Epigenetics in Gastric Cancer : Analysis of Histone Post-translation Modifications and Modifying Enzymes

By

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I, hereby declare that the investigation presented in the thesis has been carried out by me. This work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

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List of Publications arising from the thesis

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Journals

- Cell-type specificity of β-actin expression and its clinicopathological correlation in gastric adenocarcinoma**. Shafqat A Khan**, Monica Tyagi, Ajit K Sharma, Savio G Barreto, BhawnaSirohi, Mukta Ramadwar, Shailesh V Shrikhande, Sanjay Gupta**.** World J Gastroenterol 2014 September 14; 20(34): 12202-12211
- Global Histone Posttranslational Modifications and Cancer: Biomarkers for Diagnosis, Treatment and Prognosis? **Shafqat A Khan**, Divya Velga Reddy, Sanjay Gupta. World J Biol Chem. 2015 Nov 26;6(4):333-45

Book chapters

Techniques to Access Histone Modifications and Variants in Cancer. Monica Tyagi, **Shafqat A Khan**, Saikat Bhattacharya, Divya Reddy, Ajit K Sharma, Bharat Khade, Sanjay Gupta. Methods Mol Biol. 2015;1238:251-72

Under review / To be submitted

Journals

- p38-MAPK/ MSK1 mediated regulation of histone H3 Serine 10 phosphorylation defines distance dependent prognostic value of negative resection margin in gastric cancer**. Shafqat A Khan**, R Amnekar, S G Barreto, M Ramadwar, S V Shrikhade, S Gupta. *To be submitted.*

- Combinatorial effect of HDAC inhibitors and DNA-targeted chemotherapeutic drugs on gastric cancer cells**. Shafqat A Khan**, S G Barreto, M Ramadwar, S V Shrikhade, S Gupta. *To be submitted.*

Conferences

- **Oral presentation** in DBT-JRF Meet, November 21-22, 2013, ICT, Mumbai, India. Title: β-actin expression in gastric cancer: cell type specificity and correlation with clinicopathological parameters. ***Shafqat A Khan****, Monica Tyagi, Ajit K Sharma, Savio G Barreto, BhawnaSirohi, MuktaRamadwar, Shailesh V Shrikhande, Sanjay Gupta.*

- **Poster presentation** in Carcinogenesis-2015 on 'Molecular Pathways to Therapeutics: Paradigms and Challenges in Oncology', February 11-13, 2015, ACTREC, Navi Mumbai, India. Title: p38MAPK/ MSK1 pathway mediated increase in histone H3Ser10 phosphorylation leads to poor prognosis in gastric cancer. ***S A Khan**, R Amnekar, B Khade, S G Barreto, M Ramadwar, S V Shrikhande, S Gupta.*

- **Poster presentation** in 5th Meeting of Asian Forum of Chromosome and Chromatin Biology on 'Gene Networks in Chromatin/ Chromosome Function', January 15-18, 2015, JNCASR, Bengaluru, India. Title: H3S10P, a new histone oncomodification regulated through p38 MAPK/MSK1 pathway and correlates with clinicopathological characteristics in gastric cancer. ***S A Khan**, R Amnekar, B Khade, S G Barreto, M Ramadwar, S V Shrikhande, S Gupta.*

- **Poster presentation** in Kestone symposia on 'Chromatin Mechanisms and Cell Physiology', March 23-28, 2014, Oberstdorf, Germany. Title: H3S10 Phosphorylation: Regulation and Correlation with Clinicopathological Parameters in Gastric Adenocarcinoma. ***S A Khan**, A K Sharma, S G Barreto, B Sirohi, M Ramadwar, S V Shrikhande, S Gupta.*

- **Poster presentation** in 4th International Conference on Stem Cell and Cancer,October 19-22, 2013, Haffkine Institute, Mumbai, India. Title: HDAC Inhibitors Improve Chemotherapy Response in Human Gastric Cell lines. ***Shafqat Ali Khan**, Savio G Barreto, BhawnaSirohi, Shailesh V Shrikhande, Sanjay Gupta.*

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Others

Publications in Journals

- MKP1 phosphatase mediates dephosphorylation of H3Serine10P during ionization radiation induced DNA damage response in G1 phase of cell cycle. Ajit Kumar Sharma, **Shafqat A Khan**, Asmita Sharda, Divya V Reddy, Sanjay Gupta. *Mutat Res. 2015 Aug;778:71-9.*

- Expression of histone variant, H2A.1 is associated with the undifferentiated state of hepatocyte**.** Monica Tyagi, Bharat Khade, **Shafqat A Khan**, Arvind Ingle and Sanjay Gupta. *Exp Biol Med (Maywood). 2014 Oct;239(10):1335-9.*

- Dynamic alteration in H3 Serine10 phosphorylation is G1-phase specific during IR-induced DNA damage response in human cells. Ajit K. Sharma, Saikat Bhattacharyya, **Shafqat A. Khan**, and Sanjay Gupta. *Mutat Res. 2015 Mar;773:83-91.*

Dedicated

To

Ammi *(Umm-e-Salma)* *and* **Abbu** *(Shoharat Ali Khan)*

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Shafqat Ali Khan

CONTENTS

Synopsis

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Ph. D. PROGRAMME

1. **Name of the Student:** Shafqat Ali Khan
2. **Name of the Constituent Institution:** Tata Memorial Centre**,** Advanced Centre for Treatment, Research & Education in Cancer.
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4. **Title of the Thesis**: Epigenetics in gastric cancer: Analysis of histone modifications and histone modifying enzymes.
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SYNOPSIS

1. Introduction:

Carcinogenesis involves various genetic and epigenetic alterations. The overall disruption of the epigenetic landscape is one of the most common features of all human cancers which include global loss of genomic DNA methylation, local CpG island hypermethylation and a characteristic histone modification/variant pattern [1]. Histones are basic proteins and a major component of chromatin. Post-translational modifications of histones are central in the regulation of chromatin dynamics and gene regulation. Major reported histone modifications include acetylation, methylation, phosphorylation, ubiquitylation, glycosylation, ADP-ribosylation, carbonylation and SUMOylation. These covalent posttranslational modifications (PTMs) of histones singly or in distinct

combinations may alter higher-order chromatin state by affecting interaction of histone with DNA or inter and intra nucleosomes interaction or facilitates recruitment of non-histone regulatory proteins on chromatin and leads to specific chromatin related functions and processes like transcription, DNA repair, replication etc [2]. The timing of induction of different modification on different histones depends on the signaling and physiological condition within the cell.

Over the past decade accumulated evidences indicate towards the association of aberrant histone PTMs and cancer. However, only a few of the more than 60 residues of histones in which modifications have been described and linked to cancer and called as 'Histone onco-modification' [3]. Global loss of acetylation of histone H4 at lysine 16 (H4K16Ac) and loss of trimethylation of histone H4 at lysine 20 (H4K20Me3) were the first histone marks reported to be deregulated in cancer [4]. A decrease of H3K4Me2/Me3 is observed in a range of neoplastic tissues and a decrease of H3K9Ac has been linked with tumor progression in prostate and ovarian tumors. In contrast, in hepatocellular carcinoma an increase in H3K9Ac levels was reported. H3K27Me3 has been evaluated as a prognostic factor in prostate, breast, ovarian, pancreatic, esophageal cancers. Loss of H3K18Ac is correlated with poor prognosis and tumor grade in patients with prostate, pancreatic, lung, breast and kidney cancers suggesting the loss of this modification is an important event in tumor progression [3]. Therefore, available literatures have established the alteration in the global histone PTMs for multiple cancers suggesting their importance in the better management of cancer patients. However, detailed studies are required to understand how global levels of histone modifications are established and maintained and what their mechanistic links are to the cancer clinical behavior.

Gastric cancer remains the fourth most common cancer in the world and is second only to lung cancer in terms of worldwide cancer deaths [5]. It is a disease of very poor prognosis as most patients are diagnosed in advanced stages of cancer due to the delay in presentation. Adenocarcinoma is the most common malignancy of the stomach, accounting for nearly 90% of gastric tumors. Based on location of site of occurrence in stomah, gastric adenocarcinoma can be classified as: cardia or proximal, and distal or noncardia. The incidence of gastric carcinoma varies dramatically by geographic location, environmental and behavioural factors, family history and *Helicobacter pylori* infection [6]. The Asian countries with a high incidence include Japan, China, and South Korea; those with a low incidence include India, Pakistan, and Thailand [5]. In India, across the various registries, there is a wide variation in the incidence of gastric carcinoma. Among the six registries, the highest incidence in both sexes is reported from Chennai and the lowest from Barshi, Maharashtra. The incidence rate of gastric cancer is four times higher in Southern India compared with Northern India [7].

A radical D2 gastrectomy and more recently radical surgery along with preoperative chemotherapy holds the best prospect of a cure in gastric cancer [8, 9]. The most common therapeutic approach to treat locally advanced gastric adenocarcinomas is a multimodal treatment with preoperative Cisplatin/ 5-fluorouracil/ Epirubicin/ Oxaliplatin-based chemotherapy or radiochemotherapy (CRT), followed by resection. The neoadjuvant CRT approach facilitates histological tumor regression that may increase local resectability rates and eliminate chances of distant micro-metastases [10]. In surgery, achieving R0 resection where no residual disease is left behind is a challenge; therefore, distance and positivity of the resection margin becomes an important factor affecting the recurrence and prognosis of patients. 5-year survival rates for resection margin positive and negative disease being 13 versus 35% respectively [11]. Different

studies on esophageal adenocarcinoma, esophageal squamous cell carcinomas and gastric adenocarcinoma treated by preoperative CRT indicate that the degree of histopathological tumor regression can serve as a stronger prognostic marker than the current TNM system [10]].

The goal of all these strategies is to achieve curative resection (R0 resection) and thereby minimizing the chances of loco-regional recurrence and improving the prognosis of the disease. Despite of R0 resection loco regional recurrence has been encountered in 87% of patients [12]. The extent of resection based on microscopic techniques to define negative resection margin is not sufficient and is still a controversial topic. Further, other greatest obstacles to effective chemotherapy or CRT in most of cancers are differential response and the development of drug resistance [13]. Therefore, it is important to understand the cause and determine other compounds which can increase effectiveness and decrease the toxicity, if given along with chemotherapeutic drugs, and there is need of other molecular markers which can help in deciding the distance of resection margin by lowering the chances of its positivity. Therefore,

All this leads us to the point that there is need to understand in-depth the differential alteration in histones, histone modifying enzymes and to define new prognostic markers and therapeutic targets for the better management of gastric cancer patients.

2. Objectives:

I. To identify differential alterations in histones and their enzymes in gastric cancer.

II. To decipher molecular mechanism of specific alterations in histones in gastric cancer.

3. Work Plan:

Objective I: To identify differential alterations in histones and their enzymes in gastric cancer.

i. *Collection of freshly resected and paraffin embedded blocks of tissues from the site of tumor and resection margins (proximal and distal) of gastric cancer patients.*

ii. *Haematoxylin and Eosin (H&E) staining and histopathological confirmation of tissue identity and tumor content.*

iii. *PCR and Giemsa staining based screening for Helicobacter pylori infection.*

iv. *Preparation of chromatin and nucleo-cytosolic fraction from freshly resected tissues.*

v. *Pilot screening of differential site-specific histone post-translational modifications in tumor and resection margin tissues using immunoblotting.*

vi. *Immunohistochemical analysis of specific histone PTM(s) on tumor and resection margins (proximal and distal) tissues for validation in large cohort of samples.*

Objective II: To decipher molecular mechanism of specific alterations in histones in gastric cancer.

i. *Identification of specific histone modifying enzymes responsible for alteration in specific histone PTMs in cell lines and tissue samples using enzyme assay, immunoblotting and immunohistochemistry.*

ii. *Determination of effect of enzyme on site specific histone modification by exogenous overexpression and chemical inhibition followed by immunoblotting and immunofluorescence studies.*

iii. *Identification of regulatory pathway responsible for of specific histone PTM in tissues and cell lines using immunoblotting and immunofluorescence studies.*

iv. *Cell based toxicity assays to study the effect of histone modifying enzymes inhibitors for their potential application in combinatorial chemotherapy.*

4. Results

4.1.Site-specific hypo-acetylation and hyper-phosphorylation of histones in gastric cancer

Histopathologically confirmed freshly resected tumor and resection margin (proximal and distal) tissues of gastric cancer patients (n=10) were processed for studying the alteration in series of site-specific histone lysine acetylation (H3K9, H3K14, H3K18, H3K23, H3K27, H4K5, H4K8, H4K12 and H4K16), lysine methylation (H3K4Me, H3K4Me2, H4K20Me and H4K20Me3) and serine phosphorylation (H3S10). Western blot analysis showed significant decrease ($P < 0.05$) of H4K16Ac, H4K20Me3, H3K27Ac, H3K4Me2 and significant increase of H3S10P ($P < 0.001$) in tumor compared to resection margin tissues. Further, combined analysis of all acetylations revealed hypo-acetylation ($P < 0.001$) in tumor compared to resection margin tissues.

Based on these observations in-depth studies were carried out for (i) regulation and relationships of H3S10 phosphorylation with clinicopathological parameters and (ii) the significance of histone deacetylation for their prospective relevance in therapeutics, individually and/or combinatorially with standard chemotherapy.

4.2.Increase in H3S10P leads to poor prognosis in gastric cancer

The status of H3S10P was studied in validation set (n=101) among tumor, proximal and distal resection margin tissues of gastric cancer using immunohistochemistry (IHC). IHC was assessed by an experienced pathologist, intensity of staining (ranges from zero to three) and percentage of cells stained (ranges from zero to hundred) for specific intensity was calculated and expressed in term of H-score. Comparison of H-score showed significant ($p < 0.001$) higher level of H3S10P in tumor than both the resection margin tissues. Chi-square analysis H-score of tumor, proximal and distal

resection margin tissues was done to find correlation of H3S10P levels with clinicopathological parameters. H3S10P of tumor showed a significantly positive correlation with tumor grade (p= 0.0001), T stage (p= 0.005), pTNM stage (p= 0.016) and recurrence (p= 0.034). H3S10P levels of proximal and distal resection margin also showed a significant positive correlation with above said parameters. Kaplan-meier survival analysis suggested a significant negative correlation of H3S10P levels of tumor (p= 0.004 and 0.011), proximal (p= 0.014 and 0.004) and distal (p= 0.026 and 0.006) resection margin tissues with overall and disease free survival, respectively. Further, H3S10P levels of tumor tissues were also found to be an independent predictor of overall survival. Therefore, increase in H3S10P levels leads to poor prognosis in human gastric cancer.

4.2.1. Level of H3S10P in resection margin is distance dependent.

Our observation of decrease in the level of H3S10P in resection margins compared to tumor tissues lead us to study the importance of distance of resection margin from the site of tumor from which H3S10P begins to decrease significantly. To answer this, the resection margin samples were grouped as per their distance from tumor site and their mean H-score were compared with the H-score of tumor samples. We identified 4 cm as a distance of resection margin from which H3S10P showed significant reduction ($p < 0.05$) for both the margins compared to tumor tissues. In addition, H-score of tumor samples compared with H-score of resection margins with ≤4 cm and >4 cm distance also showed a significant ($p < 0.001$) reduction of H3S10P levels for the group having resection margin distance >4 cm, whereas resection margin with the distance of ≤4 cm showed no difference in H3S10P level compared to tumor tissues. Further, Chi-square analysis to investigate the effect of H3S10P dependent proximal resection margin on clinicopathological parameters showed a positive correlation of H3S10P

levels with WHO classification (p= 0.001), T-stage (p= 0.002) and TNM stage (p= 0.023) for the patients with resection margin ≤ 4 cm. In case of distal resection margin, Chi-square analysis showed a positive correlation of H3S10P levels with WHO classification (p= 0.0001) and T-stage (p= 0.009) and recurrence (p= 0.031). For both the resection margins, no correlation was found for patients with resection margin distance >4 cm. Kaplan-Meier survival analysis also did not show any significant difference between patients with resection margin distance either ≤ or > 4 cm.

4.2.2. Increase in H3S10P in gastric cancer is cell cycle independent

H3S10P levels alter throughout the cell cycle with the highest level in mitotic (G2/M) phase. Therefore, to define whether increase of H3S10P in gastric cancer is dependent or independent of cell cycle profile of the tissues samples, we compared levels of cyclins, mitotic index and cell cycle profile of tumor and resection margin tissues. Cyclin B1, D1 and E1 showed higher level in tumor tissues compared to resection margins, but their ratios were constant with the resection margin tissues. Moreover, mitotic index also did not show any significant increase in mitotic cells in tumor compared to resection margin tissues. Flow cytometry based cell cycle analysis of tissue samples showed equal percentage of G1, S and G2/M cells in tumor and resection margin tissues, though, both tumor and resection margin tissues showed more than 80% cells in G1 phase.

In interphase or G1 phase of cell cycle, H3S10P is associated with chromatin relaxation and transcriptional up-regulation of mainly immediate early (IE) genes. Therefore, using RT-PCR and immunoblotting we checked the levels of IE genes (*c-jun* and *c-fos*) which showed increase in the levels in tumor compared to resection

margin tissues. Collectively, data indicates that increase in H3S10P levels in gastric cancer is independent of cell cycle.

4.2.3. Phosphorylation of H3S10 is mediated through p38-MAPK/ MSK1 pathway:

Mitogen and stress activated kinase 1 (MSK1) at the downstream of MAPK pathway is known to phosphorylate H3S10 and required for cellular transformation. Also, overexpression of *c-jun* and *c-fos* is a result of MSK1 mediated phosphorylation of H3S10 at their promoters. Immunoblot and immunofluorescence analysis of H3S10P, MSK1, phopho-MSK1 and MAP kinases with their active phospho forms of tissues and H89 (MSK1 inhibitor) treated gastric cancer cell lines, AGS and KATOIII, indicated p38-MAPK/MSK1 mediated regulation of H3S10P in gastric cancer. Further, overexpression of MSK1 in AGS cells and treatment with specific inhibitors against phospho-EKR1/2 (PD98059) and phospho-p38 (SB203580) in gastric cancer cell lines, AGS and KATOIII, confirms p38 MAPK/MSK1 mediated regulation of H3S10P in gastric cancer.

4.2.4. Overexpression of β-actin in tumor compared to normal margins of gastric tissue:

While working with total cell lysate of gastric tissues we observed a very high level of β-actin in tumor compared to resection margin. Therefore, to detect an overall relative mRNA and protein expression of β-actin between gastric normal and tumor tissues, RT-PCR and western blot was performed on resected fresh tissues (n=5) which showed a significant higher expression of β-actin level in tumor tissues both at mRNA ($p < 0.001$) and protein level ($p < 0.01$). Existing studies also suggest high level of β-actin in number of cancer using tissue disruptive techniques; however, there was no study to provide which cell type-expression is contributing towards significant

overexpression of β-actin in cancer. Therefore, we analyzed β-actin expression and distribution in paired normal and tumor tissue samples of gastric adenocarcinoma patients using immunohistochemistry (IHC), a tissue non-disruptive technique.

4.2.5. Overexpression of β-actin in tumor is predominantly contributed by inflammatory cells

To provide histological proof of β-actin overexpression in gastric cancer, IHC was performed on formalin-fixed paraffin-embedded tissue blocks (n=26). IHC analysis showed that inflammatory cells express significantly higher level of β-actin compared to the epithelial cells in both normal ($P < 0.001$) and tumor ($P < 0.001$) tissues. Furthermore, tumor tissues express relatively higher level of β-actin compared to normal in both epithelial and inflammatory cells; however, difference between epithelial cells was not significant, whereas inflammatory cells differed significantly ($p < 0.01$). Comparison of average IHC score (sum of IHC scores of epithelial and inflammatory cells) of normal and tumor tissue also showed a significant increase of β-actin expression in tumor tissues ($p < 0.05$) compared to normal.

4.2.6. Correlation of β -actin expression with clinicopathological parameters

Univariate analysis was performed (n=26) to correlate 'total IHC score' and 'average total IHC score' of epithelial and inflammatory cells for β-actin immunostaining with clinicopathological parameters. Epithelial and 'overall' level of β-actin did not show any significant correlation with any of the clinicopathological parameters while β-actin level of inflammatory cells showed significant correlation with tumor grade or WHO classification ($p < 0.05$). Further, identification of pattern and statistical significance of β-actin level in inflammatory cells of tumor tissues of different tumor grades: moderately differentiated (MD), poorly differentiated (PD) and signet ring cell carcinoma (SRC-a type of poorly differentiated cell) was carried out. The results

showed a positive correlation of β-actin level with tumor grade with significantly higher level in PD ($p < 0.05$) and SRC ($p < 0.05$) compared to MD; however, PD to SRC difference was not significant ($p > 0.05$). In addition, low level of β-actin in SRC cell line, KATOIII compared to MD gastric adenocarcinoma cell line, AGS fascinated us to look for the pattern of β-actin expression of tissue epithelial cells with tumor grade. β-actin level in tissue epithelial cells followed a similar pattern of cell lines, i.e. decreases from MD to PD and to SRC, a negative correlation with tumor grade, though insignificant. Thus, the data indicates β-actin towards the prospective prognostic marker in gastric cancer.

4.3.*Global and site-specific hypo-acetylation is due to higher HDAC levels in gastric cancer*

The observed hypoacetylation by immmunoblot and IHC analysis in gastric cancer could be because of the low levels of histone acetyl transferase (HAT) and/or high levels of histone deacetylase (HDAC) in tumor compared to resection margin tissues in gastric cancer. Therefore, to determine the level of HAT and HDAC in tissue samples (n=5) total protein lysate was isolated and used for commercial kit based calorimetric HAT and HDAC assay. The data suggested significantly increased level of HDAC ($p< 0.01$) without alteration in HAT levels in tumor tissues compared to resection margins. The observed hypo-acetylation and increase in HDAC suggested that HDAC inhibitors (HDACi) can be explored as prospective therapeutic agent.

4.3.1. HDACi increases the amount of DNA bound chemotherapeutic drug:

Higher level of HDAC in tumor tissues prompted us to exploit HDACi, Valproic acid (VPA), Trichostatin. A (TSA) and ‘Vorinostat’ or suberoylanilide hydroxamic acid (SAHA), as drugs that can be used in combination with conventional DNA binding chemotherapy drugs like Cisplatin, Oxaliplatin and Epirubicin. HDACi leads to

increase in histone acetylation favouring chromatin relaxation and thereby may increase the binding of chemotherapeutic drugs to DNA. The binding of chemotherapy drugs to DNA at their IC_{50} values were measured by spectroscopic method in three different combinations with HDACi i.e. pre-HDACi, concurrent and post-HDACi treatment in gastric cancer cell line. Absorbance of drugs was measured at 220, 205 and 254 nm for cisplatin, oxaliplatin and epirubicin, respectively. The analysis showed increase in the amount of chemotherapy drugs bound to DNA, when HDAC inhibitors were given in pre- and concurrent combinations; however, pre-treatment of HDACi resulted in maximum increase in binding of chemotherapy drugs to DNA.

4.3.2. HDACi act synergistically in combination with chemotherapeutic drugs.

In combinatorial chemotherapy mechanism of drug interaction is an important aspect; therefore, we tested for the best combination of HDACi and chemotherapy drugs that leads to synergy at maximum effective dose for cell death. HDACi and chemotherapy drugs were tested on AGS cells for their additive, antagonistic and synergistic interaction in three different combinations (pre-HDACi, concurrent HDACi and post-HDACi) at their constant ratio for cell death using MTT assay. For each combination, affected fraction (Fa) was calculated using cell survival percentage data of MTT assay. Fa values were used to calculate combination index with the help of software CompuSyn and plotted against the dose of the individual and drugs in combination. The results showed that pre-treatment of HDACi act synergistically in combination with chemotherapy drugs. Based on these findings, we concluded that HDACi used in combination with chemotherapeutic drugs will facilitate a reduction in the effective dose of the chemotherapeutic drug without compromising on cancer cell death. This could also offer the potential for reducing chemotherapy-associated toxicity in gastric

cancer. These results offer a firm rationale for exploring these drug combinations in the clinical setting.

5. Summary and Conclusion:

In gastric cancer, the present study investigated the differential pattern of various site-specific histone PTMs, possibility of the use of HDACi in combinatorial chemotherapy and effect of microenvironment on expression of housekeeping gene, β-actin.

Salient findings:

(i) The significant increase of histone mark, H3S10P in gastric cancer leads to poor prognosis. H3S10P was also found to be independent predictor of overall survival. The correlation of H3S10P levels of resection margins with clinical parameters and survival indicate towards the involvement of histone PTMs in field cancerization. Further, mechanistic investigations also revealed that p38MAPK/MSK1 pathway is responsible for the increase of H3S10P in gastric cancer.

(ii) HDAC inhibitors, pre-treatment on gastric cancer cell line showed maximum effect in cell death as it increases the amount of chemotherapy drugs bound to DNA, and, also showed synergic effect at the fraction effect (Fa) levels 0.5, 0.75 and 0.9 compared to concurrent or post-HDACi treatment as confirmed by combination index analysis. Dose reduction index analysis also showed the reduction in dose of chemotherapy drugs in combination with HDACi may lead to decreasing the toxicity associated with chemotherapy.

(iii) The differential level of β-actin expression in inflammatory and epithelial cells of tissue microenvironment was showed as a histological evidence of β-actin overexpression in gastric cancer. The overall higher level of β-actin in tumor

tissues is mainly contributed by inflammatory cells which correlate with tumor grade.

In conclusion, our study has revealed histone hypo-acetylation and hyper-phosphorylation across a large cohort of gastric tumor samples. The identified hyper-phosphorylation of H3S10 correlates with different tumor grades, morphologic types, and phenotypic classes of gastric tumors. Additionally, hyper-phosphorylated H3S10 correlates with distance of resection margins, prognosis and clinical outcome. Further, association of histone hypo-acetylation with overexpression of HDAC enzymes lead to the use of small-molecule, HDACi as epigenetic modulators acting synergistically along with chemotherapeutic drugs for better management of gastric cancer.

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7. Publications in Refereed Journal:

a. Published

- **Shafqat A Khan**, Monica Tyagi, Ajit K Sharma, Savio G Barreto, Bhawna Sirohi, Mukta Ramadwar, Shailesh V Shrikhande, Sanjay Gupta. Cell-type specificity of β-actin expression and its clinicopathological correlation in gastric adenocarcinoma. *World Journal of Gastroenterology (PMID: 25232253).*
- **Shafqat A Khan**, Savio G Barreto, Mukta Ramadwar, Shailesh V Shrikhande, Sanjay Gupta. Global Histone Posttranslational Modifications and Cancer:

Biomarkers for Diagnosis, Treatment and Prognosis? *World Journal of Biological Chemistry (Under review)*

b. To be submitted

- p38MAPK/ MSK1 pathway mediated increase in histone H3Ser10 phosphorylation leads to poor prognosis in gastric cancer. (original research article)
- HDAC inhibitors improve chemotherapy response in human gastric cancer cell lines. (original research article)

c. Other publication

- Monica Tyagi, Bharat Khade, **Shafqat A Khan**, Arvind Ingle and Sanjay Gupta, Expression of histone variant, H2A.1 is associated with the undifferentiated state of hepatocyte. *Experimental Biology and Medicine (PMID: 24764240).*
- Ajit K. Sharma, Saikat Bhattacharyya, **Shafqat A. Khan**, and Sanjay Gupta. Dynamic alteration in H3 Serine10 phosphorylation is G1-phase specific during IR- Academic & training Program, ACTREC
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- Ajit Kumar Sharma, **Shafqat A Khan**, Divya V Reddy, Tejkiran Sagwekar, Sanjay Gupta. MKP1 phosphatase mediates dephosphorylation of H3Serine10P during ionization radiation induced DNA damage response in G1 phase of cell cycle. *Mutation Research: Fundamental and Molecular Mechanisms of Mutagenesis (Under revision).*

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Abbreviations

3D-CRT	Three-Dimensional Conformal Radiation Therapy
5-FU	5 Fluoro Uracil
β-ME	2-Mercapto Ethanol
ACT	Adjuvant chemotherapy
ADP	Adenosine Di-Phosphate
ARAC	Cytosine Arabinoside
APS	Ammonium Per Sulphate
ATP	Adenosine Tri Phosphate
AUT	Acetic Acid Urea Triton
BPB	Bromophenol Blue
BBS	Bes Buffer Saline
BSA	Bovine Serum Albumin
CagA	Cytotoxin-Associated Gene A
CBBR	Coomassie Brilliant Blue R-250
CDH1	Cadherin-1 Or E- Cadherin
CF	Chromatin Fraction
CHZ1	Nuclear Chaperon For H2a.Z
CENP-A	Centromeric Protein A
CI	Combination Index
CNE1	Calnexin
cNUCs	Circulating Nucleosome
COX-2	Cyclooxygenase-2
CT	Computed Tomographic
CTC	Copper Tartrate Carbonate
DAB	Diaminobenzidine
DEPC	Diethylpyrocarbonate
DFS	Disease Free Survival
DRM	Distal Resection Margin
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimetheyl Sulfoxide
DNMT1	Dna (Cytosine-5)-Methyltransferase 1
DTT	Dl-Dithio Threitol
DZNEP	3-Deazaneplanocin A
ECF	Epirubicin, Cisplatin And Fluorouracil
EGF	Epidermal Growth Factor
ECX	Epirubicin, Cisplatin And Capecitabine
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal Growth Factor
EGTA	Ethylene Glycol Tetraacetic Acid
EMT	Epithelial to Mesenchymal Transition
EMR	Electronic Medical Record
EOF	Epirubicin, Oxaliplatin and Fluorouracil
EGD	Esophagogastroduodenoscopy
EOX	Epirubicin, Oxaliplatin, and Capecitabine
ERK	Extracellular-Signal-Regulated Kinases

EUS	Endoscopic Ultrasonography
EZH2	Enhancer of Zeste Homolog 2
FA	Fraction Affected
FACS	Fluorescent Activated Cell Sorter
FBS	Fetal Bovine Serum
FRF	Freshly Resected Frozen
FFPE	Formalin-Fixed Paraffin-Embedded
miR	micro-RNA
GATA1	GATA binding protein 1
GC	Gastric Cancer
HFD	Histone Fold Domain
HAT	Histone Acetyl-Transferases
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HDAC	Histone Deacetylase
HDACi	HDAC Inhibitor (s)
H&E	Hematoxylene and Eosin
HIRA	Histone Regulation A
HDM	Histone Demethylases
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
HMT	Histone Methyltransferases
HP1	Heterochromatin Protein 1
H3S10ph	Histone H3 Serine10 Phosphorylation
IHC	Immunohistochemistry
IMRT	Intensity Modulated Radiation Therapy
IE	Immediate Early
JARID1B	Lysine-Specific Demethylase 5b
KDM1A	Lysine (K)-Specific Demethylase 1a
LMP1	Epstein–Barr virus latent membrane protein 1
LSD1	Lysine-Specific Demethylase 1
MBT	Malignant Brain Tumour
MOZ	Monocytic Leukemia Zinc Finger Protein
MOZ-CBP	CREB-Binding Protein
MAPK	Mitogen-Activated Protein Kinases
MD	Moderately Differentiated
MEM	Minimum Essential Medium
miR	micro-RNA
MMP15	Matrix Metallopeptidase
MMS	Methyl Methane Sulfonate
MOPS	3-(N-Morpholino) Propanesulfonic Acid
MSK1	Mitogen- and Stress-Activated Kinase 1
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-iphenyltetrazolium Bromide
NACT	Neo-adjuvant chemotherapy
NAD	Nicotinamide Adenine Dinucleotide
NCF	Nucleo-Cytosolic Fraction
NEB	New England Bioloab

NHD	Non-Histone Domain
NSAID	Nonsteroidal Anti-Inflammatory Drug
NPC	Nasopharyngeal Carcinoma
OS	Overall Survival
PBS	Phosphata Buffer Saline
PMSF	Phenylmethylsulfonyl Fluoride
PTM	Post-Translational Modification
PHD	Plant Hetero Domain
phMSK1	Phospho MSK1
PRM	Proximal Resection Margin
PRMT	Protein Arginine Methyl Transferases
PARPS	Poly-ADP-Ribose Polymerase
PIK	Phospho-Inositide Kinase
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PD	Poorly Differentiated
PKCbI	Protein kinase C beta I
PVDF	Poly Vinyliene Di-Fluoride
RM	Resection Margin
RPMI	Roswell Park Memorial Institute Medium
RT-PCR	Reverse Transcriptase PCR
SAM	S-Adenosyl Methionine
SAHA	Suberoylanilide Hydroxamic Acid
SRC	Signet Ring Cell Carcinoma
SHH	Sonic Hedgehog
SSB	Single Strand Breaks
SDS-PAGE	Sodium-Dodecyl-Sulphate –Poly-Acrylamide Gel Electrophoresis
SFRP2	Secreted Frizzled-Related Protein 2
SFRP5	Secreted Frizzled-Related Protein 2
TTBS	Tris Buffer Saline
TCL	Total Cell Lysate
TEMED	*N*,*N*,*N*′,*N*′-Tetramethylethane-1,2-Diamine
TGF-b	Transforming Growth Factor-B
TPA	Terephthalic acid
TTR	Tumor Tissue Repository
TSS	Transcriptional Start Site
TSA	Trichostatin A
TIP60	Tat Interacting Protein-60
UNC5B	Unc-5 Homolog B
VPA	Valproaic Acid
WBC CHAMBER	White Blood Cell Chamber
WHO	World Health Organizations
WNT5A	Wingless-Type Mmtv Integration Site Family, Member 5a

Chapter 1
Introduction

1.1 Background of the Work

Carcinogenesis involves various genetic and epigenetic alterations. The overall disruption of the epigenetic landscape is one of the most common features of all human cancers, which include global loss of genomic DNA methylation, local CpG island hypermethylation and a characteristic histone modification and/or variant pattern. Post-translational modifications (PTMs) of histones are central in the regulation of chromatin dynamics and regulate chromatin related processes, like transcription, DNA repair, replication, DNA damage response etc. Over the past decade accumulated evidences indicate towards the strong association of aberrant histone PTMs, termed as 'histone onco-modifications' with cancer. Further, available literatures have suggested that the alteration in the global histone PTMs in multiple cancers highlights their importance for the better management of cancer patients. However, detailed studies are required to understand '*how global levels of histone modifications are established, maintained and what their mechanistic links to the cancer clinical pathological behavior'*.

