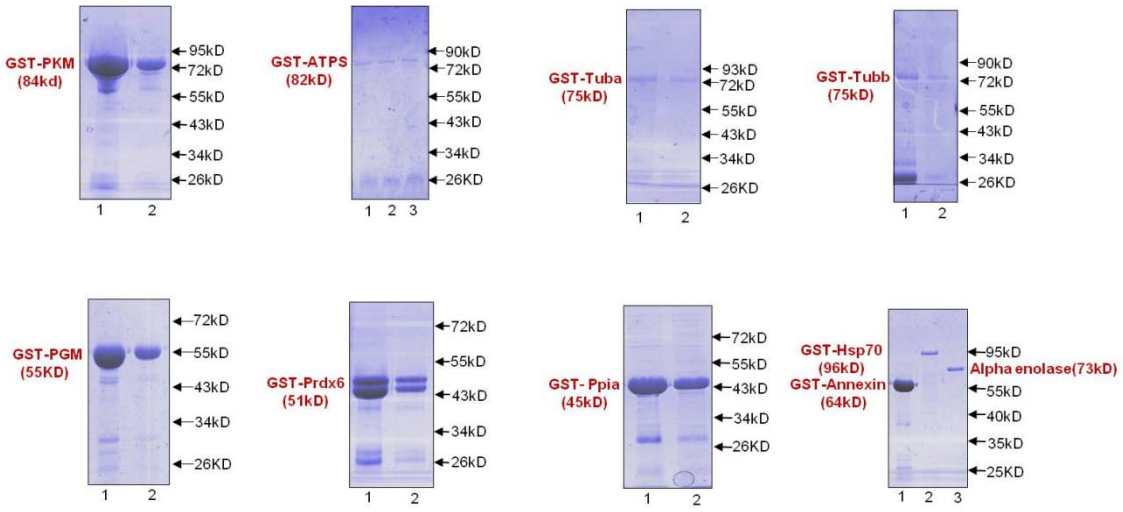


Fig. 3.23: Coomassie blue stained gels of eluted fractions of purified recombinant GST-fusion proteins run on 10 % SDS-PAGE gel. The positions of the different proteins in fraction 1 and 2 are shown in the panels. The last panel shows GST-Anx, GST-Hsp70 and GST- α Enolase in lanes 1, 2 and 3. The position of the molecular weight standards is shown on the right side of panels

3.3.3 SubCloning of Aldose keto reductase in pGEX4T1 vector via TA cloning:

Aldose reductase gene (AKR) was subcloned in pGEX4T1 via PCR cloning. AKR was amplified by PCR and subcloned in a TA vector pTZ57R/T. The cloning was confirmed by restriction digestion. Out of seven colonies screened, all were positive for the AKR gene. It was further subcloned in pGEX4T1. Cloning was confirmed by restriction digestion and DNA sequencing. Fig. 3.24 shows the PCR amplified product (A), the insert obtained after restriction enzyme digestion from the TA vector (B) and pGEX4T1 (C). The clones positive for AKR gene in pGEX4T1 vector were used for expression of recombinant protein in BL21 DE3 E.Coli cells.

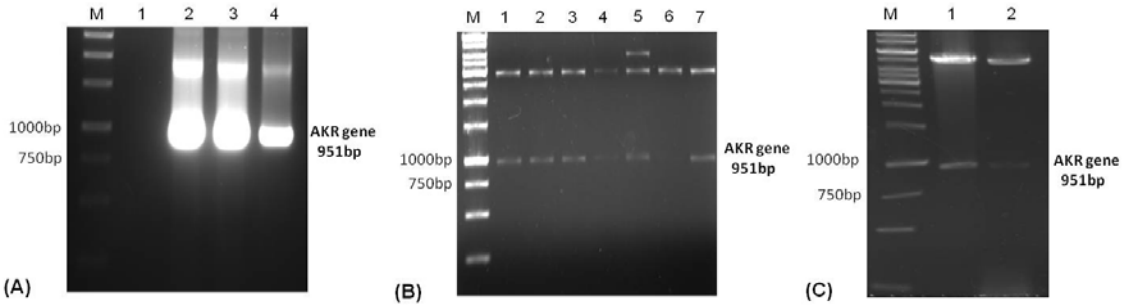


Fig. 3.24: Cloning of AKR gene in pGEX4T1 vector via TA cloning. The patterns seen on1% DNA agarose gels after electrophoresis are shown. A) PCR amplified AKR gene lanes 2,3 and 4; .B) Screening of clones positive for AKR gene in TA vector by restriction digestion with ECoR1 and Sal1 enzyme. The digested product is seen on the gel in different lanes. C) Screening of clone positive for AKR gene in pGEX4T1 vector by restriction digestion with ECoR1 and Sal1 enzyme. In all the panels, M is the 1 kb DNA ladder.

3.3.4 Expression of recombinant GST-Aldose keto reductase (AR):

Recombinant GST-aldose keto reductase was expressed and purified using affinity chromatography. The eluted fractions of the purified protein were assessed by 1DE (Fig 3.25 A). The identity of the protein was further confirmed by western blot analysis using commercial antibody to the protein (Fig. 3.25 B).

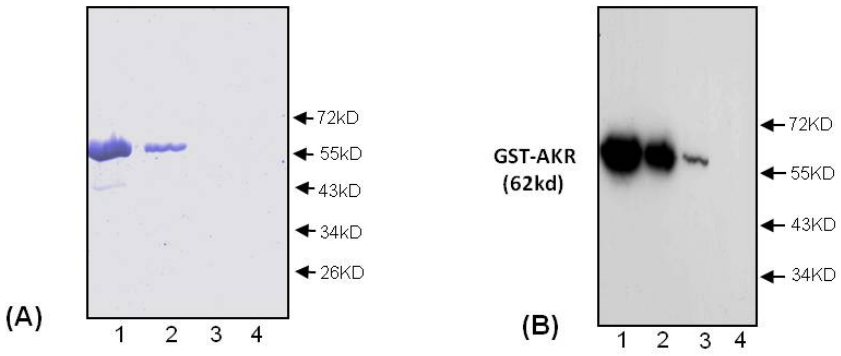


Fig. 3.25: SDS PAGE profile of purified GST-aldose keto reductase. A) Fractions of AKR protein eluted from the GST-Sepharose beads resolved by 10 % SDS-PAGE and gel stained with Coomassie blue, B) Fractions immunostained with antibody to AKR after blotting onto PVDF membrane.

3.3.5 Optimization of expression of recombinant proteins expressed mostly as insoluble protein:

In order to determine the conditions for the optimal expression of soluble ATP synthase, the recombinant clone for ATP synthase was induced at different IPTG concentrations and different temperatures. Fig. 3.27 shows the expression of soluble ATP synthase at different conditions of induction after 2 h and 3 h of induction. Protein was best expressed in soluble form at 37°C after 2 h of induction Fig 3.26 A. It was also expressed well at 30°C with both 0.05 mM and 0.1mM IPTG after 3 h of induction as seen on 1DE gel given in Fig.-3.26 B. However after 3 h of induction the protein becomes insoluble on accumulation of protein. Expression of ATP synthase and beta Tubulin improved marginally by induction at 18° C, ON with 0.1 mM IPTG and proteins were purified after induction at 18°C using affinity purification. The purified proteins were seen on 1D gel as shown in Fig. 3.27.

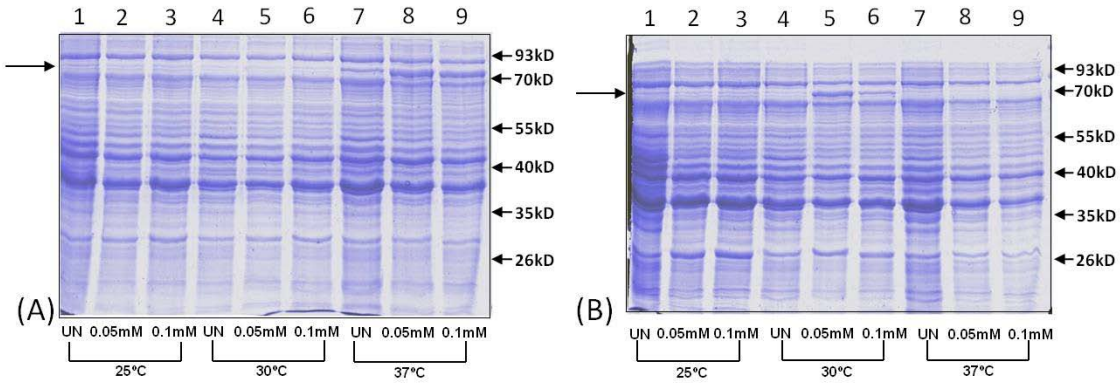


Fig. 3.26: SDS-PAGE profile of supernatants obtained from cultures induced with different IPTG concentrations and temperatures. (A) 2 h induction (B) 3 h induction. The concentration of IPTG is shown below the lanes. The arrow shows the induction of ATP synthase protein in lanes 7, 8 and 9 of A and lanes 5 and 6 of B, Un-Uninduced,

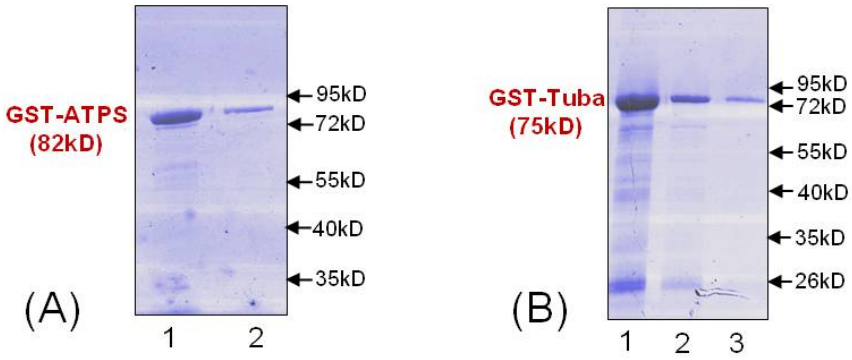


Fig. 3.27: SDS-PAGE- Coomassie stained gel profile of fractions obtained after GST-Sepharose bead purification A) GST-ATP synthase and B) GST-Tuba, obtained after induction with 0.1 mM IPTG at 18° C, ON.

3.3.6 Purification of GST fusion proteins from the inclusion bodies:

3.3.6.1 Purification of ATP synthase from the bacterial pellet: ATP synthase was poorly expressed as a soluble protein under all induction conditions. However at 18°C there was an improvement, but after purification very little protein was obtained. Major fraction of the protein expressed was seen in inclusion bodies. For solublization of protein from the inclusion bodies different concentrations of urea were used. With 4 M urea about 50% of the protein was soluble. The protein so obtained was subjected to gradient dialysis for removal of urea. The purity of the protein was verified by 1DE (Fig. 3.28) The solubilised protein was further purified using GST-Sepharose. Fig. 3.28 C shows the quality of the protein obtained.

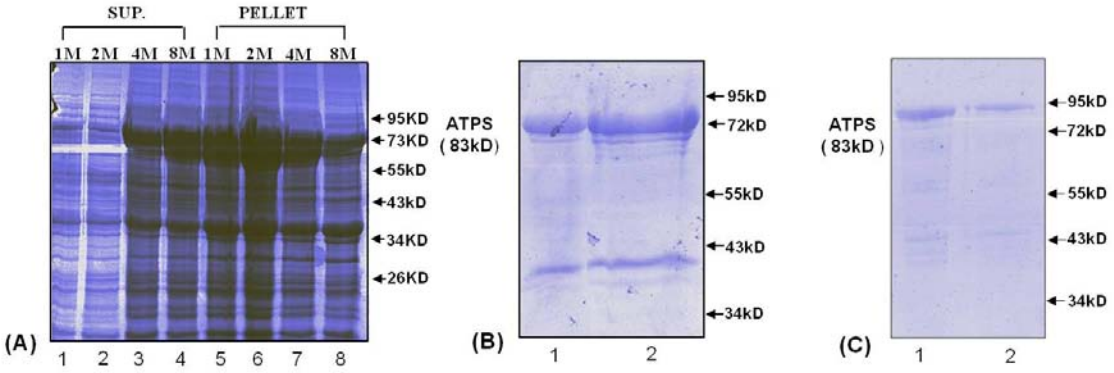


Fig. 3.28: Purification of recombinant GST-ATP synthase from inclusion bodies. Different fractions were analysed by 10% 1DE and the gel stained with Coomassie blue. (A) Supernatant fractions obtained after solublization of inclusion bodies from the cell pellet using different concentrations of Urea are shown in lane (1-4): 1M, 2M, 4M, 8M and lanes (5-8) show the protein remaining in the cell pellets after solublization of inclusion bodies in urea 1M, 2M, 4M & 8M. (B) Lane1: GST-ATP synthase solubilized from the cell pellet with 4M urea; lane 2: Insoluble protein remaining in the pellet after urea solublization (C) Lane 1 and 2, Fractions of recombinant GST-ATP synthase protein eluted from the glutathione Sepharose beads.

3.3.6.2 Purification of recombinant GST-Alpha Tubulin from the inclusion bodies:

GST- alpha Tubulin and GST- beta Tubulin proteins were mostly obtained in inclusion bodies. Alpha tubulin was solublized in 2% Empigen from the inclusion bodies and then purified further on Glutathione Sepharose beads. Along with alpha tubulin many other proteins came in to the soluble fraction and these also got bound to Glutathione Sepharose beads during purification, therefore pure alpha tubulin was not obtained. Fig. 3.29 shows the gel picture of Tuba protein and other proteins that are present in the empigen soluble fraction and bind the GST sepharose beads.

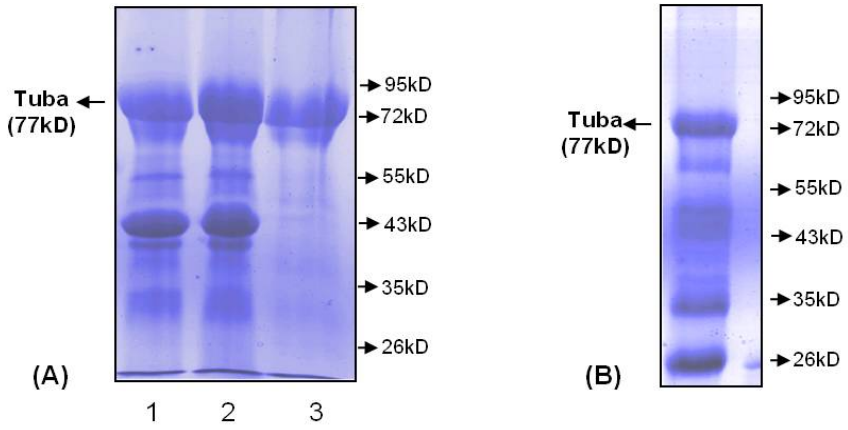


Fig. 3.29: SDS-PAGE profile of purification of insoluble recombinant GST-Tuba from the bacterial cell pellet by Empigen solublization. A) Lane 1-2: Supernatants obtained from Empigen solublization of two different bacterial cell pellets for Tuba protein lane 3: Protein remaining in bacterial cell pellet after Empigen solublization B) Tuba protein and other proteins solublized in empigen bound to glutathione sepharose beads.

3.3.7 Purification of proteins by size exclusion chromatography:

Most of the protein preparations had a 26 kD contaminant protein as seen above in the 1DE gels. To obtain proteins free of the 26 kD contaminant, FPLC was done as described in Materials and Methods section above. Fig 3.30 shows the protein patterns on 1DE of the fractions eluting from the Superdex 200 column. It is seen that except for the very early fractions with little protein, the latter fractions contained the 26 kD protein. Therefore even after FPLC, the 26 kD protein was not completely eliminated from some of the protein preparations.

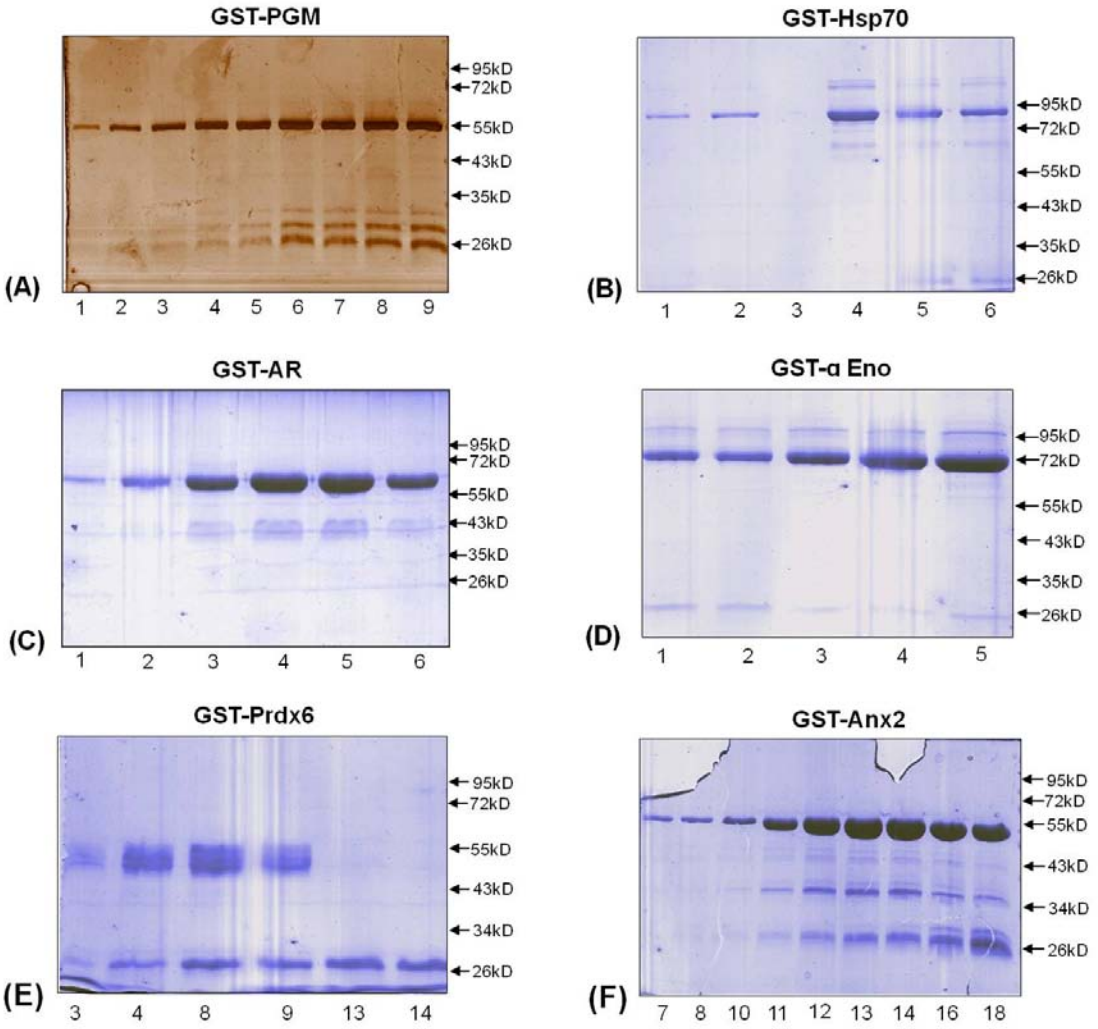


Fig. 3.30: 1DE evaluation of the fractions obtained from FPLC for the purification of different proteins. a) GST-PGM b) GST-Hsp70 c) GST-AR d) GST-alpha Enolase (e) GST-Prdx6 F) GST-Anx2

3.3.8 Purification of GST- fusion proteins from bacterial cell pellet using B-PER reagent:

To prevent the breakdown of protein during sonication of cells, fresh protein preparations were made using B-PER GST Fusion Protein Spin Purification Kit. Proteins so obtained were analysed for their purity by 1DE. Use of the B-PER kit did not prevent the retention of 26 kDa contaminant in the protein preparations Fig. 3.31. The 26 kD protein present in many of purified protein

preparations was identified as either GST protein or the protein itself indicating that there was still breakdown of proteins. Mass spectrometry data for some of the proteins is given Appendix in Fig. A2 and Table A15.

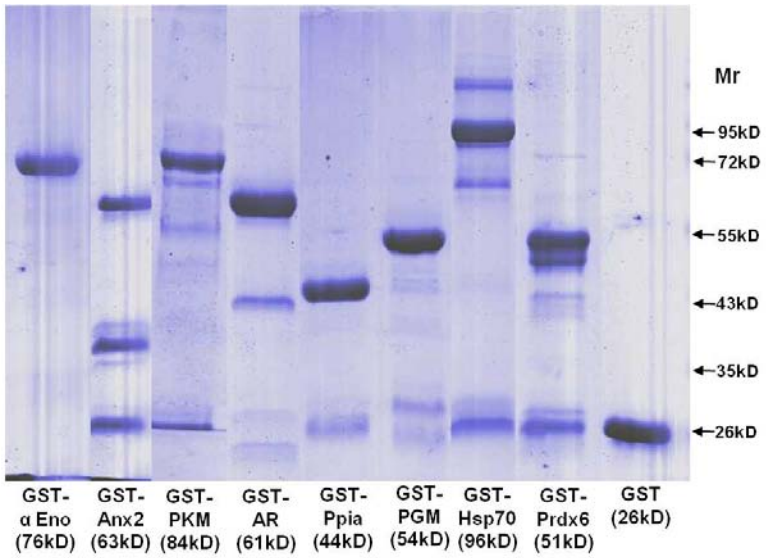


Fig.3.31: 1DE profile of recombinant GST-fusion proteins purified with the BPER spin purification kit. The proteins in the gel were stained with Coomassie blue. The position of the molecular weight markers is shown on the right hand side.

3.3.9 Purification of GST-fusion proteins without the GST tag by thrombin cleavage: Most of purified GST-Fusion protein preparations contained 26 kD GST protein even when they were purified with BPER spin purification kit. Further purification of proteins by FPLC also did not completely eliminate the 26 kD protein. In order to overcome this problem, proteins were purified without the GST tag using thrombin cleavage. Fig. 3.32 shows the 1DE profile 1qof Anx2, AR, PGM, α Eno and Hsp70 eluted from the Glutathione Sepharose beads without the GST tag after thrombin cleavage. Prdx6 was not cleaved well and most of the protein remained on the beads, Fig.3.32C.

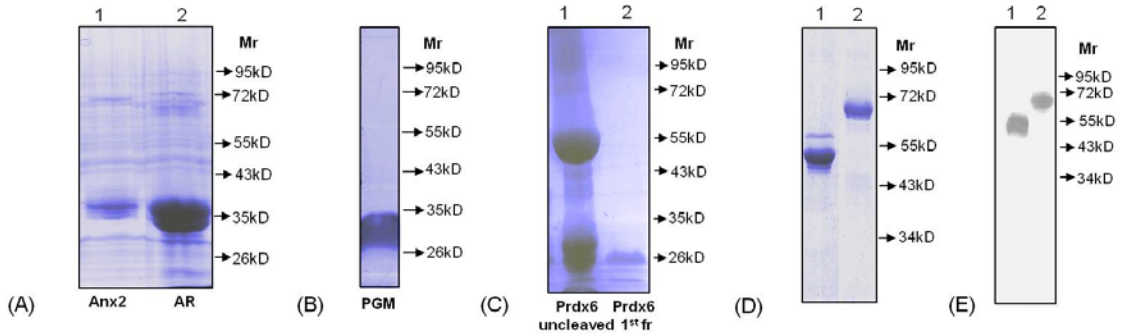


Fig. 3.32: 1DE profile of thrombin cleaved recombinant proteins (A) Lane 1: Anx2, Lane 2: AR; (B) PGM; (C) lane 1: uncleaved Prdx 6 bound to glutathione sepharose beads after thrombin cleavage, lane 2: cleaved Prdx 6; (D) Lanes 1 and 2 show the Coomassie blue stained proteins Hsp70 and alpha enolase respectively; (E) shows the western blot profile of and alpha enolase (Lane1) and Hsp70 (Lane2) probed with their respective antibodies. The position of the molecular weight standards in kD is shown alongside all the panels.

3.4 Assessment of autoantibody response on a multiplex array of the recombinant proteins

3.4.1 Dot blot assay:

3.4.1.1 Standardization of Dot blot experiments:

Towards the generation of a multiplex array, recombinant GST-fusion proteins of the identified tumor antigens were prepared and dot blot experiments were performed to standardize procedures for the development of an array. Protein content of each preparation was done using the Bradford protein assay kit. An outline of the template for the protein microarray was designed and proteins were spotted manually on the nitrocellulose membrane according to this template.

3.4.1.1.1 Volume of proteins suitable for manual spotting of proteins in an array for dot blot analysis: The recombinant proteins GST-fusion proteins GST- α Eno, GST-Anx2, GST-Tuba, GST alone, GST-Prdx6, GST-AR, GST-PKM2, GST-PGM and GST-Ppia were manually spotted in different volumes on the nitrocellulose membrane

with a micropipette. Fig. 3.33A shows the template and Fig. 3.33B shows the signals obtained when the blot was probed with anti GST antibody. Considering the density of the spot, autographic signal obtained with the spot and the feasibility of spotting, a volume of 0.5 µl was considered optimal.

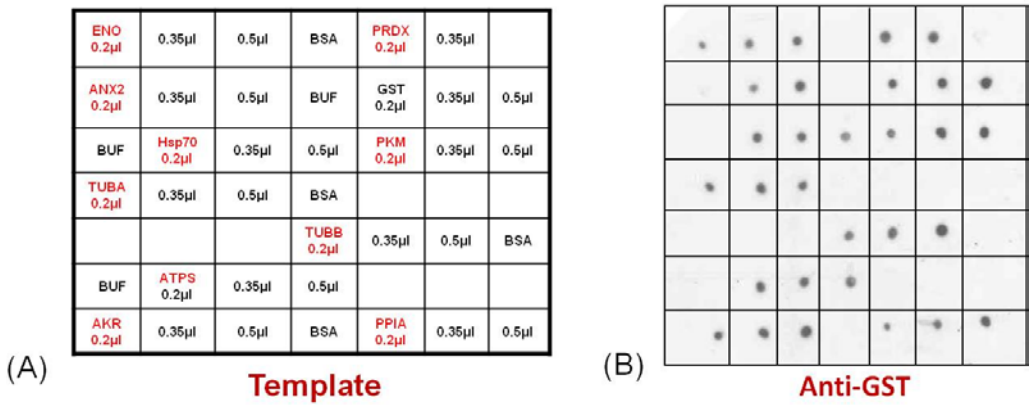


Fig. 3.33: Standardisation of manual spotting for dot blot analysis with recombinant proteins A) Template showing the order and volume of recombinant proteins manually spotted on the nitrocellulose membrane and probed with anti-GST antibody. B) Autograph obtained from the same blot after development of the signal. BUF:Buffer

3.4.1.1.2 Amount of protein suitable for dot blot analysis: The experiment was performed with 50 ng, 100 ng and 200 ng of recombinant proteins. Fig.3.34 shows the signals obtained when the blots were probed with IgG from sera of sample 824 T and CL3. It was seen that the best signals were obtained with 100 ng of spotted protein. The signals obtained were not saturated, they were distinct and did not merge with other signals on the array. Henceforth hundred ng of protein was decided as optimum for dot blot.

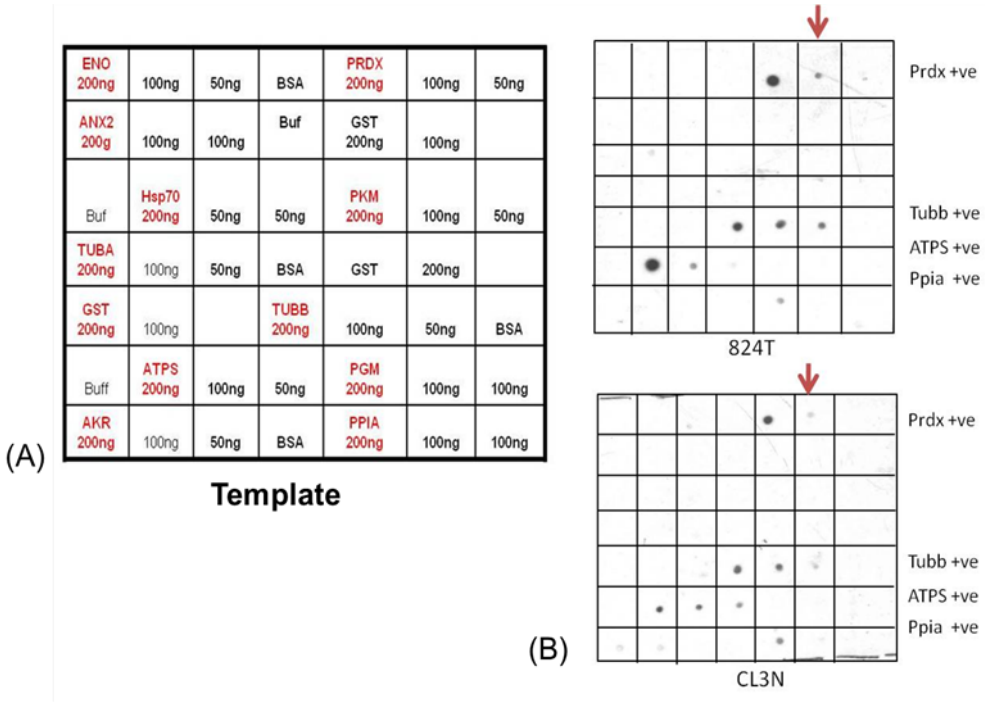


Fig. 3.34: Dot blot analysis with different concentrations of recombinant proteins (A) Template showing the order and amount of recombinant proteins manually spotted on the nitrocellulose membrane for the dot blot analysis (B) Autographs of dot blot immunostained with 5µg/ml IgG from sera of a patient (824T) and a healthy individual (CL3N)

3.4.1.1.3 Concentration of IgG / sera suitable for dot blot analysis: Three concentrations of IgG (3 µg/ml, 4 µg/ml and 5 µg/ml) were used for immunodetection of proteins in dot blot analysis. With 3µg/ml IgG signals obtained on autograph were faint, while signals were most prominent but not saturated with 5µg/ml IgG.

Instead of IgG, two different dilutions of sera 1:200 and 1:500 were also used for immunodetection of proteins on the dot blot. Figure 3.35 shows that good signals are seen with 1:200 dilution of the sera indicating that serum can be directly used for autoantibody evaluation without the need to purify IgG from them.

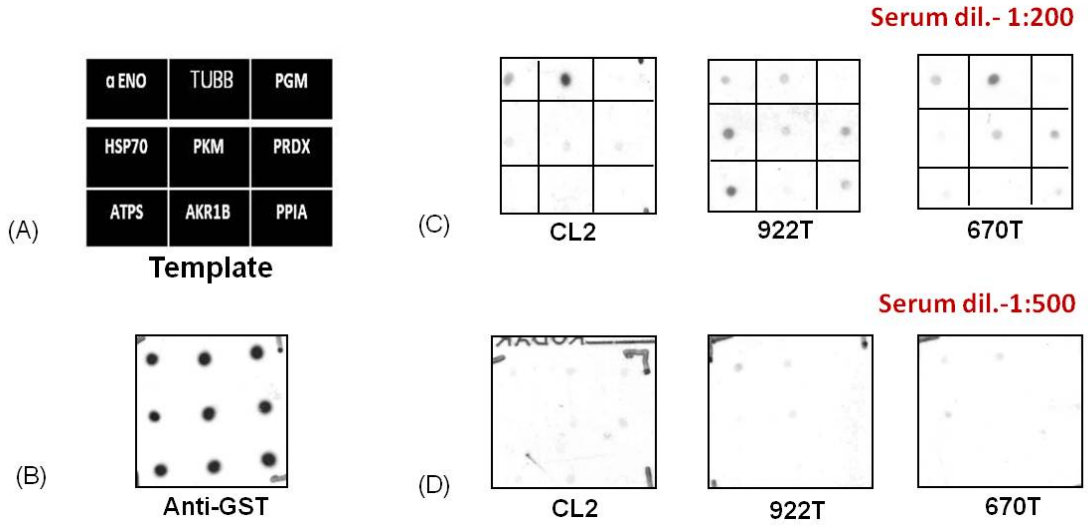
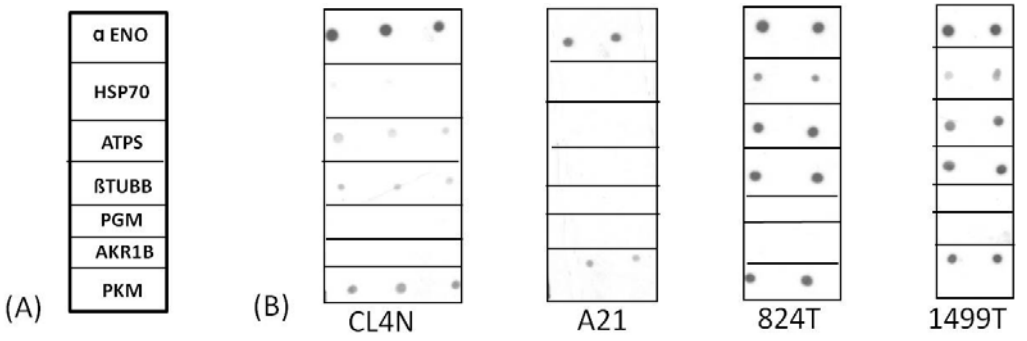


Fig. 3.35: Immunodetection with sera on dot blots of recombinant proteins: (A) Template showing the order of proteins spotted on the nitrocellulose membrane on all the blots (B) Autograph for the dot blot probed with Anti-GST antibody to show the uniform spotting of proteins (C) Autographs for the dot blots probed with two patient sera (922T and 670T) and one control sera (CL2) at 1:200 dilution (D) Autographs for the dot blots probed with same two patient sera and one control sera at 1:500 dilution.

3.4.1.2 Dot blot analysis with optimized conditions: Autoantibody response was evaluated in sera of some cancer patients and healthy individuals by dot blot analysis using the optimized conditions. Fig. 3.36 shows dot blots probed with sera. It is seen that the antibody response detected with the recombinant proteins in the sera was not identical with that obtained in the 2D immunoblot analysis with KB lysate proteins. Even some of the control sera showed reactivity with some of recombinant proteins which was not seen in 2D immunoblot analysis as shown in Fig. 3.36.