Gastric cancer is a disease of very poor prognosis and remains fourth most common cancer in terms of incident, and globally is second in terms of mortality. The most common therapeutic approach for locally advanced gastric adenocarcinoma is a multimodal treatment with pre-operative chemotherapy or radio-chemotherapy (CRT), followed by surgery. The neoadjuvant CRT approach facilitates histological tumor regression that may increase local resectability rates and eliminate chances of distant micro-metastases after surgery. In surgery achieving 'R0' resection, no residual disease is left behind, is a challenge; therefore, distance and positivity of the resection margin becomes an important factor affecting the recurrence and prognosis of patients. Despite of 'R0' resection a large numbers of gastric cancer patients show loco-recurrence, signifying the importance of assessing the currently used methods, microscopy and histology, to

define negative resection margin. Further, other greatest obstacles for effective chemotherapy or CRT in cancers are differential patient response and drug resistance. Therefore, it is important to determine effective agents/compounds which can increase effectiveness and decrease the toxicity, if given along with chemotherapeutic drugs. Also, there is a need of molecular markers which can help in deciding the distance of 'R0' resection margin.

In this presented work on human gastric cancer, histone post-translational modifications and histone modifying enzymes have been studied in association with clinic-pathological behavior. The levels of site-specific histone post-translational modifications have been compared between tumor and negative resection margin tissues. A detailed study is conducted on phosphorylation of histone H3 at serine 10 position (H3S10ph) for its regulatory mechanism and prognostic potential in gastric cancer. Further, investigation of histone deacetylase inhibitors (HDACi) has also carried out for analysis of their potential in combinatorial chemotherapy in gastric cancer.

1.2 Layout of the Thesis

Epigenetics of gastric cancer is a central theme of this thesis; therefore, the thesis starts with review of literature, chapter 2, describing gastric cancer, epigenetics, histone post-translational modifications, histone modifying enzymes and their inhibitors in detail with respect to cancer. 'Aims and Objectives' are described in chapter 3. A description on various methodologies and reagents used are described in chapter 4 as 'Materials and Methods'. The findings of the work are presented and discussed from chapter 5 to 7; each chapter is further divided in 'Introduction, Results and Discussion'. Chapter 5 *(Histone H3 Serine 10 phosphorylation: Regulation and its correlation with clinico-pathological parameters in gastric cancer)* describes our findings on H3S10ph in gastric cancer, where using statistical, histo-pathological and molecular approaches, potential of histone mark,

H3S10ph in gastric cancer prognosis and defining the 'true' negative resection margin have been investigated. Further, p38-MAPK/MSK1 was concluded as a regulatory pathway for H3S10ph in gastric cancer using biochemical and genetic manipulations approaches. While undertaking this work, I had a very interesting observation of significantly high level of β-actin in gastric tumor compared to histo-pathologically normal resection margin tissue samples, a housekeeping gene at protein level. Hence, I undertook an in-depth analysis on this observation using molecular and histopathological approaches; described in chapter 6 *(β-actin expression and its clinicopathological correlation in gastric adenocarcinoma).* This work deduces an interesting finding that β-actin has a prognostic value in gastric cancer and its high level in tumor is mainly contributed by infiltrating inflammatory or immune cell in the tumor micro-environment. The chapter 7 *(Global hypo-acetylation of histones: Combinatorial effect of HDAC inhibitors with DNA-targeted chemotherapeutic drugs on gastric cancer cell lines)* shows the correlation between hypo-acetylation of core histones, H3 and H4 with higher HDAC activity in gastric cancer. Further, this correlation is exploited to test the potential of HDAC inhibitors, VPA or TSA or SAHA in combinatorial chemotherapy with cisplatin, oxaliplatin and epirubicin. The summary and conclusion along with future prospects of this work is presented in chapter 8. The references are compiled towards the end as 'Bibliography' in chapter 9. Many of the supporting evidences for the chapter 5, 6 and 7 are compiled in the 'Appendix Section' towards the end. Published manuscripts are also added after appendix section.

Chapter 2

Review of Literature

2.1 Stomach

2.1.1 Anatomy and histology of stomach

Stomach undertakes chemical digestion and is located between the esophagus and the duodenum. It is a muscular, hollow, dilated part of the digestion system and divided into five sections, each of which has different cells and functions: Cardia, Fundus, Body, Antrum, and Pylorus. The first three parts of the stomach (cardia, fundus, and body) are called the proximal stomach, and the lower two parts (antrum and pylorus) are called the distal stomach. Further, stomach has two curves, which form its upper and lower borders are called as lesser curvature and greater curvature, respectively. The pylorus is connected to the duodenum (Figure 2.1).

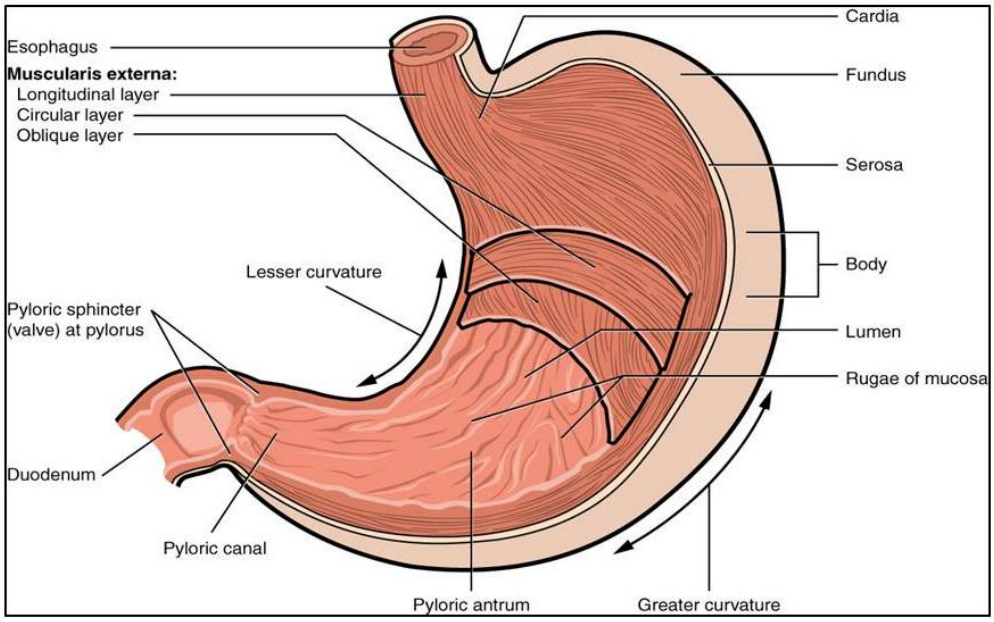

Figure 2.1: Anatomy of stomach. *Stomach is divided into five sections: Cardia, Fundus, Body, Antrum, and Pylorus. The first three parts of the stomach (cardia, fundus, and body) are called the proximal stomach, and the lower two parts (antrum and pylorus) are called the distal stomach. Source-Openstax.*

The cardia contains predominantly 'mucin- secreting cells'. The fundus contains 'mucoid cells, chief cells, and parietal cells'. The pylorus is composed of 'mucus-producing cells and endocrine cells. The stomach wall has five layers: (1) Mucosa- the innermost layer, where stomach acid and digestive enzymes are made, and where most

stomach cancers start, (2) Sub mucosa- consists of fibrous connective tissues, (3) Muscularis propria- a layer of muscle that moves and mixes the stomach contents, (4) Sub serosa- lies over the Muscularis propria, and (5) Serosa- outermost layer, act as wrapping layers for the stomach (Figure 2.2).

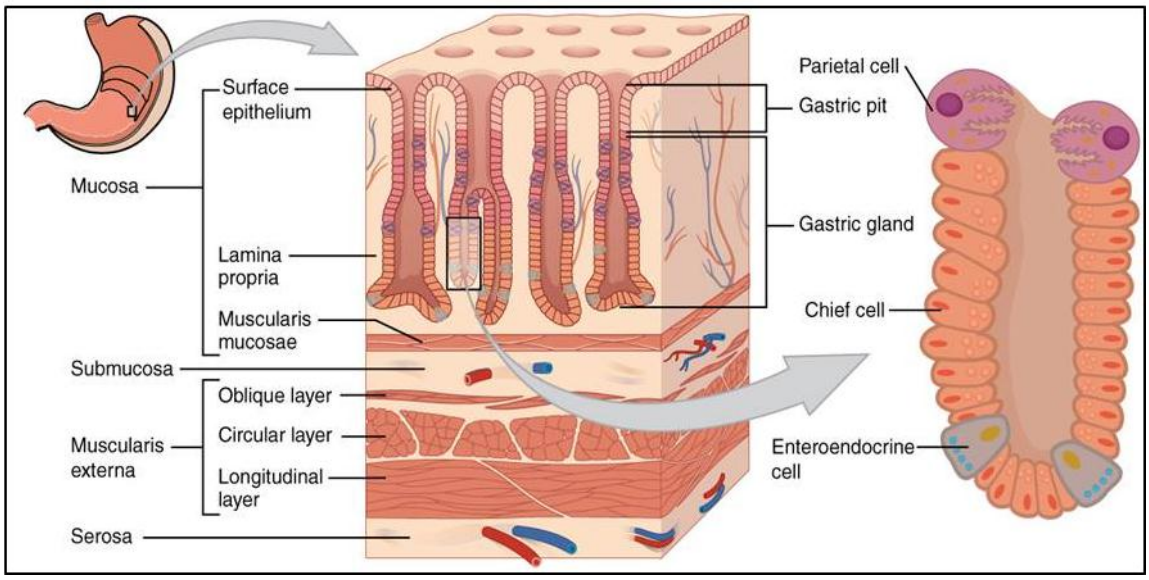

Figure 2.2: Histology of stomach*. The stomach wall is divided into five layers: Mucosa, Sub mucosa, Muscularis propriaach, Sub serosa and Serosa. Source-Openstax.*

2.1.2 Stomach/ Gastric cancer

Stomach cancers tend to develop slowly over many years. Before a true cancer develops, there are usually changes that take place in the lining of the stomach. These early changes rarely produce symptoms and therefore often are not noticed. Stomach cancers can spread in different ways. They can grow through the wall of the stomach and invade nearby organs. They can metastasize to the lymph vessels and nearby lymph nodes. At advanced stage, epithelial to mesenchymal transition of tumor cells takes place and through bloodstream primary gastric tumor spreads to other organs such as the liver, lungs, and bones and forms a secondary tumor[1].

Based on the cell type involved, gastric cancer is of following four different types.

(1) Adenocarcinomas: About 90% to 95% of stomach tumors are adenocarcinomas. This cancer develops from the cells that form the innermost lining of the stomach- the mucosa.

(2) Lymphoma: They account for about 4% of stomach tumors. These are cancers of the immune system tissue that are sometimes found in the wall of the stomach.

(3) Gastrointestinal stromal tumor: These are rare tumors that seem to start in cells in the wall of the stomach called interstitial cells of Cajal.

(4) Carcinoid tumor: These are tumor that start in hormone making cells of the stomach. Most of these tumors do not spread to other organs. About 3% of stomach cancers are carcinoid tumors.

2.2 Classification of Gastric Cancer

2.2.1 Histological classification

Several classification systems have been proposed to aid the description of gastric cancer on the basis of macroscopic or histological features, which include Borrman, Japanese system, World Health Organization (WHO) system and Laurén. However, Lauren's and WHO classification are most frequently used[1, 2].

2.2.1.1 Lauran's classification

The Laurén classification system is most commonly used and describes the tumors in relation to microscopic configuration and growth pattern. This classification system is useful in evaluating the natural history of gastric carcinoma, especially with regard to its association with environmental factors, incidence trends and its precursors. Lesions are classified into one of two major types: **intestinal** or **diffuse** (Figure 2.3 and Table 2.1).

Intestinal subtype tumors are often localized in the lower or distal part of the stomach, and are characterized by having well defined glandular formation, similar to the

microscopic appearance of colonic mucosa. The development of intestinal subtype gastric cancer follows a stepwise sequence of precursor lesions starting with superficial gastritis, continuing through chronic atrophic gastritis, intestinal metaplasia, dysplasia to, ultimately overt gastric cancer.

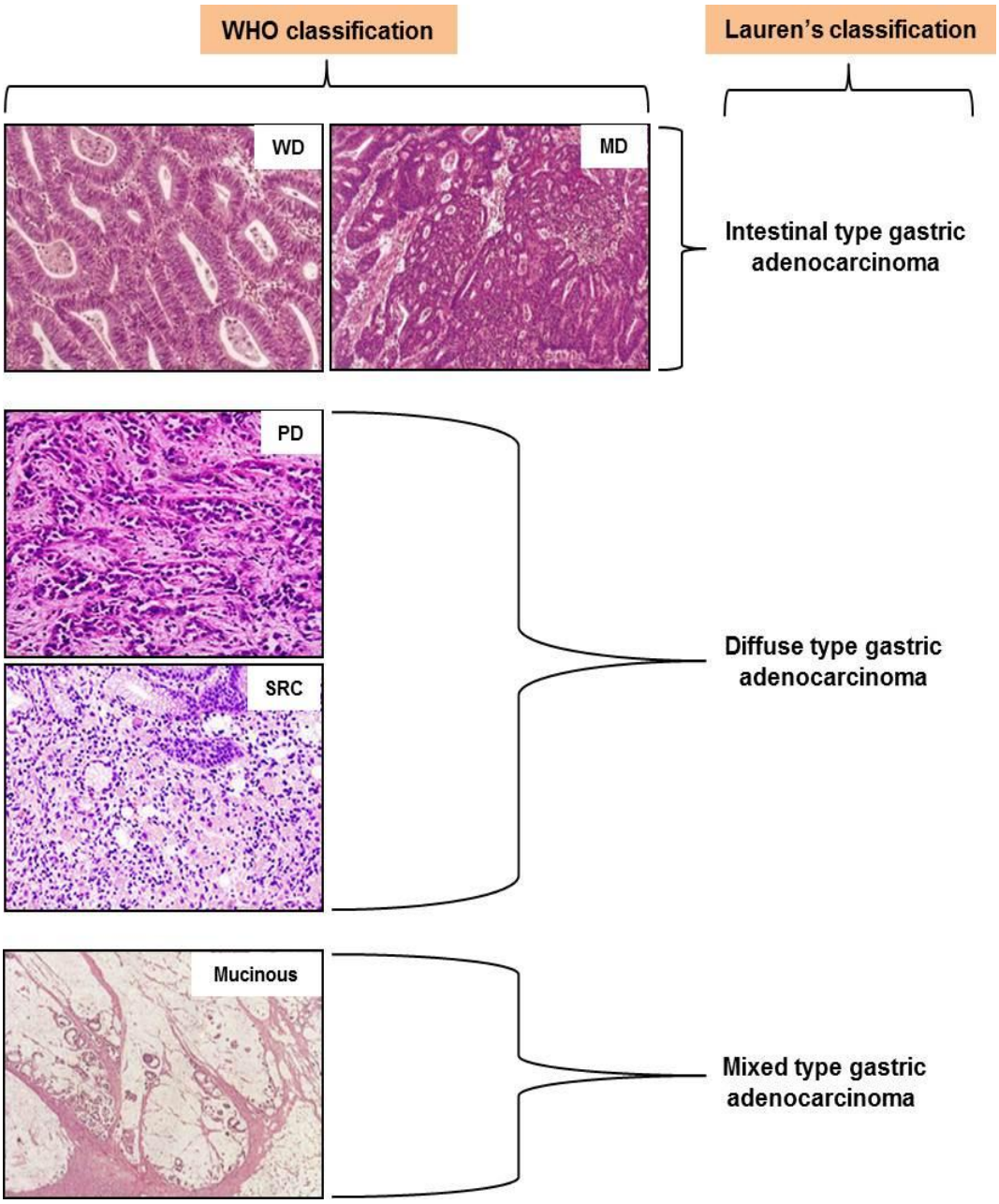

Figure 2.3: Histological classification of gastric cancer. *WHO classifies gastric tumor as per their grade of differentiation which are of 6 types- well differentiated adenocarcinoma (WD), moderately differentiated adenocarcinoma (MD), Poorly differentiated adenocarcinoma (PD), signet ring cell carcinoma (SRC) and mucinous adenocarcinoma (Mucinous). Laurens classification of gastric tumors is based on the resemblance of morphology of cells and can be broadly classified as intestinal type (WD and MD), diffuse type (PD and SRC) and mixed type (mucinous).*

The etiology of intestinal subtype gastric cancer is mainly associated to environmental factors, the tumor frequently develops late in life (after 50 years of age), and is twice more common in males than females[3] (Table 2.1)

Diffuse subtype gastric cancer more commonly develops in the corpus or upper part of the stomach which is characterized by the lack of gland formation and cellular adhesion, with single/small clusters of neoplastic cells diffusely infiltrating the stroma of the stomach wall. No recognizable pre-neoplastic lesions have been observed during the development of diffuse cancers. Diffuse subtype tumors are associated with genetic predisposition, presumably arise out of single-cell mutations in normal gastric glands. The diffuse subtype has a relatively constant or even slightly increase in incidence rates, more often occurs in young individuals, presents a similar prevalence in males and females, and is associated with a worse prognosis than the intestinal subtypea[3].

Table 2.1: Characteristic differences between intestinal and diffuse type gastric cancer[4]

Characteristics	**Intestinal type**	**Diffuse type**
Gross	Exophytic	Ulcerating, diffuse
Microscopy	Glandular	Single cells, signet-ring cells
Main co-existing	Atrophic gastritis, intestinal metaplasia	Non-atrophic gastritis
Precancer lesion	Adenoma, dysplasia; 'Correa sequence'	Foveolar hyperplasia?
Age	Old age	Young age, all age groups
Sex	Male > Female	Equal
Prevailing site	Antrum and angulus	Corpus, whole stomach
Metastasis	Lymph nodes, liver	Lymph nodes, visceral
Biology	Oestrogen protects?	Neuroendocrine differentiation?
Prior or co-existing *H. pylori*	• Common by serology (>80-90%) • False-negative results frequent with breath test, antigen stool test, biopsy-based urease test, or by microscopy	• Common (>90%) • All tests are reliable

2.2.1.2 WHO classification

The World Health Organization (WHO) classification issued in 2010 appears to be the most detailed among all pathohistological classification systems. According to WHO classification, gastric carcinoma is divided into five types (1) Well Differentiated, (2)

Moderately Differentiated, (3) Poorly Differentiated, (4) Mucinous, and (5) Signet ring cell carcinoma. In general, well and moderately differentiated cancer of WHO correspond to intestinal type according to Lauren, whereas poor differentiated or undifferentiated or signet ring cell- carcinoma to the diffuse type carcinoma respectively (Figure 2.3 and Table 2.1).

2.2.2 Anatomical classification

The anatomical location of tumors in the stomach has also been considered as an important parameter for the classification of gastric cancer. On the basis of anatomical location, two subtypes of gastric cancer can be distinguished: tumors from the distal regions of the stomach (**non-cardia cancer**) and those arising at the most proximal part of this organ (**cardia cancer**)[5] (Table 2.2).

Table 2.2: Characteristic differences between cardia and non-cardia gastric cancer[5]

Characteristics		Cardia	Non-cardia
Incidence		Increasing	Decreasing
Geographic location			
	Western countries	+	−
	East Asia	−	+
	Developing countries	−	+
Age		+ +	+ +
Male gender		+ +	+
Caucasian race		+	−
Low socio-economic status		−	+
H pylori infection		?	+
Diet			
	Preserved foods	+	+
	Fruits/vegetable	−	−
Obesity		+	?
Tobacco		+	+

NOTE: ++, strong positive association; +, positive association; -, negative association; ?, ambiguous studies.

These two anatomical subtypes of tumors present remarkable etiological differences. Non-cardia cancer is generally thought to develop as a result of the interaction between environment, host and *Helicobactor pylori*[5]. In contrast, two distinct etiological mechanisms have been proposed for cardia gastric cancer. One is associated

with atrophic gastritis and resembles the development of non-cardia malignancies. The second arises in similar fashion to esophageal carcinomas, as a result of frequent refluxing of acidic gastric juice into the distal esophageal mucosa, which leads to the transformation from squamous to columnar metaplastic epithelium to, ultimately, overt cancer. Epidemiological dissimilarities also exist between these two anatomical subtypes of gastric tumors. Non-cardia gastric cancer accounts for the majority of the cases worldwide and is the predominant type in high-risk areas. In contrast, cardia cancer is more homogeneously distributed all over the world and its incidence tends to increase[5].

2.3 Epidemiology of Gastric Cancer

2.3.1 Incidence

Gastric cancer is the fourth most frequent type of cancer worldwide, preceded by lung, breast and colorectal cancers (Figure 2.4)[6]. In India, there are limited epidemiological studies on gastric cancer which also suffers from the juvenile state of cancer registries and under-reporting of cases. However, similar to global trend, Indian registries have also observed statistically significant reducing trend in stomach cancer cases in last 20-years with approximately 35675 estimated case in 2001; about 3.91% of global incidence[7, 8] (Figure 2.4). The incidence rates of this disease present considerable variation according to age, gender, socio-economical conditions and geographical location. Thus, Gastric cancer incidence is known to increase with age with the peak incidence occurring at 60-80 years. Cases in patients younger than 30 years are very rare. The global as well as Indian incidence is twice as much in men as in women (Figure 2.4). The most substantial variations in the incidence rates of this malignancy are, however, observed in relation to geographical regions. In general, the incidence of gastric cancer is high in East Asia, Eastern Europe, and parts of Central and South America, while, low in Southern Asia,

North and East Africa, Western and Northern Europe, North America and Australia (Figure 2.5)[9].

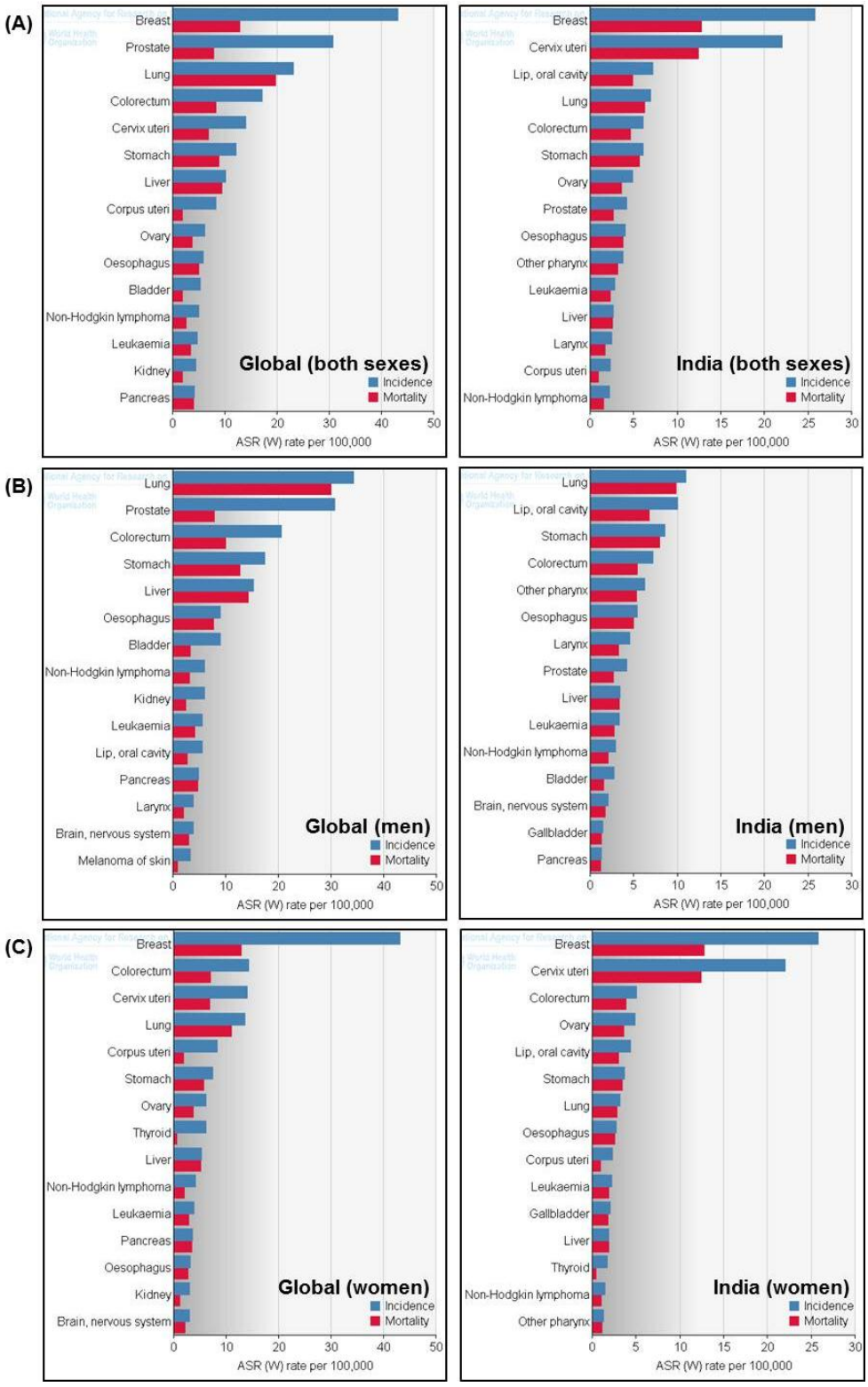

Figure 2.4: Incidence and mortality of top cancers in world and India. *(A) Incidence and mortality in both sexes. (B) Incidence and mortality in men. (C) Incidence and mortality in women. Source Globocon 2012*

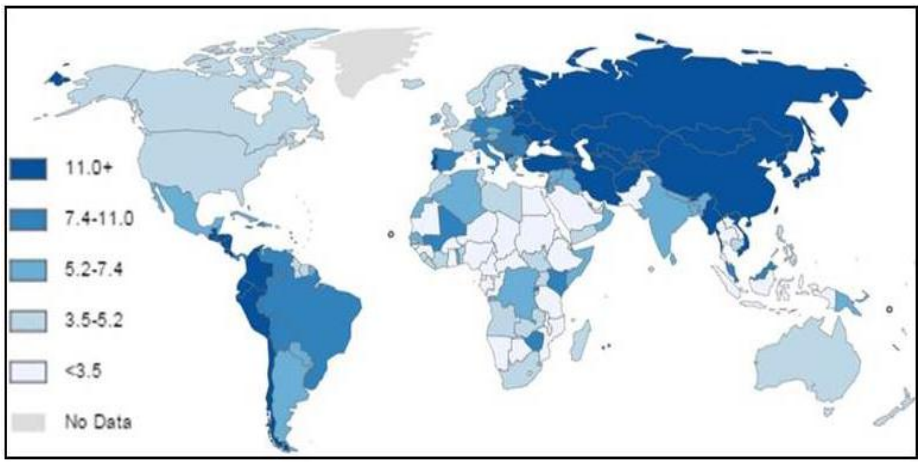

Figure 2.5: Global prevalence of gastric cancer*. Age standardized global prevalence of gastric cancer. Source, Globocon 2012.*

The incidence rates for gastric cancer have undergone a steady general decline during the past decades. Interestingly, the fall in the incidence is particularly associated to non-cardia gastric carcinoma, in contrast to cardia cancer that seems to experience a permanent slight increase. Similarly, epidemiological studies have shown that the general decrease in incidence is mainly attributed to the fall in intestinal subtype of gastric cancer, while the diffuse subtype shows a rather small change. The reasons underlying the generalized decline in the incidence of this malignancy are not well understood, however it has been hypothesized that this may be associated to improvements in the storage and preservation of foods, better nutrition and reduced transmission of *H. pylori* in childhood. Despite the notable fall in the incidence rates, the absolute number of cases of gastric cancer continues to increase globally as a result of the population growth and ageing.

2.3.2 Mortality and survival

Gastric cancer is the second most common cause of death from cancer worldwide after lung cancer, accounting for nearly 700000 deaths in 2013[10] (Figure 2.4). Wide geographical variation in mortality rates exists throughout the world, being particularly high in the developing world. Similar to the incidence, a constant decline in mortality rates in both sexes, and in low and high risk countries has occurred in the last decades[10].

Mortality rates are notably high because, in most cases, the disease is diagnosed at advanced stages when the treatment is likely to fail. In general, the five-year survival for patients of gastric cancer is below 30% in most countries, despite some variations according to the country/geographical region[11]. It is noteworthy, the relatively high 5-year survival rates of gastric cancer in Japan, which have reached more than 50% in the last decades. This is thought to be associated with the implementation of X-ray (photofluorography) based gastric cancer mass screening programs since early in 1960´s[12, 13].

2.4 Risk Factors and Prevention of Gastric Cancer

Risk factors for GC are tabulated in Table 2.3; however, some of the important risk factors strongly associated with gastric cancer are described in detail.

2.4.1 *Helicobacter pylori* infection

H. pylori is a gram-negative bacillus that colonizes the stomach and may be the most common chronic bacterial infection worldwide. In 1994, the International Agency for Research on Cancer classified *H. pylori* as a type I (definite) carcinogen in human beings as it increases the risk of gastric cancer by 2 to 16 fold compared to seronegative individuals. Gastric cancer risk is enhanced by infection with a more virulent strain of *H. pylori* carrying the cytotoxin-associated geneA (cagA). Countries with high gastric cancer rates typically have a high prevalence of *H. pylori* infection, and the decline in *H. pylori* prevalence in developed countries parallels the decreasing incidence of gastric cancer (Figure 2.6)[14].

Prevalence of *H. pylori* is closely linked to socio-economic factors, such as low income, poor education, and living conditions during childhood, such as poor sanitation and overcrowding. Public health measures to improve sanitation and housing conditions

and eradication therapy with antibiotics are the key factors in reducing the worldwide prevalence of *H. pylori* infection[15].

Figure 2.6: Global prevalence of H. pylori infection*. Age standardized rate of prevalence of H. pylori infection worldwide. Source- Globocon 2012.*

2.4.2 Dietary factors

Evidences suggest that consumption of salty foods and N-nitroso compounds, low intake of fresh fruits and vegetables increases the risk of gastric cancer. Several case-control studies have shown that a high intake of salt and salt-preserved food was associated with gastric cancer risk, but evidence from prospective studies is inconsistent. Similarly, case-control studies of polyphenol containing green tea have shown a reduced risk of gastric cancer in relation to green tea consumption; however, recent prospective cohort studies found no protective effect of green tea on gastric cancer risk. Prospective studies have reported significant reductions in gastric cancer risk arising from fruit and vegetable consumption. The worldwide decline in gastric cancer incidence may be attributable to the advent of refrigeration, which led to decreased consumption of preserved foods and increased intake of fresh fruits and vegetables. Therefore, dietary supplementation may

only play a preventive role in populations with high rates of gastric cancer and low intake of micronutrients[16].