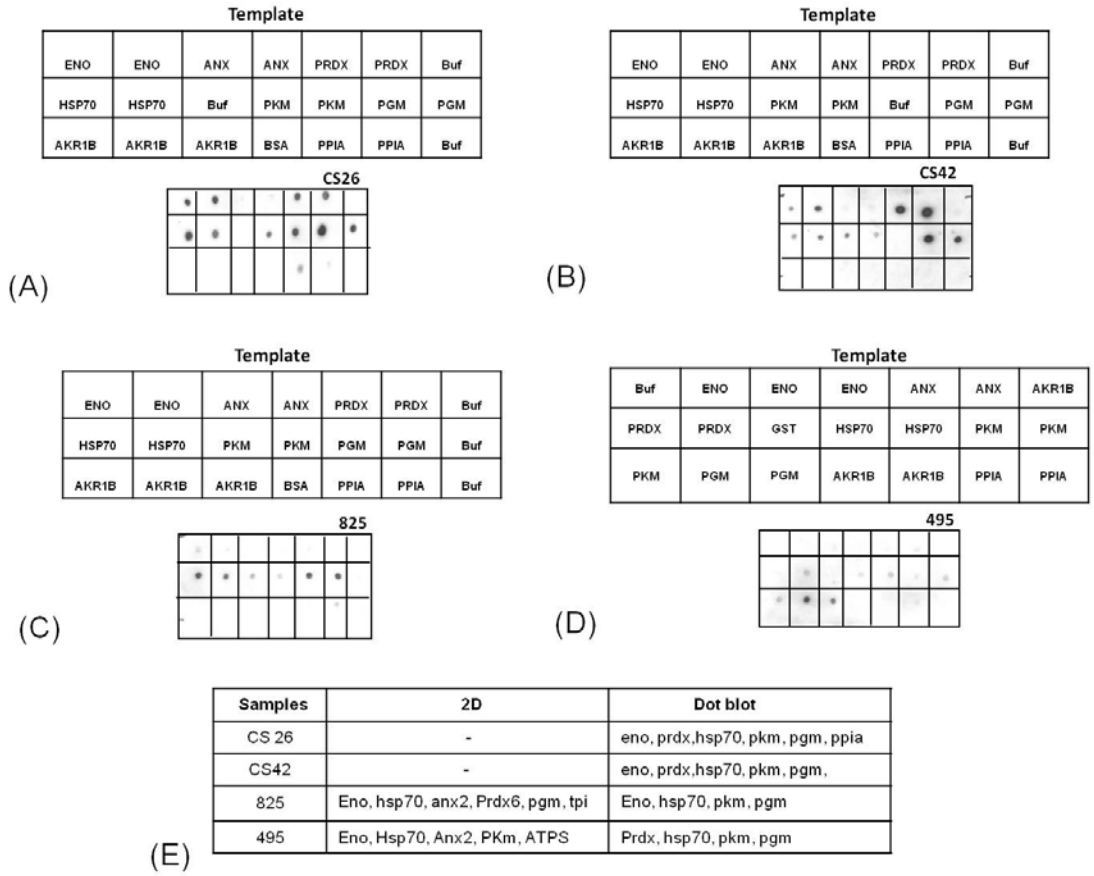


(C)

Sample	2DE	Dot blot
CL4N	Eno,	Eno, PKM
A21	Eno(f), PKM(f)	Eno, PKM
824T	Eno, ATPS, Tubb	Eno, Hsp70, ATPS, Tubb, PKM
1499T	Eno, PKM ,Hsp70(F), Tubb	Eno, Hsp70, ATPS, Tubb, PKM

Fig. 3.36: Dot blot analysis with sera using optimised conditions (100 ng protein, 5 µg/ml IgG concentration). (A) Template showing the order of proteins spotted on the dot blot. (B) Autographs for the dot blots probed with IgG from a healthy individual (CL4N) and an individual with leukoplakia (A21L) and patients with cancer (824T, 1499T). (C) Table showing comparison of autoantibody signals detected by 2D analysis and dot blot analysis. f-faint

In an another experiment where serum was used for the evaluation of autoantibody response to the recombinant proteins, pattern of antibody response was different from that obtained in 2D analysis. Fig. 3.37 shows the pattern of autoantibody obtained on autographs for dot blots probed with different sera. Control sera CS26 and CS 42 which had not shown reactivity with any of tumor antigens in 2D analysis showed reactivity with many of recombinant proteins in dot blot analysis.



Samples	2D	Dot blot
CS 26	-	eno, prdx,hsp70, pkm, pgm, ppia
CS42	-	eno, prdx,hsp70, pkm, pgm,
825	Eno, hsp70, anx2, Prdx6, pgm, tpi	Eno, hsp70, pkm, pgm
495	Eno, Hsp70, Anx2, PKm, ATPS	Prdx, hsp70, pkm, pgm

(E)

Fig. 3.37: Pattern of antibody response to recombinant proteins detected on dot blots probed with tumor sera and normal sera. Template for dot blot and the autograph for the dot blot probed with sera from (A) a healthy individual (CS26) (B) a healthy individual CS42. (C) a patient 825. (D) a patient 495. (E) Table showing comparison of results from the 2DE and dot blot analysis.

In order to understand the discrepancy in the two results, all recombinant proteins were resolved by SDS-PAGE, transferred to membrane and probed with sera from cancer patients and healthy individuals. Fig. 3.38 shows the autographs obtained from blots probed with four different sera. It was observed that the 26 kD protein contaminant present in many of the protein preparations was also immunodetected with some of the serum samples suggesting that the results obtained in dot blot analysis may be due to interference of this nonspecific interaction.

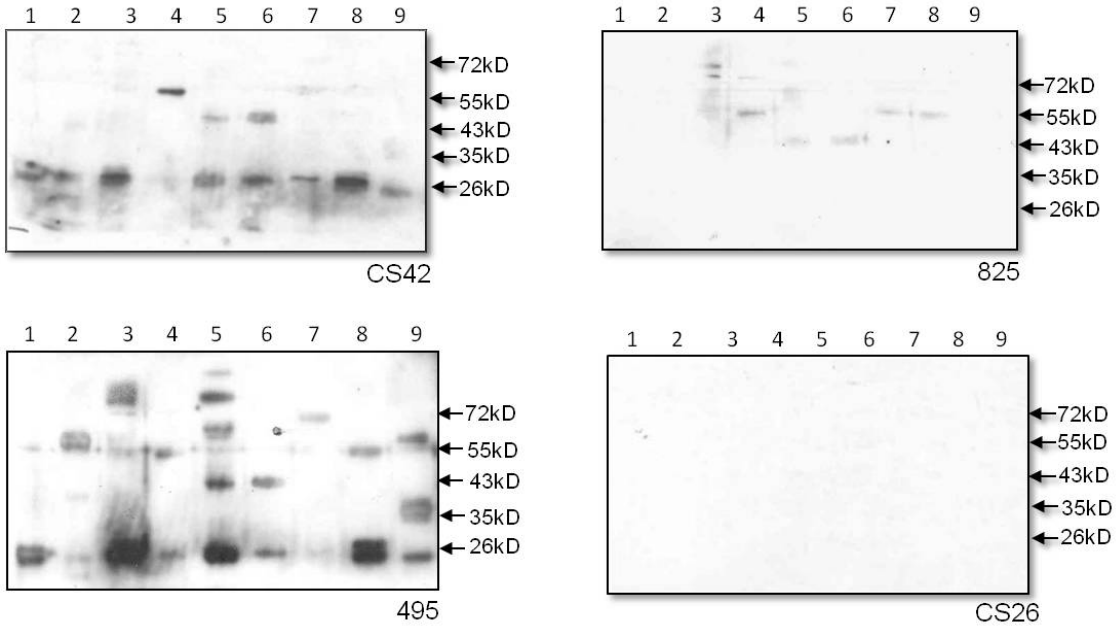


Fig: 3.38: Autographic profiles of 1DE resolved recombinant proteins probed with IgG from patient sera and normal sera. lane1: GST, lane 2: GST-AR, lane 3: GST-PKM, lane 4: GST-Prdx6, lane 5: GST-Hsp70, lane 6: GST-Ppia, lane 7: GST-Eno, lane 8: GST-PGM, lane 9: GST-Anx2. The sera used are given at the lower right hand corner of each blot.

It was also observed that some of the proteins degraded on storage. Fig: 3.39 shows the profile of the recombinant proteins used for dot blotting and the status of the same proteins after 24 days at 4° C. GST-Tubb, GST-Hsp 70, GST-Anx2, GST-Pgm, GST-PKM2, GST-Prdx6 and GST-Ppia were affected the most. The degradation would affect the antibody reactivity and in turn the signal intensity. Efforts were made to store the proteins in glycerol so that freeze thaw of proteins can be avoided as it was one of reasons for precipitation of proteins. Protease inhibitor was also added in all the purified protein preparations to avoid futher degradation of proteins stored at 4° C. However this did not prevent the degradation.

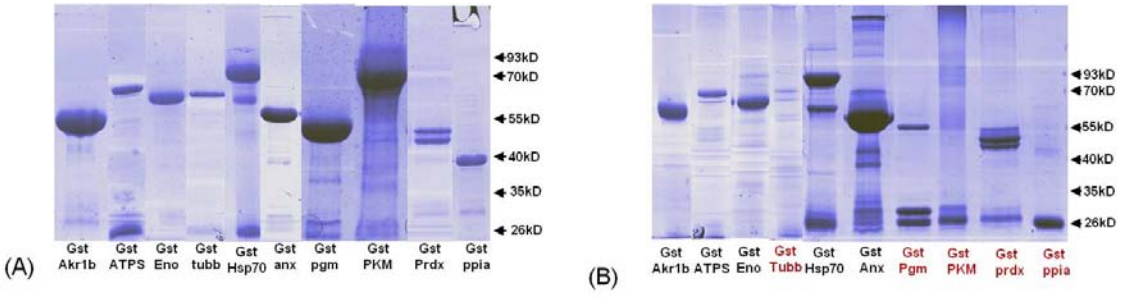


Fig. 3.39: SDS-PAGE profile of purified recombinant proteins. (A) Purified recombinant proteins used for dot blot analysis (B) Purified proteins after 24 days at 4°C

3.4.2 ELISA for α Enolase and Hsp70:

As there are inherent challenges associated with manual spotting and handling dot blots it was decided to assess the autoantibody response by ELISA. To avoid problems associated with the presence of free GST and degradation of proteins as seen in the dot blot experiments, thrombin cleaved GST-free recombinant proteins were prepared and used for the ELISA.

It is seen in the earlier Section that the autoantibody response to only α enolase and Hsp70 detected in sera by immunoproteomics showed significant correlation with the disease free survival of the patients in the Kaplan Meir survival analysis. Therefore only these two proteins were used for ELISA and Multiwestern analysis.

3.4.2.1 Assessment of Autoantibody response by ELISA:

3.4.2.1.1 Assessment of autoantibody response in sera from individuals with chewing/smoking habit and individuals with leukoplakia:

Autoantibody response to alpha enolase was evaluated in sera of individuals with leukoplakia (n=30) and individuals with and without chewing/smoking habit (n=30 each). Fig. 3.40 shows the scatter plot for OD values obtained with ELISA. The

autoantibody response was slightly higher in individuals with leukoplakia (0.29 ± 0.09) as compared to healthy individuals without (0.28 ± 0.07) and with chewing /smoking habit (0.25 ± 0.1). Median OD value of control sera 0.29 was set as a cut off value for determining sero positivity for α enolase autoantibodies.

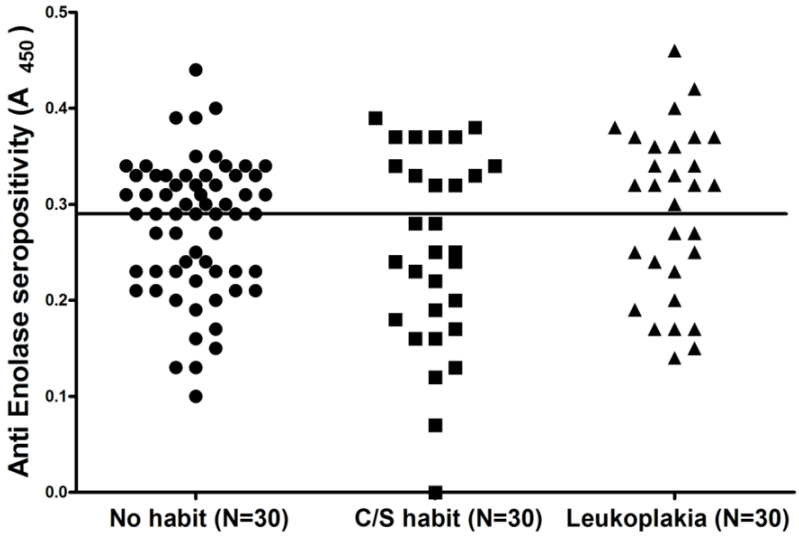


Fig. 3.40: Scattergram of the ELISA values obtained for autoantibody response to recombinant alpha enolase in sera samples of healthy individuals with and without chewing/smoking habit (C/S) and individuals with leukoplakia. The horizontal line represents the median OD value of control sera samples which is also the cut off for seropositivity.

The autoantibody response detected with ELISA in sera of individuals with chewing /smoking habit and individuals with Leukoplakia was compared with autoantibody response detected in 2D immunoproteomics analysis. Table 3.6 below shows the comparison of seropositivity of autoantibodies to α enolase by 2DE and ELISA. With ELISA 12/30 (40%) of chewing/smoking habitues and 17/30 (57 %) individuals with leukoplakia were found positive for antibody to α enolase. ELISA results for sera antibody positivity matched with 2D results in 16/30 (53%) of chewing/smoking habitues and 15/30 (50%) of the leukoplakia samples.

Chewing /smoking habit	ELISA (Enolase)	2D (Enolase)	Leukoplakia	ELISA (Enolase)	2D (Enolase)
AH1	–	–	A3	+	–
AH2	–	–	A4	+	+
AH3	–	–	A5	+	–
AH4	–	–	A6	+	–
AH5	–	+	A7	+	+
AH6	+	+	A8	–	–
AH7	+	–	A9	+	+
AH8	–	+	A10	–	+
AH9	–	–	A11	–	+
AH10	–	–	A12	–	–
AH11	+	–	A13	–	–
AH12	+	–	A14	+	–
AH13	+	–	A15	–	+
AH14	–	+	A16	+	–
AH15	+	+	A17	–	–
AH16	–	+	A18	–	+
AH17	–	–	A19	+	+
AH18	+	+	A20	+	–
AH19	+	–	A21	–	–
AH20	+	–	A22	–	–
AH21	–	+	A23	+	–
AH22	–	–	A24	+	–
AH23	+	+	A25	+	+
AH24	–	–	A26	+	+
AH25	+	–	A28	–	–
AH26	–	+	A30	+	+
AH27	+	+	A31	–	+
AH29	–	–	A32	+	–
AH28	–	–	A33	+	–
AH30	–	+	A34	–	–

Table 3.6: Comparison of antibody response to enolase detected by ELISA with that detected with 2D Western

3.4.2.1.2 Assessment of autoantibody response in sera from patients with cancer of GBC and healthy individuals:

Autoantibody response for recombinant alpha Enolase and Hsp70 was evaluated in sera of 86 patients with cancer of GBC, 68 of those evaluated by immunoproteomics in this thesis and 18 new sera. Autoantibody response to α enolase was also evaluated in sera from 63 normal healthy individuals of which 26 samples were used for the immunoproteomics analysis published earlier [151]. Median OD value of 0.29 for the

control sera was set as a cut off value for α enolase autoantibodies. For α enolase, 44/86 (51%) tumor samples and 30/63 (48%) healthy individual samples were sero positive. This provided sensitivity and specificity of 51% and 52% respectively. Fig. 3.41 shows the scattergram of the values obtained. There was no difference in the mean serum levels of autoantibodies to α enolase in patients and healthy individuals (0.29 ± 0.64 vs. 0.28 ± 0.7 respectively).

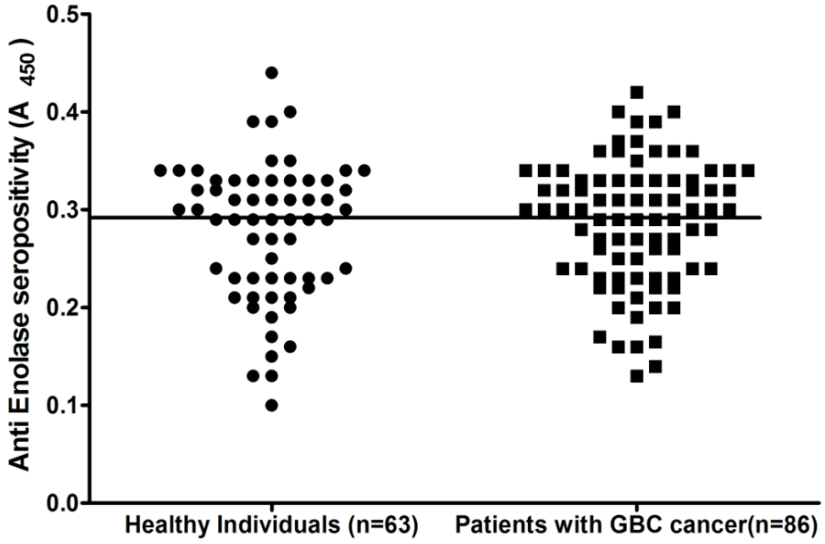


Fig. 3.41: Scattergram of the ELISA values obtained for autoantibody response to recombinant alpha enolase in sera samples of patients and healthy individuals Data points represent the normalised OD for each sample. The horizontal line represents the median OD value of control sera samples which is also the cut off for seropositivity.

Fig. 3.42 shows the scattergram of the ELISA values for autoantibody response to Hsp70. Median OD value of control sera 0.18 was set as cut off value for Hsp70 autoantibodies. For Hsp70, 31/86 (36%) and 30/67 (44%) were sero positive, providing a sensitivity and specificity of 36% and 55% respectively. There is no difference in the mean level of serum autoantibodies to Hsp70 in between patients sera and healthy individual sera (0.15 ± 0.14) vs (0.18 ± 0.14).

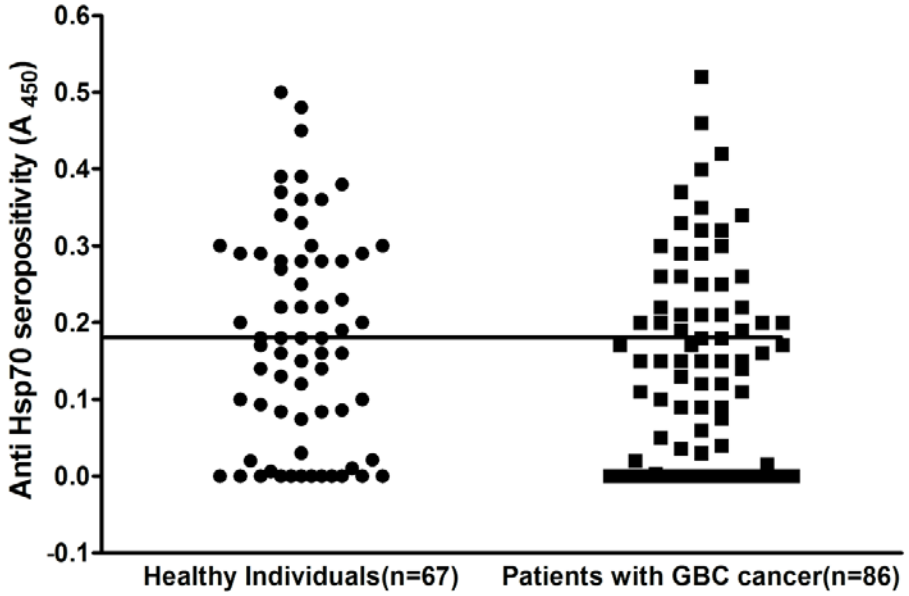


Fig. 3.42: Scattergram of the ELISA values obtained for autoantibody response to recombinant Hsp70 in sera samples of patients and healthy individuals Data points show the normalised OD the horizontal line represents the median OD value of control sera samples which is also the cut off for seropositivity.

3.4.2.2 Association of autoantibody response to α ENO and HSP70 by ELISA with disease free survival (DFS)

Fig. 3.43: shows the survival curve with the association of autoantibodies to α ENO and DFS with p = 0.517. The α ENO sera positivity was also correlated with DFS in different subgroups of patients based on the size of tumor. Patients with T3/T4 tumors who were positive for autoantibody response to α ENO showed better association with DFS at p=0.260 (Fig.3.43 C) which was not seen in patients with T1/T2 tumors (p=0.708), Fig. 3.43 B. Similar analysis with autoantibody response to Hsp70 showed that these patients did not show any significant trend. Presence of autoantibody response to Hsp 70 showed a positive trend to DFS (p =0.359).

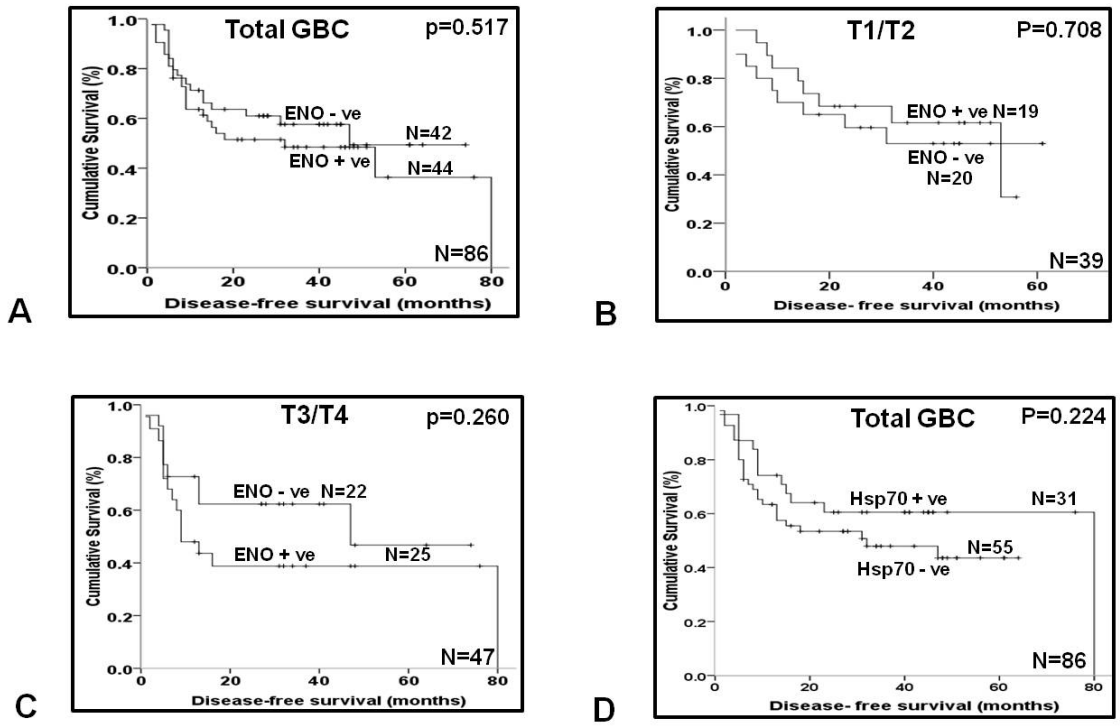
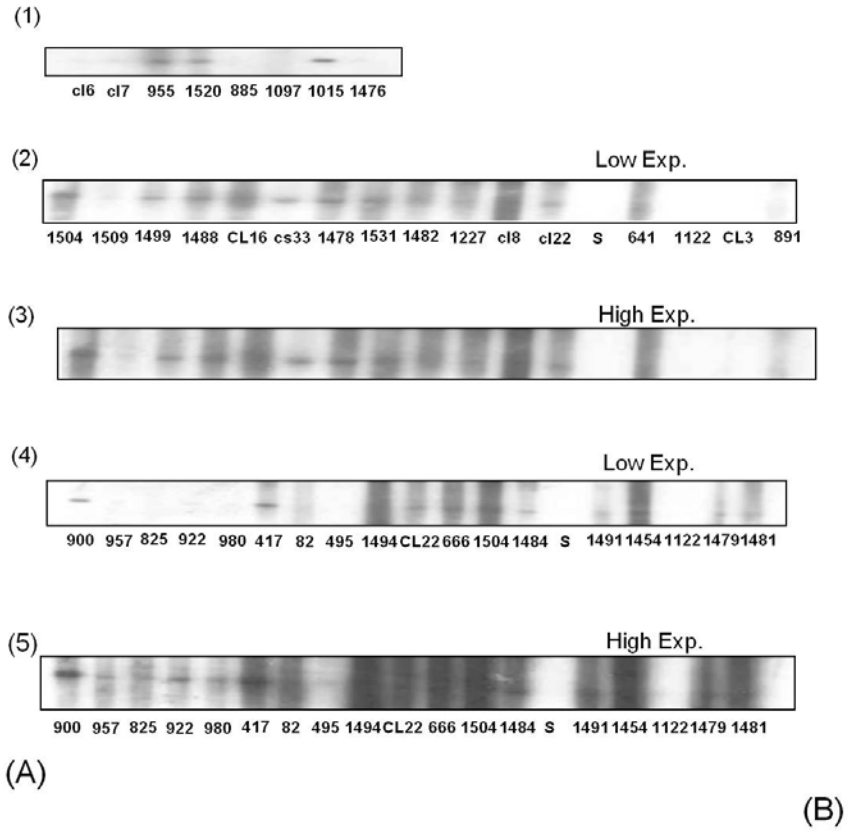


Figure 3.43: Kaplan-Meier curves with univariate analysis (log rank) for correlation of cumulative survival and DFS(in months) as assessed by ELISA for **A)** patients with cancer of GBC (T1/T2 and T3/T4 patients taken together) either negative or positive for autoantibody response to α ENO, **B)** patients with T1/T2 tumor of cancer of GBC either negative or positive for autoantibody response to α ENO, **C)** patients with T3/T4 stage of cancer of GBC either negative or positive for autoantibody response to α ENO and **D)** patients with cancer of GBC either negative or positive for autoantibody response to Hsp70. The number (N) of patients is indicated

3.4.3 Evaluation of autoantibody to response recombinant alpha Enolase and Hsp70 using Multiwestern analysis:

Free GST proteins present in some of the recombinant GST-fusion protein preparations showed reactivity with some sera samples and these interfered in the evaluation of antibody response to the recombinant proteins. Therefore recombinant proteins without the GST tag were prepared for Hsp70 and α Enolase by thrombin cleavage and used for ELISA. In ELISA and dot blot analysis proteins were in their native conformation where as in immunoproteomics analysis KB lysate proteins were in linear form. To eliminate this difference of state of protein, multiwestern analysis was performed with

the recombinant proteins α Enolase and Hsp70. Fig. 3.44 A(1-4) shows the autographs of blots screened with different sera using a Mini-PROTEAN II multiscreen apparatus for the presence of autoantibody to alpha enolase. Fig.3.44A (2 and 3) and Fig 3.44A (4 and 5) are low and high exposures of the same blots. These exposures were taken to confirm the signals. The data obtained is summarized in the accompanying table in Fig. 3.44 B .



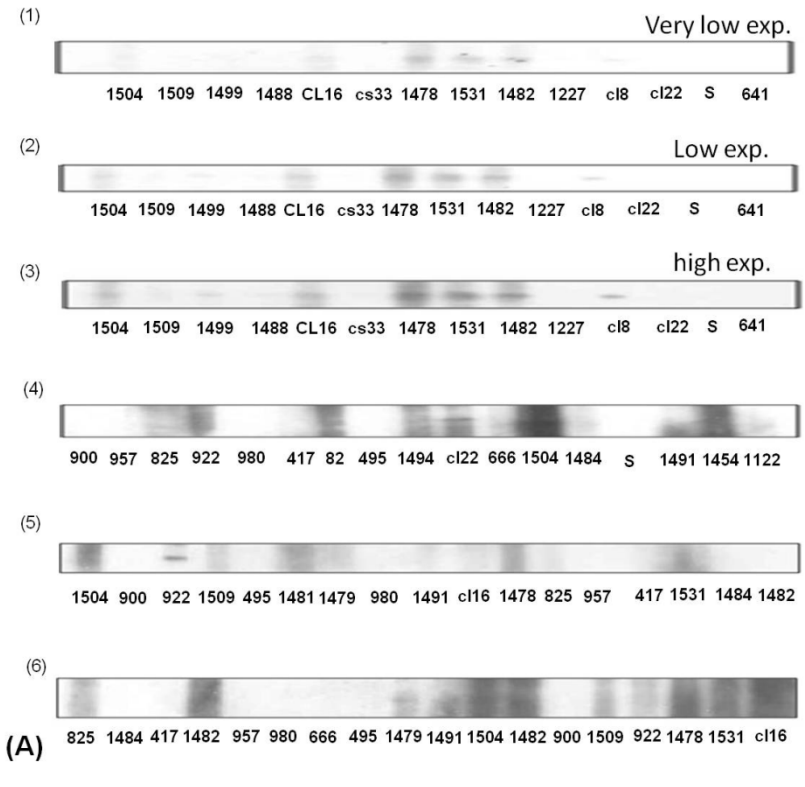
(B)

Samples	1D	High. Exp.	2D
CL6	–		–
CL7	–		–
955	+		+
1520	+		+
885	–		–
1097	–		–
1015	+		+
1476	–		+
1504	+	+	–
1509	–		–
1499	+	+	+
1488	+	+	+
CL16	–	–	–
CS33	+	+	+
1478	+	+	+
1531	+	+	–
1482	–	–	–
1227	–	–	–
CL8	–	–	–
CL22	+	+	–
641	–		–
1122	–		+
CL3	–		–
891	–		–
900	+	+	+
957	–	+	+
825	–	+	+
922	–	+	+
980	–	+	+
417	+	+	+
82	–	–	+
495	–	–	+
1494	–	–	+
666	+		+
1484	+		+
1491	+		+
1454	+		+
1479	–		–
1481	+		–

Fig: 3.44: Screening of sera samples for autoantibody to α enolase by multi western analysis: (A) Autographs of blots with equal amount of recombinant α enolase in each lane probed simultaneously with different sera samples at 1:200 dilution (B) Table shows the summary of reactivity of each sera with the recombinant α enolase in Multiwestern analysis (1D).

Fig. 3.45: shows the autographs of blots screened with sera on a Mini-PROTEAN II multiscreen apparatus for the presence of autoantibody to Hsp70. For one set of samples multiple exposures were taken and these are shown in Fig 3.45 A1, A2 and A3.

To verify whether the signal obtained is affected by the position of the protein lane on the blot, the sera used in blots shown in (A1, A2 and A3 above) were used to probe different lanes of the protein on the blots as is shown in (A4, A5, and A 6). Table in Fig. 3.45 B shows the summary of reactivity of Hsp70 with each serum. Total 39 sera samples were screened for antibody response to alpha enolase and 29 samples were screened for antibody response to Hsp70 by multiwestern analysis. Samples positive as well as negative for antibody response to alpha enolase/ Hsp 70 in 2D analysis were used in these experiments... In multiwestern analysis 23/32 (72%) tumor samples and 6/7 (86%) healthy individual samples showed similar result for alpha enolase as was obtained in 2DE analysis. For Hsp70, 13/25 (52%) tumor samples and 3/4 (75%) healthy individual samples show similar results to that obtained by 2DE analysis. There was background problem with many of sera such as 1478, 1531 and 1482 when these samples were repeated due to which signals were not visible clearly. Experiments were repeated but there was no improvement in the results. Results with alpha enolase and Hsp70 show that an optimal exposure is required to see distinct signals and this varies with each sera. Further the position of the lane on the blot does not affect the quality of the signal.



Samples	1D	2ND Exp.	3rd Exp.	2D
1504	+	D	D	+
1509	–	D		+
1499	+			+
1488	–			+
CL16	+	–	D	–
CS33	–			–
1478	+	D	D	–
1531	+	D	D	–
1482	+	D		–
1227	–			–
CL8	+			+
CL22	–	+		–
641	–			–
900	–	–		+
957	–	–		+
825	–	–		+
922	+	–		+
980	–	–	–	+
417	–	–	–	–
82	+			+
495	–	–		+
1494	–			–
666	–			–
1484	–	–		–
1491	–	–		–
1454	–			–
1122	+			–
1481	–			–
1479	–	+		–

(B)

Fig. 3.45: Screening of sera samples for autoantibody to Hsp70 by multi western analysis: (A, 1 to 6) Autographs of blots with equal amount of recombinant Hsp70 in each lane probed simultaneously with different sera samples at 1:200 dilution (B) Table showing the summary of reactivity of each sera with the recombinant Hsp70 in multiwestern analysis. 2nd Exp.: 2nd Exposure; 3rd Exp.: 3rd Exposure, 2D: Data from 2DE western blotting. D- dark.

3.4.4 Comparative analysis of autoantibody profile generated with three different techniques:

Table 3.7 shows a comparative analysis of the autoantibody response obtained using 2DE and IDE Multiwestern and ELISA with the patient IgG/sera.

Comparison of the ELISA and 2DE data showed that for α enolase, 39/68 (57%) of the tumor and 36/63 (57%) healthy individuals samples showed similar result, while for Hsp 70, 42/68 (62%) of the tumor samples and 32/67 (48%) of the healthy individuals samples showed similar results between the two techniques. The differences observed may be attributed to the difference in epitope presentation due to different state of

proteins in the two techniques.

		2D	1D	ELISA	2D	1D	ELISA
	Samples	Enolase	Enolase	Enolase	Hsp70	Hsp70	Hsp70
1	CL3	–	–	–	–		+
2	CL4	+		+	–		–
3	CS31	–		+	–		–
4	CS27	–		–	–		+
5	CS50	–		+	–		+
6	CS16	–		–	–		–
7	CL7	–	–	+	–		–
8	CS47	–		–	–		–
9	CL44	+		+	–		–
10	CS18	–		+	–		+
11	CS3	+		–	–		–
12	CS19	–		–	–		+
13	CL17	+		+	–		–
14	CS40	–		–	–		–
15	CS5	–		–	–		–
16	CS35	–		–	–		+
17	CL33	+		+	–		–
18	CL42	–		+	–		+
19	CL16	–	–	–	–	+	+
20	CL22	–	+	+	–	–	+
21	CL6	–	–	+	–		–
22	CL27	–		–	–		–
23	CS2	+		+	–		–
24	CS4	–		–	–		+
25	CS42	–		–	–		+
26	CS23	–		+	–		+
27	CS13	+		–	–		–
28	CL39	–		–	–		–
29	CS48	–		–	–		+
30	CS22	–		–	+		–
31	CS46				–		–
32	CS12				–		+

Table 3.7: Comparison of autoantibody response to α enolase and Hsp70 detected in different sera by 2D western, 1D multiwestern and ELISA, + sign denote serum positivity for the autoantibody response to the antigen while – sign denote serum negativity for the autoantibody response to the antigen.