2.4.3 Tobacco and Alcohol

Prospective studies have demonstrated a significant dose dependent relationship between smoking and gastric cancer risk. The effect of smoking was more pronounced for distal gastric cancer. There is little support for an association between alcohol and gastric cancer. Exposure to cigarette smoke, acidic conditions, and *H pylori* infection induce Cyclooxygenase-2 (COX-2) expression. Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) are thought to inhibit cancer cell growth primarily through the inhibition of COX-2, and evidence is mounting that COX-2 inhibitors may be beneficial in preventing upper gastrointestinal malignancies[5].

2.4.4 Obesity

Obesity is one of the main risk factors for gastric adenocarcinoma of cardia type. A recent prospective study has reported a significant positive association between body mass index and higher rates of stomach cancer mortality among men[5].

2.4.5 Occupation

A positive correlation has been recognized between increased stomach cancer risk and a number of occupations including mining, farming, refining, and fishing as well as in workers processing rubber, timber, and asbestos. Occupational exposure to dusty and high temperature environments such as in cooks, wood processing plant operators, food and related products machine operators was associated with a significant increased risk of gastric cancer of the diffuse subtype. A German uranium miner cohort study however found a positive statistically non-significant relationship between stomach cancer mortality and occupational exposure to arsenic dust, fine dust, and absorbed dose from α and low-linear energy transfer radiation[5].

Table 2.3: Risk factors for development of gastric cancer[17]

Precursor conditions		
1	Helicobacter pylori infection	
2	Gastric adenomatous polyps	
3	Chronic atrophic gastritis and intestinal metaplasia	
4	Pernicious anemia	
5	Partial gastrectomy for benign disease	
6	Dietary	
		Highly salted food
		Smoked foods, high fat or contaminated oil intake
		Low consumption of fruits and vegetables
7	Habits	
		Smoking
		Consumption of sake or contaminated whiskey
		Low socioeconomic status
9	Environmental	
		Acidic or peaty soil
		High nitrate content in water
		Elevated lead or zinc in water
		Volcanic rock background
		Exposure to environmental talc
		Extensive use of nitrate fertilizers
		Urban residency
10	Genetic	
		Family history of gastric cancer
		Blood type A
		Hereditary non-polyposis colon cancer syndrome
		Familial adenomatous polyposis syndrome
		Peutz–Jeghers syndrome
		Li–Fraumeni syndrome
		Hyperplastic gastric polyposis
		Familial diffuse gastric carcinoma
11	Occupational	
		Workers in mines and quarries
		Painters
		Fishermen
		Ceramic, clay, and stone workers
		Metal industry workers
		Agricultural workers
		Textile workers
		Printers and bookbinders

2.4.6 Genetic predisposition and sporadically occurring mutations

A diverse set of *de novo* genetic alterations are often found in gastric cancer (Table 2.3). Familial aggregation of gastric cancer is observed in approximately 10% of the cases, in

which two or more relatives from the same family are affected. In general, the risk for developing gastric neoplasia among relatives of gastric cancer patients is estimated to be 2 to 3-fold higher than in persons with no familial background of the disease. The germ-line mutations of the E-cadherin gene (CDH1) are the most recognized genetic aberrations found in hereditary gastric cancer, accounting for ~1-3% of the cases. Most of the gastric cancer cases attributed to CDH1 aberrations are of diffuse subtype, particularly signet-ring cell adenocarcinoma, and predominantly observed in young individuals[5].

2.4.7 Other risk factors

Less common risk factors for gastric cancer include radiation, pernicious anemia, blood type A, prior gastric surgery for benign conditions, and Epstein- Barr virus. In addition, a positive family history is a significant risk factor, particularly with genetic syndromes such as hereditary nonpolyposis colon cancer and Li- Fraumeni syndrome[5].

2.5 Pathogenesis of Gastric Cancer

Gastric cancer, like other cancers is the end result of the interplay of many risk factors as well as protective factors. Environmental and genetic factors are also likely to play a role in the etiology of the disease. Among the environmental factors, diet and infection with *H. pylori* are the most common suspects in gastric carcinogenesis[18].

Various epidemiological and pathological studies have suggested that gastric carcinogenesis develops with the following sequential steps: chronic gastritis→atrophy→ intestinal metaplasia→dysplasia (Figure 2.7). The initial stages have been linked to excessive salt intake and infection with *H. pylori*. The genetic factors play an important role in gastric carcinogenesis; leading to either abnormal gene over expression or inappropriate expression of normal genes, whose products confer the malignant phenotype. Advances have been made in the genetic changes mostly of the intestinal type; its development is probably a multi-step process. The most common genetic

abnormalities in gastric cancer tend to be loss of heterozygosity of tumor suppressor genes, particularly of p53 or 'Adenomatous Polyposis Coli (APC)' gene. The latter leads to gastric oncogenesis through changes related to E-cadherin-catenin complex, which plays a critical role in the maintenance of normal tissue architecture[18].

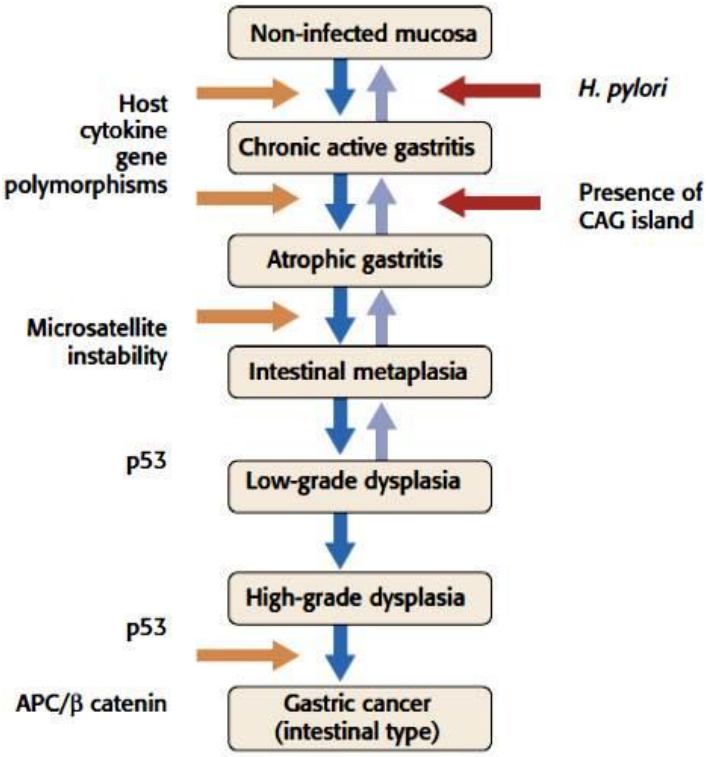

Figure 2.7: Proposed multistep pathway in the pathogenesis of gastric cancer. *Infection with Helicobacter pylori is the common initiating event in most cases, and the presence of the cag pathogenicity island is associated with more severe disease. Host genetic polymorphisms, resulting in high production of interleukin-1β and tumor necrosis factor-α and low production of interleukin-10, contribute to gastric cancer risk. Accumulation of genetic defects within gastric lesions may play a role in later steps. Gray arrows represent steps that are potentially reversible*[17].

2.6 Diagnosis of Gastric Cancer

The initial diagnosis of gastric carcinoma often is delayed because up to 80 percent of patients are asymptomatic during the early stages of stomach cancer. Weight loss, abdominal pain, nausea and vomiting, early satiety, and peptic ulcer symptoms may accompany late-stage gastric cancer. Patients presenting with the aforementioned symptoms and those with multiple risk factors for gastric carcinoma require further workup. Esophagogastroduodenoscopy (EGD) and double- contrast barium swallow is

the diagnostic imaging procedure which provides preliminary information of presence or absence and benign or malignant feature of lesion. Further confirmation is done by multiple biopsy specimens obtained from any visually suspicious areas along with computed tomographic (CT) and endoscopic ultrasonography (EUS) scanning[19-21].

2.7 Treatment of Gastric Cancer

2.7.1 Surgery

The only potentially curative treatment for localized gastric cancer is complete surgical resection. The selection of the surgical procedure in patients with gastric cancer primarily is based on the location of the tumor (proximal, middle or distal), the growth pattern seen on biopsy specimens (depth of tumor invasion, T1, T2 or T3), and the expected location of lymph node metastases; D1- perigastric lymph nodes, D2- nodes along the hepatic, left gastric, celiac, and splenic arteries or D3- removal of all D1/D2 nodes plus those within the porta hepatis and periaortic nodes[22] (Figure 2.8 and Table 2.4)

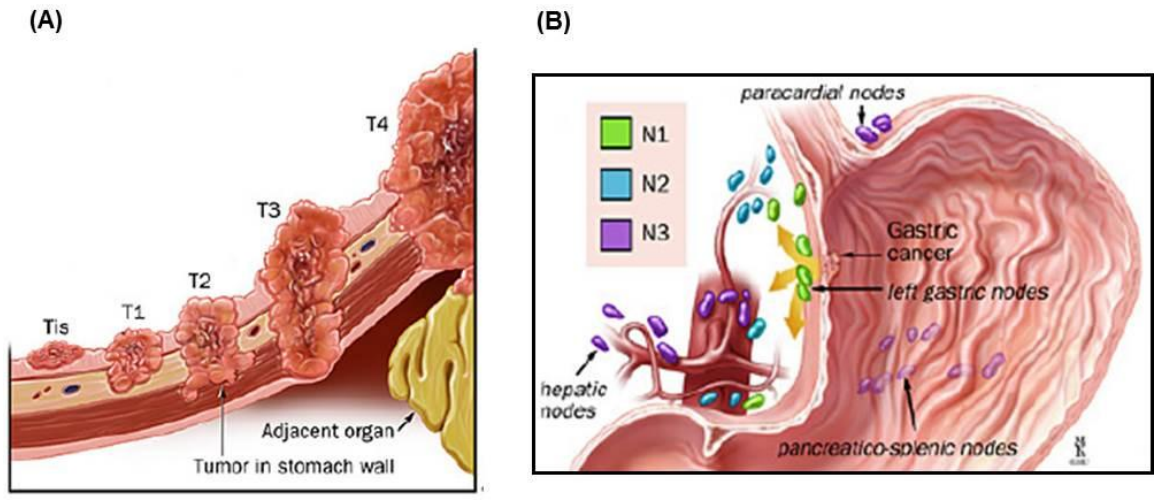

***Figure 2.8: TNM staging of gastric cancer**. **(A)** T-stages or depth of invasion. **(B)** N-stages or involvement of lymph nodes. Source-Gastroenterology and hepatology, Jhon Hokins Medicine*

The extensive lymphatic network of the stomach and the propensity for microscopic extension, the traditional surgical approach attempts to maintain a 4 to 5-cm margin proximally and distally to the primary lesion. Many studies report that nodal

involvement indicates a poor prognosis, requiring the use of more aggressive surgical approaches to attempt the removal of involved lymph nodes[23].

Table 2.4: Staging of gastric cancer as per American Joint Committee on Cancer Staging for Gastric Cancer	
Tumor (T) stage	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1s	Carcinoma in situ: intra-epithelial tumor without invasion of the lamina propia
T1	Tumor invades lamina propria or submucosa
T2	Tumor invades muscularis propria or subserosa
T2a	Tumor invades muscularis propria
T2b	Tumor invades subserosa
T3	Tumor penetrates serosa (visceral peritoneum) without invasion of adjacent structures
T4	Tumor invades adjacent structures
Nodal (N) Stage	
NX	Regional lymph node(s) cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in 1–6 regional lymph nodes
N2	Metastasis in 7–15 regional lymph nodes
N3	Metastasis in more than 15 regional lymph nodes
Metastasis (M) Stage	
MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis
Stage grouping	
Stage 0	T1s N0 M0
Stage IA	T1 N0 M0
Stage IB	T1 N1 M0; T2a/b N0 M0
Stage II	T1 N2 M0; T2a/b N1 M0; T3 N0 M0
Stage IIIA	T2a/b N2 M0; T3 N1 M0; T4 N0 M0
Stage IIIB	T3 N2 M0
Stage IV	T1-3 N3 M0; T4 N1-3 M0; Any T Any N M1

2.7.4 Chemotherapy

Several trials have shown a significant survival advantage by the use of chemotherapy; however, none of them have reported chemotherapy as a definitive treatment for gastric cancer. Chemotherapy can be given before surgery (*neoadjuvant* treatment) or after surgery (*adjuvant* treatment). Neoadjuvant treatment shrinks the size of tumor and facilitates the curative resection; whereas adjuvant treatment is given to kill any residual cancer cell that may have left behind after surgery. 5-FU, Cecitabine, Carboplatin,

Cisplatin, Docetaxel, Epirubicin, Irinotecan, Oxaliplatin and Paclitaxel are the most common drugs used as a single agent or in combination while treating the gastric cancer. Usually three cycles of chemotherapy is given before and after surgery, each cycle last for three weeks. Some of the most common combinations are: ECF (epirubicin, cisplatin and fluorouracil), EOF (epirubicin, oxaliplatin and fluorouracil), ECX (epirubicin, cisplatin and capecitabine) and EOX (epirubicin, oxaliplatin, and capecitabine).

2.7.3 Radiotherapy

A modest survival advantage has been shown to radiotherapy in patients with gastric cancer. The dosing regimen of radiation therapy is 45 to 50 Gy in 20 to 30 fractions. External beam radiation therapy is often used to treat stomach cancer. Often, special types of external beam radiation, such three-dimensional conformal radiation therapy (3D-CRT) and intensity modulated radiation therapy (IMRT) are also used. The adverse effects caused by radiation therapy include gastrointestinal toxicity from dose-limiting structures surrounding the stomach, like intestines, liver, kidneys, spinal cord, and heart.

2.7.4 Combination therapy

Studies have shown that patients receiving combined chemo-radiation therapy have demonstrated improved disease free survival and improved overall survival rates. Preoperative chemotherapy also may be useful in patients with locally advanced gastric cancer, offering a chance for surgery with curative intention in patients with an otherwise fatal long-term prognosis[24].

2.8 Epigenetics

2.8.1 Definition and mechanism of epigenetics

The field of genetics includes the study of point mutation, deletion, insertion, gene amplification, chromosomal deletion/inversion/translocation, and allelic loss/gain.

However, the appreciation of epigenetics is more recent which was originally defined by C. H. Waddington as 'the causal interactions between genes and their products, which bring the phenotype into being'[25]. Epigenetics in today's modern terms can be mechanistically defined as "*the sum of the alterations to the chromatin template that collectively establish and propagate different patterns of gene expression (transcription) and silencing from the same genome*".

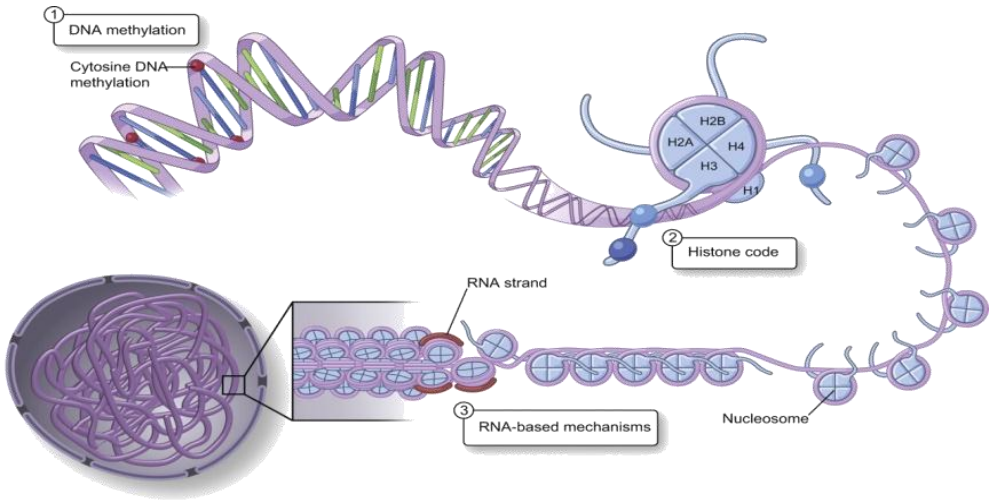

Figure 2.9: Schematic representation of fundamental mechanisms of epigenetic gene regulation[26].

Epigenetic mechanisms include DNA methylation, noncoding RNA, histone variants and histone post translational modifications[27-29] (Figure 2.9). These mechanisms work together to regulate the functioning of the genome by altering the local structural dynamics of chromatin, mostly regulating its accessibility and compactness. The interplay of these mechanisms makes an 'epigenetic landscape' that regulates the way the mammalian genome manifests itself in different cell types, developmental stages and disease states[30-33]. Failure of the proper maintenance of heritable epigenetic marks can result in inappropriate activation or inhibition of various signaling pathways and lead to disease states such as cancer[34, 35]. Epigenetic mechanisms also cooperate with genetic alteration and work together at all stages of cancer development from initiation to progression[36]. Unlike genetic alterations, epigenetic changes are reversible in nature and

can be restored to their normal state by epigenetic therapy. These findings have led to a global initiative to understand the role of epigenetics in tumorigenesis and further explore its utility in disease diagnosis, prognosis and therapy.

2.8.2 Chromatin

Chromatin is the macromolecular complex of DNA, histone proteins and non-histone proteins, which provides the scaffold for the packaging of genome. Human nuclear DNA is condensed into nucleosomes, which consist of 146 base pairs of DNA wrapped twice around an octamer core of histones (two molecules each of histones H2A, H2B, H3 and H4) (Figure 2.9 and 2.10). The core histones are predominantly globular except for their N-terminal "tails," which are unstructured[37]. In between core nucleosomes, the linker histone H1 attaches and facilitates further compaction. Each nucleosome core particle represents the basic repeating unit in chromatin and exists in the form of arrays that forms basis for higher-order chromatin structure. Nucleosomes are connected by a linker DNA of variable length (10-80 base pairs) that forms a 10nm beads on a string array. The positioning of histones along the DNA is mediated by ATP-dependent nucleosome remodeling complexes generating nucleosome free or dense chromatin. Apart from H4, all histones are known to have multiple subtypes called "variants, which undergo various covalent post-translational modifications (PTM)[29]. Combination of variants and PTMs of histones modulate the affinity of histones for DNA and DNA-associated proteins, thereby, governing the transcriptional activity and the availability of DNA for recombination, replication and repair.

2.8.3 Histone post-translational modifications

Vincent Allfrey's pioneering studies suggested histones can undergo variety of covalent post-translational modifications (PTM)[38]. Today, modifications of histones are central in the regulation of chromatin dynamics and are the target for variety of covalent

modifications at specific amino-acid residue. Reported histone modifications include acetylation, methylation, phosphorylation, ubiquitylation, glycosylation, ADP-ribosylation, carbonylation and SUMOylation[32] (Figure 2.10).

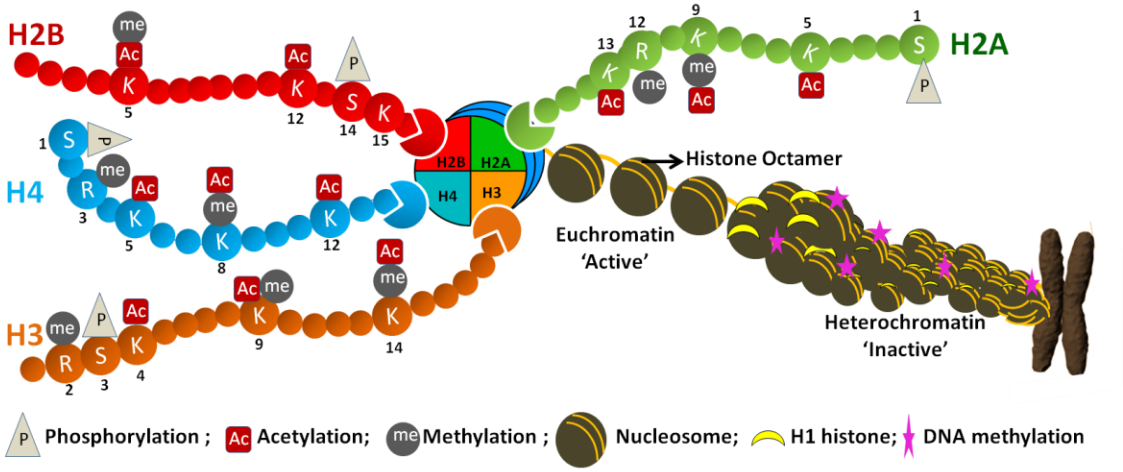

Figure 2.10: Chromatin architecture and histone modifications. *The DNA is wrapped in two turns around histone octamers (nucleosomes) at intervals of about 200 bp along the DNA. Histones within the nucleosome (two each of H2A, H2B, H3 and H4) undergo numerous post-translational modifications at their N-terminal tail which protrudes from the nucleosome. Further folding of nucleosome with linker histone H1 creates a spiral structure, the heterochromatin leading to metaphase chromosome. These modifications directly regulate the chromatin structure and thus DNA-mediated cellular processes. The diagram indicates known modifications at specific residues: M = methylation, A = acetylation, P = phosphorylation. Note- amino acid numbers depicted in the figure is only for example, they do not reflect the exact amino acid sequence of histone proteins.*[39]

These modifications occur within the histone amino- terminal tails protruding from the surface of the nucleosome as well as on the globular core region. Many studies have shown that the site- specific combinations of histone modifications correlate well with particular biological functions, such as transcription, chromatin remodeling, DNA repair and replication. Histone modifications are proposed to affect chromosome function through at least two distinct mechanisms: (1) Modifications may alter the electrostatic charge of the histone resulting in a structural change in histones or their binding to DNA. (2) These modifications act as the binding sites for protein recognition modules, such as the bromodomains or chromodomains, which recognize acetylated lysines or methylated lysines, respectively[32, 40, 41]. Histone 'modifications' or 'marks' are 'written' by specific

histone modifying enzymes known as 'writers', recognized by specific proteins referred as 'readers' and removed by enzymes referred as 'erasers' (Figure 2.11 and Table 2.5).

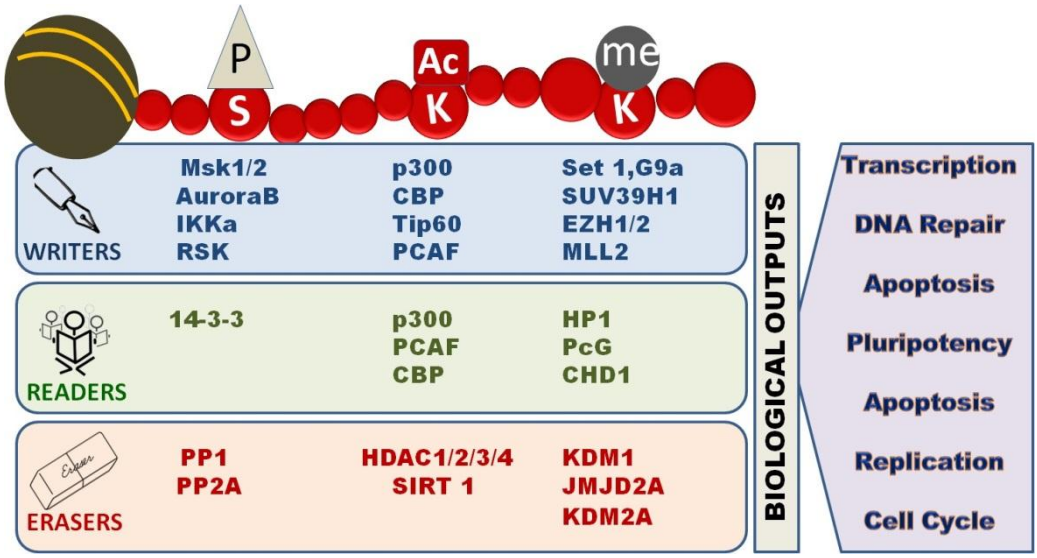

Figure 2.11: Readers, writers and erasers of chromatin marks. *Histone modifications are highly dynamic in nature. The 'writers' like histone acetyltransferases (HATs), histone methyltransferases (HMTs), protein arginine methyltransferases (PRMTs) and kinases add specific marks on specific amino acid residues on histone tails. These marks are identified by various proteins containing specific domains such as bromodomains, chromodomains and Tudor domain containing proteins called 'readers'. The written marks are removed by 'erasers' like histone deacetylases (HDACs), lysine demethylases (KDMs) and phosphatases. Addition, removal and identification of these post-translational modifications on histone tails regulate various biological processes, including transcription, DNA replication and DNA repair.*

The site-specific modification on different histones depends on the signaling and physiological condition within the cell. These multiple independent modifications enable combinatorial complexity; resulting in a large variety of functionally distinct nucleosomes. Many of the modifications can interact together or affect others, collectively constituting the '**histone code'**[42], which states that:

- Distinct modifications on core and tail regions of histone proteins generate docking sites for a large number of non-histone chromatin-associated proteins,
- Modifications on the same or different histone tails may be inter-dependent and generate various combinations and '*cross-talk*' within themselves to perform different function,

- Distinct regions of higher order chromatin, such as euchromatic or heterochromatic domains, are largely depend on the local concentration and combination of differentially modified nucleosomes,
- '*Binary switches* 'represent the differential readout of distinct combinations of marks on two neighboring residues, where one modification influence the binding of an effector protein onto another modifications on an adjacent or nearby residue
- '*Modification cassettes*' signifies combinations of modifications on adjacent sites within these short clusters lead to distinct biological readouts

2.8.3.1 Histone acetylation

Allfrey et al. first reported histone acetylation in 1964. This modification is almost invariably associated with activation of transcription. Acetylation of lysine is highly dynamic and regulated by the opposing action of two families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs)[43]. The HATs utilize acetyl Co A as cofactor and catalyse the transfer of an acetyl group to the ε- amino group of lysine side chains. In doing so, they neutralize the lysine's positive charge and this action has the potential to weaken the interactions between histones and DNA. HDAC enzymes oppose the effects of HATs and reverse lysine acetylation, an action that restores the positive charge of the lysine. This potentially stabilizes the local chromatin architecture and is consistent with HDACs being predominantly transcriptional repressors.

2.8.3.2 Histone methylation

Histone methylation mainly occurs on the side chains of lysines and arginines. Unlike acetylation and phosphorylation, histone methylation does not alter the charge of the histone protein. Histone methylation on lysines may be mono- , di- or tri- methylated, whereas arginines may be mono or di- methylated with either both methyl groups on one

terminal nitrogen (asymmetric di- methylated arginine) or one on both nitrogens (symmetric di- methylated arginine). Histone Methyltransferases (HMTs), which promote or inhibit transcription depending on the target histone residue and Histone Demethylases (HDMs), which counteract the HMTs[43].

Table 2.5: Writers, Erasers and functions of histone post-translational modifications[44]

Modifications	Nomenclature	Writers	Chromatin reader motif	Eraser	Attributed function
Acetylation	K-ac	HAT	Bromodomain	HDAC	Transcription, repair, replication and condensation
Acetylation	K-ac	HAT	Bromodomain	HDAC	Transcription, repair, replication and condensation
Methylation (K)	K-me1, K-me2, K-me3	HMT	Chromo, MBT and PHD domains	LSD1	Transcription and repair
Methylation (R)	R-me1, R-me2s, R-me2a	PRMT 1, 4, 5 and 6	Tudor domain	JMJD6	Transcription
Phosphorylation (S and T)	S-ph, T-ph	Kinase	14-3-3, BRCT	Phosphatase	Transcription, repair and condensation
Phosphorylation (Y)	Y-ph	Kinase	SH2	Phosphatase	Transcription and repair
Ubiquitylation	K-ub	E1, E2 and E3 enzymes	UIM, IUIM	Isopeptidases	Transcription and repair
Sumoylation	K-su	E1, E2 and E3 enzymes	SIM	-----	Transcription and repair
ADP ribosylation	E-ar	PARP1	Macro domain, PBZ domain	poly-ADP-ribose-glycohydrolase	Transcription and repair
O-GlcNAcylation (S and T)	S-GlcNAc, and T-GlcNAc	O-GlcNAc transferase	Unknown	β-N-acetyl glucosaminidase	Transcription

Lysine methylation: They usually modify one single lysine on a single histone and their output can be either activation or repression of transcription. Three methylation sites on histones are implicated in activation of transcription: H3K4, H3K36, and H3K79.

Three lysine methylation sites are connected to transcriptional repression: H3K9, H3K27, and H4K20[45].

Arginine methylation*:* Like lysine methylation, arginine methylation can be either activate or repress the transcription. There are two classes of arginine methyltransferases, the typeI and typeII enzymes. The two types of arginine methyltransferases form a relatively large protein family (11 members), the members of which are referred to as Protein Arginine Methyltransferases (PRMTs). All of these enzymes transfer a methyl group from SAM (S- adenosyl methionine) to the ω- guanidino group of arginine within a variety of substrates[45].

2.8.3.3 Histone phosphorylation

The phosphorylation of histones is highly dynamic. It takes place on serine, threonine and tyrosine, predominantly, but not exclusively, in the N- terminal histone tails. The levels of the modification are controlled by kinases and phosphatases that add and remove the modification, respectively. Histone kinases transfer a phosphate group from ATP to the hydroxyl group of the target amino- acid side chain. In doing so, the modification adds significant negative charge to the histone that undoubtedly influences the chromatin structure[46].

2.8.4 Cross-talk of Histone Post-translational Modifications

It is now well established that there is an intense cross-talk between histone modifications to drive distinct downstream functions. Cross regulation can occur in different flavors: on the one hand, one modification can promote/block the addition of another modification. On the other hand, one modification can stimulate/block the removal of another modification. Moreover, the cross-talk can occur on the same histone (cross-talk in cis), between histones within the same nucleosome (cross-talk in trans) or across nucleosomes (nucleosome cross-talk). An increasing number of histone modifying complexes are

found to contain more than one distinct enzymatic activities. These enzymes can act in concert to determine the functional status of chromatin by coordinating multiple histone modifications[47, 48].

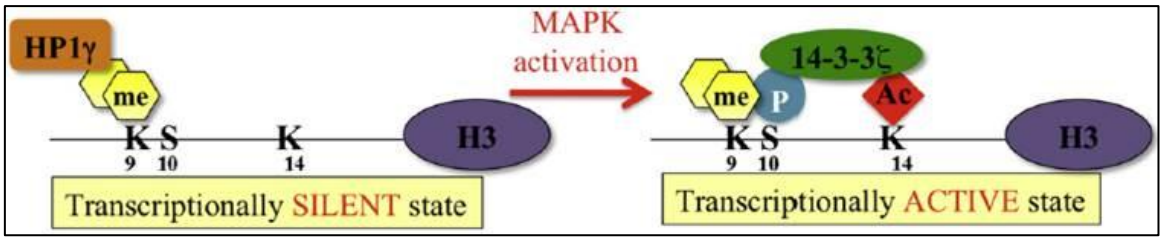

***Figure 2.12: Histone modification cross-talk**. Crosstalk among H3S10ph, H3K0ac and H3K14ac at promoters of immediate early gene[49].*

One of the first examples for cross-regulation of histone modifications in cis is between H3K9 methylation and the neighboring H3S10 phosphorylation[50] (Figure 2.12). H3S10 phosphorylation is required for chromosome condensation and segregation during mitosis[51]. H3K9me3 can be specifically bound by the chromodomain of heterochromatin protein 1 (HP1) and has a pivotal role in heterochromatin formation and propagation of pericentric heterochromatin[52]. However, in mitosis, HP1 is released from condensed chromatin despite the persistence of its recruiting mark H3K9me3[53, 54]. To explain this methyl-phospho switch model has been proposed. This methyl-phospho switch model is not limited to directly neighboring residues. For, example, H3T6 phosphorylation by PKCbI kinase can block H3K4 demethylation by the demethylases LSD1 (specific for H3K4me1/me2) and JARID1B (specific for H3K4me2/me3) and it redirects their enzymatic activity towards H3K9 methylation[55].

2.9 Histone Post-translational Modifications in Cancer

2.9.1 Dynamics of histone PTMs in cancer

In cancer, several histone PTMs have been reported to be misregulated and called as histone onco-modifications (Figure 2.13); however, their involvement in cancer patho-physiological characteristics like cellular transformation, angiogenesis and metastasis etc.

is not well understood. Moreover, there are very few studies commenting on the cancer specific regulatory mechanism behind the alterations of histone PTMs. It has been a decade when global loss of H4K16ac and H4K20me3 were reported for their association with cancer and considered as a common hallmark of tumor cells[56]. However, still there are no reports of their direct involvement in cellular transformation or any other cancer characteristics. Despite of the awareness of hMOF and HDAC4, as writer and eraser of H4K16ac, only recently low expression of hMOF has been implicated for its loss in gastric cancer[57]]. Further, Lin *et al* showed that histone lysine demethylase, KDM1A mediated loss of H3K4me2 is associated with epithelial to mesenchymal transition (EMT) in human breast cancer cells[58]. Also, loss of H3ac, H3K9me3 and H3S10ph has been observed at the promoters of Sfrp2, Sfrp5 and Wnt5a during genistein induced development of colon cancer in rat model system[59]. Alterations in H3K9 and H3K27 methylation patterns are associated with aberrant gene silencing in various forms of cancer[60, 61]. A very important association has been made in terms of phosphorylation of H3S10, as the only histone marks directly associated with cellular transformation[62, 63]. Further, Mitogen- and stress-activated kinase 1 (MSK1) has been shown to phosphorylate H3S10 in TPA and EGF mediated cellular transformation[64].

2.9.2 Histone PTM in cancer diagnosis

Decades of research have discovered a battery of markers for cancer diagnosis; however, only few could reach to clinics because of issues of sensitivity and specificity (Appendix, Table A2.5). The discovery of the presence of DNA in fecal and urine samples[65] and circulating nucleosomes in serum[66, 67] has led to the foundation of identifying epigenetic markers such as DNA methylation and histone post-translational modification for cancer diagnosis. Presence of histone proteins is not known in fecal and urine samples; therefore,

histone posttranslational modifications have been utilized as cancer diagnostic markers using circulating nucleosomes (cNUCs) in serum samples.

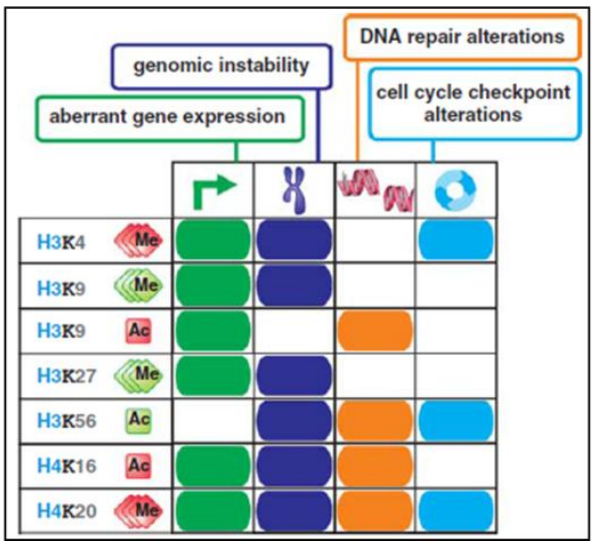

Figure 2.13: Histone onco-modifications. *Functional consequences of histone onco-modifications. Red-decrease, Greeen-increase*[68]

Two histone methylation marks, H3K9me3 and H4K20me3, the hallmarks of pericentric heterochromatin[69], were investigated in circulating nucleosomes by subsequent studies. *Ugur et al.* investigated the correlation between the H3K9me3 and H4K20me3 of cNUCs in healthy subjects and patients with colorectal cancer and multiple myeloma and found low level of these PTMs in cancer[70]. Further, the same group showed ALU115 DNA sequence associated high level of H3K9Me in multiple myeloma patients compared to healthy individuals[71]. ChIP based analysis of circulating nucleosomes in serum samples by *Gloria et al* reported a low level of H3K9me3 and H4K20me3 in patients with colorectal, pancreatic, breast and lung cancer compared to healthy control[72, 73]. Moreover, H3K9me3 and H4K20me3 have been found to be lower at the pericentromeric satellite II repeat in patients with CRC when compared with healthy controls or patients with multiple myeloma. In summary, identification of histone PTMs from serum isolated circulating nucleosomes have open the doors of immense

possibility that blood samples collected by cancer patients can also be used for histone PTM based cancer diagnosis.

2.9.3 Histone PTM in cancer prognosis

In cancer, to date, histones PTMs have been mostly studied for their potential as prognostic marker (Figure 2.14 and Appendix, Table A2.5). The first report in this area strongly suggested the utility of histone PTMs in cancer diagnosis and showed loss of H4K16ac and H4K20me3 in several cancers and establish these two marks as a hallmark of tumor and establishes the correlation of H4K16ac with tumor progression[56]. Further, loss of H4K20me3 is as well observed in animal models of carcinogenesis[74, 75]. A study on prostate cancer showed a positive correlation of H3K18Ac, H4K12Ac and H4R3Me2 with increasing tumor grade[76]. Moreover, independently of other clinical and pathologic parameters, high rate of tumor recurrence in low-grade prostate carcinoma patients is associated with low level of H3K4me2[76]. A decrease of H3K4me2/me3 is observed in a range of neoplastic tissues such as non-small cell lung cancer, breast cancer, renal cell carcinoma and pancreatic adenocarcinoma serving as a predictor of clinical outcomes[77-82].

Acetylation of histone H3K9 has shown ambiguous results with the increase in some and decrease in other cancers. Decrease of H3K9ac in prostate and ovarian tumors has been linked with tumor progression, histological grading and clinical stage. In agreement, a decrease in H3K9ac is coupled with a poor prognosis for these patients[76, 83, 84]. Patients with non-small cell lung adenocarcinoma exhibited better prognosis on the reduction of H3K9ac expression level[79, 85]. In contrast, in hepatocellular carcinoma an increase in H3K9ac levels was reported[83]. Methylation of the same residue K9 of histone H3 requires loss of H3K9ac and is also linked to number of cancers. An increase in H3K9 methylation, leading to aberrant gene silencing, has been found in various forms of cancer

and high level of H3K9me3 were associated with poor prognosis in patients with gastric adenocarcinoma[60, 86]. However, in patients with acute myeloid leukemia decrease in H3K9me3 found to be associated with better prognosis[87]. Loss of H3K18ac is correlated with poor prognosis in patients with prostate, pancreatic, lung, breast and kidney cancers, and tumor grade suggesting loss of this modification is an important event in tumor progression[76, 78, 81]. Consistent with this observation, the Kurdistani laboratory demonstrated that oncogenic transformation by the adenovirus protein E1a is accompanied by dramatic changes in the genomic location of H3K18 acetylation[88, 89]. In addition, H3K18 hypoacetylation even strongly correlated with an increased risk of tumor recurrence in patients with low-grade prostate cancer[76]. However, in contrast to the report that found that lower levels of H3K18ac predicts poor survival, low expression of this histone mark has been associated with a better prognosis for patients with esophageal squamous cell carcinoma or glioblastoma[85, 90]. This indicates, once again, that one histone modification can predict differential prognosis in different cancer types and that histone marks may possess tissue-specific features. Another histone mark, H3K27me3 has been evaluated as a prognostic factor in prostate, breast, ovarian, pancreatic and esophageal cancers, however, some of the results are perplexing and need further investigation. In esophageal cancer high level of H3K27me3 correlates with poor prognosis, whereas, in case of breast, prostate, ovarian and pancreatic cancers low level of H3K27me3 had significantly shorter overall survival time when compared with those with high H3K27me3 expression[90-93].

Using the ChIP-on-chip technique, *Zhang et al* identified candidate genes with significant differences in H3K27me3 in gastric cancer samples compared to adjacent non-neoplastic gastric tissues[94]. These genes included oncogenes, tumor suppressor genes, cell cycle regulators, and genes involved in cell adhesion. Moreover, this investigation

demonstrated that higher levels of H3K27me3 produce gene expression changes in MMP15, UNC5B, and SHH. In non-small cell lung cancer, enhanced H3K27me3 was correlated with longer overall survival (OS) and better prognosis. Moreover, both univariate and multivariate analyses indicated that H3K27me3 level was a significant and independent predictor of better survival. Recently, a study showed K27M mutations of histone H3.3 variants in 31% pediatric glioblastoma tumors suggesting another level of complexity in alteration of histone PTMs in cancer which is independent of histone modifying enzymes[95]. Mass spectrometry based analysis showed high level of H3K27ac in colorectal cancer than the corresponding normal mucosa[96]. Immunohistochemical analysis on metachronous liver metastasis of colorectal carcinomas by *Tamadawa et al* has correlated H3K4me2 and H3K9ac with the tumor histological type. In addition, lower levels of H3K4me2 correlated with a poor survival rate and also found to be an independent prognostic factor[97].

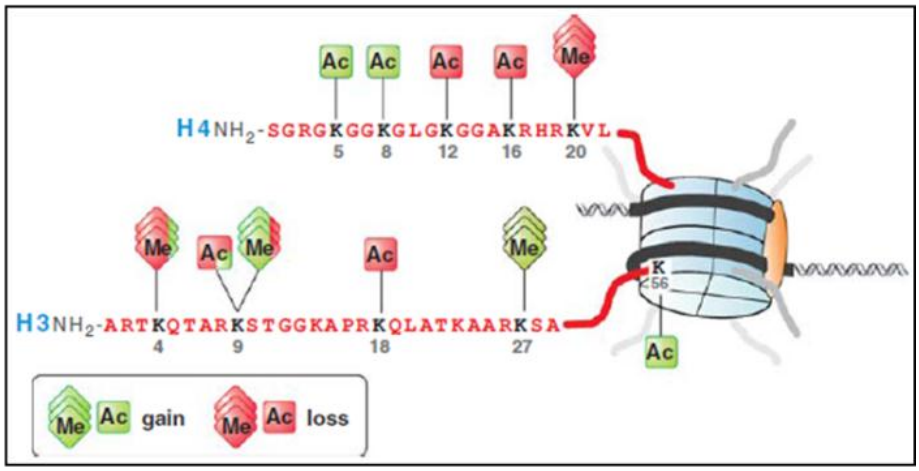

Figure 2.14: Deregulation of histone PTMs in cancer. *Histone onco-modifications; post-translation modifications on histone tails that occur in cancer cells are represented. Red-decrease, Greeen-increase*[68]

Recently, DNA damage mark YH2AX also have shown its prognostic value. In triple negative breast tumors, high level of YH2AX was associated with poor overall survival and which was further found to be associated with shorter telomere length[98, 99].

In colorectal cancer a high YH2AX expression in CRC tissues was associated with tumor stage and peri-neural invasion. Furthermore, a high YH2AX expression was associated with poor DMFS and OS. Cox regression analysis also revealed that YH2AX was an independent predictor of DMFS and OS. A high YH2AX expression in CRC tissues is associated with a more malignant cancer behavior, as well as poor patient survival[100]. ELISA based analysis in glioblastoma multiformes tumors showed the high level of H3T6ph, H3S10p and H3Y41ph as signatures associated with a poor overall survival[101]. Increase in H3S10ph has been associated with poor prognosis in several cancers including glioblastoma multiformes, cutaneous nodular melanoma, cutaneous melanoma, breast cancer, esophageal squamous cell carcinoma, gastric cancer, melanoma and nasopharyngeal carcinoma[101-109].

2.9.4 Histone PTM in cancer treatment

Reversible nature of histone modifications has drawn major attention of scientific community to study the molecular mechanism regulating the alteration in histone post-translational modifications. Such efforts have led to the discovery of several histone modifying enzymes[110] and their chemical inhibitors[111] which has emerged as an attractive strategy in cancer treatment. Targeting these enzymes can reactivate epigenetically silenced tumor-suppressor genes by modulating the levels of histone posttranslational modifications[112]. Further, these drugs have also given additional advantage in the area of combinatorial chemotherapy[113, 114].

2.9.4.1 Histone acetyl-transferases / Histone deacetylases as the targets

Histone acetyltransferases (HATs) are grouped into a few evolutionary conserved major families (Figure 2.15, Table 2.7 and Appendix, Table A2.5). The misregulation of HATs induced by mutation, translocation and overexpression has been correlated with hematological malignancies and solid tumors. In AML, translocation of CBP (CREB-

binding protein) leads to the formation of a chimeric protein fused with the monocytic leukemia zinc finger protein (MOZ), a transcriptional coactivator with intrinsic HAT activity. MOZ-CBP and MOZ-p300 cause aberrant gene expression, leading directly to malignant hematopoiesis. Similarly, mutation or deletion of p300 correlates with solid tumors, such as colorectal, gastric, breast, ovarian and epithelial cancer. Therefore, the relevance of HATs misregulation in pathology and understanding the implications of pleiotropic effects of acetylation are efforts to develop and identify a set of novel compounds that can modulate counteracting HATs-HDACs by reversing acetylation status (Table 2.8).

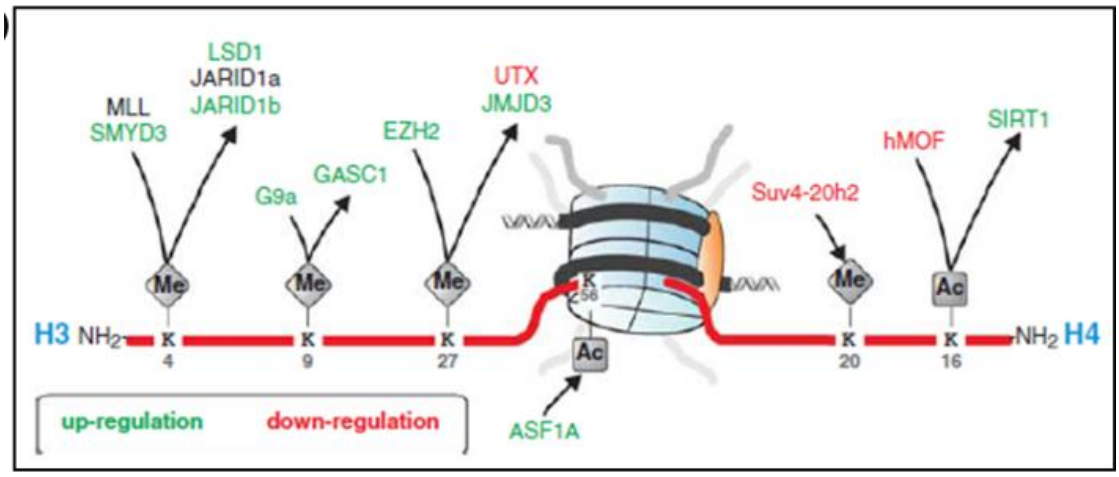

Figure 2.15: Deregulation of histone modifiers in cancer. *Enzymes for the respective histone onco-modifications are represented in green when found to be upregulated or in red if reported as downregulated in cancer cells*[68].

Eighteen distinct HDACs have been identified so far and they are classified into four groups based on their structural divergence, namely class I, II, III and IV HDACs [115](Table 2.6). Class I and II HDACs are considered as 'classical' HDACs while class III is a family of nicotinamide adenine dinucleotide (NAD+)-dependent proteins. Class IV HDAC is an atypical category of its own, based solely on its DNA sequence similarity to the others (Table 2.6). Although there are no conclusive data about the pattern of HDAC expression in human cancer, there are a number of studies showing altered expression of

individual HDACs in tumor samples. For example, there is an increase in HDAC1 expression in gastric prostate, colon, and breast carcinomas. Overexpression of HDAC2 has been found in cervical and gastric cancers, and in colorectal carcinoma with loss of APC expression. Other studies have reported high levels of HDAC3 and HDAC6 expression in colon and breast cancer specimens, respectively (Figure 2.15, Table 2.6 and Appendix, Table A2.5).

Table 2.6: Classification of known Histone deacetylases (HDACs)[115]

Class I		**Class II**		**Class III**		**Class IV**	
HDAC	**Sub-cellular Localization**	**HDAC**	**Sub-cellular Localization**	**HDAC**	**Sub-cellular Localization**	**HDAC**	**Sub-cellular Localization**
HDAC1	Nucleus	HDAC4	Nucleus/ Cytoplasm	SIRT1	Nucleus/Cytoplasm	HDAC11	nucleus/cytoplasm
HDAC2		HDAC5		SIRT2	Cytoplasm		
HDAC3		HDAC7		SIRT3	Nucleus/Mitochondria		
HDAC8		HDAC9		SIRT4	Mitochondria		
		HDAC6		SIRT5	Mitochondria		
		HDAC10		SIRT	Nucleus		
				SIRT7	Nucleus		

Aberrant gene silencing in cancer is also associated with a loss of histone acetylation. Histone acetylations are regulated through HAT (histone acetyltransferases) and HDAC (histone deacetylases). Therefore, re-establishing normal histone acetylation patterns through treatment with HAT/ HDAC inhibitors have been shown to have anti-tumorigenic effects including growth arrest, apoptosis and the induction of differentiation[116]. Some of the prominent and clinically important HAT/ HDAC inhibitors are listed in Table 2.8. Further, these antiproliferative effects of HDAC inhibitors are mediated by their ability to reactivate silenced tumor suppressor genes.

Preclinical studies have demonstrated the ability of HDACi in reversing chemoresistance in cancer cell lines and can cause the inhibition of cellular proliferation and induction of apoptosis in a number of cancer cell lines[117-122] (Figure 2.16). However, it is still unclear whether the preclinical and clinical antitumor effects of HDAC inhibitors are mainly a result of its epigenetic potency or its influence on key cellular growth regulatory pathways.

Table 2.7: Classification of known Histone acetyla-transferases (HATs)

S. No.	Family	Alias	Yeast	Human	Target histone	Complex
1	**MYST**	KAT5	Esa1	Tip60	H4K5, K8, K12, K16; Htz1K14	NuA4/TIP6
		KAT8	Sas2	MOF/MYST1	H4K16	SAS/MAF2
		KAT6	Sas3/Ybf2		H3K14, K23	
		KAT6a		MOZ/MYST3	H3K14	
		KAT6b		MORF/MYST	H3K14	
		KAT7		Hbo1/MYST2	H4K5, K8, K12; H3	
2	**GNAT**	KAT1	Hat1	Hat1	H4K5, K12	HatB
		KAT2	Gcn5		H3K9, 14, 18, 23, 27,36; H2B; yHtzl	SAGA, ADA,
		KAT2A		hGcn5	H3K9, 14, 18; H2B	STAGA,TF
		KAT2B		P/CAF	H3K9, 14, 18; H2B	PCAF
		KAT9	Elp3	Elp3	H3K14; H4K8	Elongator
		KAT10	Hpa2		H3K14; H4	
			Hpa3		H3; H4	
			Nut1		H3; H4	Mediator
3	**P300/ CBP**	KAT3B		P300	H2A-K5; H2B-K12,	
		KAT3A			H2A-K5; H2B-K12, K15; H3; H4	
4	**TFIIIC**			TFIIIC	H3; H4	
		KAT12		TFIIIC90	H3; H4	
				TFIIIC110	H3; H4	
				TFIIIC220	H3; H4	
5	**p160**	KAT13A		SRC1	H3; H4	
				ACTR/pCIP	H3; H4	
				TIF2/GRIP1	H3; H4	
6	**Orphans**	KAT11	Rtt109		H3K9, 56	
		KAT4	Taf1	Taf1	H3; H4	TFIID
				TAFII250	H3; H4	TFIID
		KAT13C		CLOCK	H3; H4	
			TFIIB	TFIIB		

As a single agent, early trials with HDACi like valproic acid and phenylbutyrate showed weak therapeutic benefit against hematologic malignancies[90]. Subsequently, more potent HDAC inhibitors such as the class-specific inhibitors (entinostat and romidepsin) and the pan HDAC inhibitors (vorinostat, belinostat and panobinostat) have been developed. In a landmark Phase IIb multicenter trial, Olsen *et al*. showed that vorinostat was effective in the treatment of patients with refractory cutaneous T-cell lymphoma[91]. Romidepsin has also been shown to have significant and durable efficacy against cutaneous T-cell lymphoma in a Phase II multi-institutional trial[92]. These and subsequent studies have led to the FDA approval of romidepsin and vorinostat for the treatment of cutaneous T-cell lymphoma, as well as the approval of romidepsin for the treatment of relapsed peripheral T-cell lymphoma[93]. There are many other HDAC inhibitors currently under Phase I and/or II study as monotherapy, including belinostat, panobinostat, entinostat, chidamide, SB939 and LAQ824 in ovarian, lung, soft tissue carcinoma, non-small-cell lung, breast and some other cancers[123-130]. However, the majority of the results from these HDAC inhibitors among solid tumor patients have been disappointing. Despite achieving only sporadic anecdotal clinical responses, their use has been associated with serious toxicities.

The interaction between different components of the epigenetic machinery has led to the exploration of effective combinatorial cancer treatment strategies. Indeed, combinations of DNA methyltransferase and histone deacetylase inhibitors appear to synergize effectively in the reactivation of epigenetically silenced genes[131-133]. Such combination treatment strategies have been found to be more effective than individual treatment approaches. For example, the derepression of certain putative tumor suppressor genes was only seen when 5-Aza-CdR and trichostatin A were combined[116]. Synergistic activities of DNA methylation and HDAC inhibitors were also demonstrated in a study

showing greater reduction of lung tumor formation in mice when treated with phenylbutyrate and 5-Aza-CdR together. Pre-treatment of HDAC inhibitor SAHA relaxes the chromatin and sensitizes cells to DNA damage induced by Topoisomerase II inhibitor[134]. Similarly pretreatment of valproic acid act in synergy with epirubicine and reduces the tumor volume in breast cancer mouse model[135].

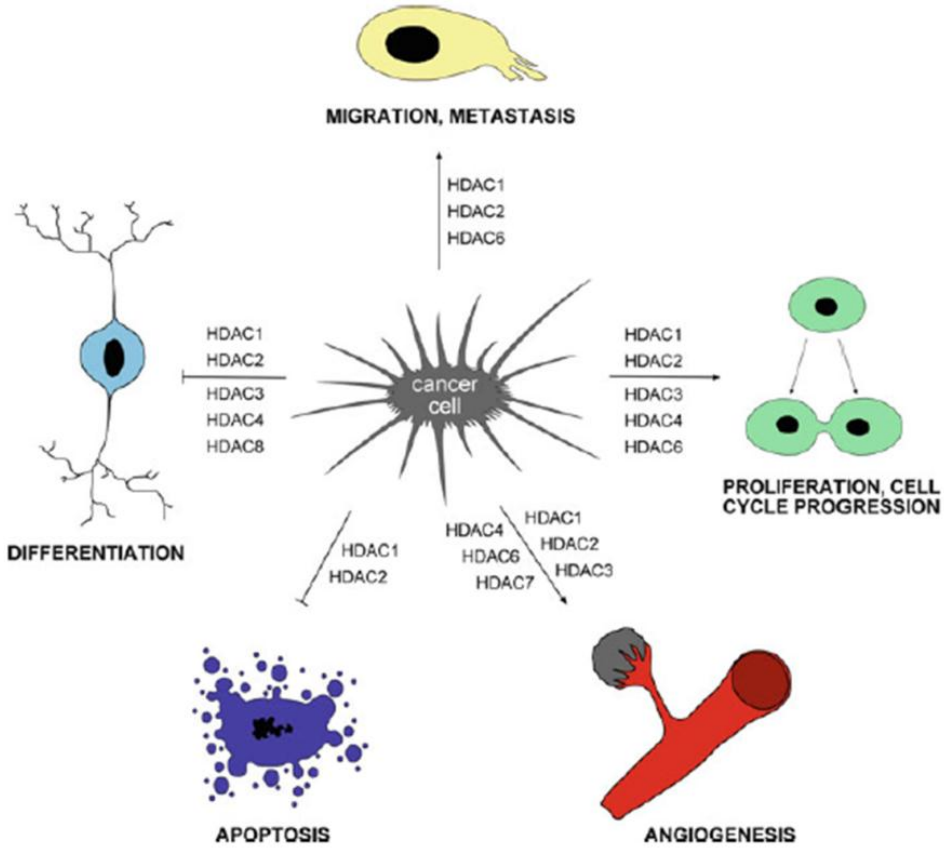

Figure 2.16: Regulation of cancer hallmarks by Histone deacetylase. *By blocking apoptosis and differentiation in addition to inducing proliferation, angiogenesis as well as metastasis, individual HDACs dictate malignant growth*[136].

Using a murine model, Belinsky *et al*. found that decitabine, when combined with the HDACi sodium phenylbutyrate, was able to decrease lung cancer formation by more than 50% in comparison with decitabine alone[133]. Another study by the same group reported that the combination of HDACi entinostat with the DNMTi azacitidine reduced tumor burden and retarded the growth of orthotopically engrafted K-ras/p53 mutant lung

adenocarcinomas in immunocompromised nude rats[137]. In another case HDACi sodium butyrate reduces the cell proliferation of MCF-7 cell when combine with vitamin-A[138].

2.9.4.2 Histone methyl transferase / Histone demethylases as the targets

Studies on histone methylation and their modifiers have been slow. Only few histone methylases (HMT) and demethylases (HDM) and their inhibitors have been discovered. However, studies on histone methylation could be more fruitful for their therapeutic potential because the less redundancy in HMTs and HDM compared to HATs and HDACs in targeting specific amino acid residue of histone[139]. This property of HMTs and HDMs provides exciting opportunities with more tailored treatment while potentially minimizing side effects.

Table 2.8: Inhibitors of histone modifiers

Class	Compound	Target enzyme	Current status
HDAC inhibitors			
Hydroxamic acid	Vorinostat (SAHA)	class I, II, IV	FDA approved
	Panobinostat	class I, II, IV	phase III CT
	Belinostat	class I, II, IV	phase II CT
	Abexinostat; Resminostat; Givinostat	class I, II	phase II CT
	Pracinostat	class I, II	phase II CT
	Dacinostat	class I, II	phase I CT
Cyclic tetrapeptide	Romidepsin	HDAC1, 2	FDA approved
	Apicidin	HDAC2, 3	Phase II CT
	Trapoxin A	HDAC1, 4, 11	ND2
Benzamide	Mocetinostat	HDAC1, 2, 11	phase II CT
	Entinostat	HDAC1, 9, 11	phase II CT
	Rocilinostat	HDAC6	phase II CT
Aliphatic acid	Valproic acid (VPA)	class I	phase III CT
	Pivanex	ND	phase II CT
	Butyrate	class I, IIa	phase II CT
Electrophilic ketone	Trifluorometchylketone	ND	ND
HAT inhibitors			
Class not yet defined	E-7438	EZH2	Phase I/II
	EPZ-5676	DOT1L	Phase I
Bromo domain inhibitors			
	GSK525762	BET	Phase I
	OTX015	BET	Phase I
	RVX208	BET	Phase II

LSD1/ KDM1 was among the first identified histone demethylases selectively targeting H3K4me1 and H3K4me2[140] and mediate gene repression. LSD1 has been found to be overexpressed in a significant number of cancers like brain, breast, and prostate, thus making it an attractive target for drug therapy[140-142]. SL11144 and tranylcypromine inhibits LSD1 and restore expression of multiple aberrantly silenced tumor suppressors, including secreted frizzled-related protein and GATA transcription factors[143, 144]. However, similar to HDACs, off-target effects on H3K9me2 and DNMT1 limit its immediate usefulness and further study is needed[145]. EZH2 is another methyltransferase responsible for H3K27me3 leads to gene silencing by promoting DNA methylation. EZH2 is found to be overexpressed in head and neck, breast, and prostate cancers and is targeted by a hydrolase inhibitor called 3-deazaneplanocin A (DZNep)[146, 147]. By countering EZH2 and inhibiting H3K27 trimethylation, DZNep induces differentiation as well as apoptosis in cancer cell lines and xenografts while sparing normal cells[148, 149].

2.9.4.3 Kinases/ Phosphatases as the targets

Compared to histone acetylation and methylation the effort of regulating histone phosphorylation by targeting kinases and phosphatases for therapeutic uses is new. High level of several histone H3 phosphorylations such as H3S10ph, H3T6ph has been reported in number of cancer. p38 MAPK pathway mediated increase in H3S10ph in response to cisplatin treatment in HeLa and MCF7 cells[150]. Romain *et al* recently reported that the kinase inhibitors like Enzastaurin (PKC-beta inhibitor), AZD1152 (Aurora-B inhibitor) and AZD1480 (Jak2 inhibitor) increases the cell death of TMZ-Irrad resistant GBM and decreases H3T3ph, H3S10ph and H3Y4ph respectively[101]. Further, H89 (MSK1 inhibitor) treatment reduces the TPA and EGF mediated cellular transformation and by decreasing H3S10ph[64].

Chapter 3

Aims and Objectives

3.1 Statement of the Problem

Gastric cancer (GC) is one of the most common malignancies worldwide. Globally, GC ranks fourth and third in terms of incidence and mortality respectively. In India, it is one of the most aggressive cancers ranking third and second in terms of incidence and mortality respectively. Surgery remains the mainstay for cure especially in early cancers, while in locally advanced GC, the addition of neo-adjuvant chemotherapy offers a better survival advantage. The NACT facilitates histological tumor regression and thereby increases the rate of curative or pathologically disease-free margin (R0). However, despite apparently curative surgery, loco regional recurrence has still been encountered in 87% of GC patients raising the doubt of current pathological techniques used in day to day practice to truly confirm the adequacy of the surgical resection margins. Therefore, there is an urgent need to identify molecular markers and investigate their expression in not only the cancerous tissues, but the surrounding resected (margin) tissue that is apparently free from disease (R0) based on histopathology.

3.2 Hypothesis

Over the past decade accumulated evidences have identified aberrant alteration in the global level of several histone post-translational modification, defined as 'histone onco-modifications'. These histone onco-modifications provide independent prognostic information for several cancers. However, relation of histone PTMs between tumor and resection margin and the regulatory mechanism for their alteration is poorly understood in cancer. Therefore, detailed studies are required to understand how global levels of histone modifications are established and maintained and what their mechanistic links are to the cancer clinical behavior. All this leads us to the point that there is need to understand in-depth the differential alteration in histones, histone modifying enzymes and to define new

prognostic markers and therapeutic targets for the better management of gastric cancer patients.

3.3 Objectives

I. To identify differential alterations in histones and their enzymes in gastric cancer

II. To decipher molecular mechanism of specific alterations in histones in gastric cancer

3.4 Experimental Plan

Objective I: To identify differential alterations in histones and their enzymes in gastric cancer.

i. *Collection of freshly resected and paraffin embedded blocks of tissues from the site of tumor and resection margins (proximal and distal) of gastric cancer patients.*

ii. *Haematoxylin and Eosin (H&E) staining and histopathological confirmation of tissue identity and tumor content.*

iii. *Preparation of chromatin and nucleo-cytosolic fraction from freshly resected tissues.*

iv. *Pilot screening of differential site-specific histone post-translational modifications in tumor and resection margin tissues using immunoblotting.*

v. *Immunohistochemical analysis of specific histone PTM(s) on tumor and resection margins (proximal and distal) tissues for validation in large cohort of samples.*

Objective II: To decipher molecular mechanism of specific alterations in histones in gastric cancer.

i. *Identification of specific histone modifying enzymes responsible for alteration in specific histone PTMs in cell lines and tissue samples using enzyme assay, immunoblotting and immunohistochemistry.*

ii. *Determination of effect of enzyme on site specific histone modification by exogenous overexpression and chemical inhibition followed by immunoblotting and immunofluorescence studies.*

iii. *Identification of regulatory pathway responsible for of specific histone PTM in tissues and cell lines using immunoblotting and immunofluorescence studies.*

iv. *Cell based toxicity assays to study the effect of histone modifying enzymes inhibitors for their potential application in combinatorial chemotherapy.*

3.5 Work Done

The result and discussion of the work carried out under above mentioned objectives are presented as three chapters with following headings:

Chapter 5: Histone H3 Serine 10 phosphorylation: Regulation and its correlation with clinico-pathological parameters in gastric cancer.

Chapter 6: β-actin expression and its clinicopathological correlation in gastric adenocarcinoma.

Chapter 7: Global hypoacetylation of histones: Combinatorial effect of HDAC inhibitors with DNA-targeted chemotherapeutic drugs on gastric cancer cell lines.