		2D	1D	ELISA	2D	1D	ELISA
	Samples	Enolase	Enolase	Enolase	Hsp70	Hsp70	Hsp70
33	CS34	–		+	–		+
34	CL12	–		+	–		+
35	CS26	–		–	–		–
36	CS20	–		+	–		–
37	CL48	–		–	–		+
38	CS8	–		–	–		+
39	CL13	–		–	–		–
40	CS28	–		–	–		+
42	CS15	–		+	–		+
42	CS10	–		+	–		+
43	CL47	–		+	–		+
44	CS30	–		–	–		–
45	CL8	–	–	–	+	+	–
46	CL19	–		–	–		–
47	CS7	+		–	–		–
48	CS9	–		+	–		+
49	CS25	–		–	–		–
50	CL14	+		+	–		+
51	CL32	–		–	–		–
52	CL2	–		–	–		–
53	CS35	–		+	–		+
54	CS14	+		+	–		–
55	CS32	–		–	–		–
56	CS17	–		+	–		–
57	CS33	+	+	–	–	–	–
58	CS45	–		+	–		+
59	CS43	–		+	–		+
60	CS36	–		+	–		–
61	CS6	+		+	–		–
62	CL15	–		+	–		–
63	CL21	–		+	–		+
64	CS29	–		–	–		–
65	CS41	+		–	–		+
66	CS37				–		–
67	CL34				–		+

Table 3.7 cont.: Comparison of autoantibody response to α enolase and Hsp70 detected in different sera by 2D western, 1D multiwestern and ELISA, + sign denote serum positivity for the autoantibody response to the antigen while – sign denote serum negativity for the autoantibody response to the antigen.

		2D	1D	ELISA	2D	1D	ELISA
	Samples	Enolase	Enolase	Enolase	Hsp70	Hsp70	Hsp70
1	642	+		+	–		–
2	891	–	–	–	–		–
3	955	+	+	–	–		–
4	974	+		–	–		–
5	1024	–		–	–		+
6	1038	+		+	–		+
7	1060	–		+	–		
8	1097	–	–	–	–		–
9	1454	+	+	–	–	–	–
10	1476	+	–	+	–		–
11	1478	+	+	+	–	+	+
12	1481	+	–	+	–		–
13	1482	–	–	+	–	+	–
14	1488	+	+	+	+	–	+
15	1491	+	+	+	–		–
16	1499	+	+	+	+	+	+
17	1504	–	+	–	+	+	–
18	1520	+	+	–	–		–
19	1531	–	–	–	–	+	+
20	82	+	–	–	+	+	–
21	660	+			+		–
22	825	+	–	–	+	–	–
23	495	+	–		+	–	–
24	417	+	+	+	–	–	–
25	365	+		–	+		–
26	756	+		+	+		–
27	358	–		+	–		+
28	867	+		+	–		–
29	922	+	+	–	+	+	+
30	925	+		–	–		–

Table 3.7 cont. : Comparison of autoantibody response to α enolase and Hsp70 detected in different sera by 2D western, 1D multiwestern and ELISA, + sign denote serum positivity for the autoantibody response to the antigen while – sign denote serum negativity for the autoantibody response to the antigen.

		2D	1D	ELISA	2D	1D	ELISA
S.No.	Samples	Enolase	Enolase	Enolase	Hsp70	Hsp70	Hsp70
31	670	+		+	+		+
32	980	+	–	+	+	–	+
33	1006	–		–	–		
34	900	+	+	+	+	–	–
35	957	+	+	+	+	–	–
36	1535	–			+		
37	1411	+		–	–		–
38	1419	+		+	–		–
39	641	–	–	+	–	–	+
40	648	+		+	–		–
41	879	+		+	–		+
42	885	–	–	–	–		–
43	1015	+	+	+	–		–
44	1035	–		+	–		
45	1064	–		–	–		–
46	1122	+	–	–	–	–	–
47	1151	+		–	–		–
48	1175	–		–	–		–
49	1227	–	–	+	–	–	–
50	1414	+		+	–		–
51	1420	–		+	–		+
52	1484	+	+	+	–	–	+
53	1486	+		–	–		+
54	1494	+	–	–	–	–	+
55	1495	–		–	–		+
56	1509	–	–	+	+	–	+
57	1522	–		–	–		–
58	1523	–		+	–		+
59	1527	–		–	–		–
60	1528	+		–	–		+

Table 3.7 cont.: Comparison of autoantibody response to α enolase and Hsp70 detected in different sera by 2DE western, 1DE multiwestern and ELISA, (+) sign denote serum positivity for the autoantibody response to the antigen while (–) sign denote serum negativity for the autoantibody response to the antigen.

		2D	1D	ELISA	2D	1D	ELISA
	Samples	Enolase	Enolase	Enolase	Hsp70	Hsp70	Hsp70
61	1533	–		–	–		–
62	493	+		+	–		–
63	697	–			–		+
64	666	+	+	+	–	–	–
65	623	+		+	–		–
66	902	+		+	–		+
67	420	–			–		
68	451	–			–		
69	883	+			–		
70	766	+			+		
71	460	+		–	+		+
72	1010	+			+		
73	1149	–		–	–		–
74	1448	+			+		
75	1467	+		–	–		–
76	1480	+		–	–		–
77	1483	+		–	–		+
78	1479	–	–	+	–		–

Table: 3.7 cont. Comparison of autoantibody response to α enolase and Hsp70 detected in different sera by 2D western, 1D multiwestern and ELISA, (+) sign denote serum positivity for the autoantibody response to the antigen while (–) sign denote serum negativity for the autoantibody response to the antigen.

Comparison of 2DE and Multiwestern analysis:

In order to minimize the difference of epitope presentation and also use another option for multiplexing, autoantibody to recombinant α enolase and HSP70 was evaluated in sera by Multiwestern analysis. In this analysis 23/32 (72%) of the tumor samples and 6/7 (86 %) of the healthy individuals samples showed similar result for α enolase as was obtained in 2DE analysis. Further the intensity of signals obtained in the two techniques was similar for greater percentage of the samples thereby validating our 2DE autoantibody results for α enolase to a large extent. For HSP70, 13/25 (52%) tumor samples and 3/4 (75%) healthy individuals samples show similar results to that obtained by 2DE analysis.

Chapter 5

Discussion

4.0 DISCUSSION:

Autoantibody response against tumor antigens has been receiving attention for its utility in early detection and prognosis of cancer. There are only a few reports which evaluate the autoantibody response in oral / Head and Neck cancers and in these studies attention has not been given to subsites of the head and neck region [147-150, 158] . The work in this thesis investigates the utility of autoantibody response to the tumor antigens Alpha enolase, Annexin II, HSP 70, Peroxiredoxin VI, ATP synthase, Alpha tubulin, Beta tubulin, Pyruvate kinase, Triose phosphate isomerase, Phosphoglycerate mutase, Aldose reductase and Cyclophilin A, reported earlier from our laboratory [151], for early detection and prognosis of cancer in a subsite of the oral cavity i.e. GBC.

The immune response to tumor associated antigens occurs early, as our immune system is capable of sensing even those alterations which appear during early tumorigenesis. Several studies have explored the utility of autoantibodies in the early detection of cancer and shown that autoantibody response is seen months before the onset of disease [100-102, 104].

To investigate the utility of the identified TAAs in ***early detection*** autoantibody response was evaluated in healthy individuals with chewing/smoking habit who are considered high risk individuals for cancer of the GBC and individuals with leukoplakia, patients with T1/T2 size tumors and T3/T4 size tumors of the GBC. The percentage autoantibody response for several of the antigens increased from individuals without cancer to those with cancer. Significant correlation of the autoantibody response to alpha Eno b, alpha Eno c , Anx 2 and Tubb was seen between healthy individuals without chewing/smoking habit and those with leukoplakia indicating that

these antigens have promising potential in the early detection of cancer. Significant correlation was also seen for autoantibody response to alpha Eno b, alpha Eno c, HSP70, Peroxiredoxin 6, PKM, ATP synthase, Tubb and AR2 between healthy individuals and patients with T1/T2 tumors. The autoantibody response to all the antigens correlated significantly between healthy individuals and patients with T3/T4 tumors showing that autoantibody response increases as the disease progresses.

The data obtained demonstrates the early occurrence of autoantibodies in circulation and the need for further validation on large number of samples along with their follow up.

There are several studies evaluating autoantibody response vis a vis disease progression or prognosis. Mudenda at el have shown that the presence of autoantibodies to p53 correlate positively with high histological grade of breast cancer [112]. Chang et al studied autoantibody response against survivin in 134 patients with head and neck cancer and found survivin antibodies in 33% patients with stage I & II while 51% patients with stage III & IV showed survivin antibodies. Autoantibodies to survivin were seen in 38 %, 46 % and 65 % of patients with well, moderately and poorly differentiated tumor respectively [159]. Mitsudomi et al found that high incidence of p53 autoantibodies is associated with high histological grade of non-small cell lung cancer but not with patient survival. They observed p53 autoantibodies in 14 % of early stage group (Stage I and II) and 30 % of advanced disease group (Stage III and IV) [160]. A similar trend was observed in our study where autoantibody response to α Enolase b and Hsp70 is significantly higher in patients with T3/ T4 tumors as compared to those with T1/T2 tumors showing correlation with the tumor size.

Evaluation of ***prognostic utility*** in this study shows that patients positive for autoantibody response to α ENO (α Enolase a, b and c taken together) and/or or Hsp70 showed faster recurrence compared to those who do not show autoantibody response to either of them. Further it is seen that autoantibody response to α ENO and Hsp70 is an additional parameter which in combination with nodal involvement and/or differentiation status of tumor improves the prognosis of cancer of the GBC. Multivariate analysis shows that the relative risk is 3.41 for patients who exhibit autoantibody response to both the antigens. This information could possibly be used by the clinician to modify treatment strategies according to the aggressiveness of the tumor.

Alpha enolase is reported to be multifunctional [161]. It can exist both in the cytoplasm as well as on the cell surface. A truncated version of α enolase MBP-1 is also expressed, which is localized in the nucleus and acts as a transcription repressor by binding to *c-Myc* promoter. Cell surface alpha enolase apart from its catalytic activity may also act as a plaminogen receptor. Binding of plasminogen to α enolase leads to its activation to plasmin by proteolytic cleavage. In breast, lung and pancreatic neoplasia, α enolase is localized on the surface of cancer cells where it may act as a plasminogen receptor [88, 162, 163]. The exact mechanism involved in the production of autoantibodies to α enolase in oral cancer is presently unclear. We have earlier reported that expression of α-enolase is increased in cancer of GBC [164] which may be a reason for the generation of autoantibody response. Tsai et.al [165] have also shown *ENO1* overexpression as a prognostic marker in head and neck cancer.

Autoantibody responses to alpha enolase have been reported in cancers of the pancreas [162], leukemia [97], melanoma [166] , head and neck/oral cavity [151, 152], breast [167] and lung [88, 96] . In a recent study, Tomaino et. al have reported that in pancreatic cancer patients, autoantibodies to ENOA are directed against two upregulated isoforms phosphorylated on Ser 419 [168].

Hsp 70 is another multifunctional protein which plays a role in regulating protein quality and turnover in normal conditions as well as under stress. Hsp 70 is reported to be overexpressed in the cancer of head and neck and oral cavity [169]. Autoantibodies to Hsp 70 have been reported in cancer of the esophagus [158], lung [170] , liver [171] , oral cavity and head and neck [146] and leukemia [97]. In a recent review Murphy et al [172] have observed that due to the changing repertoire of the antigens as the tumor progresses, there is probably a constant qualitative and quantitative change in the auto antibody response to an antigen. A similar trend is seen in this study where autoantibody response to Hsp70 detected in sera of patients with cancer of GBC correlated significantly both with the size and stage of tumor and was more in patients with advanced stage of tumor.

There are divergent reports of the prognostic relevance of Hsp70 in oral cancer [173]. Our study shows the prognostic relevance of Hsp70 wherein there is a decrease in DFS of patients showing autoantibody response to Hsp70..

Autoantibodies have been either reported to improve the prognosis of cancer patients, to worsen the clinical outcome, or even to be irrelevant for the course of the disease. For example autoantibodies to p53 have shown variable correlations with disease outcome

in different cancers. Lubin et al and Tang et al have shown autoantibody response to be associated with high-grade tumors and poor survival in breast, colon, oral, and gastric cancers [174, 175] while in another study, Saffroy et al have shown correlation of autoantibody response with enhanced overall survival in HCC patients [125]. It was anticipated that as the disease progressed the host immune surveillance mechanisms would elicit an antibody response as a measure to counter the progressing cancer and there should have been a direct reflection of this with an increase in the DFS status. It is apparent that the scenario is more complex and as described by Tan and Coussens in their review [45] enhanced humoral immunity (HI) and inflammation in combination with suppressed cell mediated immunity (CMI) are responsible for the pathogenenesis of several human cancers. Further studies are therefore required to understand the observations in this study.

It is now well accepted that a single antigen has low sensitivity for detection of cancer and there is a need of panel of biomarkers for better diagnosis and prognosis of cancer. Multiplex protein array provides the suitable platform to screen autoantibody response against large number of tumor antigens simultaneously. Towards the generation of multiplex antigen array, recombinant proteins of the identified tumor antigens were prepared and used in dot blot analysis for the evaluation of autoantibody in sera to the purified recombinant proteins. In dot blot analysis some of the control showed strong reactivity with some of the proteins which was not observed by 2D immunoproteomics. On further investigation it was found that the free GST protein present in many of the recombinant protein preparations was reacting non specifically with some of the sera.

To overcome challenges related to manual spotting, presence of free GST protein, and degradation of proteins ELISA was done for quantitative evaluation of autoantibody response against α enolase and HSP70 in serum samples of patients.

Analysis of the ELISA results for autoantibodies to recombinant enolase and Hsp70 did not match exactly with the autoantibody data evaluated with 2D immunoproteomics. Comparison of the ELISA and 2DE data showed that for α enolase, 57% of the tumor and 57% control samples showed similar result. Comparison of the ELISA and 2DE data showed that for Hsp 70, 62% of the tumor samples and 48% of the healthy individuals samples showed similar results between the two techniques. The differences observed may be attributed to the difference in epitope presentation due to different state of proteins in the two techniques.

It is seen that the autoantibody response to alpha enolase as evaluated by ELISA also shows a similar trend of correlation with prognosis of disease though it is not as significant as was found with 2DE western based autoantibody profile. Evaluation of autoantibody response to HSP70 by ELISA showed a trend of positive correlation with DFS. Therefore it is apparent that each antigen has an unique behaviour when its reactivity with sera in native state on an ELISA plate and linear denatured form as on 2DE resolved membrane blotted protein are compared.

In 2DE immunoproteomics, autoantibody positivity was assigned on the basis of presence and absence of signal on the autograph while in ELISA, autoantibody seropositivity was assigned on the basis of the cut off value which in this case was the median value of OD of normal samples. Also the two techniques differ largely in

respect to the state of protein used for autoantibody evaluation. In 2DE analysis proteins were linear and well resolved. In this state the epitopes may be more exposed while in ELISA, recombinant fusion proteins were used and they were in their native 3D conformation and same epitopes may not be equally exposed. Similar kind of observation was reported by Murphy et. al [176] in which they showed that the antigen presentation is an important determinant for detection of antigen by antibody. This may be one of the causes for the difference in detection in addition to absence of post translational modification (PTM) on the protein.

Multiwestern immunoblot analysis was used to minimize issues related to the structural difference of the proteins used for antibody evaluation. In this analysis 72% of the tumor samples and 86 % of the healthy individuals samples showed similar result for α enolase as was obtained in 2DE analysis. Further the intensity of signals obtained in the two techniques was similar for greater percentage of the samples thereby validating our 2DE autoantibody results for α enolase to a large extent. For HSP70, 52% tumor samples and 75% healthy individuals samples showed similar results to that obtained by 2DE analysis. These observations strongly indicate that epitope presentation may be an important parameter for autoantibody evaluation when different techniques are used.

It is apparent therefore that the sensitivity of detection of autoantibody response depends on the technique used to assess it since the immune response could be to a sequence in the linear protein or to an epitope presented as a consequence of the folding of the native protein [176]. These factors along with differential expression of an antigen, its post translational modifications and its relocation would provide a complex pattern which has to be deciphered to explain the autoantibody response profiles

obtained for the different antigens. The observations in this thesis provide an explanation in part for failure of validation of many tumor markers predicted as strong candidates on the basis of a single technique.

In summary, the work in the thesis has identified antigens which elicit autoantibody response with potential for early detection and prognosis in oral cancer. It has also identified the parameters which need attention while generating multiplex antigen arrays for validation of data in large sample sizes for translation into the clinics.

Bibliography

References:

1. *http://www.cancer.gov/researchandfunding/priorities/global-research-activities*.

2. Hartwell, L., et al., *Cancer biomarkers: a systems approach.* Nat Biotechnol, 2006. **24**(8): p. 905-8.

3. *http://www.sanger.ac.uk/genetics/CGP/Census/analysis.shtml*.

4. Ferlay, J., et al., *GLOBOCAN 2008,Cancer Incidence and Mortality Worldwide : IARC Cancerbase No. 10 [Internet]. Lyon, France: International Agency for Research on Cancer; 2010* Available from: http://globocan.iarc.fr, accessed on 27/06/2012.

5. Dinshaw, K. and B. Ganesh, *Hospital Based Cancer Registry, Annual report 2002-2005,Tata Memorial Hospital*, 2008.

6. Watkinson, J., M. Gaze, and J. Wilson, *Tumours of the lip and oral cavity.* Stell and Maran's head and neck surgery. 4th edn. Oxford, Butterworth-Heinemann, 2000: p. 275-318.

7. Pindborg, J., et al., *Oral submucous fibrosis as a precancerous condition.* European Journal of Oral Sciences, 1984. **92**(3): p. 224-229.

8. Murti, P., et al., *Malignant transformation rate in oral submucous fibrosis over a 17-year period.* Community dentistry and oral epidemiology, 2006. **13**(6): p. 340-341.

9. Murti, P., et al., *Etiology of oral submucous fibrosis with special reference to the role of areca nut chewing.* Journal of oral pathology & medicine, 2006. **24**(4): p. 145-152.

10. *http://www.cancer.gov/cancertopics/pdq/treatment/lip-and-oral-cavity/Patient/page1*.

11. Worsham, M.J., *Identifying the risk factors for late-stage head and neck cancer.* Expert Rev Anticancer Ther, 2011. **11**(9): p. 1321-5.

12. Argiris, A., et al., *Head and neck cancer.* The Lancet, 2008. **371**(9625): p. 1695-1709.

13. Simental, A.A., et al., *Selective neck dissection in patients with upper aerodigestive tract cancer with clinically positive nodal disease.* ANNALS OF OTOLOGY RHINOLOGY AND LARYNGOLOGY, 2006. **115**(11): p. 846.

14. Ferlito, A., et al., *Elective and therapeutic selective neck dissection.* Oral oncology, 2006. **42**(1): p. 13-24.

15. Lam, L., et al., *Retrospective study of survival and treatment pattern in a cohort of patients with oral and oropharyngeal tongue cancers from 1987 to 2004.* Oral oncology, 2007. **43**(2): p. 150-158.

16. Califano, J., et al., *Genetic progression model for head and neck cancer: implications for field cancerization.* Cancer research, 1996. **56**(11): p. 2488-2492.

17. Leemans, C.R., B.J. Braakhuis, and R.H. Brakenhoff, *The molecular biology of head and neck cancer.* Nat Rev Cancer, 2011. **11**(1): p. 9-22.

18. Chung, C.H., et al., *Molecular classification of head and neck squamous cell carcinomas using patterns of gene expression.* Cancer Cell, 2004. **5**(5): p. 489.

19. Chung, C.H., et al., *Gene expression profiles identify epithelial-to-mesenchymal transition and activation of nuclear factor-kappaB signaling as characteristics of a high-risk head and neck squamous cell carcinoma.* Cancer Res, 2006. **66**(16): p. 8210-8.

20. Ragin, C.C. and E. Taioli, *Survival of squamous cell carcinoma of the head and neck in relation to human papillomavirus infection: review and meta-analysis.* Int J Cancer, 2007. **121**(8): p. 1813-20.

21. Ang, K.K., et al., *Human papillomavirus and survival of patients with oropharyngeal cancer.* N Engl J Med, 2010. **363**(1): p. 24-35.

22. Smeets, S.J., et al., *Immortalization of oral keratinocytes by functional inactivation of the p53 and pRb pathways.* Int J Cancer, 2011. **128**(7): p. 1596-605.

23. Lindel, K., et al., *Human papillomavirus positive squamous cell carcinoma of the oropharynx: a radiosensitive subgroup of head and neck carcinoma.* Cancer, 2001. **92**(4): p. 805-13.

24. Smeets, S.J., et al., *Genome-wide DNA copy number alterations in head and neck squamous cell carcinomas with or without oncogene-expressing human papillomavirus.* Oncogene, 2006. **25**(17): p. 2558-64.

25. Reed, A.L., et al., *High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma.* Cancer Res, 1996. **56**(16): p. 3630-3.

26. Seiwert, T.Y., et al., *The MET receptor tyrosine kinase is a potential novel therapeutic target for head and neck squamous cell carcinoma.* Cancer research, 2009. **69**(7): p. 3021-3031.

27. Ralhan, R., et al., *Discovery and verification of head-and-neck cancer biomarkers by differential protein expression analysis using iTRAQ labeling, multidimensional liquid chromatography, and tandem mass spectrometry.* Molecular & Cellular Proteomics, 2008. **7**(6): p. 1162-1173.

28. Ralhan, R., et al., *iTRAQ-multidimensional liquid chromatography and tandem mass spectrometry-based identification of potential biomarkers of oral epithelial dysplasia and novel networks between inflammation and premalignancy.* Journal of Proteome Research, 2008. **8**(1): p. 300-309.

29. Schaaij-Visser, T.B., et al., *Differential Proteomics Identifies Protein Biomarkers That Predict Local Relapse of Head and Neck Squamous Cell Carcinomas.* Clin Cancer Res, 2009. **15**(24): p. 7666-7675.

30. Patel, V., et al., *Proteomic analysis of laser-captured paraffin-embedded tissues: a molecular portrait of head and neck cancer progression.* Clinical Cancer Research, 2008. **14**(4): p. 1002-1014.

31. Negishi, A., et al., *Quantitative proteomics using formalin-fixed paraffin-embedded tissues of oral squamous cell carcinoma.* Cancer Sci, 2009. **100**(9): p. 1605-1611.

32. Grandis, J.R., et al., *Levels of TGF-α and EGFR protein in head and neck squamous cell carcinoma and patient survival.* J Natl Cancer Inst Monogr, 1998. **90**(11): p. 824-832.

33. Matta, A., et al., *Prognostic Significance of Head-and-Neck Cancer Biomarkers Previously Discovered and Identified Using iTRAQ-Labeling and Multidimensional Liquid Chromatography Tandem Mass Spectrometry.* Journal of Proteome Research, 2008. **7**(5): p. 2078-2087.

34. Chang, K.P., et al., *Identification of PRDX4 and P4HA2 as metastasis-associated proteins in oral cavity squamous cell carcinoma by comparative tissue proteomics of microdissected specimens using iTRAQ technology.* Journal of Proteome Research, 2011. **10**(11): p. 4935-4947.

35. Ye, H., et al., *Proteomic based identification of manganese superoxide dismutase 2 (SOD2) as a metastasis marker for oral squamous cell carcinoma.* Cancer Genomics-Proteomics, 2008. **5**(2): p. 85-93.

36. Bleijerveld, O.B., et al., *Protein signatures associated with tumor cell dissemination in head and neck cancer.* Journal of Proteomics, 2011. **74**(4): p. 558-566.

37. Kosunen, A., et al., *CD44 expression and its relationship with MMP-9, clinicopathological factors and survival in oral squamous cell carcinoma.* Oral Oncol, 2007. **43**(1): p. 51-9.

38. Agra, I.M., et al., *Biological markers and prognosis in recurrent oral cancer after salvage surgery.* Arch Otolaryngol Head Neck Surg, 2008. **134**(7): p. 743-9.

39. Lee, J.I., et al., *Prognostic significance of CXCR-4 expression in oral squamous cell carcinoma.* Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2009. **107**(5): p. 678-84.

40. De Visser, K.E., A. Eichten, and L.M. Coussens, *Paradoxical roles of the immune system during cancer development.* Nature Reviews Cancer, 2006. **6**(1): p. 24-37.

41. Tan, T.T. and L.M. Coussens, *Humoral immunity, inflammation and cancer.* Curr Opin Immunol, 2007. **19**(2): p. 209-216.

42. Dalgleish, A.G. and K.J. O'Byrne, *Chronic immune activation and inflammation in the pathogenesis of AIDS and cancer.* Advances in cancer research, 2002. **84**: p. 231-276.

43. Soussi, T., *p53 Antibodies in the sera of patients with various types of cancer: a review.* Cancer Res, 2000. **60**(7): p. 1777-88.

44. Galon, J., et al., *Type, density, and location of immune cells within human colorectal tumors predict clinical outcome.* Science, 2006. **313**(5795): p. 1960-1964.

45. Tan, T.T. and L.M. Coussens, *Humoral immunity, inflammation and cancer.* Curr Opin Immunol, 2007. **19**(2): p. 209-16.

46. Hirasawa, Y., et al., *Natural autoantibody to MUC1 is a prognostic indicator for non-small cell lung cancer.* Am J Respir Crit Care Med, 2000. **161**(2 Pt 1): p. 589-594.

47. Reipert, B.M., et al., *Increase in autoantibodies against Fas (CD95) during carcinogenesis in the human colon: a hope for the immunoprevention of cancer?* Cancer Immunol Immunother, 2005. **54**(10): p. 1038-42.

48. Lu, S., et al., *[Anti-tumor effect of anti-dsDNA autoantibodies]*. Zhonghua Zhong Liu Za Zhi, 2005. **27**(2): p. 73-6.

49. Cordes, C., et al., *Molecular and immunological aspects of p53 and p53-autoantibodies in head and neck squamous cell carcinoma.* Oncol Rep, 2009. **22**(6): p. 1299-303.

50. Chignard, N., et al., *Cleavage of endoplasmic reticulum proteins in hepatocellular carcinoma: detection of generated fragments in patient sera.* Gastroenterology, 2006. **130**(7): p. 2010-2022.

51. Brichory, F.M., et al., *An immune response manifested by the common occurrence of annexins I and II autoantibodies and high circulating levels of IL-6 in lung cancer.* Proceedings of the National Academy of Sciences, 2001. **98**(17): p. 9824-9829.

52. von Brevern, M.C., et al., *Circulating anti-p53 antibodies in esophageal cancer patients are found predominantly in individuals with p53 core domain mutations in their tumors.* Cancer research, 1996. **56**(21): p. 4917-4921.

53. Winter, S.F., et al., *Development of antibodies against p53 in lung cancer patients appears to be dependent on the type of p53 mutation.* Cancer research, 1992. **52**(15): p. 4168-4174.

54. Cheever, M.A., et al., *Immunity to oncogenic proteins.* Immunol Rev, 1995. **145**(1): p. 33-59.

55. Ward, R.L., et al., *Antibody immunity to the HER-2/neu oncogenic protein in patients with colorectal cancer*. Human immunology, 1999. **60**(6): p. 510-515.

56. Brass, N., et al., *Role of amplified genes in the production of autoantibodies.* Blood, 1999. **93**(7): p. 2158-2166.

57. Houghton, A.N., J.S. Gold, and N.E. Blachere, *Immunity against cancer: lessons learned from melanoma.* Curr Opin Immunol, 2001. **13**(2): p. 134-140.

58. Cho, Y.S., et al., *Extracellular protein kinase A as a cancer biomarker: its expression by tumor cells and reversal by a myristate-lacking Cα and RIIβ subunit overexpression.* Proceedings of the National Academy of Sciences, 2000. **97**(2): p. 835-840.

59. Cvijic, M.E., et al., *Extracellular catalytic subunit activity of the cAMP-dependent protein kinase in prostate cancer.* Clinical Cancer Research, 2000. **6**(6): p. 2309-2317.

60. Suzuki, H., et al., *T Cell–Dependent Antibody Responses against Aberrantly Expressed Cyclin B1 Protein in Patients with Cancer and Premalignant Disease.* Clinical Cancer Research, 2005. **11**(4): p. 1521-1526.

61. Yu, M., Q. Zhan, and O.J. Finn, *Immune recognition of cyclin B1 as a tumor antigen is a result of its overexpression in human tumors that is caused by non-functional p53.* Molecular immunology, 2002. **38**(12): p. 981-987.

62. Covini, G., et al., *Immune response to cyclin B1 in hepatocellular carcinoma.* Hepatology, 2003. **25**(1): p. 75-80.

63. Ersvaer, E., et al., *Cyclin B1 is commonly expressed in the cytoplasm of primary human acute myelogenous leukemia cells and serves as a leukemia-associated antigen associated with autoantibody response in a subset of patients.* European journal of haematology, 2007. **79**(3): p. 210-225.

64. Yamamoto, A., et al., *Detection of auto-antibodies against L-myc oncogene products in sera from lung cancer patients.* Int J Cancer, 1998. **69**(4): p. 283-289.

65. Sorokine, I., et al., *Presence of circulating anti-c-myb oncogene product antibodies in human sera.* Int J Cancer, 2006. **47**(5): p. 665-669.

66. Scanlan, M.J., A. Simpson, and L.J. Old, *The cancer/testis genes: review, standardization, and commentary.* Cancer Immun, 2004. **4**(1): p. 1.

67. Zhang, J.Y., et al., *A novel cytoplasmic protein with RNA-binding motifs is an autoantigen in human hepatocellular carcinoma.* J Exp Med, 1999. **189**(7): p. 1101-1110.

68. Chen, Y.T., et al., *A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening.* Proceedings of the National Academy of Sciences, 1997. **94**(5): p. 1914-1918.

69. Stone, B., et al., *Serologic analysis of ovarian tumor antigens reveals a bias toward antigens encoded on 17q.* Int J Cancer, 2002. **104**(1): p. 73-84.

70. Stockert, E., et al., *A survey of the humoral immune response of cancer patients to a panel of human tumor antigens.* J Exp Med, 1998. **187**(8): p. 1349-1354.

71. Wang, X., et al., *Autoantibody signatures in prostate cancer.* New England Journal of Medicine, 2005. **353**(12): p. 1224-1235.

72. Qiu, J., et al., *Development of natural protein microarrays for diagnosing cancer based on an antibody response to tumor antigens.* Journal of Proteome Research, 2004. **3**(2): p. 261-267.

73. Taylor, B.S., et al., *Humoral response profiling reveals pathways to prostate cancer progression.* Molecular & Cellular Proteomics, 2008. **7**(3): p. 600-611.

74. Madoz-Gúrpide, J., et al., *Integral protein microarrays for the identification of lung cancer antigens in sera that induce a humoral immune response.* Molecular & Cellular Proteomics, 2008. **7**(2): p. 268-281.

75. Caron, M., et al., *Serological proteome analysis (SERPA) and multiple affinity protein profiling (MAPPING) to discover cancer biomarkers.* Mol. Cell. Proteomics, 2005. **4**.

76. Philip, R., et al., *Shared immunoproteome for ovarian cancer diagnostics and immunotherapy: potential theranostic approach to cancer.* J Proteome Res, 2007. **6**(7): p. 2509-17.

77. Hardouin, J., et al., *Usefulness of autoantigens depletion to detect autoantibody signatures by multiple affinity protein profiling.* J Sep Sci, 2007. **30**(3): p. 352-358.

78. Hardouin, J., et al., *Cancer Immunomics.* Ann N Y Acad Sci, 2007. **1107**(1): p. 223-230.

79. Klade, C.S., et al., *Identification of tumor antigens in renal cell carcinoma by serological proteome analysis.* Proteomics, 2001. **1**(7): p. 890-898.

80. Lichtenfels, R., et al., *Identification of metabolic enzymes in renal cell carcinoma utilizing PROTEOMEX analyses.* Biochimica et Biophysica Acta (BBA)-Proteins & Proteomics, 2003. **1646**(1): p. 21-31.

81. Gires, O., et al., *Profile identification of disease-associated humoral antigens using AMIDA, a novel proteomics-based technology.* Cellular and molecular life sciences, 2004. **61**(10): p. 1198-1207.

82. Pedersen, S.K., et al., *An Immunoproteomic Approach for Identification of Clinical Biomarkers for Monitoring Disease Application to Cystic Fibrosis.* Molecular & Cellular Proteomics, 2005. **4**(8): p. 1052-1060.

83. Prasannan, L., et al., *Identification of beta-tubulin isoforms as tumor antigens in neuroblastoma.* Clin Cancer Res, 2000. **6**(10): p. 3949-56.

84. Le Naour, F., et al., *Proteomics-based identification of RS/DJ-1 as a novel circulating tumor antigen in breast cancer.* Clin Cancer Res, 2001. **7**(11): p. 3328-35.

85. Hamrita, B., et al., *Identification of tumor antigens that elicit a humoral immune response in breast cancer patients' sera by serological proteome analysis (SERPA).* Clinica chimica acta, 2008. **393**(2): p. 95-102.

86. Brichory, F., et al., *Proteomics-based identification of protein gene product 9.5 as a tumor antigen that induces a humoral immune response in lung cancer.* Cancer Res, 2001. **61**(21): p. 7908-12.

87. Chang, J.W., et al., *Peroxiredoxin-I is an autoimmunogenic tumor antigen in non-small cell lung cancer.* FEBS Lett, 2005. **579**(13): p. 2873-7.

88. He, P., et al., *Proteomics-based identification of alpha-enolase as a tumor antigen in non-small lung cancer.* Cancer Sci, 2007. **98**(8): p. 1234-40.

89. Yang, F., et al., *Identification of tumor antigens in human lung squamous carcinoma by serological proteome analysis.* J Proteome Res, 2007. **6**(2): p. 751-8.

90. Le Naour, F., et al., *A distinct repertoire of autoantibodies in hepatocellular carcinoma identified by proteomic analysis.* Mol Cell Proteomics, 2002. **1**(3): p. 197-203.

91. Li, L., et al., *Identification of hepatocellular-carcinoma-associated antigens and autoantibodies by serological proteome analysis combined with protein microarray.* J Proteome Res, 2008. **7**(2): p. 611-20.

92. Hong, S.H., et al., *An autoantibody-mediated immune response to calreticulin isoforms in pancreatic cancer.* Cancer Res, 2004. **64**(15): p. 5504-10.

93. Xia, Q., et al., *Proteomics-based identification of DEAD-box protein 48 as a novel autoantigen, a prospective serum marker for pancreatic cancer.* Biochem Biophys Res Commun, 2005. **330**(2): p. 526-32.

94. Ho, M., et al., *Humoral immune response to mesothelin in mesothelioma and ovarian cancer patients.* Clin Cancer Res, 2005. **11**(10): p. 3814-20.

95. Fujita, Y., et al., *Proteomics-based approach identifying autoantibody against peroxiredoxin VI as a novel serum marker in esophageal squamous cell carcinoma.* Clin Cancer Res, 2006. **12**(21): p. 6415-20.

96. Nakanishi, T., et al., *Detection of eight antibodies in cancer patients' sera against proteins derived from the adenocarcinoma A549 cell line using proteomics-based analysis.* J Chromatogr B Analyt Technol Biomed Life Sci, 2006. **838**(1): p. 15-20.

97. Zou, L., et al., *Identification of leukemia-associated antigens in chronic myeloid leukemia by proteomic analysis.* Leuk Res, 2005. **29**(12): p. 1387-91.

98. Hudson, M.E., et al., *Identification of differentially expressed proteins in ovarian cancer using high-density protein microarrays.* Proceedings of the National Academy of Sciences, 2007. **104**(44): p. 17494-17499.