Chapter 4

Materials and Methods

4.1 Tissue Samples and Clinical Data

4.1.1 Inclusion criteria and collection of tissue sample

The protocol for collection of freshly resected frozen (FRF) tissues and formalin-fixed paraffin-embedded (FFPE) tissues blocks was reviewed and approved by institutional review board and ethics committee of Tata Memorial Center, Tata Memorial Hospital, Mumbai, India. All patients provided a written informed consent (Appendix-1). FRF and FFPE tissue samples were collected from gastric cancer patients based on seven inclusion criteria- *Adenocarcinoma (type of cancer), Curative surgery (intent of surgery), Indian (domicile of the patient), HBV infection negative, HCV infection negative, HIV infection negative and 1 gm (tissue weight, only for FRF tissues).*

Through Indian Council for Medical Research (ICMR) funded, Tumor Tissue Repository (TTR) at Tata Memorial Hospital, Mumbai, India; FRF tissues and FFPE blocks were collected. From each patient, tissues were collected form three different sites- Tumor (T), Proximal resection margin (PRM) and Distal resection margin (DRM). All the patients were operated between 2009 and 2012 at Tata Memorial Hospital, Mumbai, India. We first prospectively collected FRF tissues from 84 patients; tissue samples from 48 patients were excluded from the study due to either less weight and/ or tumor content (< 30%). FRF tissues for rest of the 36 patients were used in the study and FFPE blocks were also collected for the same. FRF tissues were frozen immediately in liquid nitrogen, and then stored at -80 °C until required for experimental use. Then, we retrospectively collected FFPE tissue blocks from 65 gastric cancer patients. Thus, for our study, FRF tissues were available from 36, while FFPE tissue blocks were available for 101 GC patients.

The *H. pylori* infection status in obtained FRF tissue samples were checked PCR and Giemsa staining based methods, however, all results were negative which can be

attributed to the antibiotic treatment that GC patients go through at the initial stage of treatment.

4.1.2 Preparation of tissue section slides

Both FRF tissues and FFPE tissue blocks were processed cryotome and sections of 4 µm thickness were prepared. Internal temperature of the machine was always maintained at -20ºC. Tissue sections were then transferred to poly-L Lysine coated slides and incubated overnight at 37ºC. These tissue slides were then stored at room temperature until required for experimental use.

4.1.3 Hematoxylin and eosin staining

Hematoxylin and eosin (H&E) staining was done on poly-L lysine coated glass tissues slides as per the standard protocol[151]. Slide with FFPE tissue sections were first incubated at 65°C for 20 minutes to melt paraffin, treated with Xylene twice for 10 minutes each and then treated with 100% EtOH twice for 5 minutes each. Now, FFPE and FRF tissues slides were air dried for 30 minutes at 37ºC to remove moisture. The slides were stained with 0.1% Mayers Hematoxylin (Sigma; MHS-16) for 10 minutes, rinsed in running tap water for 5 minutes and then dipped in 0.5% Eosin (1.5g dissolved in 300ml of 95% EtOH) 10 times, each lasting for 1-2 seconds. Hematoxylin and Eosin stained slides were dipped in distilled water until the Eosin stops streaking, and then washed in 50, 70 and 100% graded EtOH solutions for 5 minute each. In the end slides were cleaned by washing in Xylene and mounted with DPX mountant (Qualigens, cat#18404).

4.1.4 Histopathological analysis

Histopathological analysis was done using H&E stained tissue sections to confirm the identity of the tissues and to determine tumor content (% of tumor cells) by a blinded specialist gastrointestinal pathologist (Dr. Mukta Ramadwar, Tata Memorial Hospital, Mumbai, India). Based on histopathological analysis, FRF tissues with ≥ 70% tumor

content, FPPE tumor tissues with ≥ 10% tumor content and negative resection margins (without any tumor cell) were included in the final study. Therefore, the present study in thesis was conducted with paired tumor, PRM and DRM frozen tissues (n=10) and FFPE tissue blocks (n=101). In some of the subsequent sections negative resection margin tissues have also been referred as normal tissues.

4.1.5 Collection of clinical data

Information of clinical characteristics of the patients included in the final study was collected using Electronic Medical Record (EMR) system of Tata Memorial Hospital, Mumbai, India. Status (Dead/ Alive/ Recurrence) of patients at last follow-up date from the time of surgery was dually checked using EMR as well as by telephonic conversation with patient or patient's relative. Clinical information for all the patients is tabulated in table (Appendix-1).

4.2 Immunohistochemistry

4.2.1 Immunohistochemical staining

Immunohistochemical staining was performed using VECTASTAIN® ABC kit (Vector Lab, P6200) and as per manufacturer's protocol. Briefly, FFPE tissue blocks were sectioned at a thickness of 4 µm and mounted on poly-L-lysine coated glass slides. The sections were deparaffinized through a graded series of xylene and rehydrated through a graded series of absolute alcohol to distilled water. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol at room temperature for 30 minutes in dark. Microwave antigen retrieval was carried out with 0.01 M Sodium citrate buffer (pH 6.0). Primary antibodies (Table 4.1) were applied for 16 hours at 4°C. Immunoreactive proteins were chromogenically detected with diaminobenzidine (DAB) (Sigma, D5537). The sections were counterstained with Harris's hematoxylene and then dehydrated and mounted. In parallel, control staining was performed without adding primary antibody.

1X TBS was used to dilute blocking reagent, primary antibody, secondary antibody, tertiary reagent.

Table 4.1: List of antibodies used for IHC analysis

S. No.	Primary Antibody	Dilution
1	Anti-β-actin (Sigma, A5316)	1:200 in 1X TBS
2	H3S10ph (Abcam, 51776)	1:100 in 1X TBS
3	H3K16ac (Millipore, 07-329)	1:100 in 1X TBS
4	H4K20me3 (Abcam, 9053)	1:100 in 1X TBS
5	ph-MSK1 (Abcam, 32190)	1:100 in 1X TBS

4.2.2 Scoring of Immunohistochemical staining

The cytoplasmic immunohistochemical staining of β-actin was scored semi-quantitavely for epithelial and inflammatory cells as described in a previous study by *Yip et al*[100]. "IHC score", "Total IHC score" and "Average Total IHC score" were calculated by taking the account into percentage of immunostained cells and staining intensity (Table 4.2). Total IHC score of 2 and above was considered as positive immunoreactivity. Total IHC score ranges from 2 to 7 and further grouped into: low (score 2 and 3), intermediate (score 4 and 5) and high (score 6 and 7). The nuclear immunohistochemical staining for all the antibodies were scored using H-score which is based on intensity of staining (ranges zero to three) and percentage of stained cells using the formula, H-score= [(0 x % of cells with staining intensity of zero) + (1 x % of cells with staining intensity of one) + (2 x % of cells with staining intensity one) + (3 x % of cells with staining intensity two)]. H-score was further divided in 3 groups (i) 0-100: low level (ii) 100-200: intermediate level and (iii) 200-300: high level. The immunohistochemical staining was examined by two independent researchers one of whom is a senior consultant pathologist to ensure the evaluations were performed properly and accurately. Both the researchers were blinded to all clinicopathological and outcome variables.

Table 4.2: Scoring system for β-actin immune-staining			
Percent positivity of stained cells	**IHC score**	**Staining intensity**	**IHC score**
0%	**0**	**None**	**0**
< 25%	**1**	**Weak**	**1**
25%-50%	**2**	**Moderate**	**2**
50%-75%	**3**	**Strong**	**4**
75%-100%	**4**		
Total IHC Score = IHC score of percent positivity + IHC score of staining			

4.3 Cell Culture

4.3.1 Cell lines and culture conditions

Gastric cancer cell lines AGS (ATCC® Number: CRL-1739™; moderately differentiated) and KATO III (ATCC® Number: HTB-103™; signet ring cell carcinoma) was used. AGS and KATO III cells were cultured in RPMI1640 (Invitrogen) and F12K (Himedia) media respectively at 37 °C with 5% CO_2 supplemented with 10% FBS, 100U/ml penicillin, 100 mg/mL streptomycin (Sigma).

4.3.2 Trypsinization and sub-culturing

For trypsinization and sub-culturing standard protocol was followed with slight modifications[152]. Cell lines were passaged every 4-5 days to maintain their normal morphology and proliferation rate. Medium was removed from culture with a sterile pipette, adhered cells were washed with PBS, pH7.2-7.4 (1.9mM NaH_2PO_4, 8.1mM Na2HPO4 and 154mM NaCl) and 1ml trypsin/EDTA (0.25% w/v trypsin, 0.2% EDTA in PBS) solution was added. Cells were incubated at 37°C until cells were detached from surface. Detached cells were re-suspended in 1ml complete medium. The viable cells were counted as described below and plated in fresh culture dishes (~2X 104 cells/ml). The number of viable cells was determined by staining with 0.5ml of trypan blue (0.4% w/v in PBS). Cells were counted on haemocytometer and number of cells/ml was calculated as follows:

No. of cells/ml = average number of cells per WBC chamber × dilution factor (10) × 10^4

4.3.3 Freezing down cells for liquid nitrogen stocks

For freezing down of cells standard protocol was followed with slight modifications[152]. A near 100% confluent 90 mm dish (containing 4-6 x 10^6) was used to make stocks for storage. The cells were washed twice with 5ml 1xPBS, harvested by adding 2ml 1x trypsin for one minute at 37ºC. Trypsin was aspirated; cells were resuspended in 10 ml media, centrifuged for 5minutes at 1500 rpm. The supernatant was removed; the cells were again suspended in 1 ml of freezing media (90% serum and 10% DMSO). The date; identity of cell line; passage number were recorded. The cells were gradually frozen by incubation at-20 ºC for 2 hours and then at -80ºC for overnight. Finally, the cells were transferred to liquid nitrogen for long term storage.

4.3.4 Thawing cells from liquid nitrogen stocks

For thawing cells standard protocol was followed with slight modifications[152]. Cells were immediately thawed by immersion in 37 ºC water bath for 5 minutes. 9 ml of media was added, cells were centrifuged and resuspended in 10 ml cultural media. Cells were allowed to attach overnight (37ºC, 5% CO_2) before media was replaced and cells were passaged or sub-cultured as described above.

4.4 Genetic Manipulation

4.4.1 Cloning of MSK1

The vector DU2012 pCMV-FLAG-MSK1 wt was procured from MRC-Protein phosphorylation and ubiquitination unit of University of Dundee, UK. The empty vector pCMV5 was generated by digestion of the above vector with BglII and MluI (sites flanking Flag Msk1 gene). The 4645 kb band was gel extracted and the ends were blunted by incubating with Pfu polymerase for 20minutes at 72˚C in a PCR machine. The resulting product was purified using Fermentas PCR purification kit and ligated using

NEB's Quick Ligase as per the recommended protocol. The ligated product was transformed in ultracompetant DH5α cells. The colonies were screened by restriction enzyme digestion with NdeI and XhoI.

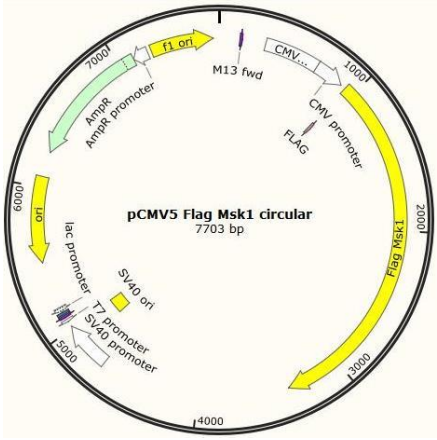

Figure 4.1: pCMV-Flag-MSK1 cloning vector map.

4.4.2 Transfection of MSK1

For transfection standard protocol was followed with slight modifications[152]. Transfection in AGS cell line was carried out by Calcium phosphate method. The cells were transfected at 50% confluency with 10µg of plasmid in a 35mm dish. The culture medium was changed to C-DMEM medium 2hours prior to transfection. Briefly the plasmid was dissolved in 125µl of TE pH 7.4 followed by addition of 125µl of 2.5M $CaCl_2$. The solution was mixed by vortexing and to this 150µl of 2X BBS (50 mM BES, 1.5 mM Na_2HPO_4 and 280 mM NaCl) was added dropwise. The solution of plasmid and Calcium phosphate was allowed to stand at RT. After 20min 200µl of this solution was spread dropwise over the cells. The cells were harvested 48hours post transfection in 2X SDS loading dye.

4.5 Biochemical Inhibition

4.5.1 Inhibition of MAP kinase pathway

PD98059 (Calbiochem, cat#LOC032021), SB203580 (Calbiochem, cat#550389) and H89 (Millipore, cat#19-141) was used to chemically inhibit MAPK kinases ERK1/2, p38 and

mitogen and stress kinase-1 (MSK1) respectively. All the inhibitors were dissolved in DMSO to prepare stock concentrations and stored at -20ºC in small aliquots. AGS and KATOIII cells were cultured in 90 mm plate till 90% confluence and chemical inhibitors were added along with with the fresh medium. Cells were treated with PD98059 and SB203580 for 1 hour at the final concentration of 10 µM; whereas, H89 treatment was done for 6 hours at the final concentration of 20 µM. After the said treatment cells were harvested and used for further experiments.

4.5.2 Inhibition of HDACs

Histone deacetylase inhibitors (HDACi), Sodium valproate/ VPA (Sigma, P4543), Trichostatin A/ TSA (Sigma, T8552) and Suberoylanilide hydroxamic acid/ SAHA (Sigma, SML0061) were dissolved in absolute ethanol to prepare stock solutions of 600, 10 and 10 mM respectively. As per the requirement of experiment, AGS and KATOIII cells were treated with range of concentrations of HDACi.

4.5.3 Chemotherapy drugs

DNA binding chemotherapy drugs, Cisplatin (Calbiochem, 232120), Oxaliplatin (Sigma, O9512) and Epirubicin (Calbiochem, 324905) were dissolved in DMSO to prepare stock solutions of 165, 63 and 14 mM respectively. As per the requirement of experiment, AGS and KATOIII cells were treated with range of concentrations of chemotherapy drugs.

4.6 Cell Viability Assay

4.6.1 Trypan blue exclusion assay

Trypan blue exclusion test for cell viability was done as per standard protocol with slight modifications[153]. The cells were stained with 0.4% Trypan Blue solution after diluting at 1:1 ratio with the cell suspension. Trypan Blue was sterile filtered before using it in order to get rid of particles in the solution that may interfere with the counting process. Manual

counting of viable (unstained cells) and non-viable cells (blue stained cells) were carried out in three independent experiments by haemocytometer.

4.6.2 MTT assay

Cell viability was quantified by its ability to reduce tetrazolium salt 3-(4,5-dimethylthiazole-2Ƴ)-2,5-diphenyl tetrasodium bromide (MTT) to colored formazan products (Sigma# m-2128) as per manufacturer's protocol[154]. MTT reagent (5mg/ml in PBS) was added to the cells at 1/10th volume of the medium to stain only viable cells and incubated at 37°C for 4hours. MTT solubilisation buffer (0.01M HCl, 10% SDS in 1X PBS) of two fold volume was added to cells, followed by incubation in the dark at 37°C for 24hours. The absorbance was measured at 570nm with Spectrostar Nano-Biotek, Lab Tech plate Reader. Cell viability was expressed as the percentage of absorbance obtained in control cultures.

4.6.3 Colony formation assay

Colony formation assay was done as per standard protocol with slight modifications[154]. The cells (n=2000) were plated in 60mm tissue culture plates and its survival was measured by clonogenic assay in monolayer after 14 days in triplicate. The cells were treated with IC_{50} concentration of chemotherapy drugs and HDAC inhibitors for 72 hour and after PBS washes, cells were incubated in complete culture medium for additional 14days, with media changes after every 2-3 days. Cells were fixed with 4% paraformaldehyde for 1 hour, stained with 0.5% crystal violet (Sigma, 0.5% in 70% ethanol) for 1hours at room temperature, rinsed and air-dried. Surviving colonies with more than 50 cells were counted and images were captured using a high-resolution Nikon D70 camera (Nikon, Tokyo, Japan). The survival data of treated cells were normalized to the plating efficiency of control.

4.7 Cell Cycle Analysis

4.7.1 Cell cycle analysis of cell line by FACS

Cell cycle was analyzed as per standard protocol with slight modification[155]. Cells were washed with PBS (twice) and fixed with 70% chilled ethanol. During fixation, ethanol was added drop-wise with vortexing to prepare a single cell suspension. After fixation, cells were stored at -20°C. Cells were further washed twice with PBS and suspended in 500µl of PBS with 0.1% Triton X-100 and 100µg/ml of RNaseA followed by incubation at 37°C for 30minutes. After incubation, propidium iodide (sigma, 25µg/ml) was added followed by with incubation at 37°C for 30 minutes. DNA content analysis was carried out in a FACS Calibur flow cytometer (BD Biosciences, USA). Cell cycle analysis was performed using the Mod-fit software from BD Biosciences.

4.7.2 Cell cycle analysis of tissue samples by FACS

Cell cycle was analyzed as per standard protocol with slight modification[155]. 50 mg of tissue was first powdered using mortar pestle in liquid nitrogen. The powder was homogenized in 2 ml of nuclear buffer A (15mM Tris-Cl pH 7.5, 60mM KCl, 15mM, 15mM NaCl, 2mM EDTA, 0.5mM EGTA, 0.34M Sucrose, 0.15mM β-ME, 0.15mM Spermine and 0.5mM Spermidin) using dounce homogenizer. The homogenate was centrifuged (5000 rpm for 15 minutes at 4ºC) to pellet nuclei; supernatant was discarded. The nuclei was washed twice in nuclear buffer A and fixed in 70% chilled absolute EtOH and stored at -20ºC until required. For cell cycle analysis by FACS rest of the steps were carried out as mentioned in section 3.6.1.

4.7.3 Mitotic index of tissue samples

On the basis of morphology of the nuclei in H&E-stained tissue sections, mitotic cells or cells which were not in G0 phase of the cell cycle were counted in 10 consecutive High Power Field (40X) and average was expressed as Mitotic index.

4.8 Microscopy Analysis

4.8.1 Immunofluorescence microscopy

Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 20 minutes. Cells were then permeabilized in PBS containing 0.5% trition X-100 for 20 minutes at RT and then blocked with PBS containing 3% BSA and 0.1% NP-40 for 1 hour. Next, cells were incubated with a primary antibody against H3S10ph and ph-MSK1 and appropriate secondary antibodies for 2 hours each. Dilution of primary (1:100) and secondary antibody (Alexa 568 or Alexa 488) was made in blocking buffer. All the steps were performed in dark and at room temperature. Finally coverslips were mounted in VECTASHIELD (Vector lab). Fluorescence intensity was analyzed using fluorescence microscope (IX81; Olympus, Tokyo, Japan).

4.9 Gene Expression Analysis

4.9.1 RNA isolation from tissue samples

Glassware was baked at 300°C for 4hours and compatible plasticware was rinsed with chloroform and washed with diethylpyrocarbonate (DEPC) treated water. Nitrile gloves were used to prevent RNase contamination. Total RNA was extracted (Thermo scientific, 0731) from 25 mg of frozen tumor and resection margin (PRM or DRM) tissue with maximum distance from the site of tumor as per the manufacturer's instructions. RNA was stored at -80°C until required. RNA was quantitated by diluting 5µl in 1ml alkaline water (1mM Na_2HPO_4) and reading at A_{260}. Quality of RNA was confirmed by A_{260}/A_{280} (1.9-2.0), A_{260}/A_{230} (2.0-2.2) and agarose formaldehyde gel electrophoresis[156].

4.9.2 Agarose formaldehyde gel electrophoresis

Agarose (0.5g) was dissolved in 36ml water and cooled to ~60°C. To prepare agarose formaldehyde gel. After cooling, 5ml of 10X MOPS running buffer (0.2M MOPS pH7.0, 0.5M sodium acetate and 0.01M EDTA) and 9ml of 12.3M formaldehyde were added.

Table 4.3: List of primers used for RT PCR				
S. No	**Gene**	**Primer Sequence**	**NCBI Ref. No**	**Product size (bp)**
1	β-actin	F: AGAAAATCTGGCACCACACC	NM_001101.3	444
		R: CCATCTCTTGCTCGAAGTCC		
2	c-Jun	F: CCCCAAGATCCTGAAACAGA	NM_002228.3	214
		R: TCCTGCTCATCTGTCACGTT		
3	c-Fos	F: CCGGGGATAGCCTCTCTTAC	NM_005252.3	365
		R: CCCTTCGGATTCTCCTTTTC		
4	cyclin-E1	F: AGCGGTAAGAAGCAGAGCAG	NM_001238.2	188
		R: TTTGATGCCATCCACAGAAA		
5	cyclin-B1	F: CGGGAAGTCACTGGAAACAT	NM_031966.3	314
		R: CCGACCCAGACCAAAGTTTA		
6	cyclin-D1	F: GATCAAGTGTGACCCGGACT	NM_053056.2	329
		R: AGAGATGGAAGGGGGAAAGA		
7	18s rRNA	F: AAACGGCTACCACATCCAAG	X03205.1	255
		R: CCTCCAATGGATCCTCGTTA		

The gel was poured into electrophoresis tray with comb and allowed to set. Comb was removed and gel was placed in gel tank. Gel tank was filled with 1X MOPS running buffer. For electrophoresis 5µg RNA was loaded per lane. RNA volume was increased to 11µl by water and 5µl of 10X MOPS buffer, 9µl of 12.3M formaldehyde and 25µl of formamide were added and sample was incubated for 15minutes at 55°C. To this mixture 10µl formaldehyde loading buffer (1mM EDTA pH8.0, 0.25% w/v BPB, 0.25% w/v xylene cyanol, 50% v/v glycerol) was added and loaded onto the gel. The gel was run at 5V/cm until dye migrated one-third to two-third length of the gel[156].

The gel was removed, transferred to RNase free glass dish with water and soaked twice for 20minutes each. After sufficient removal of formaldehyde, gel was soaked in 0.5µg/ml ethidium bromide and allowed to stain for 40minutes. The gel was destained in water for 1hour and examined on a UV transilluminator to visualize RNA.

4.9.3 c-DNA synthesis and Reverse transcription PCR

10 µg of total RNA was used for cDNA synthesis (Fermentas life sciences, K1632) using random hexamers as per the manufacturer's instructions. RT-PCR amplification was done

using specific primers with an initial denaturation step at 95°C for 2 minutes, followed by 15 cycles of denaturation at 95°C for 45 minutes, primer annealing at 55°C for 30 s, primer extension at 72°C for 30s and a final extension at 72°C for 10 minutes. Amplified products were resolved on 1.5% agarose gels and visualized by Ethidium bromide staining.

4.10 Protein Fractionation

4.10.1 Total protein lysate preparation from cell lines

Cells were harvested from 90 mm culture plates and washed twice with chilled PBS. The cell pellet was lysed in 1 ml of 1X laemmli buffer (2% SDS, 10% v/v Glycerol, 110mM Tris-Cl pH 6.8, 0.1% v.v β-ME) and stored at -20ºC until requires.

4.10.2 Nucleo-cytosolic and chromatin fraction from cell lines

Cells were harvested from 90 mm culture plates and washed twice with chilled PBS. The cell pellet was lysed in chilled MKK lysis buffer[157] (10mM Tris-Cl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 10µg/ml Leupeptin, 10µg/ml Aprotenin, 1mM PMSF, 1mM Sodium orthovanadate, 10mM Sodium fluoride, 10mM β-Glycerophosphate). The lysate was centrifuged at 100000xg for 30 minutes at 4ºC, supernatant was collected as nucleo-cytosolic fraction (NCF) and stored at -20ºC until required. The remaining pellet was dissolved and boiled in 1X laemmli buffer (section 4.10.1) and stored as chromatin fraction (CF) at -20ºC until required.

4.10.3 Nucleo-cytosolic and chromatin fraction from tissue samples

100 mg of tissue was first powdered using mortar pestle in liquid nitrogen. Using dounce homogenizer the powder was lysed and homogenized in lysis buffer (20mM Tris-Cl pH8, 2mM EDTA, 10mM EGTA, 5mM $MgCl_2$, 0.1% Triton X-100, 1mM Sodium orthovanadate, 1mM Sodium fluoride, 20mM β-Glycerophosphate, 10µg/ml Leupeptin, 10µg/ml Aprotenin, 1mM PMSF). The lysate was centrifuged at 100000xg for 30 minutes

at 4ºC, supernatant was collected as nucleo-cytosolic fraction (NCF) and stored at -20ºC until required. The remaining pellet was dissolved and boiled in 1X laemmli buffer (3.9.1) and stored as chromatin fraction (CF) at -20ºC until required.

4.10.4 Histones from cell line and tissue samples

Histones were isolated by acid extraction method as described[158]. The purified remaining chromatin pellet obtained in section 4.10.3 and 4.10.3 was used for histone isolation by acid extraction method. 0.2M H_2SO_4 was added drop-wise to the chromatin pellet with vigorous vortexing and incubated for 30minutes at 4°C. After centrifugation at 16,000 rpm at 4°C, supernatant containing histone protein was precipitated overnight with acetone at -20°C. Histone pellet was washed with acidified acetone (50mM HCl in acetone) followed by washing with chilled acetone. Total histone was dissolved in 0.1% β-mercaptoethanol in H_2O and stored at -20°C.

4.11 Protein Estimation

4.11.1 Protein estimation by Lowry's method

Histone and total protein concentrations in various samples were determined by Lowry method of protein estimation. Protein standards were prepared containing a range of 2-16µg of Bovine Serum Albumin (BSA, Sigma) and unknown samples were also prepared similarly. The freshly prepared Copper Tartrate Carbonate (CTC- 0.1% CuSO4, 0.2% Sodium potassium tartrate, 10% Na2CO3; CTC mixture: CTC, 0.8N NaOH, 10% SDS and D/W in 1:1:1:1 ratio,) mixture was added and vortexed. After incubation for 10minutes at RT, 500µl of Folin Ciocalteau reagent (1:6 dilutions with D/W, 0.33N) was added, tubes were vortexed and incubated in dark for 30 minutes at RT. Absorbance at 750 nm was measured and standard curve was prepared to determine protein concentration.

4.12 Polyacrylamide Gel Electrophoresis

4.12.1 Resolution of protein fractions by SDS-PAGE

Proteins were separated on SDS-PAGE using modification of traditional Laemmli buffer system[159]. Histones and total soluble protein lysate were separated on 18% and 10% SDS-PAGE respectively. Increased concentrations of buffers used in this modification provide better separation between the stacked histones and SDS micelles. In brief, glass plate sandwich was assembled using 0.1cm thick spacers. Separating gel solution (17.5% w/v acrylamide, 0.5% bisacrylamide, 0.75M Tris pH8.8, 0.1% w/v SDS, 0.033% w/v APS, 0.66% v/v TEMED) was prepared and poured into the glass plate sandwich and allowed to polymerize. Stacking gel solution (3.8% w/v acrylamide, 0.1% w/v bisacrylamide, 0.125M Tris-Cl pH 6.8, 0.1% w/v SDS, 0.05% w/v APS, 0.1% v/v TEMED) was then prepared and poured into the glass plate sandwich in similar manner. A 0.1cm thick Teflon comb was inserted and gel was allowed to polymerize. Histone samples to be analyzed were diluted 1:1 (v/v) with 2X SDS sample buffer (0.05M Tris-Cl pH6.8, 20% v/v glycerol, 4% w/v SDS, 2% v/v 2-ME, 0.01% w/v bromophenol blue, BPB) and incubated for 5minutes in boiling water. Teflon comb was removed, sandwich was attached to the electrophoresis chamber and filled with 2X SDS electrophoresis buffer (0.05M Tris, 0.384M glycine, 0.2% w/v SDS, pH8.3-8.6). Samples were loaded into the wells formed by comb. The gel was run at 20mA of constant current until the BPB tracking dye entered the separating gel and then at 30mA until the BPB dye reached the bottom of the gel. The power supply was then disconnected and gel was subjected to Coomassie staining or western blot analysis.

4.12.2 Coomassie staining of SDS-PAGE gels

After electrophoresis, gel was transferred to tray containing Coomassie Brilliant Blue R-250 (CBBR) staining solution (0.1% w/v CBBR, 50% methanol and 10% acetic acid in

water). The gel was stained for ~3 hours, transferred to destaining solution (50% methanol and 10% acetic acid in water) with several changes until visualization of protein bands.

4.12.3 Ammoniacal Silver nitrate staining of SDS-PAGE gels

Silver staining of SDS-PAGE gels were done as per standard protocol with slight modifications[160]. After electrophoresis of histone protein, the gel was treated with three washes of 50% methanol of 1hour followed by overnight incubation at 4°C. The gel was incubated with ammoniacal silver (2.8ml liquid ammonia in 0.38% sodium hydroxide solution (42 ml) followed by drop-wise addition of 8ml 20% silver nitrate to 200ml with D/W) staining solution for 30 minutes, followed by two washes of 5minutes with D/W. The washed gel was incubated with developer (15mg citric acid, 0.15 ml formaldehyde in 100 ml D/W) for the development of protein bands and the reaction was stopped with destaining solution (50% methanol, 10% acetic acid in D/W).

4.13 Western Blotting

4.13.1 Electroblotting from SDS-PAGE

TCL, NCF and histones were run on 18%, 10% and 18% SDS-PAGE respectively and blotted onto an adsorbent porous Polyvinylidene difluoride (PVDF) membrane, which gives a mirror image of the gel. Proteins were transferred to PVDF membrane at 4°C, employing a constant current of 300 mA for 200 minutes.

Histones (5-10µg) were electroblotted from SDS-PAGE gels to PVDF membranes for western blot analysis [146]. The transfer tank of electroblotting apparatus (Trans-Blot Cell, Bio-Rad) was filled with 1x transfer buffer (0.19 M Glycine, 25 mM Tris base, 0.01% SDS and 20% methanol). PVDF membrane was activated in 100% methanol for 5seconds. The activated membrane and SDS-PAGE gel were equilibrated in 1x transfer buffer. The gel membrane transfer sandwich was prepared and inserted into the transfer

tank with gel on cathode side and membrane on anode side. Transfer was conducted at a constant current of 300mA for 200 minutes. Proteins transferred onto the membrane were detected by staining with Fast green (0.5% w/v Fast green in destaining solution) and destaining with several changes of water.

4.13.2 Immunoblot detection

Histones transferred onto PVDF membrane were probed for global levels of acetylation, methylation and phosphorylation modifications. Antibodies and their Immunoblotting condition used in this study are list in Appendix, Table A2.2.

In general, membrane with transferred proteins was incubated in 'blocking buffer' i.e. 5% BSA in Tween20/Tris-buffered saline (TTBS, 100mM Tris-Cl pH7.5, 0.9% w/v NaCl and 0.1% v/v Tween20) for 1hr at room temperature on orbital shaker. Blocking buffer was then replaced by recommended dilutions of primary antibodies in TTBS and incubated for 1hour at room temperature in orbital shaker. The membrane was vigorously washed four times with TTBS for 15minutes each at room temperature. Further the membrane was incubated in recommended concentrations of HRPO labeled secondary antibodies in TTBS for 1hour at room temperature on orbital shaker. The membrane was again washed vigorously four times with TTBS at room temperature and developed using Immobilon Western (Miilipore, cat#P90719). The membrane was exposed to X-ray film in dark room and developed using Optimax X-ray film processor (Protec).

4.13.3 Densitometry analysis

Wherever required, the densitometry analysis was done on immunoblot and PVDF membrane to determine their mean intensities using ImageJ software. For native proteins mean intensity of immunoblot was normalized with the PVDF membrane; and, for phosphorylated forms mean intensity of immunoblot was normalized with immunoblot of

native proteins. The resulted value was used to express their mean relative levels in resection margin and tumor.

4.14 Enzyme Activity Assay

4.14.1 HAT and HDAC activity assay

Nucleo-cytosolic fractions from tissues and cell lines were estimated and 50 µg of protein was used for calorimetry based HAT activity assay (Biovision, K332-100) and HDAC activity assay (Biovision, K331-100) as per the manufacturer's instructions. Experiment was done in duplicate and average absorbance was plotted.

4.15 Drug and DNA Interaction Assay

4.15.1 Quantification of DNA bound chemotherapy drugs

AGS cells treated with chemotherapy drugs (Cisplatin, Oxaliplatin or Epirubicin) with or without different combination to HDAC inhibitors (VPA, TSA or SAHA) are washed in chilled PBS. Obtained cell pellet from one 90 mm dishes was lysed in 1 ml of chilled nuclei isolation buffer (10 mM HEPES ph7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% v/v NP-40, 2 mM EDTA, 1 mM EGTA, 0.15 mM Spermine, 0.5 mM Spermidine, 1mM Sodium orthovanadate, 10 mM Sodium fluoride, 10 mM β-Glycerophosphate, 0.2 M PMSF). The lysate was centrifuged (5000 rpm for 10 minutes at 4ºC) to obtain nuclei pellet. Nuclei pellet was digested in 200 µl 5M urea and 2M NaCl solution and used to estimate DNA at 260 nm; each sample was further diluted using 5M urea and 2M NaCl solution to make the DNA concentration of all the samples equal. Equal volume of all the samples were taken to measure the concentration of DNA bound Cisplatin, Oxaliplatin and Epirubicin at 220, 205 and 254 nm respectively[161, 162]. The absorbance at the said wavelength was considered to be in direct proportion of amount of chemotherapy drugs bound to DNA.