99. Anderson, K.S., et al., *Protein microarray signature of autoantibody biomarkers for the early detection of breast cancer.* J Proteome Res, 2011. **10**(1): p. 85-96.

100. Trivers, G.E., et al., *Anti-p53 antibodies in sera from patients with chronic obstructive pulmonary disease can predate a diagnosis of cancer.* Clin Cancer Res, 1996. **2**(10): p. 1767-75.

101. Li, Y., et al., *p53 autoantibodies predict subsequent development of cancer.* Int J Cancer, 2005. **114**(1): p. 157-160.

102. Zhong, L., et al., *Profiling tumor-associated antibodies for early detection of non-small cell lung cancer.* Journal of Thoracic Oncology, 2006. **1**(6): p. 513-519.

103. Desmetz, C., et al., *Proteomics-based identification of HSP60 as a tumor-associated antigen in early stage breast cancer and ductal carcinoma in situ.* Journal of Proteome Research, 2008. **7**(9): p. 3830-3837.

104. Pedersen, J., et al., *Early detection of cancer in the general population: a blinded case–control study of p53 autoantibodies in colorectal cancer.* British journal of cancer, 2012.

105. Chen, J.S., et al., *Combined analysis of survivin autoantibody and carcinoembryonic antigen biomarkers for improved detection of colorectal cancer.* Clin Chem Lab Med, 2010. **48**(5): p. 719-25.

106. Zhang, J.Y., et al., *Antibody detection using tumor-associated antigen mini-array in immunodiagnosing human hepatocellular carcinoma.* J Hepatol, 2007. **46**(1): p. 107.

107. Qiu, J., et al., *Occurrence of autoantibodies to annexin I, 14-3-3 theta and LAMR1 in prediagnostic lung cancer sera.* J Clin Oncol, 2008. **26**(31): p. 5060-6.

108. Lu, H., et al., *Evaluation of Known Oncoantibodies, HER2, p53, and Cyclin B1, in Prediagnostic Breast Cancer Sera.* Cancer Prevention Research, 2012. **5**(8): p. 1036-1043.

109. Desmetz, C., et al., *Identification of a new panel of serum autoantibodies associated with the presence of in situ carcinoma of the breast in younger women.* Clinical Cancer Research, 2009. **15**(14): p. 4733-4741.

110. Chang, W., et al., *Development of autoantibody signatures as biomarkers for early detection of colorectal carcinoma.* Clinical Cancer Research, 2011. **17**(17): p. 5715-5724.

111. Li, Y., et al., *Elevated serum antibodies against insulin-like growth factor-binding protein-2 allow detecting early-stage cancers: evidences from glioma and colorectal carcinoma studies.* Annals of Oncology, 2012. **23**(9): p. 2415-2422.

112. Mudenda, B., et al., *The relationship between serum p53 autoantibodies and characteristics of human breast cancer.* Br J Cancer, 1994. **69**(6): p. 1115-9.

113. Mangé, A., et al., *Serum autoantibody signature of ductal carcinoma in situ progression to invasive breast cancer.* Clinical Cancer Research, 2012. **18**(7): p. 1992-2000.

114. Anderson, K.S., et al., *p53 autoantibodies as potential detection and prognostic biomarkers in serous ovarian cancer.* Cancer Epidemiol Biomarkers Prev, 2010. **19**(3): p. 859-68.

115. Houbiers, J., et al., *Antibodies against p53 are associated with poor prognosis of colorectal cancer.* British journal of cancer, 1995. **72**(3): p. 637.

116. Kulić, A., et al., *Anti-p53 antibodies in serum: relationship to tumor biology and prognosis of breast cancer patients.* Medical Oncology, 2010. **27**(3): p. 887-893.

117. Atta, M.M., et al., *Value of serum anti-p53 antibodies as a prognostic factor in Egyptian patients with hepatocellular carcinoma.* Clin Biochem, 2008. **41**(14-15): p. 1131-1139.

118. Blixt, O., et al., *Autoantibodies to aberrantly glycosylated MUC1 in early stage breast cancer are associated with a better prognosis.* Breast Cancer Res, 2011. **13**(2): p. R25.

119. Budiu, R.A., et al., *Soluble MUC1 and serum MUC1-specific antibodies are potential prognostic biomarkers for platinum-resistant ovarian cancer.* Cancer Immunol Immunother, 2011. **60**(7): p. 975-84.

120. Barbauche, M., et al., *Prognostic significance of autoantibodies to laminin in the sera of breast cancer patients: a preliminary report.* Clinical Chemistry and Laboratory Medicine, 1994. **32**(7): p. 511-514.

121. Conroy, S., et al., *Detection of autoantibodies to the 90 kDa head shock protein in systemic lupus erythematosus and other autoimmune diseases.* Rheumatology, 1994. **33**(10): p. 923-926.

122. Fossa, A., et al., *NY-ESO-1 protein expression and humoral immune responses in prostate cancer.* Prostate, 2004. **59**(4): p. 440-7.

123. Brankin, B., et al., *Autoantibodies to the nuclear phosphoprotein nucleophosmin in breast cancer patients.* Cancer Epidemiology Biomarkers & Prevention, 1998. **7**(12): p. 1109-1115.

124. Parmigiani, R.B., et al., *Antibodies against the cancer-testis antigen CTSP-1 are frequently found in prostate cancer patients and are an independent prognostic factor for biochemical-recurrence.* Int J Cancer, 2008. **122**(10): p. 2385-2390.

125. Saffroy, R., et al., *Clinical significance of circulating anti-p53 antibodies in European patients with hepatocellular carcinoma.* Br J Cancer, 1999. **79**(3-4): p. 604-10.

126. Friedrichs, B., et al., *Humoral immune responses against the immature laminin receptor protein show prognostic significance in patients with chronic lymphocytic leukemia.* The Journal of Immunology, 2008. **180**(9): p. 6374-6384.

127. Yang, X.F., et al., *CML66, a broadly immunogenic tumor antigen, elicits a humoral immune response associated with remission of chronic myelogenous leukemia.* Proceedings of the National Academy of Sciences, 2001. **98**(13): p. 7492-7497.

128. Pallasch, C.P., et al., *Autoantibodies against GLEA2 and PHF3 in glioblastoma: Tumor-associated autoantibodies correlated with prolonged survival.* Int J Cancer, 2005. **117**(3): p. 456-459.

129. Richards, E.R., et al., *Antibodies reactive with the protein core of MUC1 mucin are present in ovarian cancer patients and healthy women.* Cancer Immunology, Immunotherapy, 1998. **46**(5): p. 245-252.

130. Kocer, B., J. McKolanis, and A. Soran, *Humoral immune response to MUC5AC in patients with colorectal polyps and colorectal carcinoma.* BMC gastroenterology, 2006. **6**(1): p. 4.

131. Vural, B., et al., *Frequency of SOX Group B (SOX1, 2, 3) and ZIC2 antibodies in Turkish patients with small cell lung carcinoma and their correlation with clinical parameters.* Cancer, 2005. **103**(12): p. 2575-2583.

132. Haidopoulos, D., et al., *Circulating anti-CEA antibodies in the sera of patientswith breast cancer.* European Journal of Surgical Oncology (EJSO), 2000. **26**(8): p. 742-746.

133. Wadle, A., et al., *Serological immune response to cancer testis antigens in patients with pancreatic cancer.* Int J Cancer, 2006. **119**(1): p. 117-125.

134. Ralhan, R., et al., *Circulating p53 antibodies as early markers of oral cancer: correlation with p53 alterations.* Clin Cancer Res, 1998. **4**(9): p. 2147-52.

135. Vidal, C.I., et al., *An HSP90-mimic peptide revealed by fingerprinting the pool of antibodies from ovarian cancer patients.* Oncogene, 2004. **23**(55): p. 8859-8867.

136. Mussolin, L., et al., *Kinetics of humoral response to ALK and its relationship with minimal residual disease in pediatric ALCL.* Leukemia, 2008. **23**(2): p. 400-402.

137. Comtesse, N., et al., *Complex humoral immune response against a benign tumor: frequent antibody response against specific antigens as diagnostic targets.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(27): p. 9601-9606.

138. Hermsen, B., et al., *Humoral immune responses to MUC1 in women with a< i> BRCA1</i> or< i> BRCA2</i> mutation.* European Journal of Cancer, 2007. **43**(10): p. 1556-1563.

139. Karanikas, V., et al., *Anti-survivin antibody responses in lung cancer.* Cancer letters, 2009. **282**(2): p. 159-166.

140. Jager, E., et al., *Humoral immune responses of cancer patients against "Cancer-Testis" antigen NY-ESO-1: correlation with clinical events.* Int J Cancer, 1999. **84**(5): p. 506-10.

141. Akcakanat, A., et al., *NY-ESO-1 expression and its serum immunoreactivity in esophageal cancer.* Cancer chemotherapy and pharmacology, 2004. **54**(1): p. 95-100.

142. Pavoni, E., et al., *A study of the humoral immune response of breast cancer patients to a panel of human tumor antigens identified by phage display.* Cancer Detect Prev, 2006. **30**(3): p. 248-256.

143. Fensterle, J., et al., *B-Raf specific antibody responses in melanoma patients.* BMC Cancer, 2004. **4**(1): p. 62.

144. Gnjatic, S., et al., *Seromic profiling of ovarian and pancreatic cancer.* Proceedings of the National Academy of Sciences, 2010. **107**(11): p. 5088-5093.

145. Gangal, S.G. and S.D. Vernekar, *Immunological studies on human tumors. Search for autoantibodies in patients with oral malignancies before and after therapy.* Indian J Cancer, 1969. **6**(4): p. 240-5.

146. Castelli, M., et al., *Anti-p53 and anti-heat shock proteins antibodies in patients with malignant or pre-malignant lesions of the oral cavity.* Anticancer Res, 2001. **21**(1B): p. 753-8.

147. Marcos, C.Á., et al., *The usefulness of new serum tumor markers in head and neck squamous cell carcinoma.* Otolaryngology--Head and Neck Surgery, 2009. **140**(3): p. 375-380.

148. Lin, H.S., et al., *Autoantibody approach for serum-based detection of head and neck cancer.* Cancer Epidemiol Biomarkers Prev, 2007. **16**(11): p. 2396-405.

149. Eto, M., et al., *Antibody responses to survivin and their clinical significance in patients with head and neck cancer.* Head & neck, 2007. **29**(12): p. 1128-1135.

150. Murase, R., et al., *Serum autoantibody to sideroflexin 3 as a novel tumor marker for oral squamous cell carcinoma.* Proteomics Clin Appl, 2008. **2**(4): p. 517-27.

151. Shukla, S., et al., *Tumor antigens eliciting autoantibody response in cancer of gingivo-buccal complex.* Proteomics Clin Appl, 2007. **1**(12): p. 1592-604.

152. Shukla, S., et al., *Immunoproteomics reveals that cancer of the tongue and the gingivobuccal complex exhibit differential autoantibody response.* Cancer Biomark, 2009. **5**(3): p. 127-35.

153. Peterson, G.L., *A simplification of the protein assay method of Lowry et al. which is more generally applicable.* Anal Biochem, 1977. **83**(2): p. 346-56.

154. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* NATURE, 1970. **227**(5259): p. 680-5.

155. Lowry, O.H., et al., *Protein measurement with the Folin phenol reagent.* J Biol Chem, 1951. **193**(1): p. 265-75.

156. Inoue, H., H. Nojima, and H. Okayama, *High efficiency transformation of Escherichia coli with plasmids.* Gene, 1990. **96**(1): p. 23-8.

157. Shukla, S., *Study of tumor antigens eliciting humoral response in Oral Cancer.* Ph.D. Thesis, University of Mumbai, 2008.

158. Fujita, Y., et al., *Proteomics-based identification of autoantibody against heat shock protein 70 as a diagnostic marker in esophageal squamous cell carcinoma.* Cancer Lett, 2008. **263**(2): p. 280-90.

159. Chang, J.T., et al., *Enzyme immunoassay for serum autoantibody to survivin and its findings in head-and-neck cancer patients.* Clin Chem, 2004. **50**(7): p. 1261-4.

160. Mitsudomi, T., et al., *Clinical implications of p53 autoantibodies in the sera of patients with non-small-cell lung cancer.* J Natl Cancer Inst, 1998. **90**(20): p. 1563-8.

161. Capello, M., et al., *alpha-Enolase: a promising therapeutic and diagnostic tumor target.* FEBS J, 2011. **278**(7): p. 1064-74.

162. Cappello, P., et al., *An integrated humoral and cellular response is elicited in pancreatic cancer by alpha-enolase, a novel pancreatic ductal adenocarcinoma-associated antigen.* Int J Cancer, 2009. **125**(3): p. 639-48.

163. Seweryn, E., et al., *Localization of enolase in the subfractions of a breast cancer cell line.* Z Naturforsch C, 2009. **64**(9-10): p. 754-8.

164. Govekar, R.B., et al., *Proteomic profiling of cancer of the gingivo-buccal complex: Identification of new differentially expressed markers.* Proteomics Clin Appl, 2009. **3**(12): p. 1451-62.

165. Tsai, S.T., et al., *ENO1, a potential prognostic head and neck cancer marker, promotes transformation partly via chemokine CCL20 induction.* Eur J Cancer, 2010. **46**(9): p. 1712-23.

166. Suzuki, A., et al., *Identification of melanoma antigens using a Serological Proteome Approach (SERPA).* Cancer Genomics Proteomics, 2010. **7**(1): p. 17-23.

167. Shih, N.Y., et al., *Anti-alpha-enolase autoantibodies are down-regulated in advanced cancer patients.* Jpn J Clin Oncol, 2010. **40**(7): p. 663-9.

168. Tomaino, B., et al., *Circulating autoantibodies to phosphorylated alpha-enolase are a hallmark of pancreatic cancer.* J Proteome Res, 2011. **10**(1): p. 105-12.

169. Lin, H.P., Y.P. Wang, and C.P. Chiang, *Expression of p53, MDM2, p21, heat shock protein 70, and HPV 16/18 E6 proteins in oral verrucous carcinoma and oral verrucous hyperplasia.* Head Neck, 2011. **33**(3): p. 334-40.

170. Zhong, L., et al., *Antibodies to HSP70 and HSP90 in serum in non-small cell lung cancer patients.* Cancer Detect Prev, 2003. **27**(4): p. 285-90.

171. Looi, K.S., et al., *Using proteomic approach to identify tumor-associated antigens as markers in hepatocellular carcinoma.* J Proteome Res, 2008. **7**(9): p. 4004-12.

172. Murphy, M.A., J.J. O'Leary, and D.J. Cahill, *Assessment of the humoral immune response to cancer.* J Proteomics, 2012.

173. Tavassol, F., et al., *Prognostic significance of heat shock protein 70 (HSP70) in patients with oral cancer.* Head Neck Oncol, 2011. **3**: p. 10.

174. Lubin, R., et al., *p53 antibodies in patients with various types of cancer: assay, identification, and characterization.* Clin Cancer Res, 1995. **1**(12): p. 1463-9.

175. Tang, R., et al., *Humoral response to p53 in human colorectal tumors: a prospective study of 1,209 patients.* Int J Cancer, 2001. **94**(6): p. 859-63.

176. Murphy, M.A., et al., *Epitope presentation is an important determinant of the utility of antigens identified from protein arrays in the development of autoantibody diagnostic assays.* J Proteomics, 2012.

Appendix

APPENDIX:

Table A1: Clinical information of healthy individuals

S. No.	Samples	Age	Sex
1	CL01	38	M
2	CL02	48	M
3	CL03	45	M
4	CL04	45	M
5	CL05	30	M
6	CL06	52	M
7	CL07	61	M
8	CL08	49	M
9	CL09	36	M
10	CL12	38	F
11	CL13	35	M
12	CL14	38	F
13	CL15	35	M
14	CL16	38	M
15	CL17	48	M
16	CL19	38	M
17	CL20	35	M
18	CL22	38	M
19	CL27	56	M
20	CL32	48	M
21	CL33	37	M
22	CL34	55	M
23	CL35	37	M
24	CL39	45	M
25	CL42	58	M
26	CL44	43	M
27	CL45	37	M
28	CL47	54	M
29	CL48	39	M
30	CL49	38	M

Table A2: Clinical information of individuals with leukoplakia

S. No.	Sample no.	Age	Sex
1	A3	53	M
2	A4	60	M
3	A5	77	M
4	A6	60	M
5	A7	42	M
6	A8	73	M
7	A9	32	M
8	A10	37	M
9	A11	37	M
10	A12	40	M
11	A13	54	M
12	A14	30	M
13	A15	37	M
14	A16	49	M
15	A17	37	M
16	A18	43	M
17	A19	40	M
18	A20	50	M
19	A21	40	M
20	A22	53	M
21	A23	53	M
22	A24	60	M
23	A25	23	M
24	A26	58	M
25	A28	63	M
26	A29	47	F
27	A30	57	M
28	A31	70	M
29	A32	63	M
30	A34	34	M

Table A3: Clinical information of individuals with chewing/ smoking habits

S. No.	Sample no.	Age	Sex
1	AH1	61	M
2	AH2	40	M
3	AH3	55	M
4	AH4	54	M
5	AH5	49	M
6	AH6	45	M
7	AH7	30	M
8	AH8	87	M
9	AH9	33	M
10	AH10	55	M
11	AH11	65	M
12	AH12	27	M
13	AH13	49	M
14	AH14	49	M
15	AH15	31	M
16	AH16	43	M
17	AH17	62	M
18	AH18	50	F
19	AH19	40	F
20	AH20	32	M
21	AH21	53	M
22	AH22	55	M
23	AH23	23	M
24	AH24	48	M
25	AH25	52	M
26	AH26	47	M
27	AH27	57	M
28	AH28	45	M
29	AH29	35	M
30	AH30	34	M

Table A4: Clinical information of patients with T1/T2 tumors of GBC

S.No.	Sample No.	Age	Sex	Pathological staging	Differentiation status
1	641	50	M	T2N1	MD
2	642	61	F	T1N0	MD
3	648	41	M	T2N2b	MD
4	882	36	F	T4N0	MD
5	1015	55	M	T2N1	PD
6	1023	58	M	T2N0	MD
7	1024	47	M	T2N1	WD-MD
8	1038	62	M	T2N2b	PD
9	1060	57	M	T1Nx	WD
10	1097	48	M	T2N2b	MD
11	1122	45	M	T1N0	VC
12	1151	56	M	T2N0	MD
13	1175	55	F	T2N0	MD
14	1225	58	M	T2N1	MD
15	1227	39	M	T2N2b	MD
16	1414	45	M	T2N0	MD
17	1420	33	F	T2N0	MD
18	1450	47	M	T2N0	WD
19	1476	36	M	T2N2b	PD
20	1478	46	M	T1Nx	WD
21	1484	58	M	T2N0	PD
22	1486	43	F	T1N0	SI
23	1493	54	M	T1N0	MD
24	1494	48	M	T2N0	PD
25	1495	46	M	T2N0	MD
26	1509	45	M	T2N2b	MD
27	1522	60	M	T1N0	MD
28	1527	57	F	T4N2b	MD
29	1531	52	M	T2N0	MD
30	1533	50	M	T2N0	WD

VC- Verrucous carcinoma, SI-Superficially invasive

Table A5: Clinical information of patients with T3/T4 tumors of GBC

S.No.	Sample No.	Age	Sex	Pathological staging	Differentiation status
1	824	75	M	T4aN2b	PD
2	879	66	M	T4aN0	MD
3	885	67	F	T4aN0	PD
4	891	72	F	T4N0	PD
5	955	40	M	T4aN0	MD-PD
6	974	35	M	T4aN0	MD
7	976	52	M	T4aN1	MD
8	1014	56	M	T3N2c	PD
9	1035	65	M	T3N2b	MD
10	1064	45	F	T4aN0	WD
11	1108	55	M	T4aN2b	MD
12	1523	58	M	T4N1	PD
13	1417	57	M	T4aN2b	MD
14	1461	74	M	T4aN0	MD
15	1481	55	F	T4N2b	PD
16	1482	35	F	T4N2b	MD
17	1488	44	M	T3N1	PD
18	1489	49	M	T4N0	MD
19	1491	55	M	T4N0	MD
20	1499	43	M	T4aN0	PD
21	1504	54	F	T4aN2b	MD
22	1505	61	M	T4N0	WD
23	1506	53	M	T4N0	
24	1520	38	M	T4N1	MD
25	1528	36	F	T4N2b	PD
26	1580	48	F	T3N0	MD
27	1606	70	M	T4aN0	WD
28	1608	49	M	T3N0	PD
29	1642	72	M	T4N2b	PD
30	1454	54	F	T4N0	PD

Table A6: Clinical information and autoantibody response to tumor antigens in healthy individuals (n=30)

Healthy Individuals	Age	Sex	No. of antigens	Tumor antigens eliciting autoantibody response													
				α Eno a	α Eno b	α Eno c	Hsp70	Anx2	Prdx6	PKM	αTub	ß Tub	ATPS	AR1	AR2	PGM	TPI
CL01	38	M	1	+													
CL02	48	M	1					+									
CL03	45	M	0														
CL04	45	M	1			+											
CL05	30	M	0														
CL06	52	M	0														
CL07	61	M	0														
CL08	49	M	2				+		+								
CL09	36	M	1										+				
CL12	38	F	0														
CL13	35	M	1					+									
CL14	38	F	1			+											
CL15	35	M	0														
CL16	38	M	0														
CL17	48	M	1			+											
CL19	38	M	1						+								
CL20	35	M	2		+	+											
CL22	38	M	0														
CL27	56	M	0														
CL32	48	M	0														
CL33	37	M	2		+	+											
CL34	55	M	2					+								+	
CL35	37	M	0														
CL39	45	M	0														
CL42	58	M	1										+				
CL44	43	M	1			+											
CL45	37	M	1										+				
CL47	54	M	0														
CL48	39	M	0														
CL49	38	M	1			+											
Autoantibody response (%)				3	7	23	3	10	7	0	0	0	10	0	0	3	0

Table A7: Autoantibody response to tumor antigens in healthy individuals (n=30) with chewing/smoking habit

Samples	No. of antigens	Tumor antigens eliciting autoantibody response													
		α Eno a	α Eno b	α Eno c	Hsp70	Anx2	Prdx6	PKM	α Tub	ß Tub	ATPS	AR1	AR2	PGM	TPI
AH01	0														
AH02	0														
AH03	0														
AH04	0														
AH05	5	Y	Y	Y	Y	Y									
AH06	3		Y	Y	Y										
AH07	0														
AH08	3	Y				Y		Y							
AH09	0														
AH10	0														
AH11	2								Y	Y					
AH12	1				Y										
AH13	0														
AH14	6	Y	Y	Y	Y			Y						Y	
AH15	2		Y	Y											
AH16	7	Y	Y	Y			Y			Y				Y	Y
AH17	0														
AH18	3		Y	Y		Y									
AH19	0														
AH20	0														
AH21	2		Y	Y											
AH22	0														
AH23	2			Y				Y							
AH24	1						Y								
AH25	1					Y									
AH26	1			Y											
AH27	4	Y	Y	Y							Y				
AH28	0														
AH29	0														
AH30	1			Y											
Autoantibody response(%)		17	27	37	13	13	7	10	3	7	3	0	0	7	3

Table A8: Autoantibody response to tumor antigens in individuals (n=30) with leukoplakia

Samples	No. of antigens	Tumor antigens eliciting autoantibody response												
		α Eno a	α Eno b	α Eno c	Hsp70	Anx2	Prdx6	PKM	α Tub	ß Tub	ATPS	AR1	AR2	PGM	TPI
A03	2					Y		Y							
A04	3	Y	Y	Y											
A05	1												Y		
A06	1					Y									
A07	2			Y						Y					
A08	2					Y					Y				
A09	1			Y											
A10	3	Y		Y	Y										
A11	2		Y	Y										Y	
A12	2						Y				Y				
A13	3				Y		Y				Y				
A14	0														
A15	4		Y	Y			Y	Y							
A16	1					Y									
A17	0														
A18	2		Y	Y											
A19	2		Y	Y											
A20	0														
A21	0														
A22	1									Y					
A23	0														
A24	0														
A25	3	Y	Y	Y											
A26	1			Y											
A30	3		Y	Y							Y				
A31	2		Y	Y											
A28	0														
A32	0														
A33	0														
A34	0														
Autoantibody response (%)		10	27	40	7	13	10	7	0	7	13	0	3	3	0

Table A9: Autoantibody response to tumor antigens in patients (n=30) with T1/T2 tumors of GBC

Samples	No. of antigens	Tumor antigens eliciting autoantibody response													
		α Eno a	α Eno b	α Eno c	Hsp70	Anx2	Prdx6	PKM	α Tub	ß Tub	ATPS	AR1	AR2	PGM	TPI
641	1							√							
642	3		√	√			√								
648	4			√				√			√		√		
882	0														
1015	4	√	√	√			√								
1023	2						√							√	
1024	1													√	
1038	1			√											
1060	0														
1097	0														
1122	2		√	√											
1151	5			√			√		√	√	√				
1175	0														
1225	0														
1227	0														
1414	3					√	√				√				
1420	2											√	√		
1450	2			√				√							
1476	4	√	√	√			√								
1478	4		√	√				√					√		
1484	4		√	√		√					√				
1486	2		√	√											
1493	2		√	√											
1494	2			√			√								
1495	2										√		√		
1509	4				√				√	√	√				
1522	2	√						√							
1527	2						√							√	
1531	1						√								
1533	0														
Autoantibody response (%)		10	27	43	3	7	30	17	7	7	20	3	13	10	0

Table A10: Autoantibody response to tumor antigens in patients (n=30) with T3/T4 tumors of GBC

Samples	No. of antigens	Tumor antigens eliciting autoantibody response													
		α Eno a	α Eno b	α Eno c	Hsp70	Anx2	Prdx6	PKM	α Tub	ß Tub	ATPS	AR1	AR2	PGM	TPI
824	0														
879	4	√	√	√			√								
885	0														
891	0														
955	3		√	√			√								
974	2			√				√							
976	0														
1014	1										√				
1035	0														
1064	0														
1108	4		√	√			√			√					
1523	0														
1417	5		√	√		√		√			√				
1461	4		√	√	√		√								
1481	2		√	√											
1482	1									√					
1488	4		√	√	√						√				
1489	2			√							√				
1491	3	√	√	√											
1499	6		√	√	√		√							√	√
1504	2				√	√									
1505	2		√	√											
1506	1								√						
1520	4	√	√	√			√								
1528	1			√											
1580	0														
1606	3						√				√			√	
1608	2		√	√											
1642	4	√	√	√							√				
1454	4		√	√							√		√		
Autoantibody response (%)		13	47	57	13	7	23	7	3	7	23	0	3	7	3

Combined Analysis:

Table A11a: Clinical information and autoantibody response to tumor antigens in healthy individuals

Healthy Individuals	Age	Sex	No. of antigens	Tumor antigens eliciting autoantibody response													
				α Eno a	α Eno b	α Eno c	Hsp70	Anx2	Prdx6	PKM	αTub	ß Tub	ATPS	AR1	AR2	PGM	TPI
CL01	38	M	1	+													
CL02	48	M	1					+									
CL03	45	M	0														
CL04	45	M	1			+											
CL05	30	M	0														
CL06	52	M	0														
CL07	61	M	0														
CL08	49	M	2				+		+								
CL09	36	M	1										+				
CL12	38	F	0														
CL13	35	M	1					+									
CL14	38	F	1			+											
CL15	35	M	0														
CL16	38	M	0														
CL17	48	M	1			+											
CL19	38	M	1						+								
CL20	35	M	2		+	+											
CL22	38	M	0														
CL27	56	M	0														
CL32	48	M	0														
CL33	37	M	2		+	+											
CL34	55	M	2					+								+	
CL35	37	M	0														
CL39	45	M	0														
CL42	58	M	1										+				
CL44	43	M	1			+											
CL45	37	M	1										+				
CL47	54	M	0														
CL48	39	M	0														
CL49	38	M	1			+											

Healthy Individuals	Age	Sex	No. of antigens	Tumor antigens eliciting autoantibody response													
				α Eno a	α Eno b	α Eno c	Hsp70	Anx2	Prdx6	PKM	α Tub	ß Tub	ATPS	AR1	AR2	PGM	TPI
CS-01	54	F	0														
CS-02	54	F	3	+	+	+											
CS-03	58	M	1			+											
CS-04	45	F	0														
CS-05	26	M	0														
CS-06	51	M	1			+											
CS-07	50	M	2		+	+											
CS-08	48	M	0														
CS-09	42	M	0														
CS-10	55	F	0														
CS-11	27	M	0														
CS-12	41	F	0														
CS-13	39	M	3	+	+	+											
CS-14	55	M	3			+				+				+			
CS-15	39	M	0														
CS-16	56	M	0														
CS-17	38	M	0														
CS-18	54	F	0														
CS-19	50	F	0														
CS-20	55	F	0														
CS-21	53	F	0														
CS-22	49	F	1				+										
CS-23	46	F	0														
CS-24	45	F	0														
CS-25	45	F	0														
CS-26	58	F	0														
CS-27	47	F	0														
CS-28	45	F	0														
CS-29	45	F	0														
CS-30	37	M	0														

Table A11a continued:

CS-31	42	F	1							+							
CS-32	44	F	2														
CS-33	42	M	3	+	+	+											
CS-34	45	M	0														
CS-35	51	M	0														
CS-36	57	M	0														
CS-37	58	M	0														
CS-38	54	M	3	+	+	+											
CS-39	54	M	0														
CS-40	40	F	0														
CS-41	49	F	2		+	+											
CS-42	60	M	0														
CS-43	44	M	0														
CS-45	28	F	1								+						
CS-46	40	M	0														
CS-47	46	M	0														
CS-48	42	M	0														
CS-49	49	M	0														
CS-50	48	M	0														
Tumor antigens				α Eno a	α Eno b	α Eno c	Hsp70	Anx2	Prdx6	PKM	α Tub	ß Tub	Atps	AR1	AR2	PGM	TPI
Autoantibody response (%)				6	10	20	2	4	2	2	1	0	4	0	0	1	0

Table A11b: Autoantibody response to tumor antigens in patients with T1/T2 tumors

Samples	No. of antigens	Tumor antigens eliciting autoantibody response												
		α Eno a	α Eno b	α Eno c	Hsp70	Anx2	Prdx6	PKM	α Tub	ß Tub	ATPS	AR1	AR2	PGM	TPI
641	1							√							
642	3		√	√			√								
648	4			√				√			√		√		
882	0														
1015	4	√	√	√			√								
1023	2						√							√	
1024	1													√	
1038	1			√											
1060	0														
1097	0														
1122	2		√	√											
1151	5			√			√		√	√	√				
1175	0														
1225	0														
1227	0														
1414	3					√	√				√				
1420	2											√	√		
1450	2			√				√							
1476	4	√	√	√			√								
1478	4		√	√				√					√		
1484	4		√	√		√					√				
1486	2		√	√											
1493	2		√	√											
1494	2			√			√								
1495	2										√		√		
1509	4				√				√	√	√				
1522	2	√						√							
1527	2						√							√	
1531	1						√								
1533	0														

Samples	No. of antigens	Tumor antigens eliciting autoantibody response												
		α Eno a	α Eno b	α Eno c	Hsp70	Anx2	Prdx6	PKM	α Tub	ß Tub	ATPS	AR1	AR2	PGM	TPI
82	7	√	√	√	√	√		√		√					
660	5	√	√	√	√	√									
190	0														
493	4	√	√	√			√								
697	4					√	√					√	√		
1006	1										√				
900	6	√	√	√	√		√					√			
1010	5	√	√	√	√		√								
1480	3			√		√				√					
1411	1			√											
1419	3			√					√	√					
1011	2		√	√											
Autoantibody response (%)		19	33	52	12	14	31	14	7	12	17	7	12	7	0

Table A11c: Autoantibody response to tumor antigens in patients with T3/T4 tumors

Samples	No. of antigens	Tumor antigens eliciting autoantibody response													
		α Eno a	α Eno b	α Eno c	Hsp70	Anx2	Prdx6	PKM	α Tub	ß Tub	ATPS	AR1	AR2	PGM	TPI
824	0														
879	4	√	√	√			√								
885	0														
891	0														
955	3		√	√			√								
974	2			√				√							
976	0														
1014	1										√				
1035	0														
1064	0														
1108	4		√	√			√			√					
1523	0														
1417	5		√	√		√		√			√				
1461	4		√	√	√		√								
1481	2		√	√											
1482	1									√					
1488	4		√	√	√						√				
1489	2			√							√				
1491	3	√	√	√											
1499	6		√	√	√		√							√	√
1504	2				√	√									
1505	2		√	√											
1506	1								√						
1520	4	√	√	√			√								
1528	1			√											
1580	0														
1606	3						√				√			√	
1608	2		√	√											
1642	4	√	√	√							√				
1454	4		√	√							√		√		

Samples	No. of antigens	Tumor antigens eliciting autoantibody response													
		α Eno a	α Eno b	α Eno c	Hsp70	Anx2	Prdx6	PKM	α Tub	ß Tub	ATPS	AR1	AR2	PGM	TPI
666	2		√	√											
825	8	√	√	√	√	√	√							√	√
623	9		√	√		√	√	√	√	√	√			√	
495	7	√	√	√	√	√		√			√				
417	3		√	√		√									
451	1									√					
365	8		√	√	√		√	√	√	√	√				
883	8		√	√		√	√	√	√	√				√	
756	13	√	√	√	√	√	√	√	√	√	√	√		√	√
766	11	√	√	√	√	√	√				√	√	√	√	√
358	1						√								
460	4	√	√	√	√										
867	9	√	√	√		√	√	√	√	√	√				
922	9	√	√	√	√		√		√	√		√	√		
670	9		√	√	√	√	√	√	√				√	√	
980	8	√	√	√	√	√			√		√		√		
957	3		√	√	√						√				
356	4	√	√	√			√								
925	5	√		√			√							√	√
1149	1										√				
1448	5		√	√	√					√			√		
1535	1				√										
1467	4	√	√	√			√								
1483	3		√	√		√									
1479	4					√		√				√	√		
136	1				√										
792	4	√	√	√	√										
828	7	√	√	√		√	√	√	√						
423	2		√									√			
1137	2					√		√							

Table A11c continued:

Samples	No. of antigens	Tumor antigens eliciting autoantibody response													
		α Eno a	α Eno b	α Eno c	Hsp70	Anx2	Prdx6	PKM	α Tub	ß Tub	ATPS	AR1	AR2	PGM	TPI
951	8	√	√	√			√	√	√	√			√		
1003	7	√	√	√		√		√	√				√		
977	6	√	√	√			√		√				√		
1534	3	√	√	√											
972	4			√	√	√					√				
1447	6	√	√	√	√		√								√
902	4		√	√		√					√				
420	1		√												
Autoantibody response (%)		32	62	68	29	28	35	20	19	16	26	7	15	13	9

Table A12: Clinical information and autoantibody response to tumor antigens in patients with cancer of GBC for which follow up information was available