4.16 Drug Combination Assay

4.16.1 MTT assay with fixed constant ratio

Cisplatin, oxaliplatin, epirubicin, VPA, TSA and SAHA was serially diluted in cell culture media in fixed constant ratio of 1:2. For each drug seven concentration keeping IC_{50} in the middle was calculated (Table 7.1). Using these concentrations MTT assay (as described in section) was done on AGS cells in three different combinations- *''Concurrent''* (24 hours HDACi and chemotherapy drug together), '*Pre'* (24 hours HDACi treatment followed by 24 hours chemotherapy drug treatment) and '*Post'* (24 hours chemotherapy drug treatment followed by 24 hours HDACi treatment) . In the end of the treatment (48 hours) percentage of cell survival was calculated.

4.16.2 Fraction affected (FA) curve analysis

Fraction affected (FA) curve is a method for growth inhibition analysis on any kind of treatment to the cells. For FA curve analysis, cell survival percentage values obtained through MTT assay was used to calculate Fraction affected (FA) values using the formula, FA value= 1−(% cell survival/100). FA values range from 0.01 to 0.99; and, FA values 0.5, 0.75 and 0.95 represents drug dose at with 50%, 75% and 95% cell death is observed respectively. Further, with the help of software compusyn which works on Chau Tally's algorithm[163]. FA values and respective dose of the drug were used generate FA curve.

4.16.3 Median effect plot analysis

Median effect plot shows combination index (CI) on Y-axis and FA values on X-axis. For a particular FA value CI value ranges from 0 to 1; CI < 0.8, CI= 0.8-1.2 and CI > 1.2 represents synergistic, additive or antagonistic nature of drug combinations respectively. FA values and total dose of drug combinations (Chemotherapy drugs and HDACi) were

used to generate median effect plot with the help of software compusyn which works Chau Tally's algorithm[163].

4.17 Statistical Analysis

4.17.1 Statistics for relative level analysis

To test the statistical significance of paired and unpaired resection margin and tumor tissues Wilcoxon matched pair and Mann-whitney test was used respectively. Wherever applicable, data is presented as mean ± SE and $P < 0.05$ was considered as statistically significant.

4.17.2 Statistics for clinicopathological correlations

To establish statistical correlation between clinicopathological parameters and β-actin expression level Mann-whitney and Krukal-wallis test with two-tailed P-value was applied. To test whether variables differed across groups, we used the Chi-square test. To test the statistical independence and significance of predictors Multivariate survival analysis was performed using the Cox proportional hazard regression model. $P < 0.05$ was considered as statistically significant.

4.17.3 Statistics for survival analysis

Survival curves were plotted using the Kaplan–Meier method, and the significance of the differences between the survival curves was determined using Univariate log-rank test. All p values were two-sided, and $p< 0.05$ was considered significant. All statistical analyses were performed with graph pad and/or SPSS software. Wherever applicable, data is presented as mean ± SE and $P < 0.05$ was considered as statistically significant.

Chapter 5

Histone H3 Serine 10 phosphorylation: Regulation and its correlation with clinico-pathological parameters in gastric cancer

5.1 Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide. Globally, GC ranks fourth and third in terms of incidence and mortality respectively[164]. In India, it is one of the most aggressive cancers ranking third and second in terms of incidence and mortality respectively[7]. Surgery remains the mainstay for cure especially in early cancers, while in locally advanced GC, the addition of perioperative chemotherapy affords a better survival advantage[165]. The NACT facilitates histological tumor regression and thereby increases the rate of curative or pathologically disease-free margin (R0)[166]. The current standard practice in GC is to submit the resected stomach for pathological examination to confirm the diagnosis and stage of the tumour as well as to assess the margins of resection (based on absence of tumor cells using haematoxylin and eosin staining and examination of the stained tissue under the light microscope). A pathologically negative resection / R0 margin affords the best chance of cure in GC with 5-year survival rates for resection margin positive and negative disease being 13 versus 35% respectively[167]. However, despite apparently curative surgery, loco regional recurrence has still been encountered in 87% of GC patients[168] raising the doubt of current pathological techniques used in day to day practice to truly confirm the adequacy of the surgical resection margins. Therefore, there is an urgent need to identify molecular markers and investigate their expression in not only the cancerous tissues, but the surrounding resected (margin) tissue that is apparently free from disease (R0) based on histpathology.

Epigenetic mechanisms like DNA methylation, microRNA, histone variants and histone post-translational modifications (PTMs) play an important role in many biological processes, including cell-cycle regulation, DNA damage and stress response, embryonic development, cellular differentiation. Global disruption of the epigenetic landscape,

resulting in aberrant gene expression and function, is a hallmark of human cancer along with genetic alteartions[169]. Global loss of acetylation of histone H4 at lysine 16 (H4K16ac) and loss of trimethylation of histone H4 at lysine 20 (H4K20me3) were the first histone marks reported to be deregulated in cancer[56]. Over the past decade accumulated evidence indicates towards the association of aberrant histone PTMs, defined as 'histone onco-modifications', provide an independent prognostic information for several cancers, including prostate, kidney, lung, ovarian, pancreatic, esophageal and breast cancers etc[68]. In gastric cancer, high level of histone methylation, H3K9me3 was found to be correlated with lympho-vascular invasion, recurrence and poor survival rate. H3K9me3 was further shown as independent prognostic marker in GC[86]. In addition to their role in disease prognosis, epigenetic alterations, specifically DNA methylation are also reported in field cancerization/defects in various types of cancer, including stomach, liver, colon, lung, breast, kidney, and esophageal[170]. However, relation of histone PTMs between tumor and resection margin and the regulatory mechanism for their alteration is poorly understood in cancer.

In this chapter of the thesis, we aimed to identify most significant and consistent differential histone PTM when tumor and negative resection margin is compared. Further, clinicopathological correlation and regulation the histone marks is studied. After initial screening, phorylation of histone H3 at serine 10 (H3S10ph) was taken-up for a detailed study.

5.2 Results

5.2.1 Level of H3S10ph levels in tumor and resection margin tissues

Histones were prepared from freshly resected paired (n=10) tumor and R0 resection margin (RM) tissues of gastric cancer patients, for a pilot study. Histones and their respective paraffin blocks were subjected to immunoblotting and IHC analysis with site-specific acetylation, methylation and phosphorylation marks of histone H3 and H4 (Appendix, Figure A3.1). H3S10ph showed most significant ($p< 0.001$) and consistent (9/10 patients) increase in tumor compared to resection margin tissues in immunoblot analysis (Figure 5.1A and Appendix, Figure A3.1). Further, the loss of H4K16ac and H4K20me3 is a hallmark of tumor[56]; however, it was not reported in GC. Our immunoblot and IHC analysis confirmed the decrease of H4K16ac and H4K20me3 in GC as well (Figure 5.1A and 5.1B). This confirmed the universality of epigenetic alterations and also validated our histopathological analysis at molecular level that defined tumor and negative resection margin. The status of H3S10ph was further studied in validation set (n= 101) among tumor, PRM and DRM tissues using IHC. IHC showed high level of H3S10ph in tumor compared to both the resection margins (Figure 5.1C). H-score based analysis of frequency distribution of tumor, PRM and DRM tissue samples showed 76, 57 and 42; 19, 40 and 44; 6, 4 and 15 samples with low, intermediate and high level of H3S10ph, respectively (Figure 5.1D). Further, comparison of H-score showed a significant high level of H3S10ph in tumor compared to PRM ($p < 0.001$) and DRM ($p < 0.001$) tissues (Figure 5.1E).

5.2.2 Correlation of H3S10ph levels of tumor, PRM and DRM with clinicopathological variables

H3S10ph levels of tumor tissues showed a significant positive correlation with World Health Organization (WHO) classification ($p= 0.0001$), T stage ($p= 0.005$), pTNM stage

(p= 0.016) and recurrence (p= 0.034). Interestingly, except recurrence, H3S10ph levels of PRM and DRM tissues also showed a significant positive correlation with the same clinical parameters as tumor tissues; WHO classification (p= 0.008 and 0.0001 for PRM and DRM respectively), T-stage (p= 0.001 and 0.003 for PRM and DRM respectively) and pTNM stage (p= 0.015 and 0.037 for PRM and DRM respectively). Only DRM showed significant correlation with recurrence (p= 0.012) (Table 5.1).

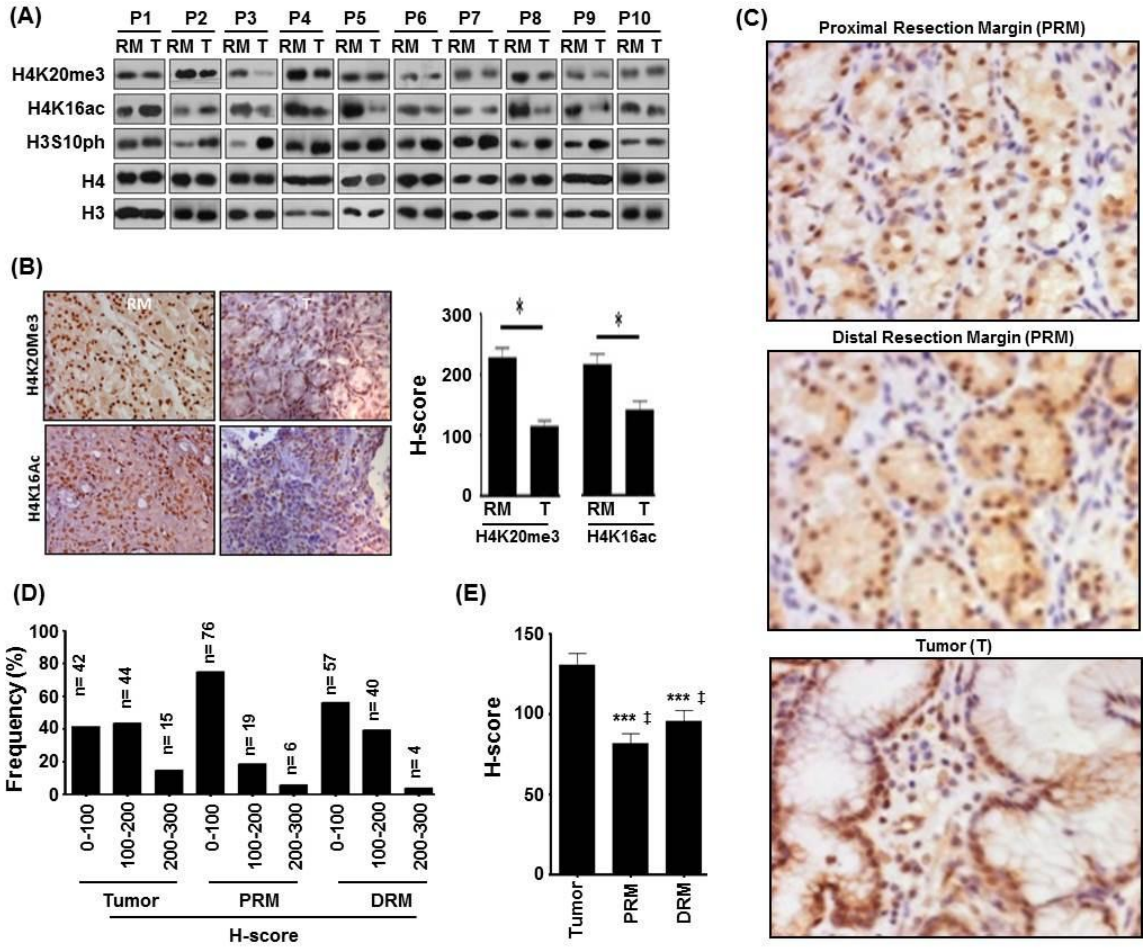

***Figure 5.1: H3S10ph level in Tumor, PRM and DRM tissues in GC**: **(A)** Immunoblot analysis of H3S10ph, H4K16ac and H4K20me3 in freshly resected paired tumor and resection margin tissues (n=10). **(B)** H4K16ac and H4K20me3 immunostaining in paired (n=10) tumor and resection margin tissues (left panel). Mean H-score of immunostaining was compared using Wilcoxon matched pair test (right panel). **(C)** H3S10ph immunostaining in paired (n=101) tumor, PRM and DRM tissues. **(D)** Frequency distribution of tumor, PRM and DRM tissues under low (H-score 0-100), intermediate (H-score 100-200) and high (H- score 200-300) level H3S10ph groups. **(E)** Comparison mean H-score of H3S10ph immunostaining revealed loss of H3S10ph in both PRM and DRM compared to tumor tissues.RM- resection margin, T- tumor, P- patient, PRM- proximal resection margin, DRM- distal resection margin, ‡-Mann-whitney test.*

Table 5.1: Correlation between H3S10 phosphorylation levels of Tumor, PRM and DRM with clinicopathological variables

Total (n= 101)		H3S10 phosphorylation level of Tumor			p-value*	H3S10 phosphorylation level of PRM			p-value*	H3S10 phosphorylation level of DRM			p-value*
		Low (%), n= 42	Inter. (%), n= 44	High (%), n= 15		Low (%), n= 76	Inter. (%), n= 19	High (%), n= 6		Low (%), n= 57	Inter. (%), n= 40	High (%), n= 4	
Age (years)													
	≤ 50	15 (35.7)	18 (40.9)	6 (40.0)	0.879‡	31 (40.8)	6 (31.6)	2 (33.3)	0.734‡	21 (36.8)	16 (40.0)	2 (50.0)	0.849‡
	> 50	27 (64.3)	26 (59.1)	9 (60.0)		45 (59.2)	13 (68.4)	4 (66.7)		36 (63.2)	24 (60.0)	2 (50.0)	
Sex													
	Male	29 (69.0)	32 (72.7)	9 (60.0)	0.652‡	53 (69.7)	11 (57.9)	6 (100.0)	0.148‡	40 (70.2)	26 (65.0)	4 (100.0)	0.343‡
	Female	13 (31.0)	12 (27.7)	6 (40.0)		23 (30.3)	8 (42.1)	0 (0.0)		17 (29.8)	14 (35.0)	0 (0.0)	
WHO classification													
	WD	2 (4.8)	0 (0.0)	0 (0.0)	**0.0001‡**	2 (2.6)	0 (0.0)	0 (0.0)	**0.008‡**	2 (3.5)	0 (0.0)	0 (0.0)	**0.0001‡**
	MD	22 (52.4)	3 (6.8)	0 (0.0)		24 (31.6)	1 (5.3)	0 (0.0)		23 (40.4)	2 (5.0)	0 (0.0	
	PD	16 (38.1)	40 (40.9)	7 (46.7)		44 (57.9)	16 (84.2)	3 (50.0)		29 (50.9)	33 (82.5)	1 (25.0)	
	SRC	2 (4.8)	1 (2.3)	8 (53.3)		6 (7.9)	2 (10.5)	3 (50.0)		3 (5.3)	5 (12.5)	3 (75.0)	
T stage													
	T1	9 (21.4)	4 (9.1)	1 (6.7)	**0.005†**	13 (17.1)	1 (5.3)	0 (0.0)	**0.001†**	11 (19.3)	3 (7.5)	0 (0.0)	**0.003†**
	T2	11 (26.2)	10 (22.7)	3 (20.0)		22 (28.9)	2 (10.5)	0 (0.0)		14 (24.6)	10 (25.0)	0 (0.0	
	T3	16 (38.1)	20 (45.5)	2 (13.3)		26 (34.2)	10 (52.6)	2 (33.3)		23 (40.4)	15 (37.5)	0 (0.0)	
	T4	6 (14.3)	10 (22.7)	9 (60.0)		15 (19.7)	6 (31.6)	4 (66.7)		9 (15.8)	12 (30.0)	4 (0.0)	
Lymph node metastasis									0.385†				
	Absent	20 (47.6)	27 (61.4)	10 (66.7)	0.136†	42 (55.3)	10 (52.6)	5 (83.3)		30 (52.6)	24 (60.0)	3 (75.0)	0.311†
	Present	22 (52.4)	17 (38.6)	5 (33.3)		34 (44.7)	9 (47.4)	1 (16.7)		27 (47.4)	16 (40.0)	1 (25.0)	
pTNM stage													
	I	14 (33.3)	7 (15.9)	1 (6.7)	**0.016†**	20 (26.3)	2 (10.5)	0 (0.0)	**0.015†**	17 (29.8)	5 (12.5)	0 (0.0)	**0.037†**
	II	15 (35.7)	22 (50.0)	6 (40.0)		33 (43.4)	8 (42.1)	2 (33.3)		22 (38.6)	20 (50.0)	1 (25.0)	
	III	13 (31.0)	14 (31.8)	7 (46.7)		22 (28.9)	8 (42.1)	4 (66.7)		17 (29.8)	14 (35.0)	3 (75.0)	
	IV	0 (0.0)	1 (2.3)	1 (6.7)		1 (1.3)	1 (5.3)	0 (0.0)		1 (1.8)	1 (2.5)	0 (0.0)	
Recurrence													
	Absent	32 (76.2)	28 (63.6)	7 (46.7)	**0.034†**	54 (71.1)	8 (42.1)	5 (83.3)	0.351†	43 (75.4)	23 (57.5)	1 (25.0)	**0.012†**
	Present	10 (23.8)	16 (36.4)	8 (53.3)		22 (28.9)	11 (57.9)	1 (16.7)		14 (24.6)	17 (42.5)	3 (75.0)	
Treatment modality													
	Surgery	24 (57.1)	21 (47.7)	12 (80.0)	0.093‡	43 (56.6)	11 (57.9)	3 (50.0)	0.943‡	28 (49.1)	26 (65.0)	3 (75.0)	0.087‡
	NACT + Surgery	18 (42.9)	23 (52.3)	3 (20.0)		33 (43.4)	8 (42.1)	3 (50.0)		29 (50.9)	14 (35.0)	1 (25.0)	

* All three columns are compared in each category,† Chi-square test by two-sided linear-by-linear association, † Chi-square test by two-sided linear-by-linear association
‡ Chi-square test by two-sided Fischer's exact test, Bold indicates values that are statistically significant (<0.05), Int.- Intermediate, PRM- proximal resection margin, DRM- distal resection margin

5.2.3 Correlation of H3S10ph levels of tumor and resection margins with survival

Overall survival (OS) and disease free survival (DFS) rate among groups with low, intermediate and high level of H3S10ph was compared by log-rank test/ Kaplan-meier survival analysis (Figure 5.2).

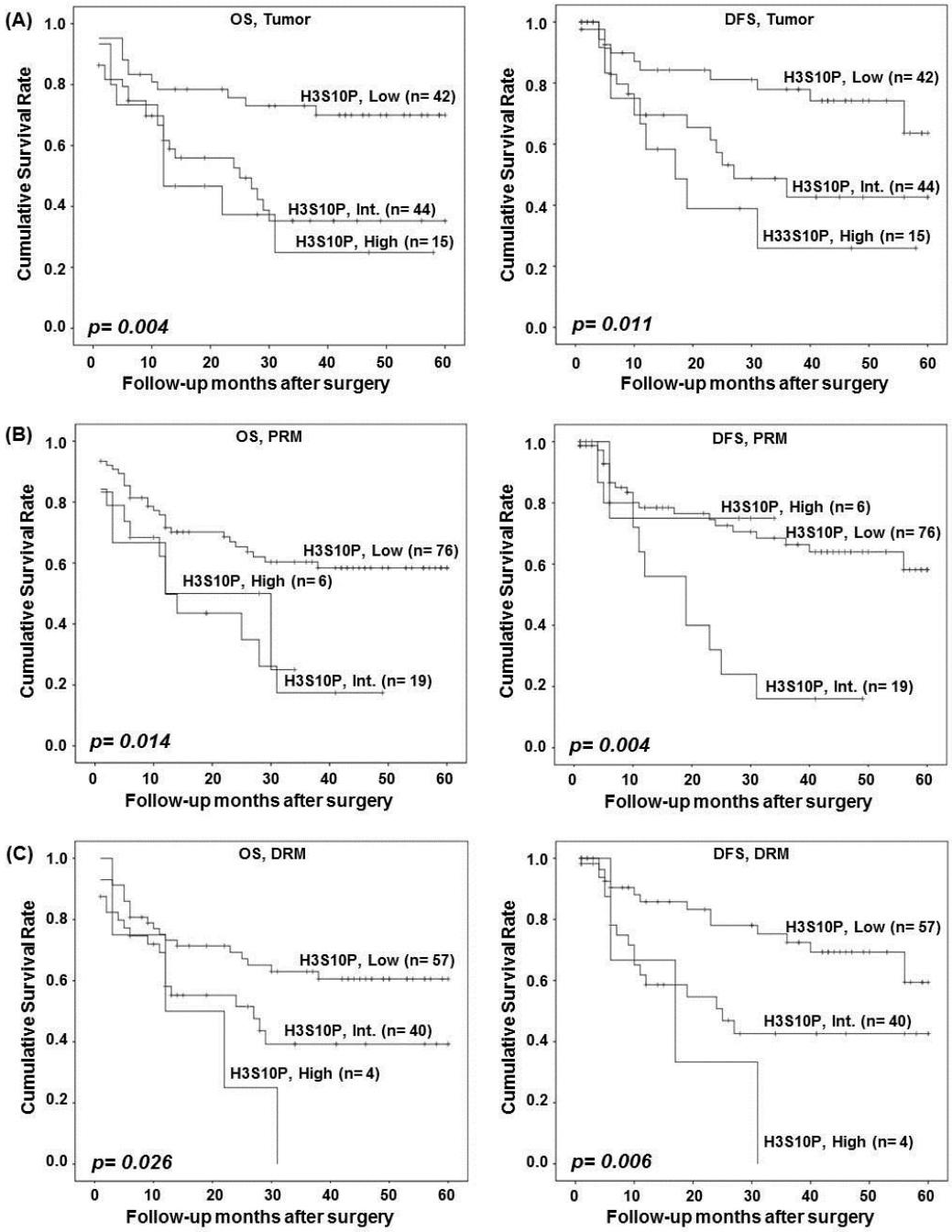

Figure 5.2: Effect of H3S10ph levels of Tumor, PRM and DRM on patients' survival. *Kaplan–Meier survival analysis according to H3S10ph staining H-score. High level of H3S10P of tumor, PRM and DRM is associated with both poor overall survival (OS) and disease free survival (DFS).* ***(A)*** *OS and DFS based on H3S10P levels of tumor tissues* ***(B)*** *OS and DFS based on H3S10P levels of PRM tissues* ***(C)*** *OS and DFS based on H3S10P levels of DRM tissues. Int. – Intermediate, PRM- proximal resection margin, DRM- distal resection margin*

Table 5.2: Survival analysis of variables predicting the risk of death for patients with gastric cancer						
Variables	**Overall survival (n= 101)**			**Disease free survival (n= 101)**		
	Univariate†	**Multivariate‡**	**HR (CI)**	**Univariate†**	**Multivariate‡**	**HR (CI)**
H3S10 phosphorylation status of **Tumor** (Low vs Intermediate vs High)	**0.004**	**0.03**	2.145 (1.067-4.275)	**0.011**	0.411	1.437 (0.605-3.409)
H3S10 phosphorylation status of **PRM** (Low vs Intermediate vs High)	**0.014**	0.567	1.159 (0.700-1.918)	**0.004**	0.353	0.746 (0.402-1.384)
H3S10 phosphorylation status of **DRM** (Low vs Intermediate vs High)	**0.026**	0.592	1.2 (0.615-2.344)	**0.006**	0.402	1.393 (0.642-3.025)
WHO Classification (WD vs MD vs PD vs SRC)	0.707	0.156	0.605 (0.301-1.212	0.362	0.51	1.374 (0.544-3.467)
T stage (T1 vs T2 vs T3 vs T4)	0.062	0.375	0.783 (0.4561.344)	**0.038**	0.495	1.268 (0.641-2.505)
Lymphovascular invasion (Negative vs positive)	**0.011**	0.115	1.719 (0.877-3.3771)	0.137	0.303	1.532 (0.681-3.444)
Treatment Modality (Surgery vs NACT+Surgery)	0.511	0.267	1.414 (0.767-2.604)	**0.023**	0.004	3.197 (1.460-7.002)
pTNM stage (I vs II vs III vs IV)	0.062	0.169	1.614 (0.816-3.191)	**0.038**	0.654	1.214 (0.519-2.842)

† Log rank test, ‡ Cox proportional hazard regression, HR- Hazard ration, CI- 95% confidence interval, Bold indicates values that are statistically significant (<0.05). PRM- Proximal resection margin; DRM- Distal resection margin

Analysis showed a significant negative correlation of H3S10ph levels of tumor (p= 0.004 and 0.011), PRM (p= 0.014 and 0.004) and DRM (p= 0.026 and 0.006) with OS and DFS respectively (Figure 5.2A, B and C). Moreover, H3S10ph levels of tumor, but not the PRM and DRM, were found to be independent predictors of overall survival (Table 5.2). Therefore, data of this and previous sections confirm the association of high level of H3S10ph of resection margins along with tumor tissues with poor prognosis of gastric cancer.

5.2.4 Relation of H3S10ph levels of resection margins and their distance from the site of tumor

Our observation of low level of H3S10ph in resection margin compared to tumor tissues led us to examine whether the decrease had any relation with the distance of resection margin from the tumor. To answer this, we first grouped the resection margin samples as per their distance from tumor site and compared the mean H-score of H3S10ph immunostaining of each group with the mean H-score of tumor samples (Figure 5.3). For both PRM and DRM, a significant reduction in HS10ph ($p < 0.05$) was observed for patient's group with resection margin distance is > 4 cm (Figure 5.3A and B, left panel).

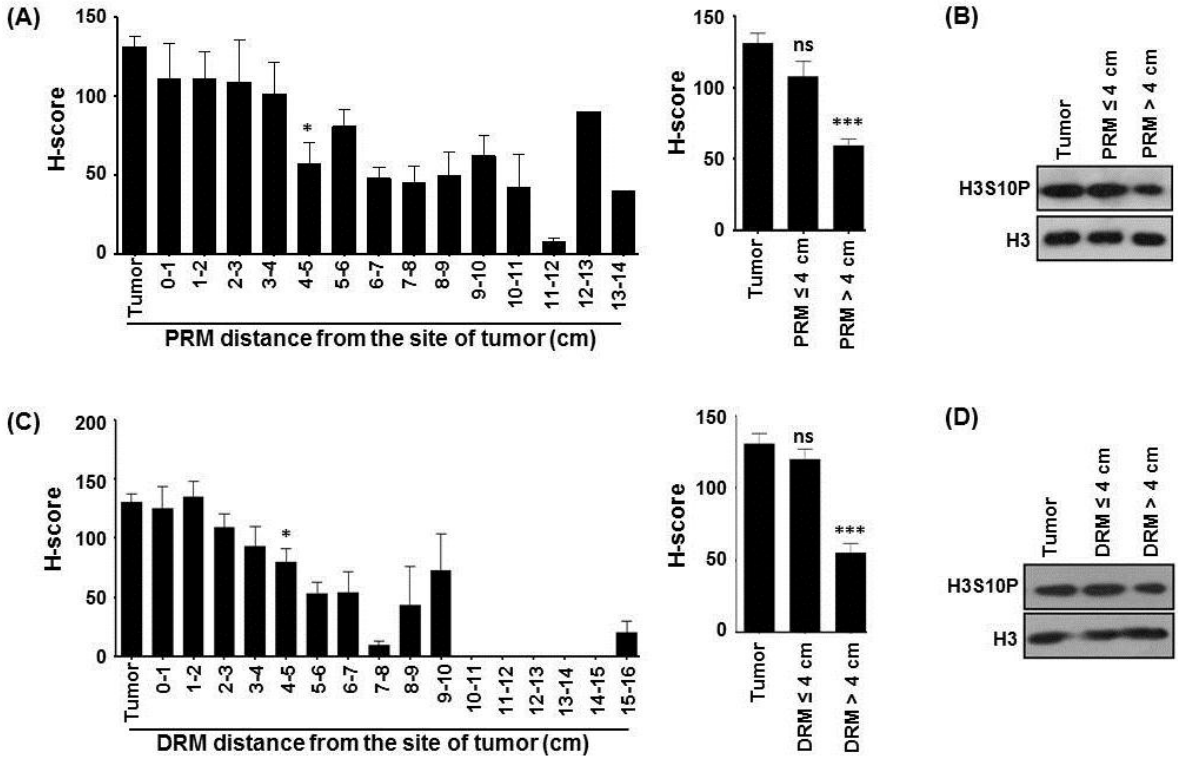

Figure 5.3: Association of H3S10ph with the distance of resection margin. (A) *and* ***(C)*** *To identify the distance of resection margin from where H3S10ph start decreasing significantly compared to tumor tissues, accordingly resection margins were grouped as per their distance from the site of tumor with 1 cm interval and mean H-score (H3S10ph) of each group was compared with tumor. In case of both PRM and DRM, analysis showed a significant decrease in H3S10ph levels as the margin length reaches more than 4 cm (left panel). Comparison of mean H-score of all resection margins with margin distance ≤ 4 cm and >4 cm with tumor confirms the significant reduction of H3S10ph if the margin distance is > 4 cm (right panel).* ***(B)*** *and* ***(D)*** *Confirmation of reduction of H3S10ph, if the margin length is > 4 cm by immunoblotting.*

Further, patients were divided into two groups based on the distance of resection margin- ≤ 4 cm or > 4 cm and their mean H-scores were compared with tumor. Comparison showed H3S10ph levels of resection margins with the distance ≤ 4 cm were almost equal to tumor tissues, however, resection margins with > 4cm distance showed a significant ($p < 0.001$) reduction both in case PRM and DRM (Figure 5.3A and B, middle panel). Additionally, immunoblot analysis also confirmed the reduction of H3S10ph levels of resection margin if the distance is > 4cm from the site of tumor (Figure 5.3A and B, right panel).

5.2.5 Effect of resection margin distance on prognostic value of H3S10ph

To investigate the effect of resection margin distance from the site of tumor on the prognostic value of H3S10ph, its association with clinicopathological variables and survival were compared between the group with the resection margin ≤ 4 cm and > 4 cm. Chi-square analysis showed a positive correlation of H3S10ph levels with WHO classification ($p= 0.001$), T-stage ($p= 0.002$) and TNM stage ($p= 0.023$) for the patients with resection margin ≤ 4 cm. In case of DRM, Chi-square analysis showed a positive correlation of H3S10ph levels with WHO classification ($p= 0.0001$) and T-stage ($p= 0.009$) and recurrence ($p= 0.031$) for the patients with resection margin ≤ 4 cm. For both the resection margins, no correlation was found for patients with > 4 cm resection margin distance (Table 5.3).

In case of OS, patients with PRM ≤ 4 cm showed significant ($p= 0.003$) difference among the group of high, intermediate and low level of H3S10ph (Figure 5.4A) and no difference was observed in case of DRM (Figure 5.4C). However, in case of DFS, distance seems to have no effect as patients with both ≤ or > 4 cm resection showed significant difference in survival among the group of high, intermediate and low level of H3S10ph for both PRM ($p= 0.028$ vs 0.006) and DRM ($p= 0.041$ vs 0.005).