Samples	Age	Sex	DS	PS	DFS (months)	Status	No. of antigens	Tumor antigens eliciting autoantibody response												
								α Eno a	α Eno b	α Eno c	Hsp 70	Anx2	Prdx 6	Pkm	α tub	ß tub	Atps	AR1	AR2	Pgm	TPI
642	50	M	MD	T1N0	18	R	3		+	+			+								
891	72	F	PD	T4N0	13	R	0														
955	40	M	MD-PD	T4aN0	6	R	3		+	+			+								
974	35	M	MD	T4aN0	5	R	2			+				+							
1024	47	M	WD-MD	T2N1	15	R	1													+	
1038	56	M	PD	T2N2b	9	R	1			+											
1060	57	M	MD	T4aN1	53	R	0														
1097	48	M	MD	T2N2b	31	R	0														
1454	54	F	PD	T4N0	13	R	4		+	+							+		+		
1476	36	M	PD	T2N2b	6	R	4	+	+	+			+								
1478	46	M	WD	T1Nx	14	R	4		+	+				+					+		
1481	55	F	PD	T4N0	9	R	2		+	+											
1482	35	F	MD	T4N2B	5	R	1									+					
1488	44	M	PD	T3N1	5	R	4		+	+	+						+				
1491	55	M	MD	T4N0	7	R	3	+	+	+											
1499	43	M	PD	T4aN0	9	R	6		+	+	+		+							+	+
1504	54	F	MD	T4aN2b	4	R	2				+	+									
1520	38	M	MD	T4N1	47	R	4	+	+	+			+								
1531	52	M	MD	T2N0	23	R	1						+								
82	68	F	MD	T2N2	15	R	7	+	+	+	+	+		+		+					
660	37	M	MD	T2N0	6	R	5	+	+	+	+	+									
825	40	F	MD	T4N2	5	R	8	+	+	+	+	+	+							+	+
495	43	M	PD	T4N0	18	R	7	+	+	+	+	+		+			+				
417	35	F	MD	T4N0	6	R	3		+	+		+									
365	50	F	PD	T3N2	3	R	8		+	+	+		+	+	+	+	+				
756	60	F	MD	T4N0	13	R	14	+	+	+	+	+	+	+	+	+	+	+	+	+	+
358	65	M	WD	T4N0	80	R	1						+								
867	56	M	PD	T4N2	1	R	9	+	+	+		+	+	+	+	+	+				
670	80	F	MD	T4N2	9	R	10		+	+	+	+	+	+	+				+	+	+
980	40	M	MD	T4N2	5	R	8	+	+	+	+	+			+		+		+		
1006	46	M	MD	T2N2	3	R	1										+				

Sample	Age	Sex	DS	PS	DFS (months)	Status	No. of antigens	Tumor antigens eliciting autoantibody response													
								Eno a	Eno b	Eno c	hsp 70	Anx2	prdx 6	Pkm	α tub	ß tub	Atps	AR 1	AR 2	Pgm	TPI
900	43	M	PD	T2N2	8	R	6	+	+	+	+		+					+			
922	55	M	PD	T4N2	1	R	9	+	+	+	+		+		+	+		+	+		
925	55	F	PD	T2N2	2	R	5	+		+			+							+	+
957	38	M	MD	T4N1	5	R	3		+	+	+						+				
1535	55	M	PD	T4N2	4	R	1				+										
1411	38	M	PD	T2N2	4	R	1			+											
1419	47	M	MD	T2N0	32	R	3			+					+	+					
641	50	M	MD	T2N1	25	NED	1							+							
648	41	M	MD	T2N2b	22	NED	4			+				+			+		+		
879	66	M	MD	T4aN0	76	NED	4	+	+	+			+								
885	67	F	PD	T4aN0	74	NED	0														
1015	55	M	PD	T2N1	49	NED	4	+	+	+			+								
1035	65	M	MD	T3N2b	31	NED	0														
1064	45	F	WD	T4aN0	64	NED	0														
1122	45	M	VC	T1N0	51	NED	2		+	+											
1151	56	M	MD	T2N0	61	NED	5			+			+		+	+	+				
1175	55	F	MD	T2N0	61	NED	0														
1227	39	M	MD	T2N2b	35	NED	0														
1414	45	M	MD	T2N0	51	NED	3					+	+				+				
1420	33	F	MD	T2N0	45	NED	2											+	+		
1484	58	M	PD	T2N0	46	NED	4		+	+		+					+				
1486	43	F	SI	T1N0	40	NED	2		+	+											
1494	48	M	PD	T2N0	45	NED	2			+			+								
1495	46	M	MD	T2N0	45	NED	2										+		+		
1509	45	M	MD	T2N2b	41	NED	4				+				+	+	+				
1522	60	M	MD	T1N0	42	NED	2	+						+							
1523	58	M	PD	T4N0	13	NED	0														
1527	57	F	MD	T4N2b	27	NED	2						+							+	
1528	36	F	PD	T4N2b	31	NED	1			+											

Table A12 continued:

1533	50	M	WD	T2N0	28	NED	0													
493	29	F	MD	T2N0	56	NED	4	+	+	+			+								
697	56	M	PD	T2NX	49	NED	4					+	+					+	+		
666	38	M	MD	T4N0	34	NED	2		+	+											
623	51	M	MD	T4N0	37	NED	9		+	+		+	+	+	+	+	+			+	
902	60	F	WD	T1N0	46	NED	4		+	+		+					+				
420	46	M	MD	T2N0	50	NED	1		+												
451	35	M	WD	T4N0	58	NED	1									+					
883	65	F	MD	T4N0	43	NED	8		+	+		+	+	+	+	+				+	
766	50	F	MD	T4NO	16	NED	11	+	+	+	+	+	+				+	+	+	+	+
460	52	M	PD	T4N0	41	NED	4	+	+	+	+										
1010	55	M	WD	T1N0	42	NED	5	+	+	+	+		+								
1149	61	M	MD	T4N0	27	NED	1										+				
1448	35	F	MD	T3N2	22	NED	5		+	+	+					+			+		
1467	38	M	PD	T4N2	7	NED	4	+	+	+			+								
1480	52	F	MD	T1N0	19	NED	3			+		+				+					
1483	67	M	MD	T4N0	40	NED	3		+	+		+									
1479	58	F	MD	T3N1	12	NED	4					+		+				+	+		
Percentage of autoantibody response in 78 samples								27	53	64	26	26	36	18	14	18	24	9	17	13	8
Tumor antigens								α Eno a	α Eno b	α Eno c	hsp 70	Anx2	prdx 6	Pkm	α tub	ß tub	Atps	AR 1	AR 2	Pgm	TPI

Table A13a: Correlation of autoantibody response to tumor antigens between healthy individuals and individuals with leukoplakia and individuals with chewing/smoking habit

Autoantibody response to Tumor antigens		Sample type		P Value	Sample type		P value
		Healthy individuals N=79 (100%)	Leukoplakia N=42 (100%)		Healthy individuals N=79 (100%)	C/S habit N=31 (100%)	
Eno a	+ve	N=5 (6.3%)	N=6 (14.3%)	0.187	N=5 (6.3%)	N=5 (16.1%)	0.141
	-ve	N=74(93.7%)	N=36(85.7%)		N=74(93.7%)	N=26(83.9%)	
Eno b	+ve	N=8 (10.1%)	N=10(23.8%)	**0.044**	N=8 (10.1%)	N=8(25.8%)	0.067
	-ve	N=71(89.9%)	N=32(76.2%)		N=71(89.9%)	N=23(74.2%)	
Eno c	+ve	N=16 (20.3%)	N=21(50%)	**0.001**	N=16 (20.3%)	N=11(35.5%)	0.095
	-ve	N=63(79.7%)	N=21(50%)		N=63(79.7%)	N=20(64.5%)	
Hsp70	+ve	N=2 (2.5%)	N=4(9.5%)	0.181	N=2 (2.5%)	N=5(16.1%)	**0.018**
	-ve	N=77(97.5%)	N=38(90.5%)		N=77(97.5%)	N=26(83.9%)	
Anx2	+ve	N=3 (3.8%)	N=7(16.7%)	**0.031**	N=3 (3.8%)	N=4(12.9%)	0.097
	-ve	N=76(96.2%)	N=35(83.3%)		N=76(96.2%)	N=27(87.1%)	
Prdx6	+ve	N=2 (2.5%)	N=4(9.5%)	0.181	N=2 (2.5%)	N=2(6.5%)	0.315
	-ve	N=78(97.5%)	N=38(90.5%)		N=78(97.5%)	N=29(93.5%)	
Pkm	+ve	N=2 (2.5%)	N=5(11.9%)	**0.048**	N=2 (2.5%)	N=3(9.7%)	0.135
	-ve	N=77(97.5%)	N=37(88.1%)		N=77(97.5%)	N=28(90.3%)	
TUBB	+ve	N=0 (0%)	N=4(9.5%)	**0.013**	N=0 (0%)	N=2(6.5%)	0.078
	-ve	N=79(100%)	N=38(90.5%)		N=79(100%)	N=29(93.5%)	
TUBA	+ve	N=1(1.2%)	N=1(2.4%)	1	N=1(1.2%)	N=1(3.2%)	0.486
	-ve	N=78(98.7%)	N=41(97.6%)		N=78(98.7%)	N=30(96.8%)	
ATPS	+ve	N=3(3.8%)	N=5(11.9%)	**0.124**	N=3(3.8%)	N=1(3.2%)	1
	-ve	N=76(96.2%)	N=37(88.1%)		N=76(96.2%)	N=30(96.8%)	
AR1	+ve	N=0(0%)	N=2(4.8%)	0.119	N=0(0%)	N=0(0%)	
	-ve	N=79(100%)	N=40(95.2%)		N=79(100%)	N=31(100%)	
AR2	+ve	N=0(0%)	N=1(3.3%)	0.347	N=0(0%)	N=0(0%)	
	-ve	N=79(100%)	N=41(97.6%)		N=79(100%)	N=31(85.3%)	
PGM	+ve	N=2(2.5%)	N=2(4.8%)	0.609	N=2(2.5%)	N=2(6.5%)	0.315
	-ve	N=77(97.5%)	N=40(95.2%)		N=77(97.5%)	N=29(93.5%)	
TPI	+ve	N=0(0%)	N=1(2.4%)	0.347	N=0(0%)	N=1(3.2%)	0.282
	-ve	N=79(100%)	N=41(97.6%)		N=79(100%)	N=30(96.8%)	

C/S-chewing/smoking

Table A13b: Correlation of autoantibody response to tumor antigens between healthy individuals and patients with T1/T2 tumors and T3/T4 tumors

Autoantibody response to Tumor antigens		Sample type		P Value	Sample type		P value
		Healthy individuals N=79 (100%)	T1/T2 N=42 (100%)		Healthy individuals N=79 (100%)	T3/T4 N=68 (100%)	
Eno a	+ve	N=5 (6.3%)	N=8(19%)	**0.060**	N=5 (6.3%)	N=22(32.4%)	**0.000**
	-ve	N=74(93.7%)	N=34(81%)		N=74(93.7%)	N=46(67.6%)	
Eno b	+ve	N=8 (10.1%)	N=13(31%)	**0.004**	N=8 (10.1%)	N=43(63.2%)	**0.000**
	-ve	N=71(89.9%)	N=29(69%)		N=71(89.9%)	N=25(36.8%)	
Eno c	+ve	N=16 (20.3%)	N=22(52.4%)	**0.000**	N=16 (20.3%)	N=46(67.6%)	**0.000**
	-ve	N=63(79.7%)	N=20(47.6%)		N=63(79.7%)	N=22(32.4%)	
Hsp70	+ve	N=2 (2.5%)	N=5(11.9%)	**0.048**	N=2 (2.5%)	N=20(29.4%)	**0.000**
	-ve	N=77(97.5%)	N=37(88.1%)		N=77(97.5%)	N=48(70.6%)	
Anx2	+ve	N=3 (3.8%)	N=6(14.3%)	0.063	N=3 (3.8%)	N=19(27.9%)	**0.000**
	-ve	N=76(96.2%)	N=36(85.7%)		N=76(96.2%)	N=49(72.1%)	
Prdx6	+ve	N=2 (2.5%)	N=13(31%)	**0.000**	N=2 (2.5%)	N=24(35.3%)	**0.000**
	-ve	N=78(97.5%)	N=29(69%)		N=78(97.5%)	N=44(64.7%)	
Pkm	+ve	N=2 (2.5%)	N=6(14.3%)	**0.020**	N=2 (2.5%)	N=14(20.6%)	**0.000**
	-ve	N=77(97.5%)	N=36(85.7%)		N=77(97.5%)	N=54(79.4%)	
TUBB	+ve	N=0 (0%)	N=5(11.9%)	**0.004**	N=0 (0%)	N=11(16.2%)	**0.000**
	-ve	N=79(100%)	N=37(88.1%)		N=79(100%)	N=57(83.8%)	
TUBA	+ve	N=1(1.2%)	N=3(7.1%)	0.120	N=1(1.2%)	N=13(19.1%)	**0.000**
	-ve	N=78(98.7%)	N=39(92.9%)		N=78(98.7%)	N=55(80.9%)	
ATPS	+ve	N=3(3.8%)	N=7(16.7%)	**0.031**	N=3(3.8%)	N=18(26.5%)	**0.000**
	-ve	N=76(96.2%)	N=35(83.3%)		N=76(96.2%)	N=50(73.5%)	
AR1	+ve	N=0(0%)	N=3(7.1%)	**0.040**	N=0(0%)	N=5(7.4%)	**0.020**
	-ve	N=79(100%)	N=39(92.9%)		N=79(100%)	N=63(92.6%)	
AR2	+ve	N=0(0%)	N=5(11.9%)	**0.004**	N=0(0%)	N=10(14.7%)	**0.000**
	-ve	N=79(100%)	N=37(88.1%)		N=79(100%)	N=58(85.3%)	
PGM	+ve	N=2(2.5%)	N=3(7.1%)	0.340	N=2(2.5%)	N=9(13.2%)	**0.014**
	-ve	N=77(97.5%)	N=39(92.9%)		N=77(97.5%)	N=59(86.8%)	
TPI	+ve	N=0(0%)	N=0(0%)		N=0(0%)	N=6(8.8%)	**0.009**
	-ve	N=79(100%)	N=42(100%)		N=79(100%)	N=62(91.2%)	

Table A13c: Correlation of autoantibody response to tumor antigens between healthy individuals with chewing/smoking habit and leukoplakia and between patients with T1/T2 and T3/T4 tumors

Autoantibody response to Tumor antigens		Sample type		P Value	Sample type		P value
		C/S habit N=31 (100%)	Leukoplakia N=42 (100%)		T1/T2 N=42 (100%)	T3/T4 N=68 (100%)	
Eno a	+ve	N=5(16.1%)	N=6(14.3%)	0.540	N=8(19%)	N=22(32.4%)	0.128
	-ve	N=26(83.9%)	N=36(85.7%)		N=34(81%)	N=46(67.6%)	
Eno b	+ve	N=8(25.8%)	N=10(23.8%)	0.845	N=13(31%)	N=43(63.2%)	**0.001**
	-ve	N=23(74.2%)	N=32(76.2%)		N=29(69%)	N=25(36.8%)	
Eno c	+ve	N=11(35.5%)	N=21(50%)	0.217	N=22(52.4%)	N=46(67.6%)	0.109
	-ve	N=20(64.5%)	N=21(50%)		N=20(47.6%)	N=22(32.4%)	
Hsp70	+ve	N=5(16.1%)	N=4(9.5%)	0.481	N=5(11.9%)	N=20(29.4%)	**0.033**
	-ve	N=26(83.9%)	N=38(90.5%)		N=37(88.1%)	N=48(70.6%)	
Anx2	+ve	N=4(12.9%)	N=7(16.7%)	0.750	N=6(14.3%)	N=19(14.3%)	0.097
	-ve	N=27(87.1%)	N=35(83.3%)		N=36(85.7%)	N=49(72.1%)	
Prdx6	+ve	N=2(6.5%)	N=4(9.5%)	1.0	N=13(31%)	N=24(35.3%)	0.640
	-ve	N=29(93.5%)	N=38(90.5%)		N=29(69%)	N=44(64.7%)	
Pkm	+ve	N=3(9.7%)	N=5(11.9%)	1.0	N=6(14.3%)	N=14(20.6%)	0.405
	-ve	N=28(90.3%)	N=37(88.1%)		N=36(85.7%)	N=54(79.4%)	
TUBB	+ve	N=2(6.5%)	N=4(9.5%)	1.0	N=5(11.9%)	N=11(16.2%)	0.537
	-ve	N=29(93.5%)	N=38(90.5%)		N=37(88.1%)	N=57(83.8%)	
TUBA	+ve	N=1(3.2%)	N=1(2.4%)	1.0	N=3(7.1%)	N=13(19.1%)	0.084
	-ve	N=30(96.8%)	N=41(97.6%)		N=39(92.9%)	N=55(80.9%)	
ATPS	+ve	N=1(3.2%)	N=5(11.9%)	0.232	N=7(16.7%)	N=18(26.5%)	0.233
	-ve	N=30(96.8%)	N=37(88.1%)		N=35(83.3%)	N=50(73.5%)	
AR1	+ve	N=0(0%)	N=2(4.8%)	0.505	N=3(7.1%)	N=5(7.4%)	1.0
	-ve	N=31(100%)	N=40(95.2%)		N=39(92.9%)	N=63(92.6%)	
AR2	+ve	N=0(0%)	N=1(3.3%)	1.0	N=5(11.9%)	N=10(14.7%)	0.677
	-ve	N=31(85.3%)	N=41(97.6%)		N=37(88.1%)	N=58(85.3%)	
PGM	+ve	N=2(6.5%)	N=2(4.8%)	1.0	N=3(7.1%)	N=9(13.2%)	0.367
	-ve	N=29(93.5%)	N=40(95.2%)		N=39(92.9%)	N=59(86.8%)	
TPI	+ve	N=1(3.2%)	N=1(2.4%)	1.0	N=0(7.1%)	N=6(8.8%)	0.081
	-ve	N=30(96.8%)	N=41(97.6%)		N=42(100%)	N=62(91.2%)	

C/S- chewing/smoking

Table A14a: Correlation of antibody response to Annexin 2 and Peroxiredoxin 6 with clinical parameters

Clinicopathological parameters		Autoantibody response against each of tumor antigens						
		N=78	Anx 2		p Value	PRDX 6		p Value
			Yes	No		Yes	No	
Age (Median, 50 yrs) (29-80 Yrs)	≤50	N=43(55%)	N=6(14%)	N=37(86%)	0.009	N=11(25%)	N=32(68%)	0.035
	>50	N=35(45%)	N=14(40%)	N=21(60%)		N=17(48%)	N=18(52%)	
Sex	Male	N=52(67%)	N=10(19%)	N=42(81%)	0.067	N=17(33%)	N=35(67%)	0.404
	Female	N=26(33%)	N=10(26%)	N=16(74%)		N=11(42%)	N=15(58%)	
Stages	I/II	N=23(29%)	N=6(26%)	N=17(74%)	0.953	N=8(35%)	N=15(65%)	0.894
	III/IV	N=55(71%)	N=14(25%)	N=41(75%)		N=20(36%)	N=35(64%)	
Tumor Size	T1/T2	N=37(47%)	N=7(19%)	N=30(81%)	0.196	N=12(32%)	N=25(68%)	0.544
	T3/T4	N=41(53%)	N=13(32%)	N=28(68%)		N=16(39%)	N=25(61%)	
Nodal Status	+ve	N=34(44%)	N=8(23%)	N=26(77%)	0.790	N=13(38%)	N=21(62%)	0.657
	-ve	N=42(55%)	N=11(26%)	N=31(74%)		N=14(33%)	N=28(67%)	
Differentiation	WD/MD	N=52(67%)	N=16(31%)	N=36(69%)	0.166	N=16(31%)	N=36(69%)	0.141
	PD	N=25(32%)	N=4(16%)	N=21(84%)		N=12(48%)	N=13(52%)	
Recurrence	Yes	N=38(48%)	N=10(26%)	N=28(74%)	0.894	N=15(39%)	N=23(61%)	0.521
	No	N=40(52%)	N=10(25%)	N=30(70%)		N=13(32%)	N=27(68%)	

Table A14b: Correlation of antibody response to ATP synthase and Phosphoglycerate mutase with clinical parameters

Clinicopathological parameters		Autoantibody response against each of tumor antigens						
		N=78	ATP Synthase		p Value	PGM		p Value
			Yes	No		Yes	No	
Age (Median, 50 yrs) (29-80 Yrs)	≤50	N=43(55%)	N=11(25%)	N=32(75%)	0.780	N=3(7%)	N=40(93%)	0.087
	>50	N=35(45%)	N=8(23%)	N=27(77%)		N=7(20%)	N=28(80%)	
Sex	Male	N=52(67%)	N=14(27%)	N=38(73%)	0.456	N=3(6%)	N=49(94%)	0.008
	Female	N=26(33%)	N=5(19%)	N=21(81%)		N=7(27%)	N=19(73%)	
Stages	I/II	N=23(29%)	N=5(22%)	N=18(78%)	0.727	N=0	N=23(100%)	0.029
	III/IV	N=55(71%)	N=14(25%)	N=41(75%)		N=10(18%)	N=45(82%)	
Tumor Size	T1/T2	N=37(47%)	N=8(22%)	N=29(78%)	0.593	N=2(5%)	N=35(95%)	0.063
	T3/T4	N=41(53%)	N=11(27%)	N=30(73%)		N=8(19%)	N=33(81)	
Nodal Status	+ve	N=34(44%)	N=9(26%)	N=25(74%)	0.790	N=6(18%)	N=28(82%)	0.298
	-ve	N=42(55%)	N=10(24%)	N=32(76%)		N=4(9%)	N=38(91%)	
Differentiation	WD/MD	N=52(67%)	N=13(25%)	N=39(75%)	0.924	N=8(15%)	N=44(85%)	0.367
	PD	N=25(32%)	N=6(24%)	N=19(81%)		N=2(8%)	N=23(92%)	
Recurrence	Yes	N=38(48%)	N=9(24%)	N=29(76%)	0.892	N=6(16%)	N=32(84%)	0.445
	No	N=40(52%)	N=10((25%)	N=30(75%)		N=4(10%)	N=36(90%)	

Table A14c: Correlation of antibody response to Aldose reductase 1 and Aldose reductase 2 with clinical parameters

Clinicopathological parameters		Autoantibody response against each of tumor antigens						
		N=78	AR1		p Value	AR2		p Value
			Yes	No		Yes	No	
Age (Median, 50 yrs) (29-80 Yrs)	≤50	N=43(55%)	N=2(5%)	N=41(95%)	0.139	N=6(14%)	N=37(86%)	0.476
	>50	N=35(45%)	N=5(14%)	N=30(86%)		N=7(20%)	N=28(80%)	
Sex	Male	N=52(67%)	N=3(6%)	N=49(94%)	0.161	N=6(11%)	N=47(89%)	0.086
	Female	N=26(33%)	N=4(15%)	N=22(85%)		N=7(27%)	N=19(73%)	
Stages	I/II	N=23(29%)	N=2(9%)	N=21(91%)	0.956	N=4(17%)	N=19(83%)	0.912
	III/IV	N=55(71%)	N=5(9%)	N=50(91%)		N=9(16%)	N=46(84%)	
Tumor Size	T1/T2	N=37(47%)	N=3(8%)	N=34(92%)	0.799	N=5(13%)	N=32(87%)	0.478
	T3/T4	N=41(53%)	N=4(10%)	N=37(90%)		N=8(19%)	N=33(81%)	
Nodal Status	+ve	N=34(44%)	N=4(12%)	N=30(88%)	0.260	N=7(20%)	N=27(80%)	0.173
	-ve	N=42(55%)	N=2(5%)	N=40(95%)		N=4(9%)	N=38(91%)	
Differentiation	WD/MD	N=52(67%)	N=4(8%)	N=48(92%)	0.538	N=10(19%)	N=42(81%)	0.428
	PD	N=25(32%)	N=3(12%)	N=22(88%)		N=3(12%)	N=22(88%)	
Recurrence	Yes	N=38(48%)	N=3(8%)	N=35(92%)	0.745	N=6(16%)	N=32(84%)	0.839
	No	N=40(52%)	N=4(10%)	N=36(90%)		N=7(17%)	N=33(83%)	

Table A14d: Correlation of antibody response to Alpha tubulin and Beta tubulin with clinical parameters

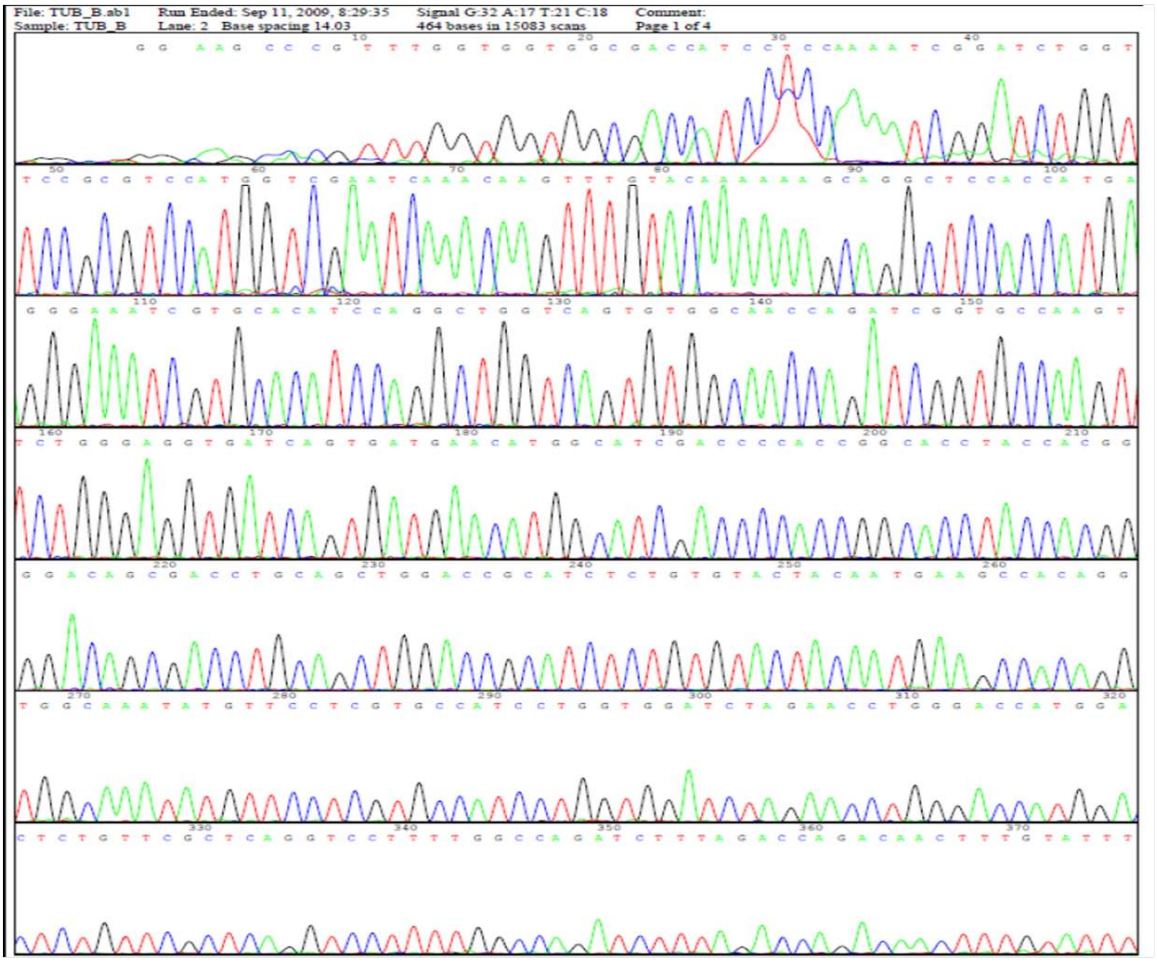
Clinicopathological parameters		Autoantibody response against each of tumor antigens						
		N=78	Alpha tubulin		p Value	Beta tubulin		p Value
			Yes	No		Yes	No	
Age (Median, 50 yrs) (29-80 Yrs)	≤50	N=43(55%)	N=4(9%)	N=39(91%)	0.177	N=6(14%)	N=37(86%)	0.308
	>50	N=35(45%)	N=7(20%)	N=28(80%)		N=8(23%)	N=27(77%)	
Sex	Male	N=52(67%)	N=7(13%)	N=45(87%)	0.818	N=7(13%)	N=45(87%)	0.144
	Female	N=26(33%)	N=4(15%)	N=22(85%)		N=7(27%)	N=19(73%)	
Stages	I/II	N=23(29%)	N=2(9%)	N=21(91%)	0.375	N=3(13%)	N=20(87%)	0.465
	III/IV	N=55(71%)	N=9(16%)	N=46(84%)		N=11(20%)	N=44(80%)	
Tumor Size	T1/T2	N=37(47%)	N=3(8%)	N=34(92%)	0.148	N=5(7%)	N=32(93%)	0.332
	T3/T4	N=41(53%)	N=8(19%)	N=33(81%)		N=9(22%)	N=32(78%)	
Nodal Status	+ve	N=34(44%)	N=6(18%)	N=28(82%)	0.479	N=7(20%)	N=27(80%)	0.661
	-ve	N=42(55%)	N=5(12%)	N=37(88%)		N=7(17%)	N=35(83%)	
Differentiation	WD/MD	N=52(67%)	N=8(15%)	N=44(85%)	0.691	N=11(21%)	N=41(79%)	0.329
	PD	N=25(32%)	N=3(12%)	N=22(88%)		N=3(12%)	N=22(88%)	
Recurrence	Yes	N=38(48%)	N=3(8%)	N=35(92%)	0.745	N=6(16%)	N=32(84%)	0.839
	No	N=40(52%)	N=4(10%)	N=36(90%)		N=7(17%)	N=33(83%)	

Table A14e: Correlation of antibody response to Triose phosphate isomerase and Pyruvate kinase 2 with clinical parameters

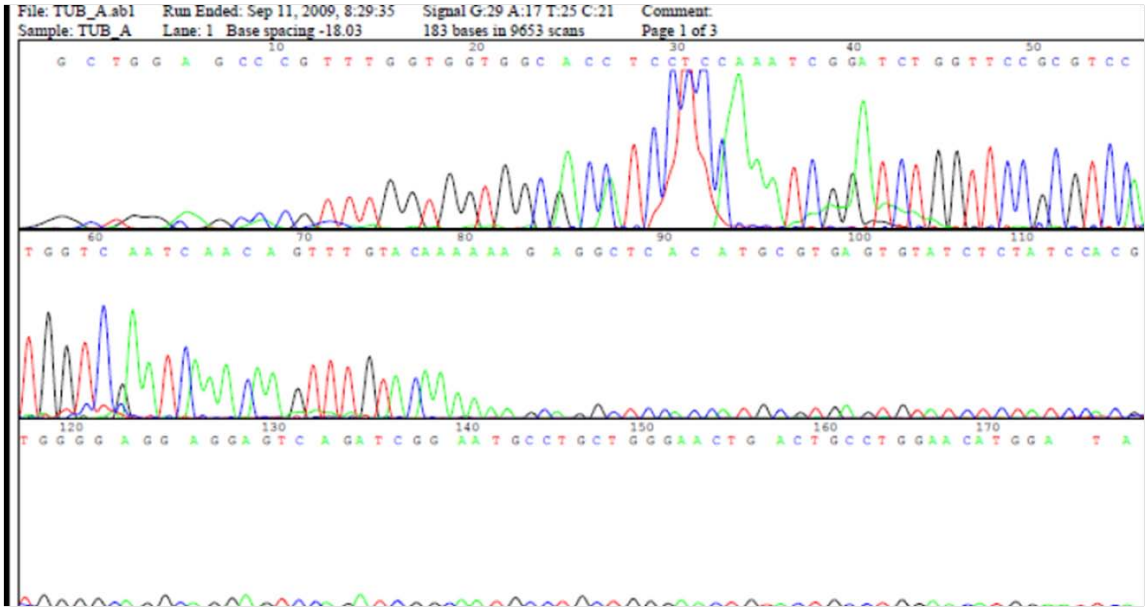
Clinicopathological parameters		Autoantibody response against each of tumor antigens						
		N=78	TPI		p Value	PKm 2		p Value
			Yes	No		Yes	No	
Age (Median, 50 yrs) (29-80 Yrs)	≤50	N=43(55%)	N=2(5%)	N=41(95%)	0.264	N=6(14%)	N=37(86%)	0.308
	>50	N=35(45%)	N=4(11%)	N=31(89%)		N=8(23%)	N=27(77%)	
Sex	Male	N=52(67%)	N=1(2%)	N=51(98%)	0.007	N=7(13%)	N=45(87%)	0.144
	Female	N=26(33%)	N=5(19%)	N=21(81%)		N=7(27%)	N=19(73%)	
Stages	I/II	N=23(29%)	N=0	N=23(100%)	0.099	N=2(9%)	N=21(91%)	0.168
	III/IV	N=55(71%)	N=6(11%)	N=49(89%)		N=12(22%)	N=43(78%)	
Tumor Size	T1/T2	N=37(47%)	N=1(3%)	N=36(97%)	0.116	N=5(14%)	N=32(86%)	0.332
	T3/T4	N=41(53%)	N=5(12%)	N=36(88%)		N=9(22%)	N=32(78%)	
Nodal Status	+ve	N=34(44%)	N=4(12%)	N=30(88%)	0.260	N=7(20%)	N=27(80%)	0.468
	-ve	N=42(55%)	N=2(5%)	N=40(95%)		N=6(14%)	N=36(86%)	
Differentiation	WD/MD	N=52(67%)	N=4(8%)	N=48(92%)	0.962	N=11(21%)	N=41(79%)	0.329
	PD	N=25(32%)	N=2(8%)	N=23(92%)		N=3(12%)	N=22(88%)	
Recurrence	Yes	N=38(48%)	N=5(13%)	N=33(87%)	0.077	N=8(21%)	N=30(79%)	0.486
	No	N=40(52%)	N=1(2%)	N=39(98%)		N=6(15%)	N=34(85%)	

Fig. A1: DNA sequence for genes cloned in pDEST15:

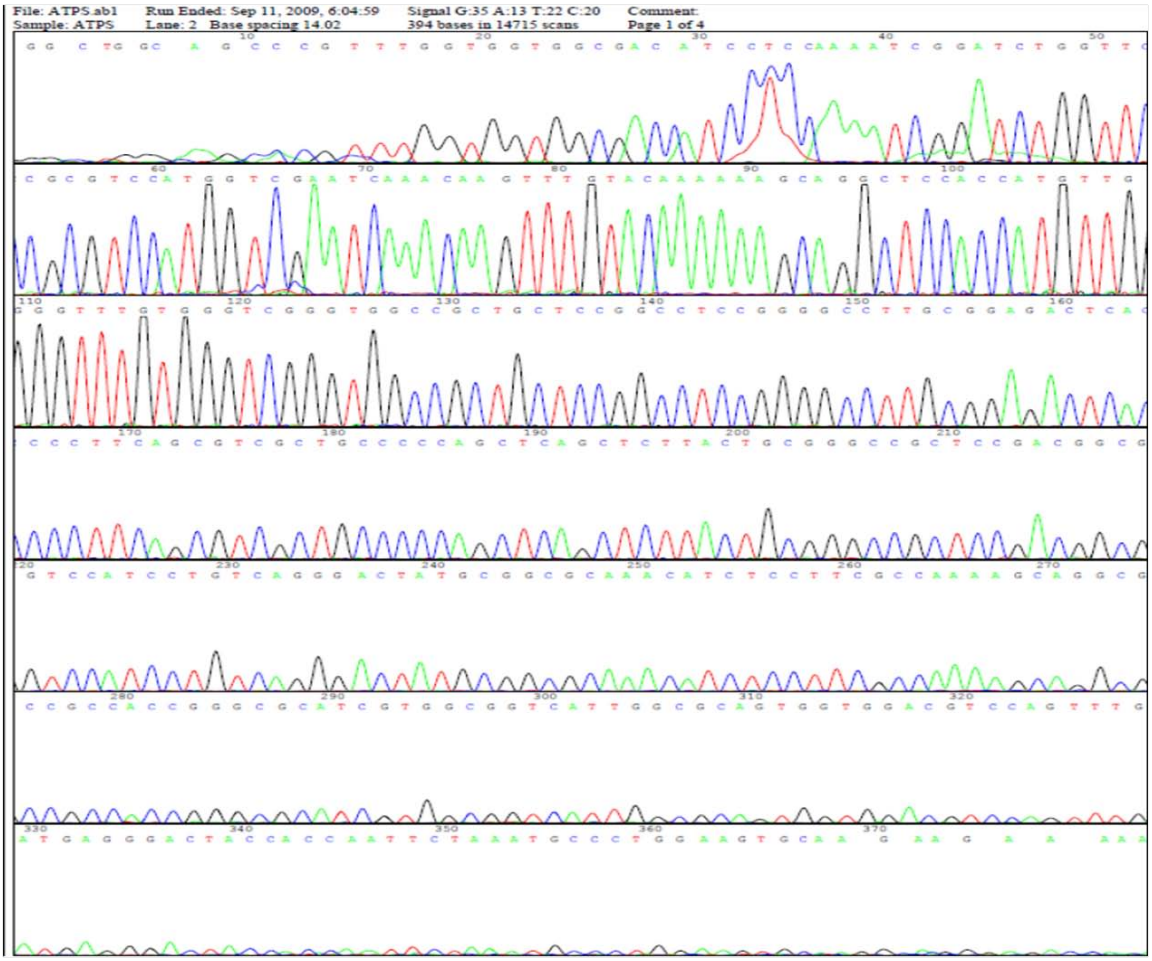
1) **Beta Tubulin (TUBB)**



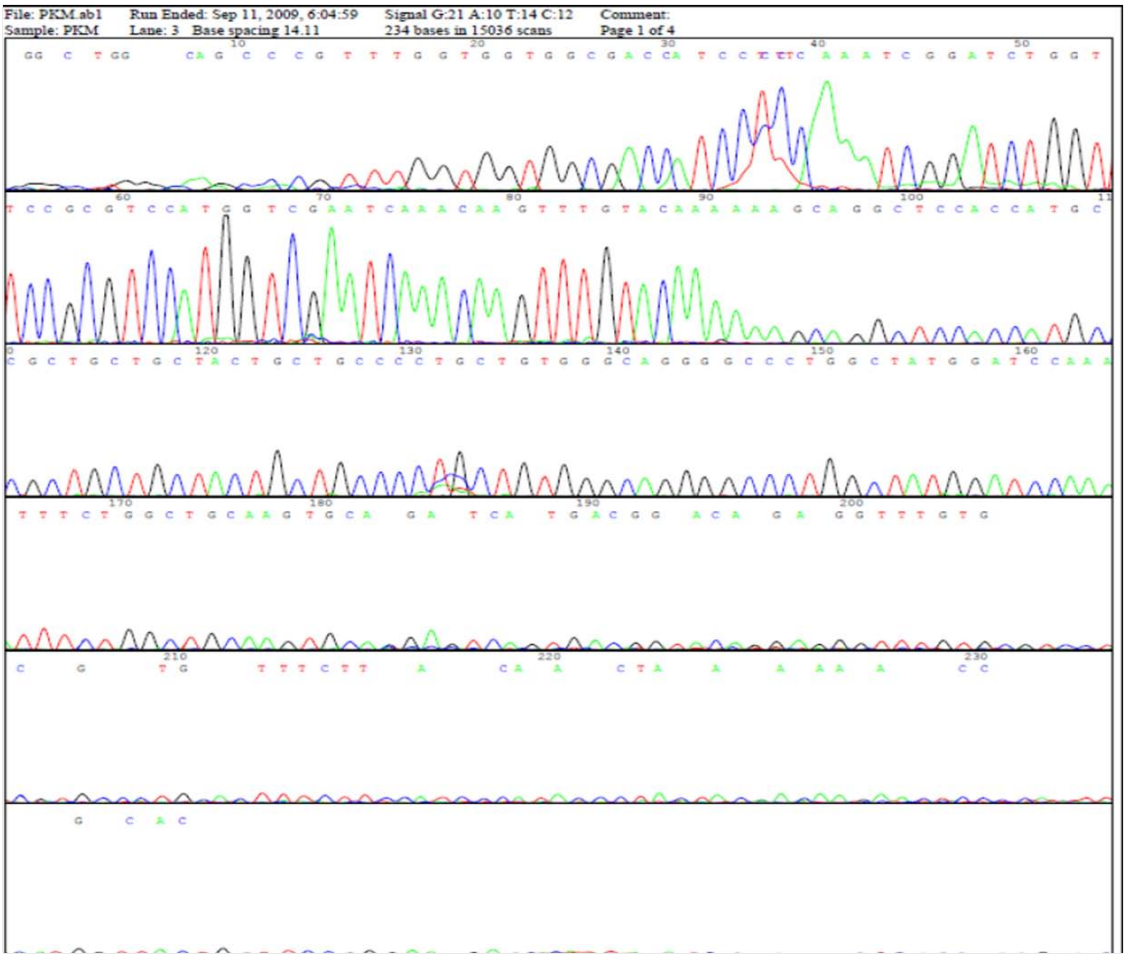
2) **Alpha Tubulin (TUBA)**



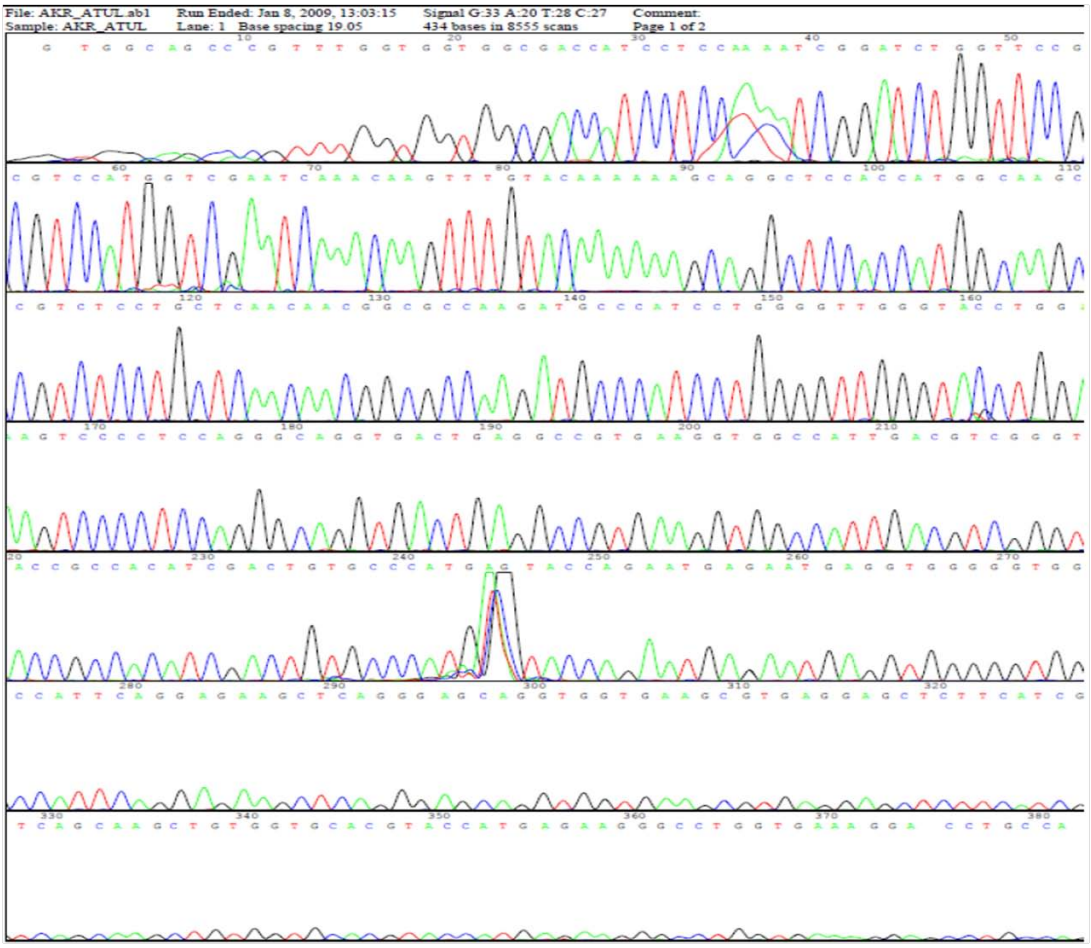
3) ATP synthase (ATPS)



4) Pyruvate kinase (PKM)



5) Aldose reductase (AKR)



6) Phosphoglycerate Mutase (PGM)

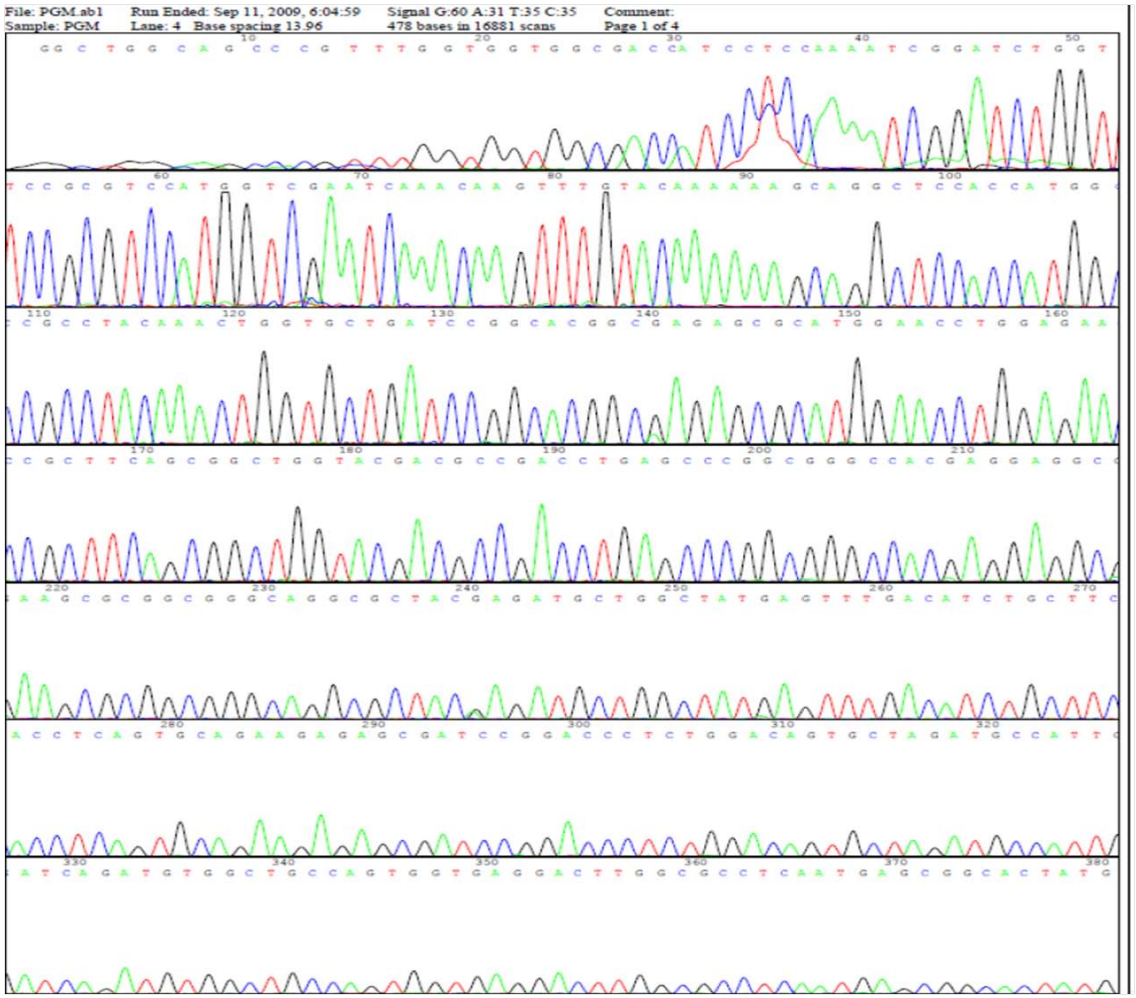
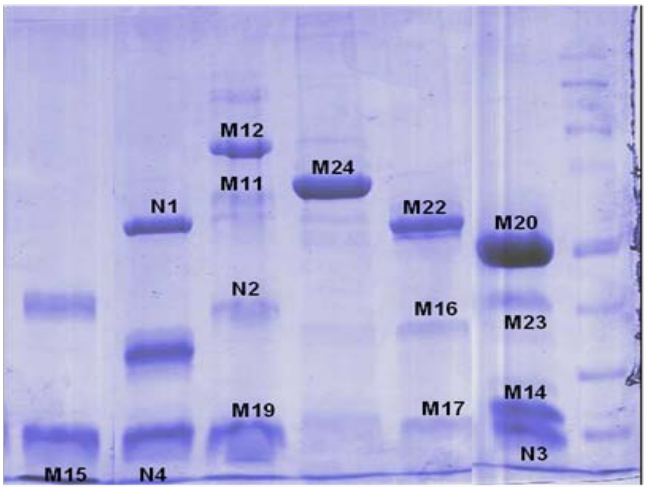


Fig. A2: 1DE-gel profile of recombinant proteins used for mass spectrometry



Spot no.	Protein Id
M15	GST
N1	Annexin A2,GST
N4	GST
M12	Hsp70,GST
M11	Hsp70
N2	Hsp70
M19	GST
M24	alpha-Enolsae,GST
M22	ALDR,GST
M16	ALDR,GST
M17	GST
M20	PGM1,GST
M23	PGM1
M14	PGM1
N3	PGM1,GST

Table A15: Mass-spectrometry data for recombinant proteins shown in Fig. A2:

Spot No.	Protein Name	Protein ID	Accession No.	Score	Mass (Da)	Intensity coverage (%)	Sequence coverage (%)	Tolerance (ppm)	Searched Peaks	Matched Peaks
M15	Glutathione S-transferase class-mu 26 kDa isozyme	GST26_SCHJA	P08515	108	25710	59	53	100	77	15
N1	Annexin A2	ANXA2_HUMAN	P07355	184	38808	46	53	100	68	23
	Glutathione S-transferase class-mu 26 kDa isozyme	GST26_SCHJA	P08515	70	25710	22	44	100	68	10
N4	Glutathione S-transferase class-mu 26 kDa isozyme	GST26_SCHJA	P08515	108	25710	56	44	100	49	11
M12	Heat shock 70 kDa protein 1A/1B	HSP71_HUMAN	P08107	183	70294	56	41	100	59	22
	Glutathione S-transferase class-mu 26 kDa isozyme	GST26_SCHJA	P08515	58	25710	23	44	100	59	9
M11	Heat shock 70 kDa protein 1A/1B	HSP71_HUMAN	P08107	164	70294	48	36	100	67	21
N2	Heat shock 70 kDa protein 1A/1B	HSP71_HUMAN	P08107	71	70294	19	20	100	19	8
M19	Glutathione S-transferase class-mu 26 kDa isozyme	GST26_SCHJA	P08515	107	25710	70	54	100	61	15
M24	Alpha-enolase	ENOA_HUMAN	P06733	77	47481	75	52	100	64	19

	Glutathione S-transferase class-mu 26 kDa isozyme	**GST26_SCHJA**	P08515	58	25710	7	44	100	64	10
M22	**Aldose reductase**	**ALDR_HUMAN**	P15121	114	36230	34	57	100	67	13
	Glutathione S-transferase class-mu 26 kDa isozyme	**GST26_SCHJA**	P08515	71	25710	25	44	100	67	11
M16	**Aldose reductase**	**ALDR_HUMAN**	P15121	53	36230	11	31	100	56	7
	Glutathione S-transferase class-mu 26 kDa isozyme	**GST26_SCHJA**	P08515	80	25710	31	48	100	56	11
M17	**Glutathione S-transferase class-mu 26 kDa isozyme**	**GST26_SCHJA**	P08515	71	25710	32	44	100	44	8
M20	**Phosphoglycerate mutase 1**	**PGAM1_HUMAN**	P18669	81	28900	68	46	100	40	9
	Glutathione S-transferase class-mu 26 kDa isozyme	**GST26_SCHJA**	P18669	51	25710	21	37	100	40	6
M23	**Phosphoglycerate mutase 1 OS=Homo sapiens**	**PGAM1_HUMAN**	P18669	134	28900	38	61	100	74	19
M14	**Phosphoglycerate mutase 1**	**PGAM1_HUMAN**	P18669	226	28900	87	73	100	39	19
N3	**Phosphoglycerate mutase 1**	**PGAM1_HUMAN**	P18669	151	28900	19	60	100	43	16
	Glutathione S-transferase class-mu 26 kDa isozyme	**GST26_SCHJA**	P08515	69	25710	27	44	100	43	8

Publications

DOI 10.1002/prca.201200081 *Proteomics Clin. Appl.* 2013, *7*, 392–402

RESEARCH ARTICLE

Prognostic utility of autoantibodies to α-enolase and Hsp70 for cancer of the gingivo-buccal complex using immunoproteomics

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Received: August 9, 2012
Revised: October 11, 2012
Accepted: October 26, 2012

Purpose: Studies from our laboratory have reported 14 tumor antigens that elicit an autoantibody response in patients with cancer of the gingivobuccal complex (GBC) In this study, utility of the autoantibody response has been evaluated for prognosis of cancer of the GBC.
Experimental design: Autoantibody response was evaluated using immunoproteomics and the prognostic significance was assessed by Kaplan-Meier survival and multivariate analysis.
Results: Autoantibody response against α-enolase isoforms a, b, and c and Hsp70 was detected in 27, 53, 64, and 26% of the 78 patients, respectively. Patients positive for autoantibody response to α-ENO and Hsp70 individually and in combination, showed significantly reduced disease-free survival (DFS) compared to those who do not show autoantibody response to either of them. Further the patients, who exhibit autoantibody response to α-ENO and Hsp70 in combination with nodal involvement and/or differentiation status, have significantly lowered DFS. The relative risk of recurrence is 3.41 for patients who exhibit autoantibody response to both the antigens.
Conclusions and clinical relevance: Autoantibody response against α-ENO and Hsp70 provides an additional parameter and may be utilized along with nodal involvement and differentiation status for better prognosis of cancer of GBC.

Keywords:
Alpha-enolase / Autoantibodies / Hsp70 / Oral cancer / Prognosis

Additional supporting information may be found in the online version of this article at the publisher's web-site

1 Introduction

Oral cancer is a major cause of cancer morbidity and mortality in males in India [1]. Squamous cell carcinoma of the oral cavity ranks as the 15th most common cancer in the world and 10th most frequent in males [1]. In India, the gingivobuccal complex of the oral cavity is the common site of cancer in males due to the prevalent habit of chewing tobacco [2]. A major hurdle in the management of oral cancer is high rate of locoregional recurrence. Currently, diagnosis, treatment, and prognosis of the disease are entirely based on clinical and radiologic and standard histopathologic findings. Such techniques do not allow for recognition of genotypically different tumors with identical morphology and different prognosis. Therefore identification of biological markers is necessary to complement clinicopathological findings for a more accurate prediction of individual patient's prognosis and to help clinicians in planning more effective therapeutic strategies.

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Abbreviations: DFS, disease-free survival; **α-ENO**, α-enolase isoforms a, b, c together; **GBC**, Gingivobuccal complex; **MD**, moderately differentiated; **PD**, poorly differentiated; **TPI**, triose phosphate isomerase; **WD**, well differentiated

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A large number of biomarkers are being reported for cancer of the head and neck region [3, 4] and their diagnostic and prognostic utility are being assessed [5–9]. Autoantibody response is also receiving increasing attention as a molecular sieve for identifying specific biomarkers for cancer for detection, prognosis, progression, and response to therapy [10–20]. It is apparent that autoantibodies to tumor antigens have potential for clinical evaluation, as their levels can be determined in blood using less invasive techniques without the need for biopsies.

Various approaches have been used for identification of autoantibody response in head and neck cancer. These include, ELISA [14, 21–24], immunoproteomics [16, 20, 25], phage display library [15], and AMIDA-autoantibody-mediated identification of antigens [26]. In most studies, except ours [16, 20] wherein biomarkers are being identified, attention has not been given to subsite specificity of the oral cavity/head and neck region.

Using immunoproteomics, many tumor antigens and their cognate antibodies have been identified and reported as biomarkers in different cancers [27–31]. We have also reported a set of proteins that elicit autoantibody response in patients with cancer of gingivo-buccal complex (GBC), a subsite of the oral cavity [16]. Their site specificity is evident, as there is negligible autoantibody response to these antigens in patients with cancer of tongue [20]. The tumor antigens reported in our study [16, 20] include α-enolase and Hsp70. Autoantibodies to α-enolase and Hsp70 have been reported in several cancers including those in the head and neck region [16, 21, 32–35]. These studies have focused on the diagnostic utility of these tumor markers. In the present study, the utility of these autoantibodies in prognosis of cancer of GBC has been investigated.

2 Materials and methods

2.1 Blood samples from patients with cancer of GBC

This study was approved by the Hospital ethics committee of the Tata Memorial Centre, Mumbai, India. Blood was collected from 78 patients with cancer of GBC with due written consent and serum was isolated as reported earlier [16]. Clinical information of the patients was obtained from hospital records and is summarized in Supporting Information Table S1.

2.2 Autoantibody response

Autoantibody response to the tumor antigens identified in our earlier study [16], namely α-enolase (isoforms a, b, and c), peroxiredoxin VI, annexin II, Hsp70, pyruvate kinase, alpha tubulin, beta tubulin, ATP synthase, phosphoglycerate mutase, aldose reductase, and triose phosphate isomerase (TPI) were evaluated in sera of 47 patients with cancer of GBC and 50 healthy individuals using immunoproteomics. Autoantibody response to the same antigens in 31 patients reported in our earlier investigations [16, 20] were also included in this study. Briefly, IgGs were isolated from serum using the Melon gel IgG spin purification kit (Pierce, USA, Cat. No. 45206) as discussed earlier [16]. Proteins from KB cell line were resolved by 2DE and blotted on to PVDF membranes and probed with IgGs purified from patients and healthy individuals. The IgGs bound to the antigens were detected with horseradish peroxidase-labeled antihuman IgG and the Enhanced Chemiluminescence (ECL) Detection system (RPN-2132, GE Healthcare, USA) and the image was captured on an x-ray film. Multiple gels and their corresponding blots were processed simultaneously to minimize experimental differences. The presence of a spot was scored as positive when the signal on the autograph was clearly defined by its shape that overlapped with the colloidal gold/amido black pattern for the protein on the PVDF membrane. Details of the 2DE, immunoblotting, and scoring procedures are given in the Supporting Information MMS1.

2.3 Statistical analysis

Statistical analysis was performed using the SPSS 16.0 software package. Clinicopathological parameters were correlated with autoantibody response detected in sera against each tumor antigen using Chi square test. The association of followup information with autoantibody response observed in the patients was evaluated by Kaplan-Meier analysis and multivariate analysis by Cox-regression method.

3 Results

3.1 Assessment of autoantibody response against the tumor antigens by immunoproteomics

The autoantibody response against the antigens identified in our earlier study [16], namely α-enolase (isoforms a, b, and c), peroxiredoxin VI, annexin II, Hsp70, pyruvate kinase, alpha tubulin, beta tubulin, ATP synthase, phosphoglycerate mutase, aldose reductase, and TPI was evaluated in 78 patients with cancer of GBC using the 2DE immunoproteomics approach. Of these, the autoantibody response to 31 patients has been reported in our earlier publications [16, 20]. Autoantibody response was also evaluated in 50 healthy individuals as control, Supporting Information Table S2. Representative autographs of KB 2DE immunoblots with signals obtained when probed with purified IgG from patients with cancer of GBC and for the control sera are given in Supporting Information Fig. S1 (A–L).

Supporting Information Table S3 shows the final percentage of autoantibody response to each of the tumor antigens in patients and healthy individuals. Autoantibody response to the antigens varied between 8% for TPI to 64% for α-enolase c

and the combination of antigens eliciting an autoantibody response was different among patients. Details of autoantibody response to the antigens by each of the patients considered in this study are given in Supporting Information Table S1. The intensity of the signal for each protein spot was distinct for each patient that reflects the specific autoantibody response of the individual to each antigen. Autoantibody response against α-enolase isoforms a, b, and c was detected in 27, 53, and 64% of the 78 patients, respectively. The autoantibody response to Hsp70 was seen in 26% of the patients. Autoantibody response to three isoforms of α-enolase and Hsp70 was much lower and was seen in 6, 10, 20, and 4%, respectively of normal healthy individuals.

3.2 Correlation of the autoantibody profile for each of the tumor antigens with clinicopathological parameters

Clinical significance of the autoantibody response to tumor antigens and clinicopathologic parameters were assessed using Chi-square test. Significant correlations were seen with Hsp70 and α-ENO. Supporting Information Table S4 shows the data for these two antigens and those for the other antigens is given in the Supporting Information Table S5. Autoantibody response to Hsp70 shows significant correlation with stage, tumor size, nodal status, and recurrence while autoantibody to α-ENO correlates only with differentiation at $p = 0.055$. The autoantibody response for both the antigens did not correlate with age and sex of the patients.

3.3 Association of autoantibody response with disease-free survival (DFS)

Kaplan-Meier survival analysis was performed for autoantibody response against each tumor antigen reported earlier to assess their independent association with DFS. Fig. 1 (A–D) and Fig. 2 (A–C) show the data for each of the isoforms of α-enolase and all isoforms taken together (α-ENO) and Hsp70. The data for the other antigens is given in the Supporting Information Fig. S2 (A–J). Significant association of autoantibody response with DFS was seen with α-enolase and Hsp70 and this is described in detail below. For the other antigens, the association was not significant, except for TPI for which only 6 of 78 patients showed autoantibody positivity, Supporting Information Fig. S2-J.

3.3.1 Association of autoantibody response to α-ENO isoforms and Hsp70 individually with DFS

Correlation of autoantibody response to each of the three isoforms of α-enolase individually, with DFS of the patients is shown in Fig. 1(A–C). Among the three isoforms, autoantibody response to the isoform α-enolase-c showed significant correlation with DFS of patients ($p = 0.0187$), Fig. 1C. A similar trend was seen with autoantibody response to α-ENO at $p = 0.053$, Fig. 1D and for Hsp70 at $p = 0.0002$, Fig. 2A. It is seen that patients who exhibit autoantibody response to each of the antigens recur significantly faster than those who are negative for autoantibody response.

3.3.2 Association of autoantibody response to Hsp70 and α-ENO in combination with DFS

Patients were grouped in those, who do not show antibody response to either of the antigens, those who exhibit autoantibody response to either Hsp70 or α-ENO, those who show antibody response to both Hsp70 and α-ENO. Autoantibody response seen in these groups was correlated with DFS of patients. The survival curves are shown in Fig. 2B. It is seen that patients with autoantibody response to both antigens recur faster than those without an autoantibody response, $p = 0.0013$. Similarly presence of autoantibody response to Hsp70 and α-enolase isoform c in combination significantly correlates with DFS of patients ($p = 0.0017$), Fig. 2C.

From the above, it is clear that presence of an autoantibody response to the two antigens has correlation with DFS and this can be an added parameter for prognosis of cancer of the GBC.

3.4 Nodal and differentiation status of the tumor and DFS

The nodal and differentiation status of the tumor are well-established prognostic clinical parameters. Supporting Information Fig. S3 (A and B) show the survival curves for the patients in this study with respect to these parameters.

3.4.1 Autoantibody response to α-ENO, nodal status, and DFS

The autoantibody response to Hsp70 and α-ENO independently correlate with DFS. The association of each of these parameters in combination with nodal status was evaluated with DFS.

Data from 76 patients (for two patients pathological information was not available to assess nodal status) were divided into four groups based on the presence of antibody response to α-ENO and nodal involvement. The survival curve obtained for each group is shown in Fig. 3A. The overall trend is statistically significant at $p = 0.0002$. Importantly, there is a clear decrease in DFS in patients who have node negative tumors and exhibit an autoantibody response to α-ENO and those who do not. Similarly among node positive patients, individuals who exhibit autoantibody to α-ENO recur faster. A similar significant trend was observed when autoantibody

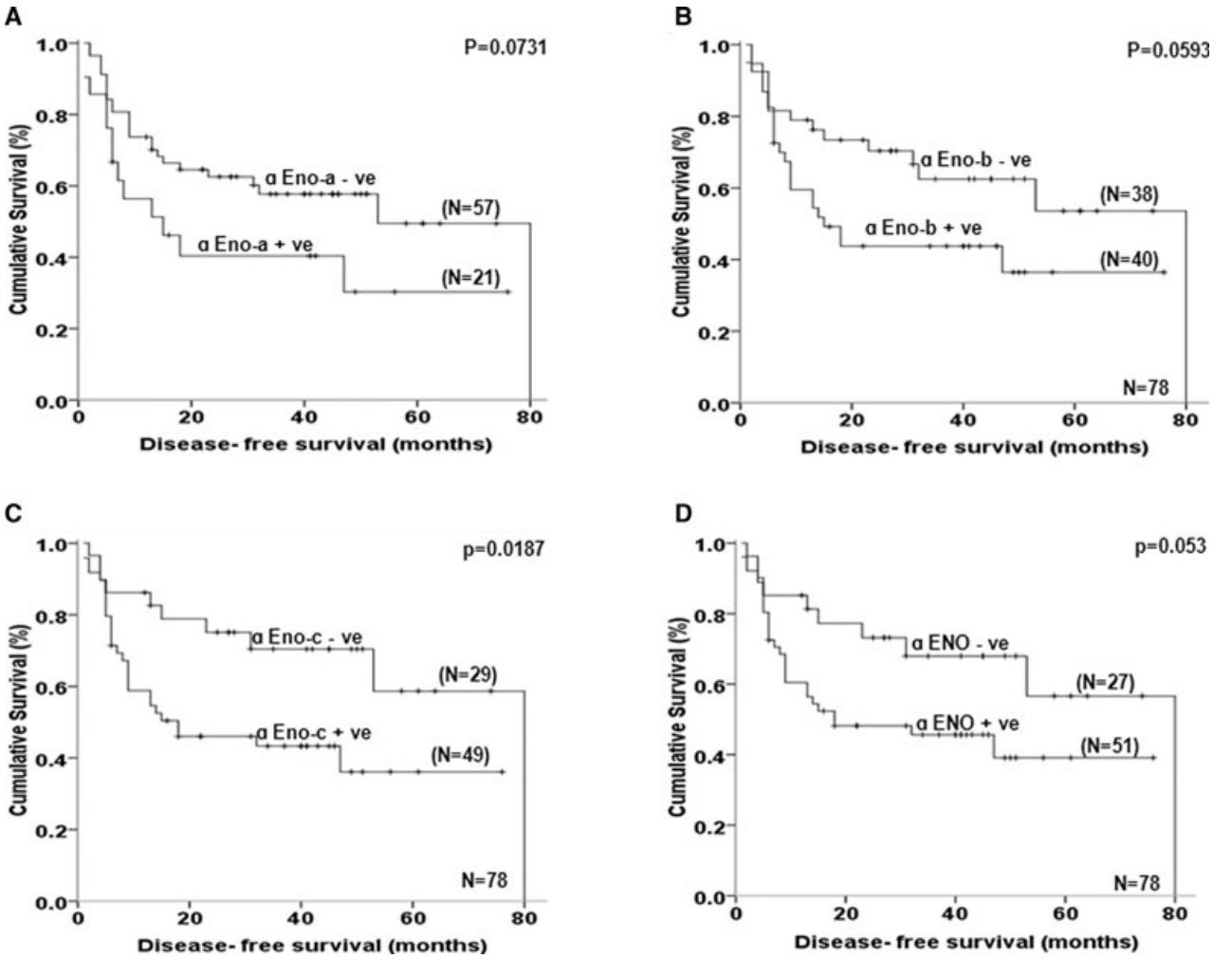

Figure 1. Kaplan-Meier curves with univariate analysis for correlation of cumulative survival and DFS (in months) with autoantibody response in groups of patients: negative or positive for autoantibody response to each of the antigens shown (A) α-enolase a; (B) α-enolase-b; (C) α-enolase c; (D) α-ENO (α-enolase a, b, and c taken together). The number of patients in each group is given as (N) against each curve.

response to only α-enolase c isoform was correlated with DFS of patients along with the nodal involvement ($p = 0.0003$), Fig. 3B.

3.4.2 Autoantibody response to Hsp70, nodal status, and DFS

Data from 76 patients were divided into four groups based on the presence of antibody response to Hsp70 and nodal involvement. The survival curve obtained for each group is shown in Fig. 3C. The overall trend is significant at $p = 0.0001$. It is noted that there is a significant decrease in DFS ($p = 0.049$) in patients positive for autoantibody response to Hsp70 and nodal involvement as compared to patients positive only for node. In addition, there is also a significant difference in DFS of patients ($p = 0.050$) between the group negative for both node and antibody response to Hsp70 and the group positive for antibody response to Hsp70 but negative for node.