Table 5.3: Correlation between H3S10ph levels of PRM and DRM, ≤ 4 cm vs > 4 cm

Total (n= 101)		H3S10 phosphorylation level of PRM ≤ 4 cm (n= 48)			p-value*	H3S10 phosphorylation level of PRM > 4cm (n= 53)			p-value*
		Low (%), n= 28	Int. (%), n= 14	High (%), n= 6		Low (%), n= 48	Int. (%), n= 5	High (%), n= 0	
WHO classification									
	WD	1 (3.6)	0 (0.0)	0 (0.0)	**0.001‡**	1 (2.1)	0 (0.0)	0 (0.0)	0.632‡
	MD	14 (50.0)	1 (7.1)	0 (0.0)		10 (20.8)	0 (0.0)	0 (0.0)	
	PD	12 (42.9)	12 (85.7)	3 (50.0)		32 (66.7)	4 (80.0)	0 (0.0)	
	SRC	1 (3.6)	1 (7.1)	3 (50.0)		5 (10.4)	1 (20.0)	0 (0.0)	
T stage									
	T1	6 (21.4)	1 (7.1)	0 (0.0)	**0.002†**	7 (14.6)	0 (0.0)	0 (0.0)	0.121†
	T2	10 (35.7)	2 (14.3)	0 (0.0)		12 (25.0)	0 (0.0)	0 (0.0)	
	T3	8 (28.6)	7 (50.0)	2 (33.3)		18 (37.5)	3 (60.0)	0 (0.0)	
	T4	4 (14.3)	4 (28.6)	4 (66.7)		11 (22.9)	2 (40.0)	0 (0.0)	
pTNM stage									
	I	10 (35.7)	2 (14.)	0 (0.0)	**0.023†**	10 (20.8)	0 (0.0)	0 (0.0)	0.068†
	II	10 (35.7)	6 (42.9)	2 (33.3)		23 (47.9)	2 (40.0)	0 (0.0)	
	III	8 (28.6)	6 (42.9)	4 (66.7)		14 (29.2)	2 (40.0)	0 (0.0)	
	IV	0 (0.0)	0 (0.0	0 (0.0)		1 (2.1)	1 (10.0)	0 (0.0)	
Recurrence									
	Absent	20 (71.4)	8 (57.1)	5 (57.1)	0.956†	34 (70.8)	2 (40.0)	0 (0.0)	0.193†
	Present	8 (28.6)	6 (42.9)	1 (16.7)		14 (29.2)	3 (50.0)	0 (0.0)	
Total (n= 101)		H3S10 phosphorylation level of DRM ≤ 4 cm (n= 62)			p-value*	H3S10 phosphorylation level of DRM > 4cm (n= 39)			p-value*
		Low (%), n= 24	Int. (%), n= 34	High (%), n= 4		Low (%), n= 33	Int. (%), n= 6	High (%), n= 0	
WHO classification									
	WD	1 (4.2)	0 (0.0)	0 (0.0)	**0.0001‡**	1 (3.0)	0 (0.0)	0 (0.0)	0.6‡
	MD	10 (41.7)	1 (2.9)	0 (0.0)		13 (39.4)	1 (16.7)	0 (0.0)	
	PD	12 (4.2)	29 (85.3)	1 (25.0)		17 (51.5)	4 (66.7)	0 (0.0)	
	SRC	1 (4.2)	4 (11.8)	3 (75.0)		2 (6.1)	1 (16.7)	0 (0.0)	
T stage									
	T1	5 (20.8)	3 (8.8)	0 (0.0)	**0.009**	6 (18.2)	0 (0.0)	0 (0.0)	0.287†
	T2	6 (25.0)	8 (23.5)	1 (25.0)		8 (24.2)	2 (33.3)	0 (0.0)	
	T3	9 (37.5)	13 (38.2)	3 (75.0)		14 (42.4)	2 (33.3)	0 (0.0)	
	T4	4 (16.7)	10 (29.4)	0 (0.0)		5 (15.2)	2 (33.3)	0 (0.0)	
pTNM stage									
	I	5 (20.8)	5 (14.7)	0 (0.0	0.361†	12 (36.4)	0 (0.0)	0 (0.0)	0.107†
	II	9 (37.5)	17 (50.0)	1 (25.0)		13 (39.4)	3 (50.0)	0 (0.0)	
	III	10 (41.7)	11 (32.4)	3 (75.0)		7 (21.2)	3 (50.0)	0 (0.0)	
	IV	0 (0.0)	1 (2.9)	0 (0.0)		1 (3.0)	0 (0.0)	0 (0.0)	
Recurrence									
	Absent	19 (79.2)	13 (38.2)	1 (25.0)	**0.031†**	24 (72.7)	4 (66.7)	0 (0.0)	0.063†
	Present	5 (20.8)	21 (61.8)	3 (75.0)		9 (27.3)	2 (33.3)	0 (0.0)	

* All three columns are compared in each category,† Chi-square test by two-sided linear-by-linear association, † Chi-square test by two-sided linear-by-linear association ‡ Chi-square test by two-sided Fischer's exact test, Bold indicates values that are statistically significant (<0.05)

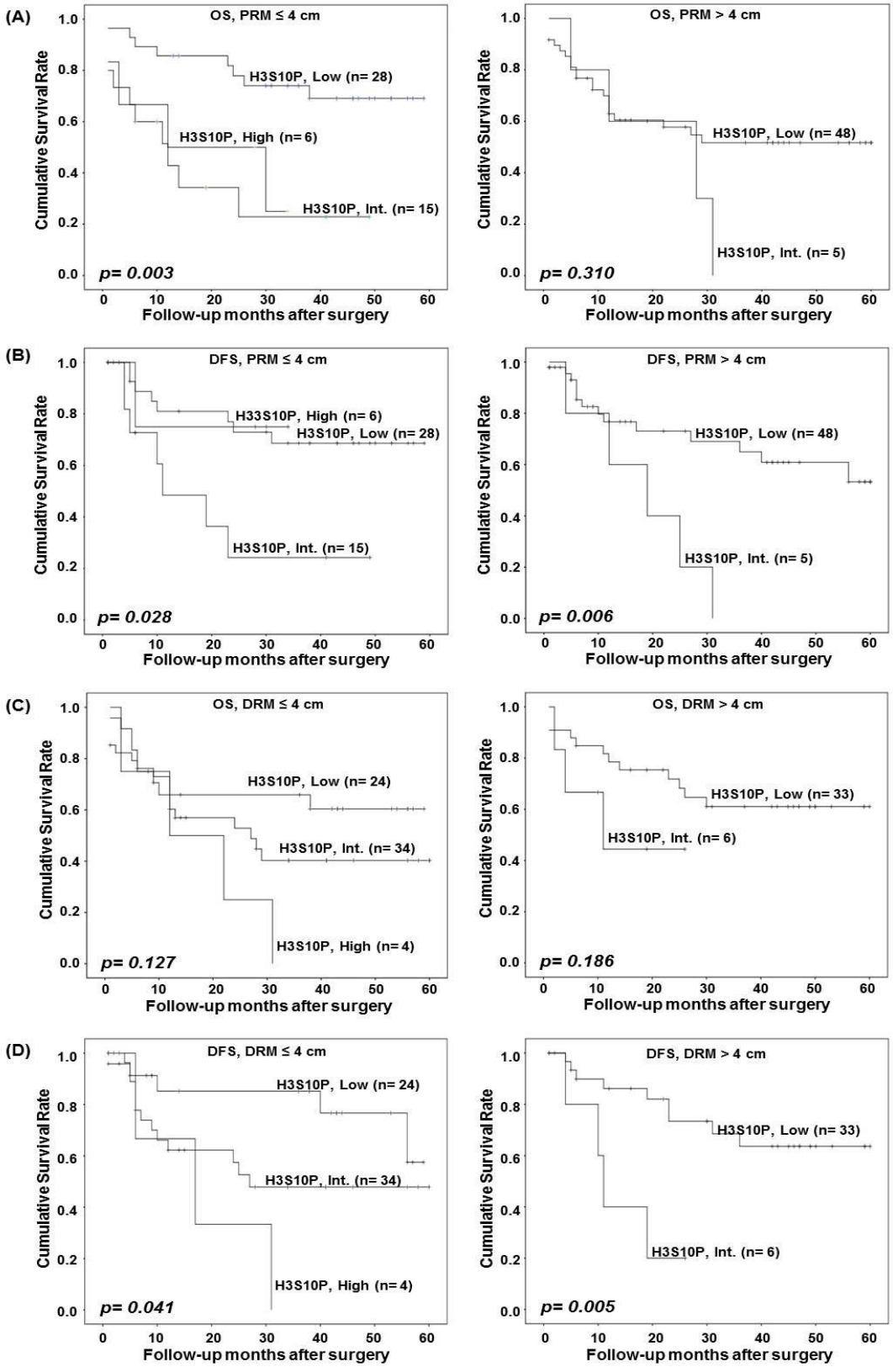

Figure 5.4: Effect of distance of resection margin on patients' survival. *Kaplan–Meier survival analysis according to H3S10ph staining H-score.* ***(A)*** *and* ***(B)*** *Low level of H3S10ph associates with better overall survival (OS) of the patients with PRM ≤ 4cm; however it does not affect disease free survival (DFS).* ***(C)*** *and* ***(D)*** *Low level of H3S10ph associates with better OS and DFS, however, distance does not affects this association.*

Taken together, these data indicate that the distance of resection margin is an important factor in GC prognosis and H3S10ph could be a potential biomarker in predicting the association between distance of resection margin and clinical parameters. However, H3S10ph cannot be used to predict the survival difference based on the distance of resection margin for both PRM and DRM.

5.2.6 Association of increase of H3S10ph with phase of cell cycle in GC

H3S10ph is a very dynamic histone marker and its level changes throughout the cell cycle with the highest level in mitotic, and the lowest level in the interphase of the cell cycle[51, 171]. To determine whether increase of H3S10ph in gastric cancer is dependent on the cell cycle profile of the tissues samples, cyclin levels, mitotic index and cell cycle profile of tumor and resection margin tissues were studied (Figure 5.5). Cyclin B1, D1 and E1 levels are known to peak at the time of G2/M phase transition, mid-S phase and G1/S phase transition, respectively. RT-PCR analysis showed the increase in the mRNA levels of all the cyclins in tumor than the resection margin tissues; however no change were observed in their ratios between tumor and resection margin tissues (Figure 5.5A). Mitotic index also did not show any significant increase in mitotic cells in tumor compared to resection margin tissues (Figure 5.5B). Flow cytometry based cell cycle analysis of tissue samples showed equal percentage of G1, S and G2/M cells in tumor and resection margin tissues with maximum population of cells in G1 phase (Figure 5.5C). These results indicate that the observed increase of H3S10ph in GC is not because of the enrichment of cells in any cell cycle phase in tumor compared to resection margin tissues.

In mitotic phase H3S10ph is associated with chromatin condensation and transcription silencing while in interphase of cell cycle increase of H3S10ph is associated with chromatin relaxation and transcription up-regulation of mainly immediate early (IE) genes[172]. Cell cycle analysis revealed about 80% cells of tumor and resection margin

tissues were in G1 phase (Figure. 5.5C). Therefore, to determine whether increase in H3S10ph in GC is an interphase-associated phenomenon or not, we checked the levels of IE genes (c-jun and c-fos) using RT-PCR and immunoblotting. The data showed increase in the levels of c-jun and c-fos in tumor compared to resection margin tissues (Figure. 5.5D). Therefore, taken together, these data confirm that increase in H3S10ph levels in GC is independent of cell cycle, but an interphase associated phenomenon.

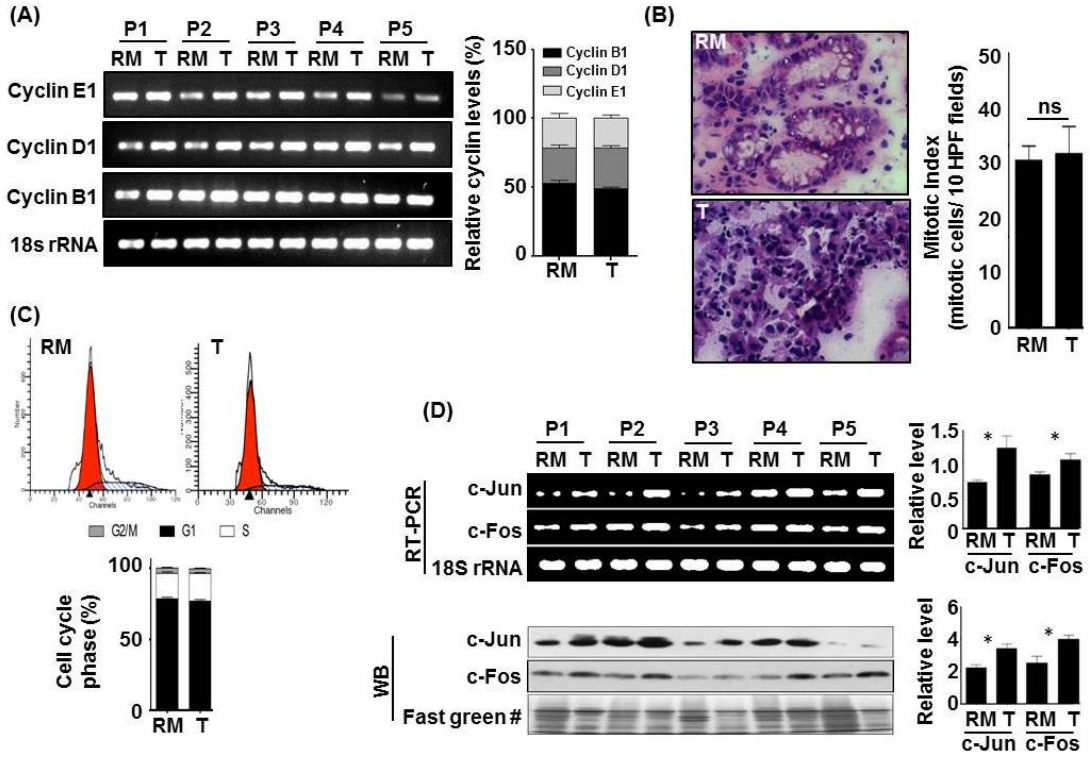

Figure 5.5: Association of H3S10ph with cell cycle profile of gastric tumor and resection margin tissues. (A) *RT-PCR analysis shows high mRNA level of cyclin B1, D1 and E1 in tumor than resection margin tissues (left panel). mRNA level of cyclins were normalized with 18s rRNA, combined % was calculated for each cyclin in tumor and resection margin tissues and their relative % levels were compared showing no significant difference in cell cycle profile of tumor and resection margin tissues (right panel).* ***(B)*** *Arrow heads showing mitotic cells in H&E stained resection margin and tumor tissue sections (left panel). On H&E stained tissue sections mitotic index was calculated for paired samples (n=40) and compared between tumor and resection margin tissues showing no significant difference (right panel).* ***(C)*** *FACS based cell cycle profile showed most of the cells of both tumor and resection margin tissues are in G1 phase (upper panel). Comparison of mean % (n=10) of G2/M, G1 and S phase of cell cycle showed no difference in cell cycle profile of tumor and resection margin tissues (lower panel).* ***(D)*** *RT-PCR (left upper panel) and immunoblot (left lower panel) analysis showed high level of immediate early genes, c-jun and c-fos in tumor than resection margin tissues. After normalization, comparison of relative level also showed significant increase of c-jun and c-fos in tumor tissues, both at transcript (right upper panel) and protein (right lower panel) level.* *Wilcoxon matched pair test.*

5.2.7 MSK1 phosphorylates H3S10 through p38-MAPK pathway in GC

Several kinases are known to phosphorylate H3S10[51]; however, only mitogen- and stress-activated protein kinase-1 (MSK1) mediated phosphorylation of H3S10 is known to be involved in cellular transformation[63] which is activated through p38 and/ or ERK1/2 MAP kinase pathway[173]. In addition, overexpression of c-jun and c-fos, as observed in our experiments (Figure. 5.5D) has also been linked to MSK1 mediated phosphorylation of H3S10 at their promoters[172]. Therefore, Levels of ph-MSK1 and ph-p38 and ph-ERK1/2 levels in tumor and resection margin tissues of GC patients were analysed (Figure 5.6A). Immunoblot (Figure 5.6A, upper panel) as well as densitometry analysis (Figure 5.6A, lower panel) showed the increase of ph-MSK1 and ph-p38 levels while ph-ERK1/2 levels decrease in tumor compared to resection margin tissues. Thus, indicating p38 mediated activation of MSK1 in GC. The increase of ph-MSK1 levels in GC was further confirmed by IHC analysis of the same tissues (Figure 5.6B). The observed increase of H3S10ph on overexpression of MSK1 in AGS cells by immunoblot (Figure 5.6C) and moreover, decrease of H3S10ph on H89 mediated biochemical inhibition of MSK1 by immunoblot studies in AGS and KATOII cell lines (Figure 5.6D) and immunofluorescence studies in AGS cells (Figure 5.6E) confirmed MSK1 mediated phosphorylation of H3S10 in GC. Further, immunoblot analysis with specific antibodies showed decrease of ph-MSK1 and H3S10ph only on the treatment of p38 inhibitor (SB203580) in AGS and KATOIII cells but not on the treatment of ERK1/2 inhibitor (PD89059) (Figure. 5.6F). And, immunofluorescence studies on inhibitor treated AGS cells validated that p38 is responsible for phosphorylation of MSK1 in GC. Thus, confirming p38-MAPK/ MSK1 mediated increase of H3S10ph in GC.

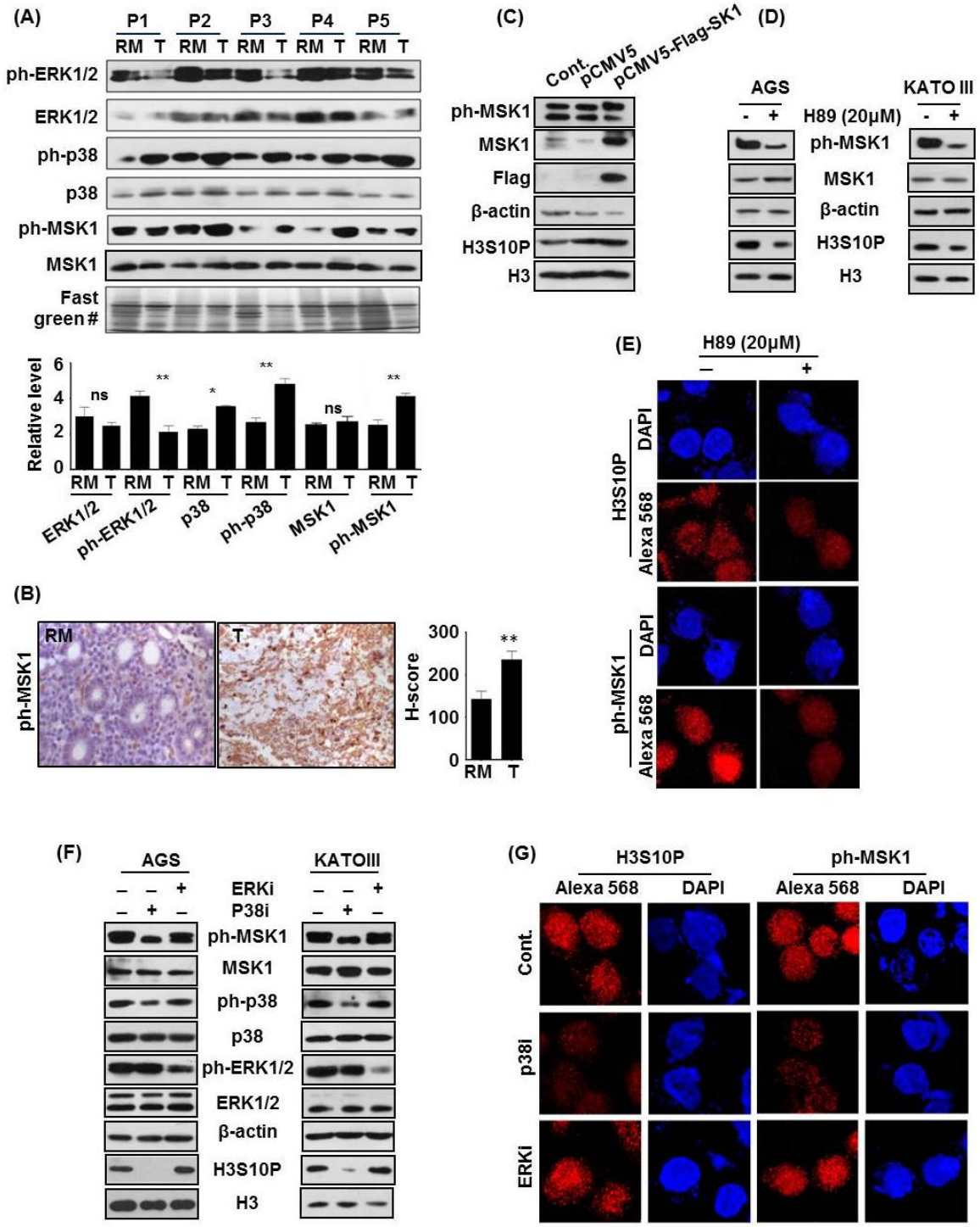

Figure 5.6: Regulatory mechanism for differential levels of H3S10ph in GC. (A) *Immunoblot and its densitometry based analysis of relative levels of ph-MSK1, ph-p38 and ph-ERK1/2 levels in tumor and resection margin tissues.* ***(B)*** *Immunohistochemistry (left panel) analysis in paired tissue samples (n=10) and comparison of their relative H-score of ph-MSK1 levels in tumor than resection margin.* ***(C)*** *Immunoblot analysis of ph-MSK1 and H3S10ph in AGS cells transiently over-expressing MSK1.* ***(D)*** *And* ***(E),*** *Analysis of ph-MSK1 and H3S10ph by immunoblot in AGS and KATOII cells and immunofluorescence analysis of AGS cells after H89 treatment.* ***(F)*** *And* ***(G)*** *Analysis of ph-MSK1 and H3S10ph levels in AGS and KATOII cells by immunoblot and in AGS cell by immunofluorescence after treatment of p38 and ERK1/2 inhibitors.*

5.3 Discussion

Histone post-translational modifications (PTMs) play an important role in the regulation of gene expression, including those involved in cancer development and progression. The histone modifications like acetylation (i.e., H3K9ac, H3K14ac, H3K23ac, H4K5ac and H4K16ac) is generally associated with relaxed chromatin and transcriptional activation, whereas histone methylation is associated with both transcriptional activation (*i.e.*, H3K4, H3K36, and H3K79) and repression (*i.e.*, H3K9, H3K27, and H4K20)[32]. Also, phosphorylation of histone like H3S10ph and H3S28ph are associated with the regulation of proto-oncogenes such as c-fos, c-jun and c-myc[172]. However, despite of more than a decade, no global scale comparative analysis of histone PTM levels for large cohort study with clinical samples has been carried out between tumor and R0 resection margin in gastric cancer tissues. Moreover, studies related to their regulatory pathways are also very limited. Such studies present novel avenues for tackling the significance of global histone modification patterns in human cancer. From this study on human GC, we identified most consistent alteration in H4K16ac, H4K20me3 and H3S10ph in paired tumor and negative resection margin tissue samples (Figure. 5.1A and B). H3S10ph showed highly significant difference between tumor and R0 resection margin. To the best of our knowledge, several cell lines and animal model based studies have shown increase in H3S10ph, as the only histone marker responsible in carcinogenesis and cellular transformation[62, 63, 102, 174]. However, there is no report on its relative level (tumor vs resection margin) and regulatory pathway in GC. Our IHC analysis in paired samples (n=101) demonstrated increase of H3S10ph in gastric tumor compared to both negative PRM and DRM, for the first time. This observation also corroborated with earlier study in nasopharyngeal carcinoma (NPC) where H3S10ph was found to be significantly higher in the poorly differentiated NPC tissues than normal nasopharynx tissues[102]. The histone

modifications, H4K16ac and H4K20me3, reported as hallmarks of tumor[56, 86], also showed the novel finding of decrease in GC compared to resection margin tissues. This observation also validates our histopathological analysis to define tumor and negative resection margin tissues. On further analysis with clinical parameters, we identified that increase of H3S10ph in tumor tissues is a marker of poor prognosis and independent prognostic marker for OS in GC (Table. 5.1, 5.2 and Figure. 5.2A).

Currently, surgery is a main treatment for GC and achieving adequate margin length for R0 resection is a major challenge. With a 9-21% false negative result, palpation, gross inspection and even assessment of tumor and resection margin by frozen section examination are seemingly unreliable methods to judge the adequacy of resection[175, 176]. Studies in esophageal, pancreatic, rectal, soft tissue sarcoma and oral value; however, positive resection margin and its length affects recurrence and survival of patients[177-181]. The alarmingly high loco-regional recurrence rate of gastric cancer in patients with R0 resection[182], which point towards the fact that defined negative resection margin, is not 'true' negative resection margin. In our study H3S10ph of both PRM and DRM showed association with clinical parameters and poorly affects OS and DFS (Table. 4.1; Figure. 5.1B and C). Additionally, H3S10ph levels of DRM showed positive correlation with recurrence were disease reverted back in 75% patients of high level H3S10ph group compared to 42.5% and 24.6% in intermediate and low level of H3S10ph groups, respectively. Our data implicate that high levels of phosphorylation is prognostically relevant. Thus, this study for the first time identified, H3S10ph as a potential molecular marker in predicting prognosis of R0 resected GC patients using their histopathologically confirmed negative resection margins. Further, observed loss of H3S10ph and association with clinical parameters including recurrence for the patient group with the resection margin length > 4 cm (Table. 5.3) in determining the 'true'

negative resection margin in GC. Demarcation of 4 cm as an optimal margin length in our study rationalizes the recommendations of National Comprehensive Cancer Network at molecular level, which state that 'the resection margin of more than 4 cm is necessary to achieve a negative microscopic margin[183]. Therefore, H3S10ph could be helpful in limiting the extent of resection and thereby preventing post-surgery loco-recurrence of disease. The distance dependent relation of H3S10ph with clinical parameters strongly suggests its association with field cancerization defects. Moreover, various epigenetic factors like chromatin state, histone deacetylase, microRNA and DNA methylation and chromatin remodeling factors have shown their involvement in field cancerization in number of cancers including GC[184-187]. The occurrence of such epigenetic field defects may predispose the tissue to go through oncogenic transformation. Further, earlier *in vitro* study has shown that higher level of H3S10ph alone is responsible for cellular transformation. The altered epigenome in the histopathological normal appearing cells may permit for more permissive environment for the growth of newly transformed cells. This may provide a possible explanation for high loco-recurrence after R0 resection. Our results of distance dependent alteration in H3S10ph level of negative resection margin (Figure. 5.3) and its association with clinical parameters provides the first proof of histone PTMs in field effect. However, further profiling studies of early GC lesions will enable us to establish role of H3S10ph in risk assessment and recurrence of GC.

Most of the earlier reports have shown H3S10ph as a better marker for assessing proliferation and mitotic index than Ki-67, and also have established the increase of H3S10ph as a marker for poor prognosis in several cancers including GC[100, 103, 107-109, 188]. However, except glioblastoma study, none of these studies have used paired normal mucosa or negative resection margin along with tumor tissues; therefore, it is difficult to comment on whether the high proliferation and/or mitotic index or G2/M phase cells is

the reason for the elevated level of H3S10ph in cancer. H3S10ph is known to regulate protein–protein interactions to favour chromatin condensation as cells enter the M-phase, whereas, favours expression of immediate early genes in G1 phase of cell cycle. In background of these cell cycle specific functions, our data (Figure. 5.5 A, B and C) have shown no difference in relative cyclin levels, mitotic index and cell cycle profile between tumor and negative resection margin tissues, thus strongly suggesting that the increase of H3S10ph in GC is independent of G2/M cell cycle phase. A recent report have also shown cell cycle independent cigarette sidestream smoke induced increase of H3S10ph leads to the overexpression of proto-oncogenes c-jun and c-fos and tumor promotion[174]. Further, our study also showed presence of maximum percentage of cells in G1 phase of cell cycle (Figure. 5.5C) and overexpression of c-jun and c-fos in tumor than negative resection margin tissues lead us to believe that increase of H3S10ph in GC is a cell cycle interphase-specific phenomenon.

Interestingly, global H3S10ph modification levels were lower in non-malignant resection margin tissue, and increased dramatically in GC. This indicates that the action of the histone modifying enzymes differs in paired R0 resection margin as compared to gastric cancer sample. In our study, cell cycle independent increase of H3S10ph and high expression of IE genes c-jun and c-fos (Figure. 5.5) suggests us to believe that an interphase specific kinase, MSK1 phosphorylates H3S10. Moreover, MSK1 is also the only known kinase of H3S10 whose direct role has been implicated in cellular transformation[171, 172]. This notion was further strengthened by the observed high level of ph-MSK1 (active form of MSK1) in GC tumor tissues (Figure. 5.6A and B). MSK1 is phosphorylated by MAP kinases, ERK1/2 or p38 in context dependent manner[173, 189]. In GC, ph-ERK1/2 reported to have no association with clinical parameters[190]. On the other hand several studies in prostate cancer, breast cancer, bladder cancer, liver cancer, lung

cancer, transformed follicular lymphoma and leukemia have suggested direct role of p38 MAPK in cancer patho-physiological characteristics like proliferation, metastasis and angiogenesis etc[191-197]. Our study demonstrates for the first time that p38 MAPK cascade is responsible for MSK1 mediated H3S10 phosphorylation in gastric or any other cancer (Figure. 5.6). Further, chronic inflammation is a characteristic of GC[198] which manifest itself by overexpression of pro-inflammatory cytokines like IL-1 and IL-6[199]; therefore, along with above stated facts p38 MAPK being a key regulator of inflammatory response[200] justifies p38 MAPK/ MSK1 but not ERK1/2 MAPK/ MSK1 pathway mediated regulation of H3S10ph in GC.

In summary, present study provides the first evidence that p38/MSK1 regulated increase in H3S10ph is strongly correlated with resection margins and concomitantly with patients' prognosis. The MSK1-mediated nucleosomal response via H3S10ph in gastric cancer might be associated with aberrant gene expression. Further, the coherence of H3S10ph in GC with two well-known reported altered histone modifications in human cancers, H4K16ac and H3K20me3 suggests that combination of epigenetic modifications may serve as molecular biomarkers for gastric cancer. Importantly, our data provide a new rationale for using MSK1 as a molecular target to alter the epigenetic landscape in GC.

Chapter 6

β- actin expression and its clinicopathological correlation in gastric adenocarcinoma

6.1 Introduction

In the last section while investigating the regulatory mechanism of H3S10ph in GC using total cell lysate and nuleo-cytosolic fraction we observed a very high level of β-actin in tumor tissues in immunoblot studies. The observation was very consistent and reproducible; therefore, we persuaded this observation to investigate clinicopathological importance of β-actin in GC.

Gastric cancer (GC) incidence and mortality is decreasing over several decades, however, it still remains the fourth most common type of cancer and the second leading cause of cancer related deaths worldwide[6]. In India, there are limited epidemiological studies on gastric cancer which also suffers from the juvenile state of cancer registries and under-reporting of cases. However, similar to global trend, Indian registries have also observed statistically significant reducing trend in stomach cancer cases in last 20-years with approximately 35675 estimated case in 2001; about 3.91% of global incidence[7, 8]. A radical D2 gastrectomy and more recently radical surgery along with perioperative chemotherapy holds the best prospect of a cure in gastric cancer[165, 201]. However, delayed presentation and thus diagnosis owing to the non-specific symptoms often preclude the possibility of a curative surgical resection making palliative chemotherapy and other measures as the treatment mainstay in these patients. The development of chemoresistance[202] is also an increasingly appreciated phenomenon contributing to the poor outcomes in the disease. Therefore, an improved understanding of GC molecular biology to ascertain new potential tumor biomarkers would be useful to guide patient management and develop new therapeutic options is essential.

β-actin is a housekeeping gene and an obligatory part of the cell cytoskeleton. It expresses in almost all eukaryotic cells and is involved in controlling basic housekeeping functions such as development and maintenance of cell shape, cell migration, cell

division, growth and signaling. It also plays a critical role in transcriptional regulation, mRNA transport, mRNA processing and chromatin remodeling[203-205]. Further, β-actin is also one of the most commonly used endogenous reference loading controls in laboratory techniques to normalize gene and protein expressions as it is believed to have constant expression levels in different cellular, experimental and physiological conditions. However, growing evidences have demonstrated its differential expression in certain situations like growth, ageing, differentiation, developmental stages and diseases like asthma, Alzheimer's disease, congenital heart disease and cancer[205].

In comparison to normal, an overall differential expression of β-actin is reported in multiple cancers[206-212]. The methodologies used in earlier tissue based studies make it difficult to answers, which specific cell type out of the heterogeneous population of cells in a tissue, is responsible for altered expression of β-actin in cancer. To date, no histological studies have been conducted to provide informations about the pattern of β-actin expression and distribution in different cell types of the normal and tumor tissues. Such information of β-actin expression in a tissue will provide a better understanding of its role in carcinogenesis, its correlation with clinicopathological parameters and its potential to be used as a tumor biomarker or therapeutic target. β-actin polymerization or remodeling plays a crucial role in a cell's physiology and drugs altering the dynamics of β-actin have been studied as potential chemotherapeutic agent, however, clinical implications of these drugs are yet to be established[213-215].