3.4.3 Autoantibody response to both α-ENO and Hsp70, nodal status, and DFS

Presence of autoantibody response to either Hsp70 or α-ENO or both in sera correlates independently to the DFS of patients. To determine their potential as an additional prognostic marker in combination, survival analysis was performed with nodal involvement. Data from 76 patients were divided into six groups based on the presence of antibody response to either α-ENO or Hsp70 or both and nodal status. Fig. 3D shows the survival curves obtained for each group. It is seen that patients who are negative for both autoantibody response to Hsp70 and α-ENO and node fare better than those who

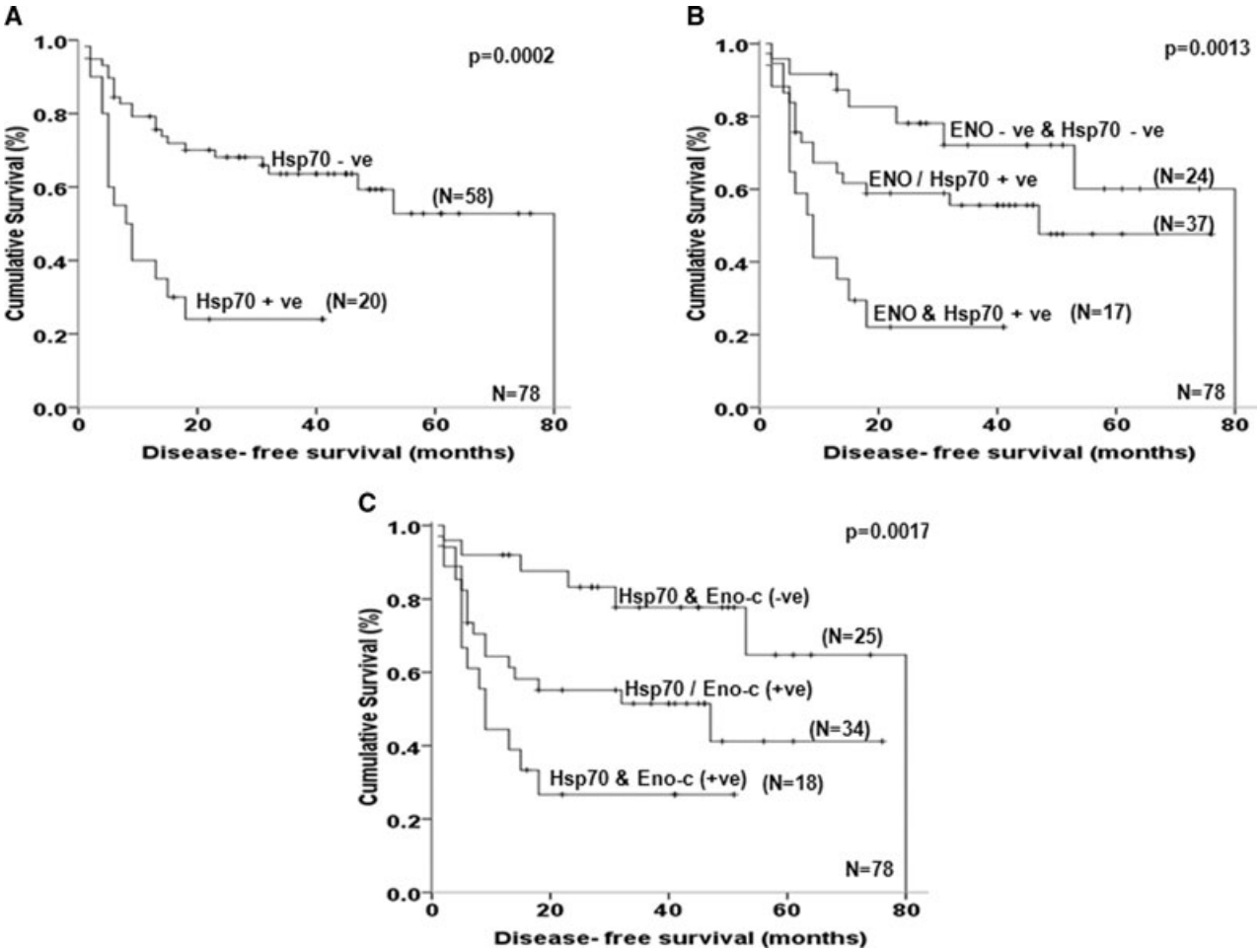

Figure 2. Kaplan-Meier curves with univariate analysis for correlation of cumulative survival and DFS (in months) with autoantibody response in: (A) group of patients positive and negative for autoantibody response to Hsp70; (B) group of patients positive and negative for either autoantibody response to α-ENO (α-enolase- a, b, and c taken together) or Hsp70 or both; (C) group of patients positive and negative for either autoantibody response α-enolase c or anti Hsp70 antibody or both. The number of patients in each group is given as (N) against each curve.

are positive for antibody response to either Hsp70 or α-ENO or both and negative for node. Similarly, the patients negative for antibody response to both Hsp70 and α-ENO and positive for node fare better than patients positive for antibody response to either Hsp70 or α-ENO or both as well as node.

In summary, autoantibody response to both antigens is an added parameter to node positivity for prognosis of cancer of GBC.

3.4.4 Autoantibody response to α-ENO and Hsp70 individually, differentiation status, and DFS

The parameters, differentiation, and autoantibody response to α-ENO alone or Hsp70 alone independently correlated with DFS as shown above. To determine whether the two parameters have any additive effect on DFS of the patients, data from 77 patients (for one patient differentiation status was not available) were divided into six groups based on the presence of autoantibody response to α-ENO and differentiation status of the tumors. The survival curves obtained are shown in Fig. 4A. The group with well differentiated (WD) or moderately differentiated (WD/MD) tumors and positive for antibody response to α-ENO has reduced DFS relative to the group with WD/MD tumors and negative for antibody response to α-ENO.

The group with poorly differentiated (PD) tumors and positive for antibody response to α-ENO has significantly reduced DFS from group with PD tumor and negative for autoantibody to α-ENO. The overall trend is significant at $p = 0.012$. Similarly, presence of antibody response to Hsp70 showed significant correlation with DFS along with the differentiation status of tumor ($p = 0.0002$), Fig. 4B.

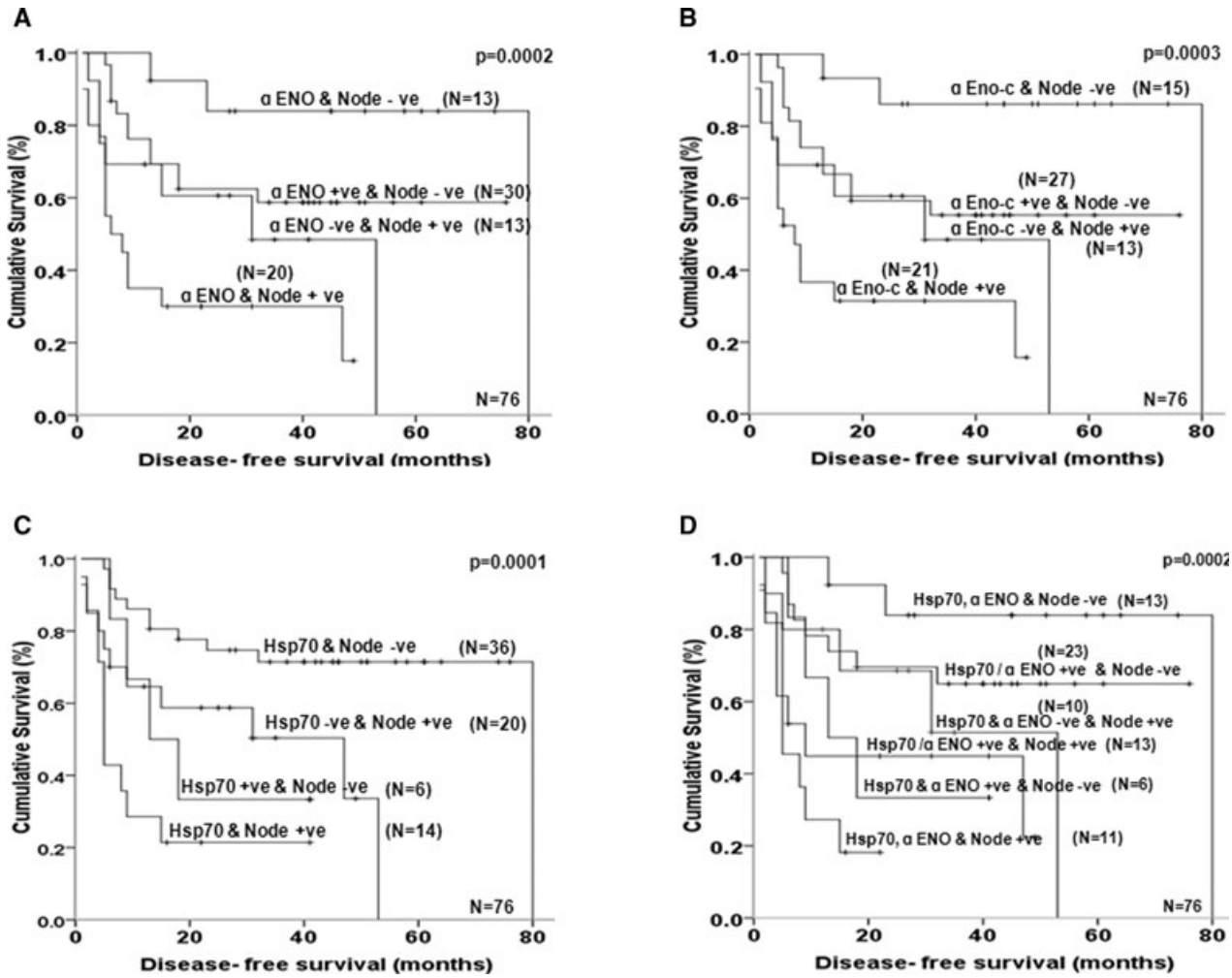

Figure 3. Kaplan-Meier curves with univariate analysis for correlation of cumulative survival and DFS (in months) with autoantibody response in: (A) Four groups of patients viz those negative for autoantibody response to both α-ENO and node; those positive for autoantibody response to α-ENO but negative for node; those negative for autoantibody response to α-ENO but positive for node; those positive for both autoantibody response to α-ENO and node; (B) Four groups of patients viz those negative for both autoantibody response to α-enolase-c antibody and node; those positive for autoantibody response to α-enolase-c but negative for node; those negative for autoantibody response to α-enolase-c but positive for node; those positive for both autoantibody response to α-enolase-c and node; (C) Four groups of patients viz those negative for both autoantibody response to Hsp70 and node; those negative for autoantibody response to Hsp70 but positive for node; those positive for autoantibody response to Hsp70 but negative for node; those positive for both autoantibody response to Hsp70 and node (D) Six groups of patients viz those negative for autoantibody response to Hsp70 and α-ENO and node; those positive for autoantibody response to Hsp70/α-ENO and negative for node; those positive for autoantibody response to both Hsp70 and α-ENO and negative for node; those negative for autoantibody response to both Hsp70 and α-ENO and positive for node; those positive for autoantibody response to Hsp70/α-ENO and positive for node; those positive for autoantibody response to Hsp70 and α-ENO and node. The number of patients in each group is given as (N) against each curve.

3.4.5 Autoantibody response to α-ENO and Hsp70, differentiation status, and DFS

The data from 77 patients were divided into six groups based on autoantibody positivity and differentiation status. The survival curves in Fig. 4C show that those patients with PD tumors who exhibit autoantibody to α-ENO and Hsp70 recur faster than those who are negative for an autoantibody response to both or either antigens. Similarly, patients with MD/WD tumors who exhibit antibody response to both antigens recur faster than those who are negative for autoantibody response. The overall trend is significant at $p = 0.001$.

The emerging data shows that autoantibody response to α-ENO and Hsp70 alone and in combination is an added parameter, which with differentiation status of the tumor can improve the prognosis of cancer of GBC.

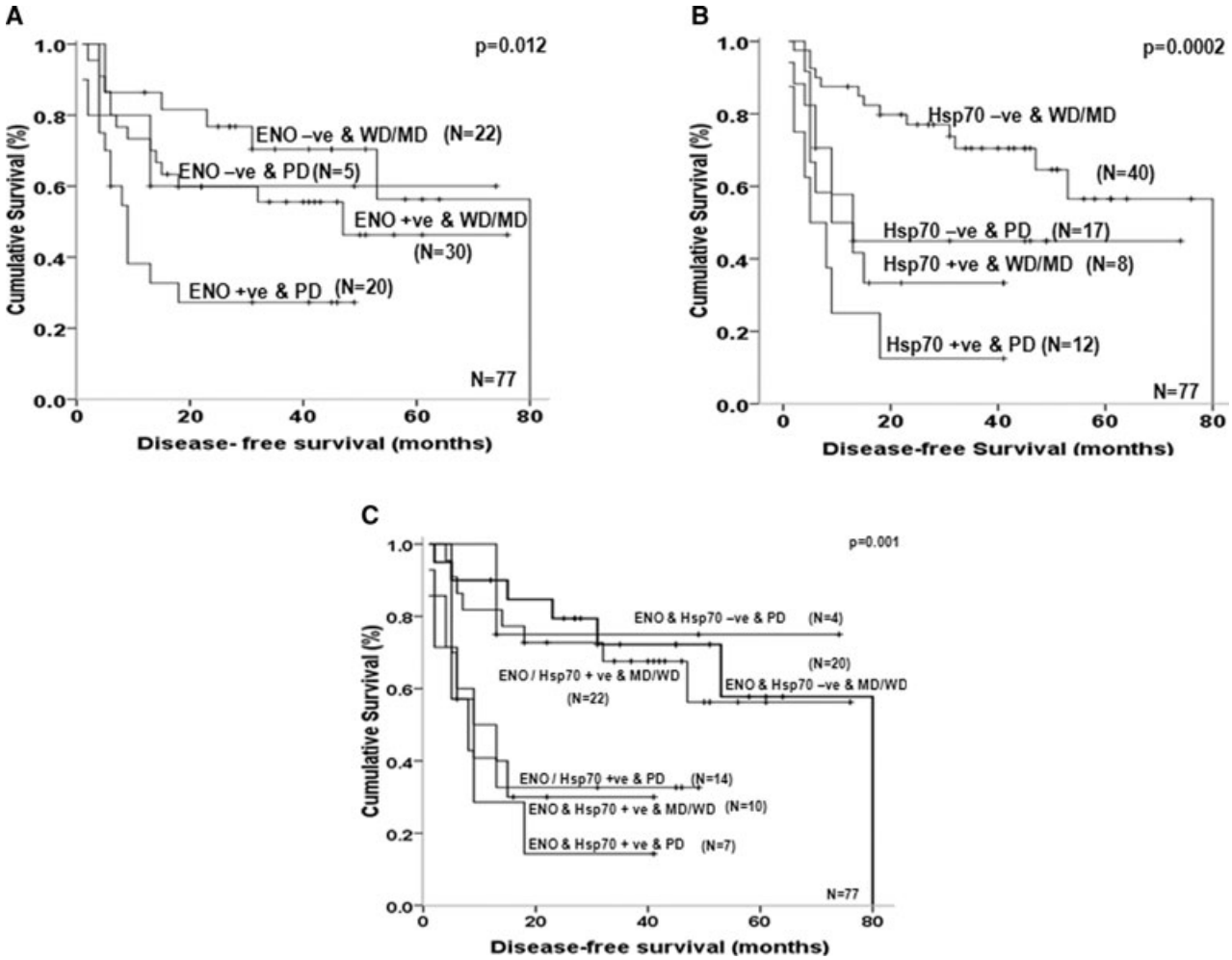

Figure 4. Kaplan-Meier curves with univariate analysis (log rank) for correlation of cumulative survival and DFS (in months) in: (A) Four groups of patients viz those with WD (well differentiated) tumor/MD (moderately differentiated) tumor and negative for autoantibody response to α-ENO; those with WD tumor/MD tumor and positive for autoantibody response to α-ENO antibody; those with PD (poorly differentiated) tumor and negative for autoantibody response to α-ENO; those with PD tumor and positive for autoantibody response to α-ENO; (B) Four groups of patients viz those with WD tumor/MD tumor and negative for autoantibody response to Hsp70; those with WD tumor/MD tumor and positive for autoantibody response to Hsp70; those with PD and negative for autoantibody response to Hsp70 antibody; those with PD tumor and positive for autoantibody response to Hsp70; (C) Six groups of patients viz those negative for autoantibody response to α-ENO and Hsp70 and with WD/PD tumor; those positive for autoantibody response to α-ENO/Hsp70 and with WD/PD tumor; those positive for autoantibody response to both α-ENO and Hsp70 and with WD/PD tumor; those negative for autoantibody response to both α-ENO and Hsp70 and with PD tumor; those positive for autoantibody response to α-ENO/Hsp70 and with PD tumor; those positive for autoantibody response to both α-ENO and Hsp70 and with PD tumor. The number of patients in each group is given as (N) against each curve.

3.5 Multivariate analysis

Cox regression analysis was performed to determine the prognostic potential of autoantibody response to α-ENO and Hsp70 for cancer of GBC in combination with the clinical parameters, differentiation, and nodal status. Table 1 shows that the relative risk of recurrence is 3.41 when the patients exhibit autoantibody response to both the antigens supporting their potential as added parameters that influence the prognosis of the disease.

4 Discussion

Autoantibody response against tumor antigens has been receiving attention for its utility in early detection and prognosis of cancer. There are several studies evaluating autoantibody response vis a vis disease progression or prognosis. Mudenda et al. have shown that the presence of autoantibodies to p53 correlate positively with high histological grade of breast cancer [10]. Chang et al. studied autoantibody response against survivin in 134 patients with head and neck cancer and found

Table 1. Multivariate analysis evaluating the relative risk of recurrence vis a vis nodal status, differentiation status of the tumor and autoantibody response using Cox regression analysis

		Number of patients	Relative risk	95% CI for relative risk		Significance *(p)*
				Lower	Upper	
Differentiation status	WD/MD	51	2.29	1.14	4.6	0.020
	PD	24				
Nodal involvement	−ve	41	2.98	1.49	5.96	0.002
	+ve	34				
Autoantibody response to Hsp70 and α-Enolase	(−ve/−ve)	23	1.6	0.63	4.1	0.318
	(−ve/+ve)	35				
	(+ve/+ve)	17	3.41	1.28	9.0	0.014

WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated; CI: confidence interval.

survivin antibodies in 33% patients with stage I and II while 51% patients with stage III and IV showed survivin antibodies. Autoantibodies to survivin were seen in 38, 46, and 65% of the patients with well, moderately, and poorly differentiated tumor, respectively [14]. Mitsudomi et al. found that high incidence of p53 autoantibodies is associated with high histological grade of nonsmall cell lung cancer but not with patient survival. They observed p53 autoantibodies in 14% of early stage group (Stage I and II) and 30% of advanced disease group (Stage III and IV) [13].

In the present study, we have examined the prognostic utility of presence of autoantibody response to the tumor antigens reported earlier from our laboratory [16] in sera of patients with cancer of GBC using immunoproteomics. Patients positive for autoantibody response to α-ENO or Hsp70 showed faster recurrence compared to those who do not show autoantibody response to either of them. Further, we show that autoantibody response to α-ENO and Hsp70 is an additional parameter that in combination with nodal involvement and/or differentiation status of tumor improves the prognosis of cancer of the GBC. This information could possibly be used by the clinician to modify treatment strategies according to the aggressiveness of the tumor.

α-Enolase is reported to be multifunctional [36]. It can exist both in cytoplasm as well on the cell surface. A truncated version of α-ENO MBP-1 is also expressed, which is localized in the nucleus and acts as a transcription repressor by binding to *c-Myc* promoter. Cell surface α-ENO apart from its catalytic activity may also act as a plaminogen receptor. Binding of plasminogen to α-enolase leads to its activation to plasmin by proteolytic cleavage. In breast, lung, and pancreatic neoplasia, α-enolase is localized on the surface of cancer cells where it may act as a plasminogen receptor [35, 37, 38]. The exact mechanism involved in the production of autoantibodies to α-enolase in oral cancer is presently unclear. We have earlier reported that expression of α-enolase is increased in cancer of GBC [39] that may be a reason for the generation of autoantibody response. Tsai et al. [9] have shown *ENO1* overexpression as a prognostic marker in head and neck cancer.

Autoantibody responses to α-enolase have been reported in cancers of the pancreas [37], leukemia [32], melanoma [40], head and neck/oral cavity [16, 20], breast [41], and lung [35, 42]. In a recent study, Tomaino et al. have reported that in pancreatic cancer patients, autoantibodies to ENOA are directed against two upregulated isoforms phosphorylated on Ser 419 [43].

Hsp70 is another multifunctional protein that plays a role in regulating protein quality and turnover in normal conditions as well under stress. Autoantibodies to Hsp70 have been reported in cancer of the esophagus [25], lung [34], liver [33], oral cavity, and head and neck [21] and leukemia [32]. Hsp70 is reported to be overexpressed in the cancer of head and neck and oral cavity [44]. There are divergent reports of its prognostic relevance in oral cancer [45]. Autoantibody response to Hsp70 detected in sera of patients with cancer of GBC correlated significantly both with the size and stage of tumor and was more in patients with advanced stage of tumor. Further, there is a decrease in DFS with increase in autoantibody response.

In a recent review, Murphy et al. [46] have observed that due to the changing repertoire of the antigens as the tumor progresses, there is probably a constant qualitative and quantitative change in the autoantibody response to an antigen. In addition, the autoantibody response will depend on the technique used to assess it since the immune response could be to a sequence in the linear protein or to an epitope presented as a consequence of the folding of the native protein [47]. These factors along with differential expression of an antigen, its PTMs and its relocation would provide a complex pattern that has to be deciphered to explain the autoantibody response profiles obtained for the different antigens.

Autoantibodies have been either reported to improve the prognosis of cancer patients, to worsen the clinical outcome, or even to be irrelevant for the course of the disease. For example autoantibodies to p53 have shown variable correlations with disease outcome in different cancers. Lubin et al. and Tang et al. have shown autoantibody response to

Clinical Relevance

Squamous cell carcinoma of the oral cavity ranks as the 15th most common cancer in the world and 10th most frequent in males. In India, the gingivo-buccal complex (GBC) a subsite of the oral cavity, is the common site of cancer in males due to the prevalent habit of chewing tobacco. A major hurdle in the management of oral cancer is high rate of locoregional recurrence. Biological markers are necessary to complement clinicopathological findings for better prognosis.
Using immunoproteomics, we have reported earlier a set of tumor antigens that elicit autoantibody response in patients with cancer of GBC. The reported antigens include α-enolase isoforms a, b and c and Hsp70. Autoantibodies to α-enolase and Hsp70 have been reported in several cancers, wherein they have focused on the diagnostic utility of these biomarkers. In the present study, the utility of the autoantibody response to the antigens in prognosis of cancer of GBC has been investigated.
Our data show that autoantibody response against α-ENO and Hsp70 provides an additional parameter for predicting disease-free survival and recurrence and may be utilized along with nodal involvement and differentiation status for better prognosis of cancer of GBC.

be associated with high-grade tumors and poor survival in breast, colon, oral, and gastric cancers [48,49] while in another study, Saffroy et al. have shown correlation of autoantibody response with enhanced overall survival in HCC patients [50]. It was anticipated that as the disease progressed the host immune surveillance mechanisms would elicit an antibody response as a measure to counter the progressing cancer and there should have been a direct reflection of this with an increase in the DFS status. It is apparent that the scenario is more complex and as described by Tan and Coussens in their review [51] enhanced humoral immunity and inflammation in combination with suppressed cell-mediated immunity are responsible for the pathogenenesis of several human cancers. Further studies are therefore required to understand the observations in this study.

In summary, autoantibody response against α-ENO and Hsp70 provides an additional parameter for predicting DFS and recurrence and may be utilized along with nodal involvement and differentiation status for better prognosis of cancer of GBC.

The work was supported by a grant from Council of Scientific and Industrial Research, (27(0151)/06/EMR-II) and institutional funds from ACTREC to Dr. Surekha Zingde. Sanjeev Shukla was supported by a fellowship from ACTREC during 2003 to 2007 and Lady Tata Memorial Trust, (LTMT) during 2007–2008, India. Atul Pranay was supported by a fellowship from ACTREC, India.

All authors have declared no conflicts of interest.

5 References

[1] Ferlay, J., Shin, H., Bray, F., Forman, D. et al., *GLOBOCAN 2008, Cancer Incidence and Mortality Worldwide: IARC Cancerbase No. 10*, International Agency for Research on Cancer: Lyon, France 2010 Available at http://globocan.iarc.fr, accessed on 27/06/2012.

[2] Dinshaw, K., Ganesh, B., *Hospital Based Cancer Registry, Annual Report 2002–2005, Tata Memorial Hospital 2008*, Tata Memorial Centre, Mumbai, India.

[3] Schaaij-Visser, T. B., Brakenhoff, R. H., Leemans, C. R., Heck, A. J., Slijper, M., Protein biomarker discovery for head and neck cancer. *J. Proteomics.* 2010, *73*, 1790–1803.

[4] Leemans, C. R., Braakhuis, B. J., Brakenhoff, R. H., The molecular biology of head and neck cancer. *Nat. Rev. Cancer* 2011, *11*, 9–22.

[5] Wang, Z., Jiang, L., Huang, C., Li, Z. et al., Comparative proteomics approach to screening of potential diagnostic and therapeutic targets for oral squamous cell carcinoma. *Mol. Cell Proteomics* 2008, *7*, 1639–1650.

[6] Meyer, F., Samson, E., Douville, P., Duchesne, T. et al., Serum prognostic markers in head and neck cancer. *Clin. Cancer Res.* 2010, *16*, 1008–1015.

[7] Schaaij-Visser, T. B., Graveland, A. P., Gauci, S., Braakhuis, B. J. et al., Differential proteomics identifies protein biomarkers that predict local relapse of head and neck squamous cell carcinomas. *Clin. Cancer Res.* 2009, *15*, 7666–7675.

[8] Bleijerveld, O. B., Brakenhoff, R. H., Schaaij-Visser, T. B., Damen, J. M. et al., Protein signatures associated with tumor cell dissemination in head and neck cancer. *J. Proteomics* 2011, *74*, 558–566.

[9] Tsai, S. T., Chien, I. H., Shen, W. H., Kuo, Y. Z. et al., ENO1, a potential prognostic head and neck cancer marker, promotes transformation partly via chemokine CCL20 induction. *Eur. J. Cancer* 2010, *46*, 1712–1723.

[10] Mudenda, B., Green, J. A., Green, B., Jenkins, J. R. et al., The relationship between serum p53 autoantibodies and characteristics of human breast cancer. *Br. J. Cancer* 1994, *69*, 1115–1119.

[11] Trivers, G. E., De Benedetti, V. M., Cawley, H. L., Caron, G. et al., Anti-p53 antibodies in sera from patients with chronic obstructive pulmonary disease can predate a diagnosis of cancer. *Clin. Cancer Res.* 1996, *2*, 1767–1775.

[12] Disis, M. L., Pupa, S. M., Gralow, J. R., Dittadi, R. et al., High-titer HER-2/neu protein-specific antibody can be detected in

patients with early-stage breast cancer. *J. Clin. Oncol.* 1997, *15*, 3363–3367.

[13] Mitsudomi, T., Suzuki, S., Yatabe, Y., Nishio, M. et al., Clinical implications of p53 autoantibodies in the sera of patients with non-small-cell lung cancer. *J. Natl. Cancer Inst.* 1998, *90*, 1563–1568.

[14] Chang, J. T., Wong, F. H., Liao, C. T., Chen, I. H. et al., Enzyme immunoassay for serum autoantibody to survivin and its findings in head-and-neck cancer patients. *Clin. Chem.* 2004, *50*, 1261–1264.

[15] Lin, H. S., Talwar, H. S., Tarca, A. L., Ionan, A. et al., Autoantibody approach for serum-based detection of head and neck cancer. *Cancer Epidemiol. Biomarkers Prev.* 2007, *16*, 2396–2405.

[16] Shukla, S., Govekar, R. B., Sirdeshmukh, R., Sundaram, C. S. et al., Tumor antigens eliciting autoantibody response in cancer of gingivo-buccal complex. *Proteomics Clin. Appl.* 2007, *1*, 1592–1604.

[17] Qiu, J., Choi, G., Li, L., Wang, H. et al., Occurrence of autoantibodies to annexin I, 14–3-3 theta and LAMR1 in prediagnostic lung cancer sera. *J. Clin. Oncol.* 2008, *26*, 5060–5066.

[18] Tjalsma, H., Schaeps, R. M., Swinkels, D. W., Immunoproteomics: from biomarker discovery to diagnostic applications. *Proteomics Clin. Appl.* 2008, *2*, 167–180.

[19] Qiu, J., Hanash, S., Autoantibody profiling for cancer detection. *Clin. Lab. Med.* 2009, *29*, 31–46.

[20] Shukla, S., Pranay, A., D'Cruz, A. K., Chaturvedi, P. et al., Immunoproteomics reveals that cancer of the tongue and the gingivobuccal complex exhibit differential autoantibody response. *Cancer Biomark.* 2009, *5*, 127–135.

[21] Castelli, M., Cianfriglia, F., Manieri, A., Palma, L. et al., Anti-p53 and anti-heat shock proteins antibodies in patients with malignant or pre-malignant lesions of the oral cavity. *Anticancer Res.* 2001, *21*, 753–758.

[22] Ralhan, R., Nath, N., Agarwal, S., Mathur, M. et al., Circulating p53 antibodies as early markers of oral cancer: correlation with p53 alterations. *Clin. Cancer Res.* 1998, *4*,xbrk 2147–2152.

[23] Cordes, C., Von Lingen, J., Gorogh, T., Ambrosch, P. et al., Molecular and immunological aspects of p53 and p53-autoantibodies in head and neck squamous cell carcinoma. *Oncol. Rep.* 2009, *22*, 1299–1303.

[24] Murase, R., Abe, Y., Takeuchi, T., Nabeta, M. et al., Serum autoantibody to sideroflexin 3 as a novel tumor marker for oral squamous cell carcinoma. *Proteomics Clin. Appl.* 2008, *2*, 517–527.

[25] Fujita, Y., Nakanishi, T., Miyamoto, Y., Hiramatsu, M. et al., Proteomics-based identification of autoantibody against heat shock protein 70 as a diagnostic marker in esophageal squamous cell carcinoma. *Cancer Lett.* 2008, *263*, 280–290.

[26] Gires, O., Munz, M., Schaffrik, M., Kieu, C. et al., Profile identification of disease-associated humoral antigens using AMIDA, a novel proteomics-based technology. *Cell Mol. Life Sci.* 2004, *61*, 1198–1207.

[27] Hong, S. H., Misek, D. E., Wang, H., Puravs, E. et al., An autoantibody-mediated immune response to calreticulin isoforms in pancreatic cancer. *Cancer Res.* 2004, *64*, 5504–5510.

[28] Fujita, Y., Nakanishi, T., Hiramatsu, M., Mabuchi, H. et al., Proteomics-based approach identifying autoantibody against peroxiredoxin VI as a novel serum marker in esophageal squamous cell carcinoma. *Clin. Cancer Res.* 2006, *12*, 6415–6420.

[29] Yang, F., Xiao, Z. Q., Zhang, X. Z., Li, C. et al., Identification of tumor antigens in human lung squamous carcinoma by serological proteome analysis. *J. Proteome Res.* 2007, *6*, 751–758.

[30] Pereira-Faca, S. R., Kuick, R., Puravs, E., Zhang, Q. et al., Identification of 14–3-3 theta as an antigen that induces a humoral response in lung cancer. *Cancer Res.* 2007, *67*, 12000–12006.

[31] Li, L., Chen, S. H., Yu, C. H., Li, Y. M., Wang, S. Q., Identification of hepatocellular-carcinoma-associated antigens and autoantibodies by serological proteome analysis combined with protein microarray. *J. Proteome. Res.* 2008, *7*, 611–620.

[32] Zou, L., Wu, Y., Pei, L., Zhong, D. et al., Identification of leukemia-associated antigens in chronic myeloid leukemia by proteomic analysis. *Leuk. Res.* 2005, *29*, 1387–1391.

[33] Looi, K. S., Nakayasu, E. S., Diaz, R. A., Tan, E. M. et al., Using proteomic approach to identify tumor-associated antigens as markers in hepatocellular carcinoma. *J. Proteome. Res.* 2008, *7*, 4004–4012.

[34] Zhong, L., Peng, X., Hidalgo, G. E., Doherty, D. E. et al., Antibodies to Hsp70 and HSP90 in serum in non-small cell lung cancer patients. *Cancer Detect. Prevent.* 2003, *27*, 285–290.

[35] He, P., Naka, T., Serada, S., Fujimoto, M. et al., Proteomics-based identification of alpha-enolase as a tumor antigen in non-small lung cancer. *Cancer Sci.* 2007, *98*, 1234–1240.

[36] Capello, M., Ferri-Borgogno, S., Cappello, P., Novelli, F., Alpha-enolase: a promising therapeutic and diagnostic tumor target. *FEBS J.* 2011, *278*, 1064–1074.

[37] Cappello, P., Tomaino, B., Chiarle, R., Ceruti, P. et al., An integrated humoral and cellular response is elicited in pancreatic cancer by alpha-enolase, a novel pancreatic ductal adenocarcinoma-associated antigen. *Int. J. Cancer* 2009, *125*, 639–648.

[38] Seweryn, E., Pietkiewicz, J., Bednarz-Misa, I. S., Ceremuga, I. et al., Localization of enolase in the subfractions of a breast cancer cell line. *Z. Naturforsch C.* 2009, *64*, 754–758.

[39] Govekar, R. B., D'Cruz, A. K., Alok Pathak, K., Agarwal, J. et al., Proteomic profiling of cancer of the gingivo-buccal complex: identification of new differentially expressed markers. *Proteomics Clin. Appl.* 2009, *3*, 1451–1462.

[40] Suzuki, A., Iizuka, A., Komiyama, M., Takikawa, M. et al., Identification of melanoma antigens using a Serological Proteome Approach (SERPA). *Cancer Genom. Proteome.* 2010, *7*, 17–23.

[41] Shih, N. Y., Lai, H. L., Chang, G. C., Lin, H. C. et al., Anti-alpha-enolase autoantibodies are down-regulated in advanced cancer patients. *Jpn. J. Clin. Oncol.* 2010, *40*, 663–669.

[42] Nakanishi, T., Takeuchi, T., Ueda, K., Murao, H., Shimizu, A., Detection of eight antibodies in cancer patients'

sera against proteins derived from the adenocarcinoma A549 cell line using proteomics-based analysis. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2006, *838*, 15–20.

[43] Tomaino, B., Cappello, P., Capello, M., Fredolini, C. et al., Circulating autoantibodies to phosphorylated alpha-enolase are a hallmark of pancreatic cancer. *J. Proteome Res.* 2011, *10*, 105–112.

[44] Lin, H. P., Wang, Y. P., Chiang, C. P., Expression of p53, MDM2, p21, heat shock protein 70, and HPV 16/18 E6 proteins in oral verrucous carcinoma and oral verrucous hyperplasia. *Head Neck* 2011, *33*, 334–340.

[45] Tavassol, F., Starke, O. F., Kokemuller, H., Wegener, G. et al., Prognostic significance of heat shock protein 70 (Hsp70) in patients with oral cancer. *Head Neck Oncol.* 2011, DOI: 10.1186/1758-3284-3-10.

[46] Murphy, M. A., O'Leary, J. J., Cahill, D. J., Assessment of the humoral immune response to cancer. *J. Proteomics* 2012, 4573–4579.

[47] Murphy, M. A., O'Connell, D. J., O'Kane, S. L., O'Brien, J. K. et al., Epitope presentation is an important determinant of the utility of antigens identified from protein arrays in the development of autoantibody diagnostic assays. *J. Proteomics* 2012, 4668–4675.

[48] Lubin, R., Schlichtholz, B., Teillaud, J. L., Garay, E. et al., p53 antibodies in patients with various types of cancer: assay, identification, and characterization. *Clin. Cancer Res.* 1995, *1*, 1463–1469.

[49] Tang, R., Ko, M. C., Wang, J. Y., Changchien, C. R. et al., Humoral response to p53 in human colorectal tumors: a prospective study of 1,209 patients. *Int. J. Cancer* 2001, *94*, 859–863.

[50] Saffroy, R., Lelong, J. C., Azoulay, D., Salvucci, M. et al., Clinical significance of circulating anti-p53 antibodies in European patients with hepatocellular carcinoma. *Br. J. Cancer* 1999, *79*, 604–610.

[51] Tan, T. T., Coussens, L. M., Humoral immunity, inflammation and cancer. *Curr. Op. Immunol.* 2007, *19*, 209–216.

Cancer Biomarkers 5 (2009) 127–135
DOI 10.3233/CBM-2009-0604
IOS Press

Immunoproteomics reveals that cancer of the tongue and the gingivobuccal complex exhibit differential autoantibody response

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Abstract. Autoantibody response to tumor antigens has been widely used to identify novel tumor markers for different cancers, including that of the head and neck. The oral cavity, which is in the head and neck region, comprises of many sub sites with distinct biologies and incidence of cancer of each sub site of the oral cavity is different. It is anticipated therefore that each sub site of the oral cavity may elicit a differential autoantibody response. This report evaluates the autoantibody response in 15 patients with cancer of gingivo-buccal complex and in 15 patients with cancer of tongue using Immunoproteomics, and shows that the autoantibody response to alpha-enolase, HSP 70, peroxiredoxin-VI, annexin II, pyruvate kinase, alpha-tubulin, beta-tubulin, ATP synthase, triose phosphate isomerase and aldose reductase seen in patients with cancer of gingivo-buccal complex is absent in patients with cancer of tongue. This suggests that cancer of these sub sites should be studied separately because of their different biology and emerging site specific molecular signatures including autoantibody responses to ensure unambiguous clinical interpretations.

Keywords: Autoantibodies, tongue, gingivo-buccal complex, tumor antigen, immunoproteomics

1. Introduction

Autoantibody response has been widely considered as a molecular sieve for identifying specific biomarkers for cancer, which can be used for early detection, prognosis and cancer management [1–5]. Many laboratories are combining different proteomic technologies with serological analysis to identify autoantibodies [6–9]. This has lead to the term "Immunoproteomics". Autoantibody response is reported to occur against tumor antigens because of their over expression, mutation, post translational modifications or change in location [1]. The mechanisms involved in the process of tumorogenesis in cancers from different tissues are specific. This occurs due to alterations in different proteins in each cancer, which in turn may elicit immune responses that are specific for each cancer [6–9].

In an ongoing study in our laboratory, we have identified autoantibodies against several antigens in cancer of gingivo-buccal complex (GBC) [10], which is a sub site of the oral cavity. In recent years it has become apparent that each sub site of the region has specific biological characteristics which respond to anti cancer therapy differently.

The oral cavity consists of the gingivo-buccal complex (buccal mucosa, lower alveolus, retromolar trigone and gingiva), tongue, lip, palate, and floor of mouth. The epithelial lining of each of these sub sites is site specific consisting of keratinizing and non keratiniz-

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ISSN 1574-0153/09/$17.00

ing stratified epithelium. The epithelium of the lining mucosa (buccal mucosa, soft palate, upper lip, lower lip, alveolar region, gingiva) is nonkeratinized stratified squamous, whereas that of the masticatory mucosa (hard palate) is ortho- or para keratinized, to protect it from the shearing forces of mastication. Specialized mucosa of the tongue can be represented as a mosaic of keratinized and nonkeratinized epithelium [11]. The oral mucosa participates in the immunological defense of the oral cavity through the presence of Langerhans cells and antigen presenting cells in the epithelium [12]. The incidence of lymph node metastasis is distinct for each sub site [13]. Further, the two sub sites i.e. GBC and tongue are embryologically different, and arise from ectoderm and endoderm respectively [14].

A recent report analyzing different molecular parameters has shown that genetically tongue and buccal mucosa exhibit different expressions of molecules involved in cell cycle regulation [15]. The proteomic profile of each of these sites is also being reported as distinct [16,17]. Schliephake et al. [18] have reviewed the prognostic ability of several molecules implicated in cancer of the head and neck. Their analysis reveals that there is inconsistency in the clinical relevance of the molecules, which they mainly attribute to sample size. We propose that this may also be due to lack of consideration for sub sites.

Different studies have reported autoantibody responses against p53, HSP 70, and sideroflexin 3 [19–22] in oral cancer and keratin 8, vimentin, beta tubulin, cyclophilin-A etc in head and neck cancer [23]. Lin et al. have screened a phage display library with sera from head and neck cancer patients and identified multiple myeloma overexpression gene 2, ubiquinone binding protein, NADH dehydrogenase subunit 1, C10 protein, and a hypothetical protein as molecules eliciting an autoantibody response [24]. None of these reports focus on a particular sub site of the oral cavity or the head and neck region.

Immunoproteomic studies from our laboratory have identified α-enolase, peroxiredoxin-VI, annexin-II, HSP70, pyruvate kinase, α-tubulin, β-tubulin, ATP-synthase, phosphoglycerate mutase, aldose reductase, triosephosphate isomerase (TPI), and cyclophilin-A as antigens which elicit an autoantibody response in cancer of the GBC [10].

In this study we have compared the autoantibody response in patients with cancer of the tongue and GBC. Our data shows that auto antibody response is sub site specific, thereby suggesting that cancer of different sub sites of the oral cavity/head & neck region should be analyzed separately.

2. Materials and methods

2.1. Sera

This study was approved by the Institutional scientific review committee and approved by the Hospital Ethics Committee of the Tata Memorial Hospital. Blood was collected from 15 patients each with cancer of GBC, cancer of anterior tongue and 15 age and sex matched healthy individuals, after obtaining informed consent and the isolated serum was stored in aliquots at -80°C until further use. The clinical information for the patients is given in Table 1A and 1B

2.2. Cells and their maintenance

The KB cell line originally derived from epidermal carcinoma of the mouth, has been used in this study as a source of enriched tumor antigens. Cells were grown in DMEM (Gibco Invitrogen Corporation, USA) supplemented with 10% fetal bovine serum (JRH Biosciences,USA), streptomycin (Nicholas Piramal, India) (1 g/l), gentamicin (Nicholas Piramal, India) (80 mg/l), amphotericin B (Himedia RM 468) (2.5 mg/l) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Sample preparation

Cells were harvested and washed with chilled phosphate-buffered saline (PBS), and 1×10^7 cells were solubilized in 200 μl of buffer consisting of 8 M urea (Sigma U-5378), 2 M thiourea (Sigma T-7875), 2% CHAPS (USB-13361), 1% dithiothrietol (USB-15397), and the same centrifuged at 1,00,000 g for 1 h at 4°C in the Beckman TLD ultracentrifuge. The supernatant was removed and stored in aliquots at -80° C until used for 2DE analysis. Protein was estimated in one of the aliquots by the Peterson's modified Lowry method [25].

2.4. Purification of IgG from serum

IgGs were purified from serum using the Melon Gel IgG Spin Purification Kit (45206) from Pierce Biotechnology and the protein estimated according to the Lowry method [26]. Purity of the IgG preparation was assessed by 1D SDS-PAGE.

Table 1A Clinical information of the patients with cancer of gingivo-buccal complex

Sr. No	Sample number	Age	Sex	Pathological staging	Differentiation status
1	900	43	M	T2N2	PD
2	951	37	M	T4N1	MD
3	957	38	M	T4N1	MD
4	977	70	F	T4N0	MD
5	980	40	M	T4N2	MD
6	1003	49	M	T3N0	MD
7	1006	46	M	T2N2	MD
8	1010	55	M	T1N0	WD
9	1137	63	M	T3N2b	MD
10	1149	61	M	T4N0	MD
11	1447	55	M	T3N0	MD
12	1448	35	F	T3N2	MD
13	1467	38	M	T4N2	PD
14	1534	74	M	T4N0	PD
15	1535	55	M	T4N2	PD

Table 1B Clinical information of the patients with cancer of tongue

Sr. No	Sample number	Age	Sex	Pathological staging	Differentiation status
1	284	45	M	T2N0	MD
2	293	74	F	T3N0	MD
3	472	38	M	T4N0	PD
4	473	61	F	T2N0	MD
5	496	65	M	T4N0	MD
6	658	64	M	T4N2c	PD
7	716	40	M	T1N0	MD
8	723	60	F	T2N0	MD
9	757	31	F	T2N0	MD
10	764	35	F	T1N0	MD
11	783	40	F	T4N2	PD
12	785	44	M	T4N1	MD
13	1473	28	M	T2N0	MD
14	1474	59	M	T1N0	PD
15	1477	29	M	T2N2	MD

MD-Moderately differentiated, PD-Poorly differentiated, WD-well differentiated.

2.5. Electrophoresis and Western blotting

2-D SDS-PAGE of the proteins was done essentially according to Laemmli [27]. Seven cm (pH 3–10) immobilized pH gradient (IPG) dry strips (BioRad 163-2000) were rehydrated with 40 μg proteins dissolved in 125 μl rehydration buffer (8 M urea, 2 M thiourea, 1% dithiothrietol and 2% CHAPS) and focused in the BioRad Protean IEF Cell. Separation in the second dimension was carried out in the BioRad PROTEAN 3 Dodeca Cell on a 12% SDS polyacrylamide gel. After electrophoresis, proteins in the gels were transferred to PVDF membranes or visualized by staining with silver or Coomassie blue R-250 (Sigma B-0149). The membranes were incubated for 1 h in blocking buffer (TBST), containing 5% milk powder in 20 mM Tris-Cl, (pH 7.5), 0.15 M NaCl, 0.1% Tween 20 (USB-20605). The membranes were incubated overnight at 4°C with IgG isolated from serum of patients or healthy individuals as a source of primary antibody, at a concentration of 5 μg IgG /ml TBST. After three washes with washing buffer (Tris buffered saline containing 0.05% Tween 20), the membranes were incubated with horseradish peroxidase-conjugated sheep anti-human IgGs from Amersham Biosciences, (NA-933) at a dilution of 1:10,000 for 1 h at RT. Immuno-detection was done using the enhanced chemiluminescence ECL plus kit from Amersham Biosciences (RPN 2132) and the signals captured on X-ray film (Kodak). After immunodetection, the membranes were stripped with destainer (40% methanol and 10% acetic acid) washed with washing buffer and stained with Colloidal Gold (BioRad 170-6527) / Amido black to obtain the pattern of the separated proteins. The antigens eliciting an autoantibody response were identified by overlaying the autograph directly on the stained PVDF membrane with landmarks made on the membranes at specific positions prior to immunostaining so as to eliminate ambiguity of positioning the autograph. The colloidal gold patterns were also matched to the pattern on the silver

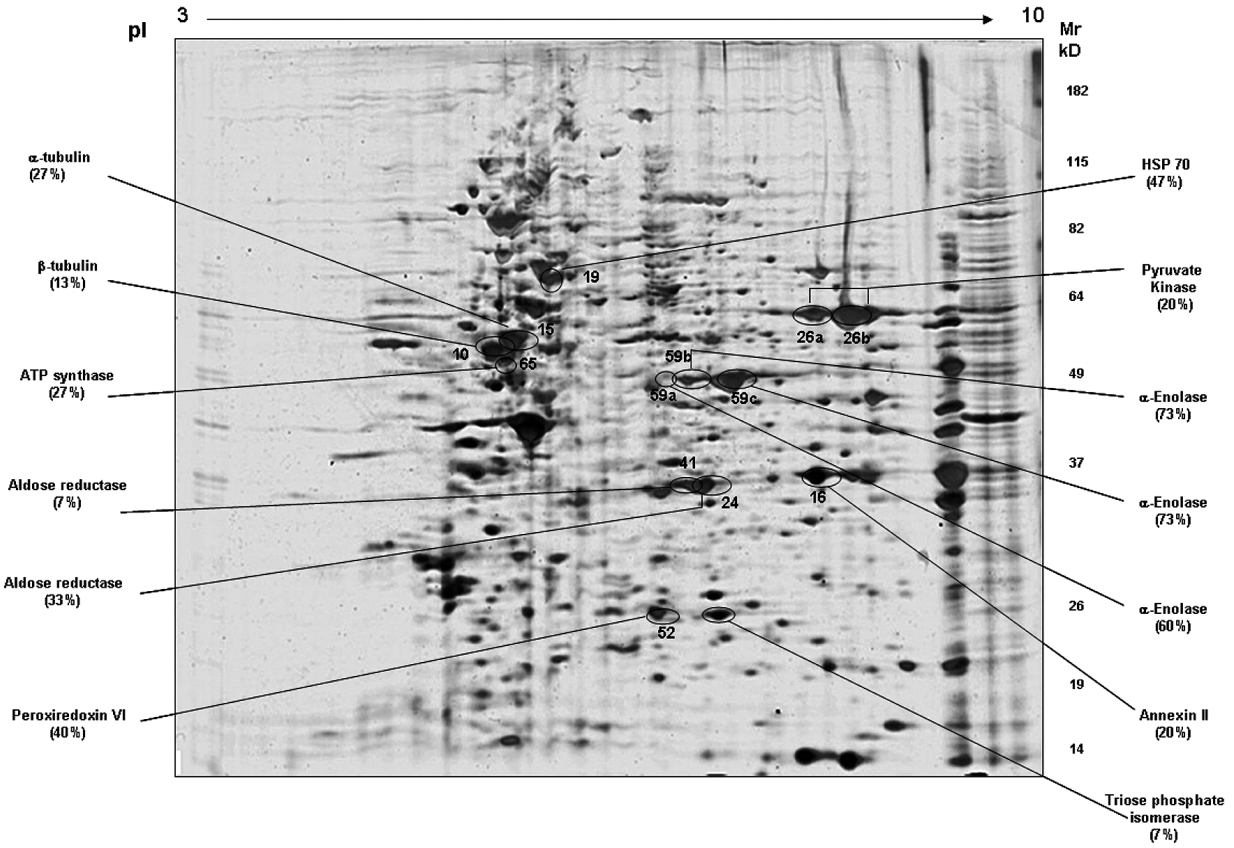

Fig. 1. Figure shows the silver stained 2-DE profile of KB cell lysate, with the position of identified tumor antigens, which elicit an antibody response. The pH gradient of the first dimension electrophoresis is shown on top of the gel and migration of molecular weight markers on SDS-PAGE in second dimension is shown on the right side. The numbers given to the spots and the identities of the proteins are same as those reported in our earlier paper [10].

stained gel run simultaneously. This was further confirmed with the assistance of Microsoft PhotoDraw V2 software. The presence of a spot was scored as positive when the signal on the autograph was clearly defined by its shape which overlaps with the colloidal gold/amido black pattern for the protein on the membrane. The intensity of the signal for each protein spot was specific for each patient. The identity of the spots which was obtained by mass spectrometry has been reported earlier from our laboratory [10].

2.6. Preparation of recombinant GST-α enolase and GST-HSP 70 protein

pCMV-SPORT6 alpha enolase construct was obtained from Dr. Peter J Hornsby, University of Texas, USA and pSV-HSP 70 construct was obtained from Dr Marja Jaattela, Danish Cancer Institute, Denmark through Dr Santosh Kumar, RGCB, India as a kind gift. Alpha enolase and HSP 70 were sub cloned into pGEX4T1 vector and GST, GST-α enolase and GST-HSP 70 proteins were expressed and purified as described in our earlier report [10].

2.7. Confirmation of autoantibody response

Recombinant GST-α enolase, GST-HSP 70, and GST proteins (500 ng) were spotted on PVDF membrane. The ability of the IgG from sera of patients and of healthy individuals to detect the proteins was assessed by immunoblotting as described in Section 2.5.

3. Results

The presence of autoantibodies in sera from patients with cancer of GBC and tongue was investigated by immunoproteomics as described above. For every exper-

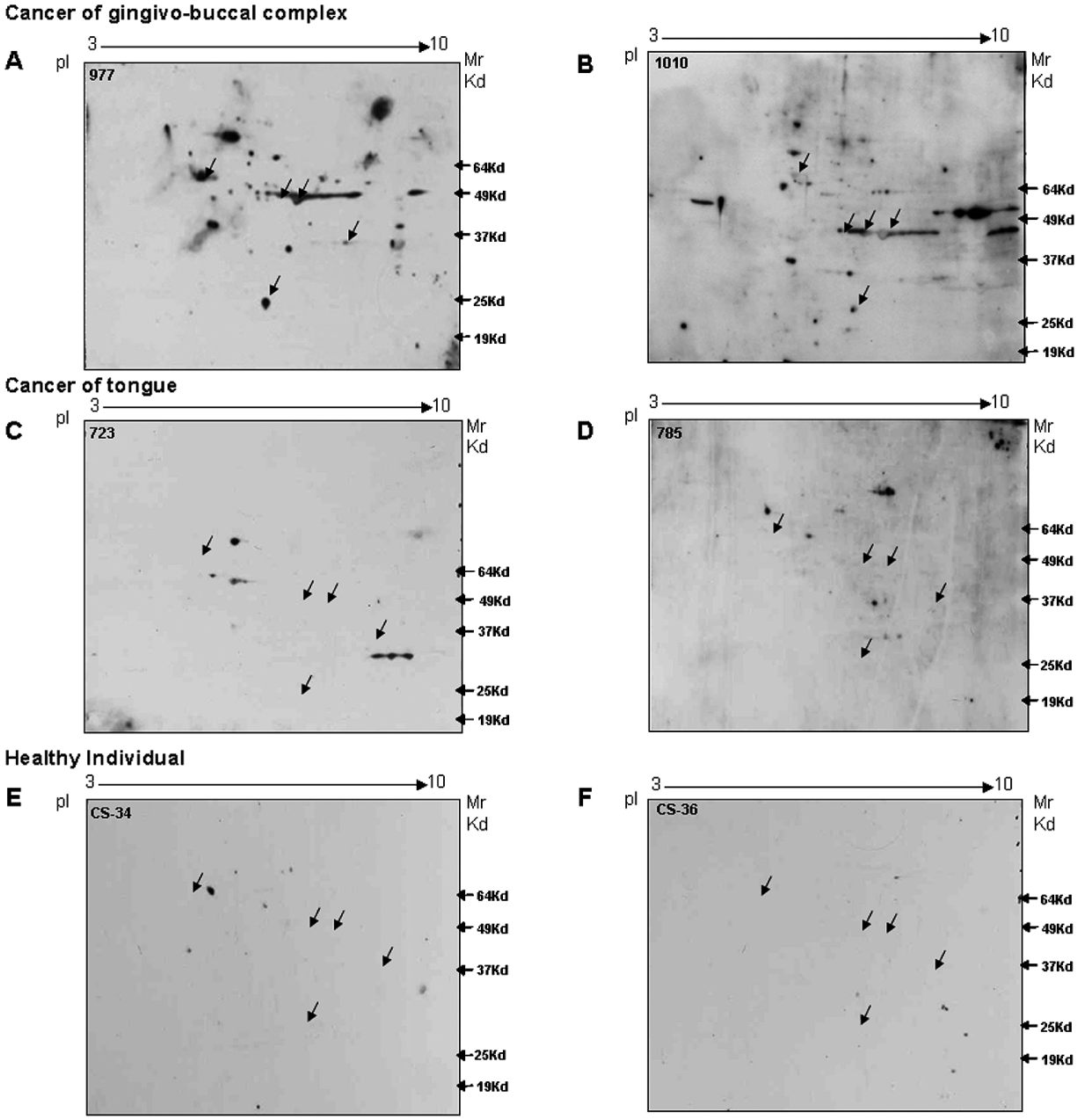

Fig. 2. Figure shows the representative autographs of KB cell lysate proteins separated by 2-DE and probed with serum IgG from two patients with cancer of gingivo-buccal complex (2A, 2B), two patients with cancer of tongue (2C, 2D), and two healthy individuals (2E, 2F). Protein spots, which elicit an antibody response in several patients with cancer of GBC, are indicated by arrows. The number of the sera sample used is given on the top left corner of each autograph.

iment, sets of 9 gels were run simultaneously. One gel was used for visualization of proteins by silver staining; the remaining gels were used for immunoblotting.

Figure 1 shows the pattern of separation of the KB cell lysate proteins and the identity of the proteins as reported earlier [10]. Figure 2 shows the patterns of signals obtained on the autographs for two patients with cancer of GBC, for two patients with tongue cancer and for two healthy individuals. Table 2 shows autoantibody response to the proteins in the KB cell lysate protein profile in Fig. 1.

In patients with cancer of GBC, autoantibody re-

Table 2
Autoantibody response in healthy individuals, patients with cancer of tongue and cancer of the gingivo-buccal complex

Spot	Protein identity	% occurrence		
		Healthy Individual* (n =15)	Tongue cancer* (n = 15)	GBC cancer* (n = 15)
59b	Alpha enolase	20 (3)	7 (1)	73 (11)
59c	Alpha enolase	20 (3)	7 (1)	73 (11)
59a	Alpha enolase	13 (2)	7 (1)	60 (9)
52	Peroxiredoxin VI	–	–	40 (6)
16	Annexin II	–	7 (1)	20 (3)
19	HSP70	7 (1)	–	47 (7)
26	Pyruvate kinase	7 (1)	–	20 (3)
15	Alpha tubulin	7 (1)	7 (1)	27 (4)
10	Beta tubulin	–	–	13 (2)
65	ATP synthase	–	7 (1)	27 (4)
41	Aldose reductase-1	–	–	7 (1)
24	Aldose reductase-2	–	–	33 (5)
69b	Triose phosphate isomerase	–	–	7 (1)

*Figures in parenthesis are the number of patients.

Table 3
Mass Spectroscopic analysis of spot 24

Spot	ID	Accession number	Theoretical Mr/pI	MS analysis				MS/MS analysis	
				Observed Mr/pI	Score	Sequence coverage	Peptide matched	Peptides	Score
24	Aldose reductase 2	P15121	36/6.56	35/7.1	310	68	21/35	LIQYCQSK MPILGLGTWK LWCTYHEK YKPAVNQIECHPYLTQEK	110

sponse was detected against three forms of alpha-enolase spots 59a, 59b and 59c in 60%, 73% and 73% respectively, HSP 70 (47%), annexin II (20%), peroxiredoxin-VI (40%), pyruvate kinase (20%), alpha tubulin (27%), beta tubulin (13%), ATP synthase (27%), TPI (7%), aldose reductase (spot 41) (7%), aldose reductase (spot 24) (33%) (Table 2). This autoantibody response matched with our earlier reported data seen in this cancer [10]. These were the only antigens which elicited autoantibody response consistently. In our earlier study, the autoantibody response against spot 24 was seen in 14 % but was not reported. Spot 24 has now been identified as aldose reductase (Table 3).

The autoantibody response in patients with tongue cancer was very different. Only 7% (1/15) patients with tongue cancer showed autoantibody response against either alpha enolase (59a, 59b, 59c), annexin II, alpha tubulin or ATP synthase. Autoantibodies against these antigens were observed in different patients i.e. none of these antigens showed antibody response in combination.

Sera of healthy individuals had autoantibodies against alpha enolase, 59a, 59b, and 59c in 13%, 20% and 20% respectively. Autoantibody response against pyruvate kinase, HSP 70 and alpha tubulin was also seen in one healthy individual (Table 2).

The auto antibody response was confirmed by dot blotting. Recombinant alpha enolase and Hsp 70 were spotted on PVDF membrane and probed with sera from healthy individuals, patients with cancer of the tongue and cancer of the GBC. Figure 3 shows the autoantibody response to these antigens. Majority of the patients with cancer of GBC, who showed autoantibody response against alpha enolase and HSP 70 on 2D blots of KB cell lysate proteins, also detected recombinant alpha enolase and HSP 70 on the dot blots, which were done in duplicate. GST protein alone did not show any signal with any of the sera (data not shown).

4. Discussion

In this study the autoantibody response in patients with cancer of two different sub sites of the oral cavity i.e. GBC and anterior tongue was compared using immunoproteomics. The proteins eliciting an autoantibody response in cancer of GBC reported earlier [10], belong to diverse groups consisting of metabolic en-

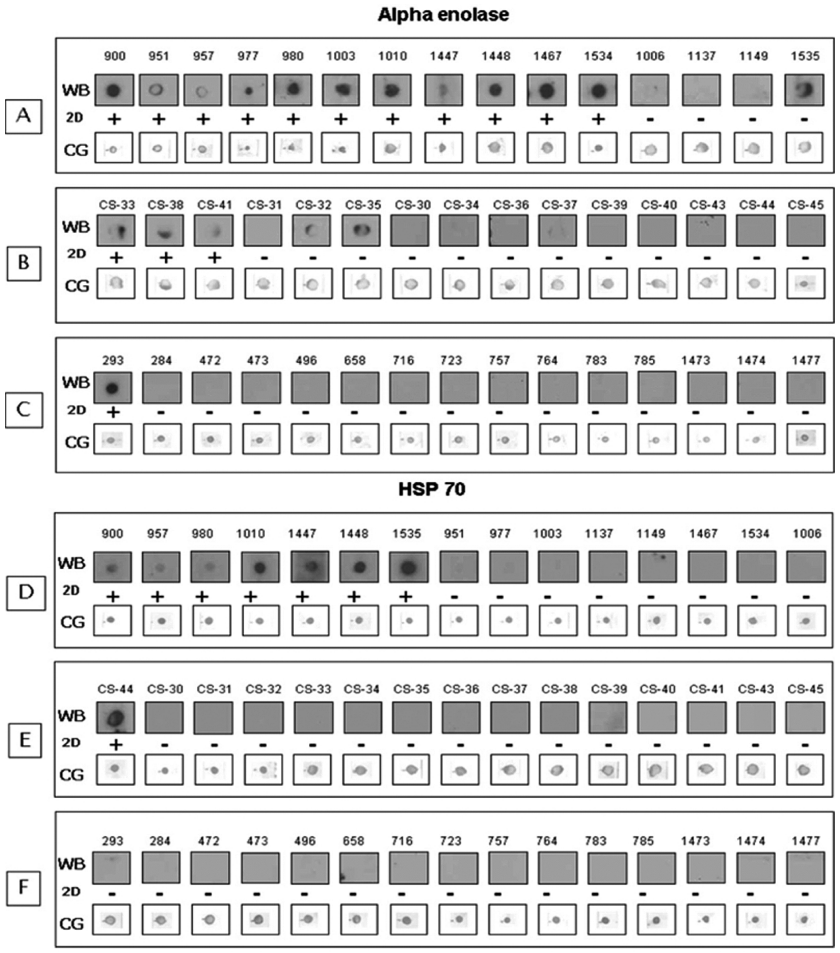

Fig. 3. Figure shows the signals obtained on the dot blots (WB) wherein recombinant proteins GST-α enolase and GST-HSP 70 were probed with sera IgG from 15 patients with cancer of GBC (A and D), healthy individuals (B and E) and cancer of tongue (C and F) respectively. Below each autographic signal (WB), the status of the presence or absence of the signal on the 2D blots is given as $+/-$ and below that the image of the respective colloidal gold (CG) stained dot blot is shown. The sera samples used in the analysis are shown by the numbers above each of the blots.

zymes (alpha enolase, ATP synthase, pyruvate kinase, TPI, aldose reductase), chaperones (HSP 70), microtubular proteins (alpha and beta tubulin), cell adhesion proteins (annexin II), and antioxidants (peroxiredoxin VI). Some of these proteins have been shown to elicit an autoantibody response in other cancers, except for pyruvate kinase and aldose reductase, which we have reported for the first time in our earlier study [10].

Table 2 shows that the autoantibody response in cancer of GBC varied from 7–73% for different tumor antigens in different individuals. On the other hand, there was no significant autoantibody response to these antigens in patients with tongue cancer. Autoantibody response against alpha enolase, annexin-II and ATP synthase were each seen in one out of fifteen patients only. The difference observed for autoantibody response in patients with cancer of these two sub sites i.e. GBC and tongue may be because of different clinicopathological features, and different biological origin.

The immunoproteomics approach used in this study and our earlier report [10] has certain limitations as any other technique, in that only autoantibodies which identify an epitope on a linear protein on western blots are detected. Within this framework, the autoantibody response between the two subsites of the oral cavity is distinct.

Autoantibody response in cancer of GBC has been earlier reported by us [10] to correlate with differentiation status of the tumors for some of the antigens. In this study, there were almost equal number of patients in each cohort vis a vis differentiation status of the tumor, i.e. 4 vs 4 PD and 10 vs 11 MD, 1 vs 0 WD

in GBC cancer and tongue cancer respectively. Even then there was very minimal autoantibody response to the antigens in patients with tongue cancer. Hence the differential auto antibody response is not due to the differentiation status of the tumor.

From the above it is becoming apparent that the immune surveillance mechanisms in tongue cancer appear to be more complex and suppressed than those in cancer of the GBC and normal healthy individuals, as even the minimal autoantibody response to alpha enolase as seen in the latter is not seen in tongue cancer patients. Though the reason for differential autoantibody response is not clear but the data suggests that different pathways may be involved in the carcinogenesis of GBC and tongue. Both these need further attention.

Evaluation of autoantibody responses is becoming a well accepted approach for the identification of biomarkers for cancer which can be used for early detection, prognosis and follow up of therapy through less invasive measures. The observations in this study indicate that cancer of each sub site of the oral cavity must be studied separately because of their different biology and emerging site specific molecular profiles including autoantibody responses to ensure unambiguous clinical interpretations.

Acknowledgement

The work was supported by a grant from Council of Scientific and Industrial Research, (27(0151)/06/EMR-II). Sanjeev Shukla was supported by a fellowship from Lady Tata Memorial Trust, (LTMT). Atul Pranay was supported by a fellowship from ACTREC. We thank Bhagyashree V Rajeshwar, social worker, ACTREC, for her help in collection of blood from healthy individuals.

Conflicts of interests

None.

References

[1] K.S. Anderson and J. LaBaer, The sentinel within: exploiting the immune system for cancer biomarkers, *J Proteome Res* **4**(4) (2005), 1123–1133.

[2] Y.S. Cho-Chung, Autoantibody biomarkers in the detection of cancer, *Biochim Biophys Acta* **1762**(6) (2006), 587–591.

[3] M. Nesterova et al., Autoantibody biomarker opens a new gateway for cancer diagnosis, *Biochim Biophys Acta* **1762**(4) (2006), 398–403.

[4] S.H. Shoshan and A. Admon, Novel technologies for cancer biomarker discovery: Humoral proteomics, *Cancer Biomark* **3**(3) (2007), 141–152.

[5] M. Caron, G. Choquet-Kastylevsky and R. Joubert-Caron, Cancer immunomics using autoantibody signatures for biomarker discovery, *Mol Cell Proteomics* **6**(7) (2007), 1115–1122.

[6] S.R. Pereira-Faca et al., Identification of 14-3-3 theta as an antigen that induces a humoral response in lung cancer, *Cancer Res* **67**(24) (2007), 12000–12006.

[7] L. Li et al., Identification of hepatocellular-carcinoma-associated antigens and autoantibodies by serological proteome analysis combined with protein microarray, *J Proteome Res* **7**(2) (2008), 611–620.

[8] B.S. Taylor et al., Humoral response profiling reveals pathways to prostate cancer progression, *Mol Cell Proteomics* **7**(3) (2008), 600–611.

[9] A. Gagnon et al., Use of a combination of approaches to identify and validate relevant tumor-associated antigens and their corresponding autoantibodies in ovarian cancer patients, *Clin Cancer Res* **14**(3) (2008), 764–771.

[10] S. Shukla et al., Tumor antigens elciting autoantibody response in cancer of gingivo-buccal complex, *Proteomics Clinical Applications* **1**(12) (2007), 1592–1604.

[11] C.A. Squier and M.J. Kremer, Biology of oral mucosa and esophagus, *J Natl Cancer Inst Monogr* (29) (2001), 7–15.

[12] D.M. Walker, Oral mucosal immunology: An overview, *Ann Acad Med Singapore* **33** (2004), 27S–30S.

[13] S.R. Moore et al., The epidemiology of tongue cancer: a review of global incidence, *Oral Dis* **6**(2) (2000), 75–84.

[14] T.A. Winning and G.C. Townsend, Oral mucosal embryology and histology, *Clin Dermatol* **18**(5) (2000), 499–511.

[15] K.M. Sathyan et al., Carcinoma of tongue and the buccal mucosa represent different biological subentities of the oral carcinoma, *J Cancer Res Clin Oncol* **132**(9) (2006), 601–609.

[16] J. Chen et al., Proteomics of buccal squamous cell carcinoma: the involvement of multiple pathways in tumorigenesis, *Proteomics* **4**(8) (2004), 2465–2475.

[17] Q.Y. He et al., Identification of tumor-associated proteins in oral tongue squamous cell carcinoma by proteomics, *Proteomics* **4**(1) (2004), 271–278.

[18] H. Schliephake, Prognostic relevance of molecular markers of oral cancer – a review, *Int J Oral Maxillofac Surg* **32**(3) (2003), 233–245.

[19] J. Kaur, A. Srivastava and R. Ralhan, Serum p53 antibodies in patients with oral lesions: correlation with p53/HSP70 complexes, *Int J Cancer* **74**(6) (1997), 609–613.

[20] R. Ralhan et al., Circulating p53 antibodies as early markers of oral cancer: correlation with p53 alterations, *Clin Cancer Res* **4**(9) (1998), 2147–2152.

[21] M. Castelli et al., Anti-p53 and anti-heat shock proteins antibodies in patients with malignant or pre-malignant lesions of the oral cavity, *Anticancer Res* **21**(1B) (2001), 753–758.

[22] R. Murase et al., Serum autoantibody to sideroflexin 3 as a novel tumor marker for oral squamous cell carcinoma, *Proteomics Clinical Applications* **2** (2008), 517–527.

[23] O. Gires et al., Profile identification of disease-associated humoral antigens using AMIDA, a novel proteomics-based technology, *Cell Mol Life Sci* **61**(10) (2004), 1198–1207.

[24] H.S. Lin et al., Autoantibody approach for serum-based detec-

tion of head and neck cancer, *Cancer Epidemiol Biomarkers Prev* **16**(11) (2007), 2396–2405.
[25] G. Peterson, A simplification of the protein assay method of Lowry, et al which is more generally applicable, *Anal Biochem* **83** (1977), 346–356.
[26] O.H. Lowry et al., Protein measurement with the Folin phenol reagent, *J Biol Chem* **193**(1) (1951), 265–275.
[27] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **227**(5259) (1970), 680–685.