The present study aimed to provide histological evidence of β-actin expression and distribution in specific cell types of gastric adenocarcinoma and its correlation with clinicopathological parameters.

6.2 Results

6.2.1 Overexpression of β-actin in tumor compared to normal gastric tissue

To detect an overall relative mRNA and protein expression of β-actin between gastric normal and tumor tissues, RT-PCR and western blot were performed on curatively resected fresh tissues from 5 randomly selected gastric cancer patients. Relative β-actin mRNA and protein levels were expressed after normalizing their intensities with the intensity of 18S rRNA and total protein respectively. Intensities were calculated by using ImageJ software[216]. Compared to normal, RT-PCR and western blot analysis showed a significant higher expression of β-actin level in tumor tissues both at mRNA (1.47 ± 0.13 vs 2.36 ± 0.16; $P < 0.001$) and protein level (1.92 ± 0.26 vs 2.88 ± 0.32; $P < 0.01$) as confirmed by paired t-test (Figure 6.1A and B).

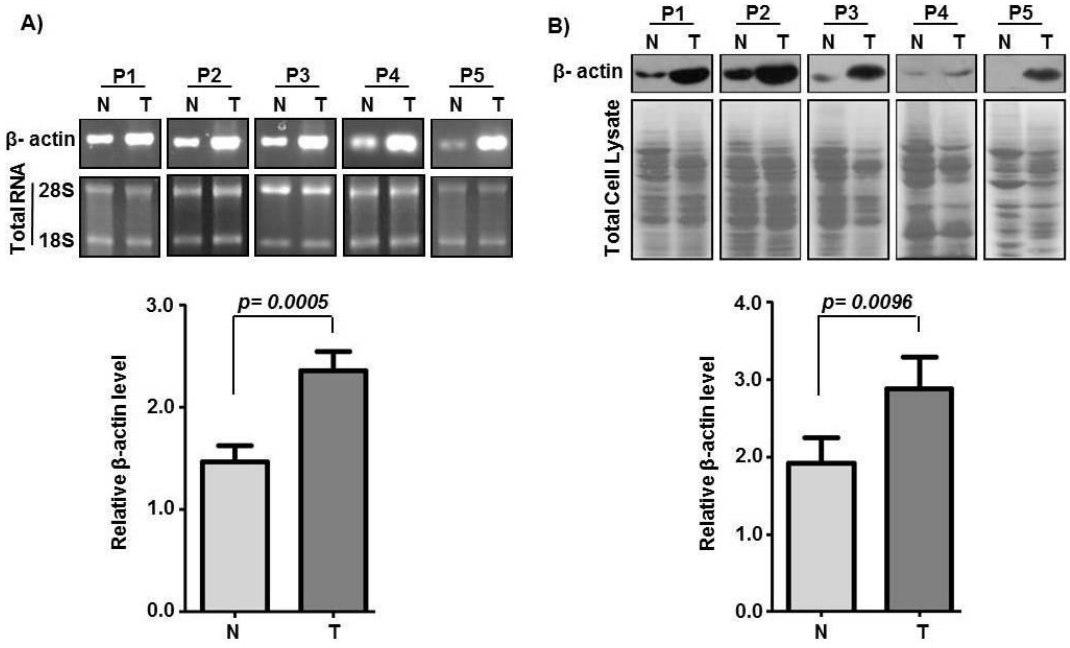

Figure 6.1: Comparison of β-actin level in gastric normal and tumor tissue (n = 5). *A: RT-PCR analysis of β-actin and 18S rRNA was used as an internal loading control (upper panel). Band intensities of β-actin mRNA were normalized with 18S rRNA band intensity of respective lanes and obtained values were plotted (lower panel); B: Western blot analysis of β-actin (upper panel). Band intensity of blot was normalized with the total protein lysate intensity of respective lanes and obtained values were plotted (lower panel). Statistical significance was tested using "paired t-test". N: Normal; T: Tumor.*

6.2.2 Overexpression of β-actin in tumor tissue is predominantly contributed by inflammatory cells

After confirming an overall higher expression of β-actin in tumor compared normal gastric tissues, distribution of β-acting expression was studied in different cell types of the tissues on formalin-fixed paraffin-embedded tissue blocks using IHC. Study was carried out in paired normal and tumor tissues from 24 gastric adenocarcinoma patients. Analysis of immunostained tissue sections revealed that the β-actin immunostaining was majorly distributed between epithelial and inflammatory cells (Figure 6.2A). "Total IHC score" for β-actin immunostaining was calculated for both epithelial and inflammatory cells and frequency of tissue sample for a particular total IHC score was determined (Table 6.2). For both normal and tumor tissues, analysis of frequency table showed that the most of the samples scored low to intermediate "total IHC score" for β-actin immunostaining of epithelial cells while in case of inflammatory cells most of the samples scored Intermediate to high "total IHC score".

Table 6.1: Frequency of samples with respect to total immunohistochemistry score of β-actin

β-actin immune-positive cells in tissues		Total IHC score (*n*= 24)					
		Low, n (%)		Intermediate, n (%)		High, n (%)	
		2	3	4	5	6	7
Epithelial cells	Normal tissue	15 (63)	7 (29)	2 (8)	0 (0)	0 (0)	0 (0)
	Tumor tissue	16 (67)	2 (8)	3 (13)	3 (13)	0 (0)	0 (0)
Inflammatory cells	Normal tissue	0 (0)	0 (0)	3 (13)	7 (29)	3 (13)	11 (46)
	Tumor tissue	0 (0)	0 (0)	1 (4)	0 (0)	4 (17)	19 (79)

IHC: Immunohistochemistry.

Comparison of 'total IHC scores' showed that inflammatory cells express significantly higher level of β-actin compared to the epithelial cells in both normal (2.46 ± 0.13 vs 5.29 ± 0.23, $P < 0.001$) and tumor (2.76 ± 0.24 vs 6.70 ± 0.14, $P < 0.001$) tissues as confirmed by Mann-whitney test (Figure 6.2B). Furthermore, tumor tissues express relatively higher level of β-actin compared to normal in both epithelial and

inflammatory cells, however, difference between epithelial cells was not significant (2.46 ± 0.13 vs 2.79 ± 0.24, P > 0.05) whereas inflammatory cells differed significantly (5.92 ± 0.23 vs 6.71 ± 0.14, P < 0.01) as confirmed by Wilcoxon matched-pair test (Figure 6.2B).

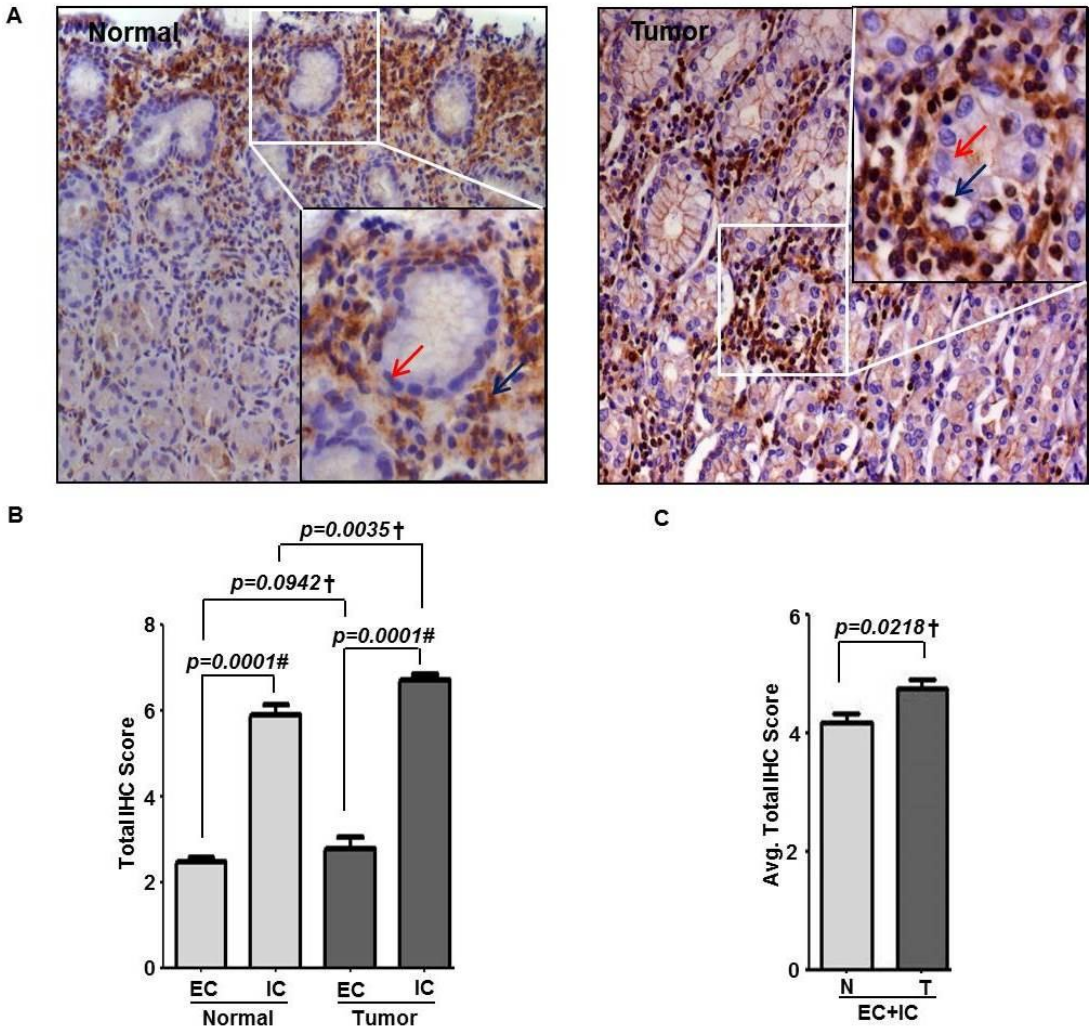

Figure 6.2: Histological analysis of β-actin in gastric normal and tumor tissues (n = 24). *"Total IHC score" and "Average total IHC score" were calculated as described in Table 4.1.* ***A****: Representative pictures of β-actin immuno-staining of normal (left panel) and tumor (right panel) tissues showed β-actin expression is majorly distributed between epithelial (red arrow) and inflammatory (blue arrow) cells. The image is taken at 20 X magnification;* ***B****: "Total IHC score" of EC and IC of normal (N) and tumor (T) tissues were plotted;* ***C****: "Average total IHC score" for normal and tumor tissues were plotted. #Mann-Whitney test; †Wilcoxon matched pair test. IHC: Immunohistochemistry; EC: Epithelial cells; IC: Inflammatory cells.*

As overall β-actin level in a tissue will be a combined result of its expression in all cell types of the tissue, therefore, we asked, whether our IHC analysis corroborates with our RT-PCR and western blot data showing an overall higher expression of β-actin in

tumor tissues? To answer this, we compared "average total IHC score" (average of "total IHC scores" of epithelial and inflammatory cells) of normal and tumor tissue. IHC analysis supports the results of RT-PCR and western blotting and also showed a significant increase of β-actin expression in tumor tissues (4.19 ± 0.15 vs 4.75 ± 0.14, $P < 0.05$) compared to normal (Figure 6.2C).

6.2.3 Correlation of β-actin expression with clinicopathological parameters

A total 26 non-metastatic gastric adenocarcinoma cases were examined and analyzed. Although, only inflammatory cells showed significant increase in β-actin level of tumor tissues; for correlational studies, epithelial cells were also considered because they have also shown an increase in tumor compared to normal tissues (Figure 6.2B). Univariate analysis was performed to correlate "total IHC score" and "average total IHC score" of epithelial and inflammatory cells for β-actin immunostaining with clinicopathological parameters like age, sex, tumor grade, depth of invasion, lymph node status and mode of treatment. The associations between β-actin expression and clinicopathological parameters are shown in Table 6.2. Epithelial and overall level of β-actin did not show any significant correlation with any of the clinicopathological parameters while β-actin level of inflammatory cells showed significant correlation with tumor grade or WHO classification ($P < 0.05$). Further, identification of pattern and statistical significance of β-actin level in inflammatory cells of tumor tissues of different tumor grades: moderately differentiated (MD), poorly differentiated (PD) and signet ring cell carcinoma (SRC) was carried out. The results showed a positive correlation of β-actin level with tumor grade (Figure 6.3A) with significantly higher level in PD (6.25 ± 0.22 vs 6.79 ± 0.21, $P < 0.05$) and SRC (6.25 ± 0.22 vs 6.88 ± 0.14, $P < 0.05$) compared to MD; however, PD to SRC difference was not significant (6.79 ± 0.21 vs 6.88 ± 0.14, $P > 0.05$).

Table 6.2: Univariate analysis of β-actin immunostaining with clinicopathological parameters *n* (%)

Clinicopathological parameters	Groups	N (%)	Epithelial Cells (total IHC score)	Inflammatory cells (total IHC score)	Epithelial + Inflammatory cells (avg. total IHC score)
			***P* value**	***P* value**	***P* value**
Age (yr)					
	≤ 50	11 (42)	0.4933[1]	0.2724[1]	0.2941[1]
	> 50	15 (58)			
Sex					
	Male	20 (77)	0.9721[1]	0.2724[1]	0.5275[1]
	Female	6 (23)			
Tumor grade					
	WD	0 (0)	0.6089[2]	**0.0168[2]**	0.8393[2]
	MD	4 (15)			
	PD	14 (54)			
	Mucinous	0 (0)			
	SRC	8 (31)			
Depth of invasion[3]					
	T1	2 (8)	0.5446[2]	0.6618[2]	0.8804[2]
	T2	2 (8)			
	T3	13 (52)			
	T4	8 (32)			
Lymph Node status[3]					
	N0	6 (24)	0.751[2]	0.6293[2]	0.5426[2]
	N1	8 (32)			
	N2	8 (32)			
	N3	3 (12)			
Treatment Modality[3]					
	Surgery	14 (56)	0.3542[1]	0.8135[1]	0.291[1]
	NACT+ Surgery	11 (44)			

[1]Mann Whitney Test; [2]Kruskal Wallis Test; [3]TNM staging and Treatment modality information was available for only 25 (out of 26) patients. $P < 0.05$ indicates statistically significant difference. IHC: Immunohistochemistry; MD: Moderately differentiated; PD: Poorly differentiated; SRC: Signet ring cell carcinoma.

In addition, low level of β-actin in signet ring cell carcinoma (a type of poorly differentiated cell) cell line KATO III compared to moderately differentiate gastric adenocarcinoma cell line AGS (Figure 6.3B) attracted us to look for the pattern of β-actin expression of tissue epithelial cells with tumor grade. β-actin level in tissue epithelial

cells followed a similar pattern of cell lines and decreases from MD to PD and to SRC (Figure 6.3C), a negative correlation with tumor grade, though insignificant.

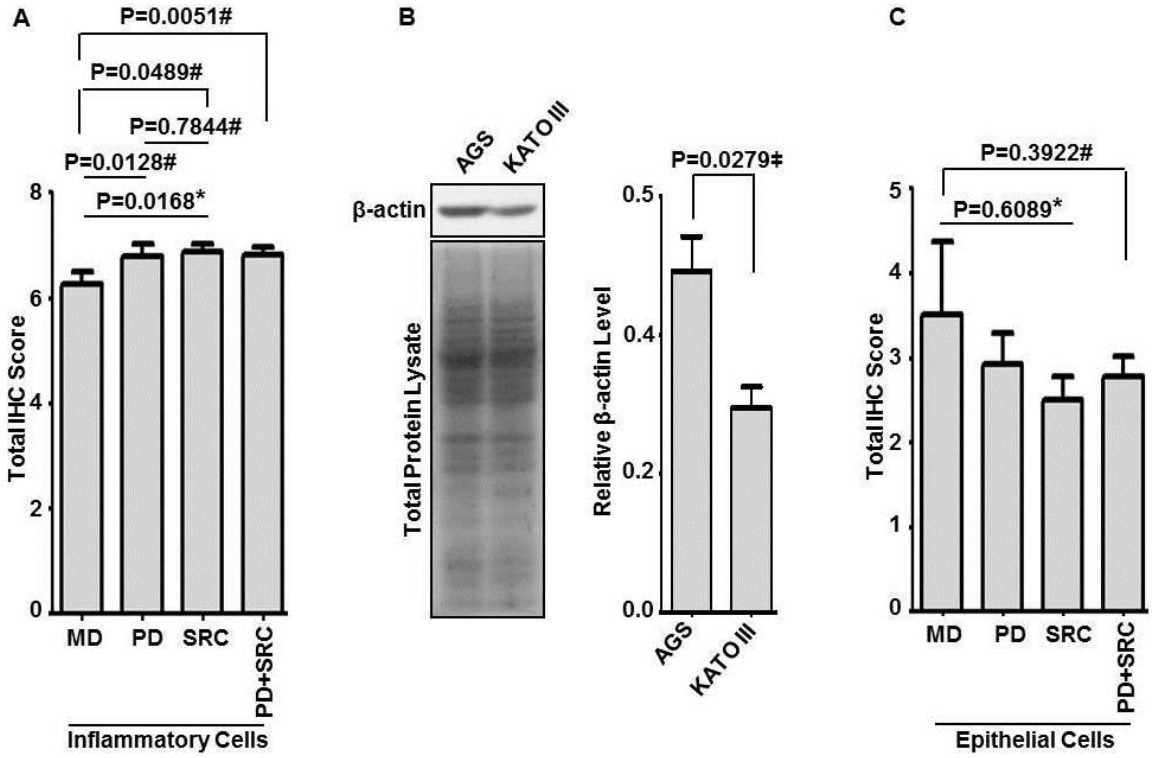

Figure 6.3: Correlation of β-actin expression with tumor grade*. A: "Total IHC scores" of β-actin immunostaining in inflammatory cells were correlated with tumor grade; B: β-actin expression between gastric cancer cell lines AGS and KATO III was analyzed using western blotting (right panel). Blot intensities were normalized with the intensity of total protein lysate of respective lanes and obtained values from three independent experiments were plotted (right panel); C: "Total IHC scores" of β-actin immunostaining in epithelial cells were correlated with tumor grade. #Mann-Whitney test; *Kruskal-Wallis test.*

The SRC is a type of poorly differentiated adenocarcinoma, therefore, SRC and PD was combined together and analyzed for their β-actin expression in epithelial and inflammatory cells compared to MD (Figure 6.3A and 6.3C). The significance of differential expression of β-actin was increased both in case of inflammatory cells (P = 0.0168 to P = 0.0051) and epithelial cells (P = 0.6089 to P = 0.3922), further confirming the association of β-actin expression with tumor grade in gastric adenocarcinoma.

6.3 Discussion

β-actin has been reported to be differentially expressed in multiple cancers[206-211] and suggested as a possible target for chemotherapy[213-215]. These studies signify the potential of β-actin to be considered as a tumor biomarker. Till date, only overall level of varying expression of β-actin in cancer has been reported at the mRNA and protein level by "tissue disruptive techniques", where whole tissue with heterogeneous population of cells crushed and lysed, therefore, observed differential level of β-actin can't be attributed to a specific cell type. The present study, along with tissue disruptive techniques (RT-PCR and western blotting) provides histological evidences (IHC) of differential expression and distribution of β-actin in different cell types of gastric adenocarcinoma.

β-actin overexpression in tumor compared to normal tissues at mRNA level was most consistent and significant as evident by comparing *P*-values of RT-PCR (1.47 ± 0.13 *vs* 2.36 ± 0.16; $P < 0.001$) and western blot (1.92 ± 0.26 *vs* 2.88 ± 0.32; $P < 0.01$) analysis (Figure 6.1A and B). Therefore, the significant overexpression of β-actin at mRNA level in gastric cancer suggests its deregulation at the level of transcription or mRNA turnover. Earlier reports have also shown β-actin overexpression in colorectal, pancreatic, esophageal, hepatic and gastric cancers patients using tissue disruptive techniques. Molecular mechanism of β-actin transcription control is still unclear, however, CpG island hypermethylation of β-actin promoter has been found to be a negative regulator of expression[217]. Further, rapid upregulation in β-actin transcription in response to mitogenic stimuli including epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and platelet derived growth factor[218-220] have also been reported. In addition, miR-145, miR-206 and miR-466a are known to target and degrade β-actin mRNA, therefore, playing a critical role in altering its mRNA turn over[221-224]. Functionally, β-actin plays a predominant role in cell migration as its overexpression is

observed in cells with metastatic potential compared to non-metastatic or cells with less metastatic potential; for example, metastatic variants of human colon adenocarcinoma cell line LS180[211], hepatoma morris 5123[225] and human invasive melanoma cells[226] overexpress β-actin. Thus, our results along with existing literature suggest that β-actin deregulation may have an important role in carcinogenesis.

Immunohistochemistry analysis (n = 24) shows an overall increase (4.19 ± 0.15 *vs* 4.75 ± 0.14, P< 0.05) in β-actin expression in tumor compared to normal gastric adenocarcinoma tissues (Figure 6.2C), this is in conjunction with β-actin profile observed by western blotting (Figure 6.1B). Further, the expression of the β-actin is mainly distributed between epithelial and inflammatory cells of the tissues with significantly higher level in inflammatory cells than their corresponding epithelial cells both in normal (2.46 ± 0.13 *vs* 5.92 ± 0.23, P <0.001) and tumor tissues (2.79 ± 0.24 *vs* 6.71 ± 0.14, P < 0.001) (Figure 6.2A and 2B). Both epithelial and inflammatory cells of tumor overexpressed β-actin compared to normal tissues, however, only inflammatory cells showed significant increase (5.92 ± 0.23 *vs* 6.71 ± 0.14, P < 0.01). The increased expression of β-actin of inflammatory cells is in strong correlation with chronic inflammation in gastric cancer[198] which leads to the homing of large number of inflammatory cells with higher level of β-actin required for immediate cytoskeleton rearrangement for the formation of membrane protrusions at the time of their migration[227-229]. This observation is important as inflammation is a key component of the tumor microenvironment, promotes tumor development and being considered as a hallmark of cancer[230, 231].

Further, univariate analysis showed β-actin level of tumor inflammatory cells positively correlates (P < 0.05) with tumor grade or poorer differentiation of gastric cancer while epithelial cells showed an inverse correlation (P > 0.05) (Figure 6.3A and

C). The insignificant correlation of epithelial cells can be attributed to low number of moderately differentiated gastric adenocarcinoma tissue samples (n = 4) with high range of "total IHC score" (3.5 ± 1.5). This correlation indicates toward an important role of β-actin in tumor dedifferentiation. The chronic inflammation in gastric cancer, predominantly caused by *Helicobacter pylori* infection, is known to promote poorer tumor differentiation and CpG-island hypermethylation[198, 232, 233] and β-actin promoter hypermethylation downregulates the gene expression[217]. Therefore, the positive correlation of β-actin level of tumor inflammatory cells with tumor grade may be due to the persistent inflammation in tumor micro-environment. On the other hand, hypermethylation of β-actin promoter may be a cause of negative correlation of β-actin level of tumor epithelial with tumor grade. Low level of β-actin in gastric adenocarcinoma cell line KATO III (signet ring cell carcinoma, a type of poorly differentiated cell) compared to AGS (moderately differentiated) (Figure 6.3B), further strengthens the observation that β-actin level of tumor epithelial cells negatively correlates with poorer tumor differentiation.

In summary, to the best of our knowledge, present study provides first histological evidence of cell type specific distribution of β-actin in normal and tumor gastric tissues. The significant increase in β-actin expression in tumor tissues is due to inflammation, an initial characteristic in the stage of gastric cancer progression and positively correlates with tumor grade. Therefore, β-actin may represent a promising biomarker in early diagnosis and prognosis of gastric cancer. However, further studies are needed to explore the relationship of cell type specific differential expression of β-actin with its functional implications in carcinogenesis and to be used as a chemotherapeutic target.

Chapter 7

Global hypo-acetylation of histones: Combinatorial effect of HDAC inhibitors with DNA-targeted chemotherapeutic drugs on gastric cancer cell lines

7.1 Introduction

Gastric cancer (GC) is the second leading cause of cancer related deaths in the world and the one of the top lethal cancer in Asia[164]. In India, it is one of the most aggressive cancers ranking third and second in terms of incidence and mortality respectively[7]. The management of gastric cancer is usually a multi-approach involving surgery, chemotherapy and radiotherapy. For operable GC, surgery along with neoadjuvant and adjuvant chemotherapy (NACT and ACT) holds the best prospects of cure[165]. The NACT facilitates histological tumor regression and thereby increases the rate of curative or R0 resection where no residual disease is left behind, whereas, ACT is given to kill the cancer cells if left behind after surgical resection[166]. In case of inoperable GC, chemo and radiotherapy based palliative care is the only treatment. Therefore, in both the cases chemotherapy is a major aspect of GC treatment, mostly given in combination with different drugs. Some of the most commonly used drug combinations are- ECF (epirubicin, cisplatin and fluorouracil), EOF (epirubicin, oxaliplatin and fluorouracil), ECX (epirubicin, cisplatin and capecitabine) and EOX (epirubicin, oxaliplatin, and capecitabine)[202]. In all these combinations, drugs such as cisplatin, oxaliplatin and epirubicin are important part, which exert their cytotoxic effect by DNA intercalation/ binding and thereby causing DNA damage and inhibition of DNA related processes[234, 235]. Based on some of the reports where inhibitors of chromatin remodelers such as Valproic acid and Butyric acid have increased the efficacy of chemotherapy drugs[236-238], it has now been hypothesized that chromatin confirmation affects the amount of DNA bound chemotherapy drugs. Therefore, chemical compounds which can interfere with the activity of chromatin modifiers and alter the dynamics of chromatin confirmation could be of immense potential if combined with conventional DNA binding chemotherapy drugs[239].

Post-translational modifications of histone proteins are one of the major epigenetic mechanisms regulating chromatin confirmations[46]. Acetylation of histones have been most studied and shown to have a positive correlation with chromatin relaxation. Dynamics of histone acetylation is regulated through enzymes, histone acetyl-transferases (HAT) and histone deacetylases (HDAC)[240]. Alteration in the levels of several histone acetylations such as H3K12ac, H3K18ac, H3K9ac and H4K16 has been reported in cancers like liver, kidney, prostate, breast and gastric etc[68]. Moreover, aberrant expression of HAT like CBP and p300, and HDAC like HDAC1 and HDAC2 has been observed in hematological malignancies along with colorectal, gastric, breast, ovarian and epithelial cancers[68]. Such findings have led to the exponential growth in research area of HAT and HDAC inhibitors and their anti-cancer properties. HAT inhibitors like E-7438 and EPZ-5676 are in phase II and in phase I clinical trials respectively while Sodiuam butyrate is in phase II and, Panobinostat and Valproic acid (VPA) are in phase III clinical trials. Additionally, HDAC inhibitors like Vorinostat (SAHA) and Romidepsin are now FDA approved for cancer treatment[241]. These HAT/HDAC inhibitors have shown potential therapeutic benefit in combinatorial chemotherapy than as single agent; however, success is very limited in case of solid tumors[242, 243]. Therefore, in-depth investigations are required to identify the most potential combination and the sequence of HAT/ HDAC inhibitors and chemotherapy drugs for the treatment of solid tumors.

Here, in human GC, we studied histone H3 and H4 acetylation status of tumor and resection margin tissues. Further, we used HDAC inhibitors VPA, TSA and SAHA; and DNA binding chemotherapy drugs Cisplatin, Oxaliplatin and Epirubicin to identify best sequence specific combination for enhanced cytotoxicity of gastric cancer cells.

7.2 Results

7.2.1 Hypo-acetylation in GC associates with high HDAC activity

Histones and nucleo-cytosolic fraction (NCF) were prepared from paired (n=5) tumor and negative resection margin (RM) frozen tissues. Histones were subjected to immunoblot analysis to assess the level of acetylation using anti-acetyl lysine antibodies (Figure. 7.1A).

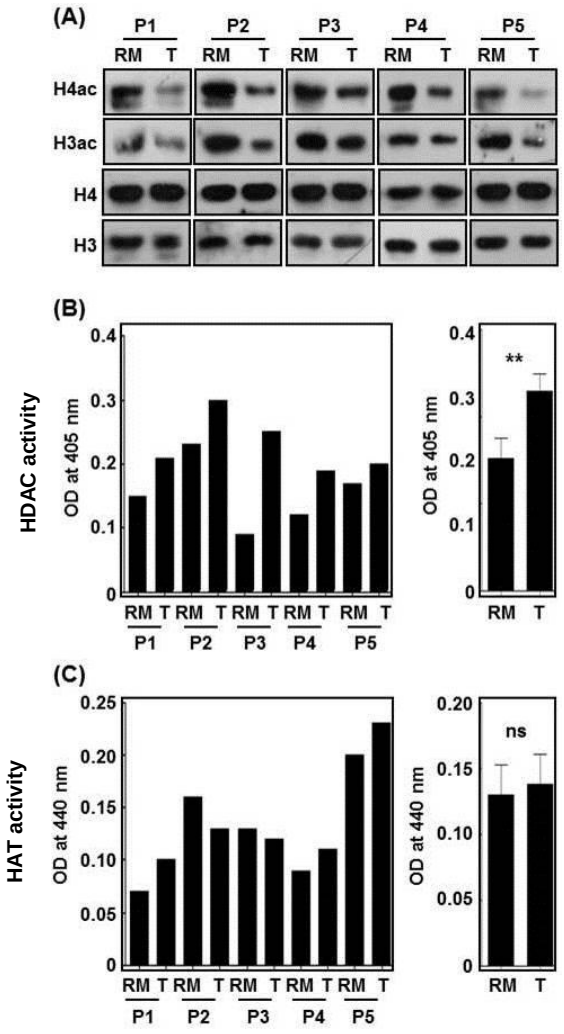

***Figure 7.1: Histone acetylation, HAT and HDAC levels in GC**. **(A)** Immunoblot analysis for the comparison of pan-acetyl levels of histone H3 and H4 between paired (n=5) negative resection margin (RM) and tumor (T) tissues. **(B)** and **(C)** nucleo-cytosolic fraction was used to compare HDAC and HAT levels in paired (n=5) negative resection margin and tumor tissues using calorimetry based assay respectively (B and C left panel). Combined relative levels between negative resection margin and tumor tissues showed high level of HDAC activity but no change in HAT activity in GC (B and C right panel).*

Immunoblot analysis showed low level of histone H3 and H4 acetylation in all the tumor tissues compared negative RM tissues. This observed loss in acetylation level of histone H3 and H4 could be result of low or high level of HAT (histone acetyl transferase) or HDAC (histone deacetylase) activity respectively. Therefore, NCF was used to assess HAT and HDAC activity using calorimetric assay (Figure. 7.1B and C). Tumor and RM tissues showed differential level of HAT and HDAC activity; however, all the tumor tissues showed high HDAC activity (Figure. 7.1B, left panel) compared to their paired RM tissues, but HAT activity (Figure. 7.1C, left panel) did not show any consistent pattern. Further, on statistical analysis showed a significant ($p< 0.001$) high level of HDAC activity in tumor compared negative RM (Figure.7.1B, right panel). However, no significant difference was found in HAT activity (Figure. 7.1C, right panel). Taken together, our data indicates a positive association between hypo-acetylation and HDAC activity in GC.

7.2.2 Dose response of chemotherapy drugs and HDAC inhibitors on GC cells

Dose response curve for chemotherapy drugs (Cisplatin, Oxaliplatin and Epirubicin) and HDAC inhibitors (VPA, TSA and SAHA) was generated using MTT assay (Figure 7.2A). All the experiments were done in duplicate and average values were plotted. Analysis of dose response curve for Cisplatin, Oxaliplatin and Epirubicin showed differential behavior or AGS (well differentiated) and KATOIII (poorly differentiated) gastric adenocarcinoma cells (Fig. 7.2A). AGS showed resistance behavior towards cisplatin, oxaliplatin and epirubicin than KATOIII with less percentage of cell death at all the doses. Moreover, in case of AGS cells, IC_{50} of cisplatin, oxaliplatin and epirubicin was identified as 12µM, 10µM and 0.2µM (200nM) respectively (Figure 7.2A, upper panel), whereas for KATOIII it was 7µM, 8µM and 0.05µM (50nM) respectively (Figure 7.2A, upper panel).

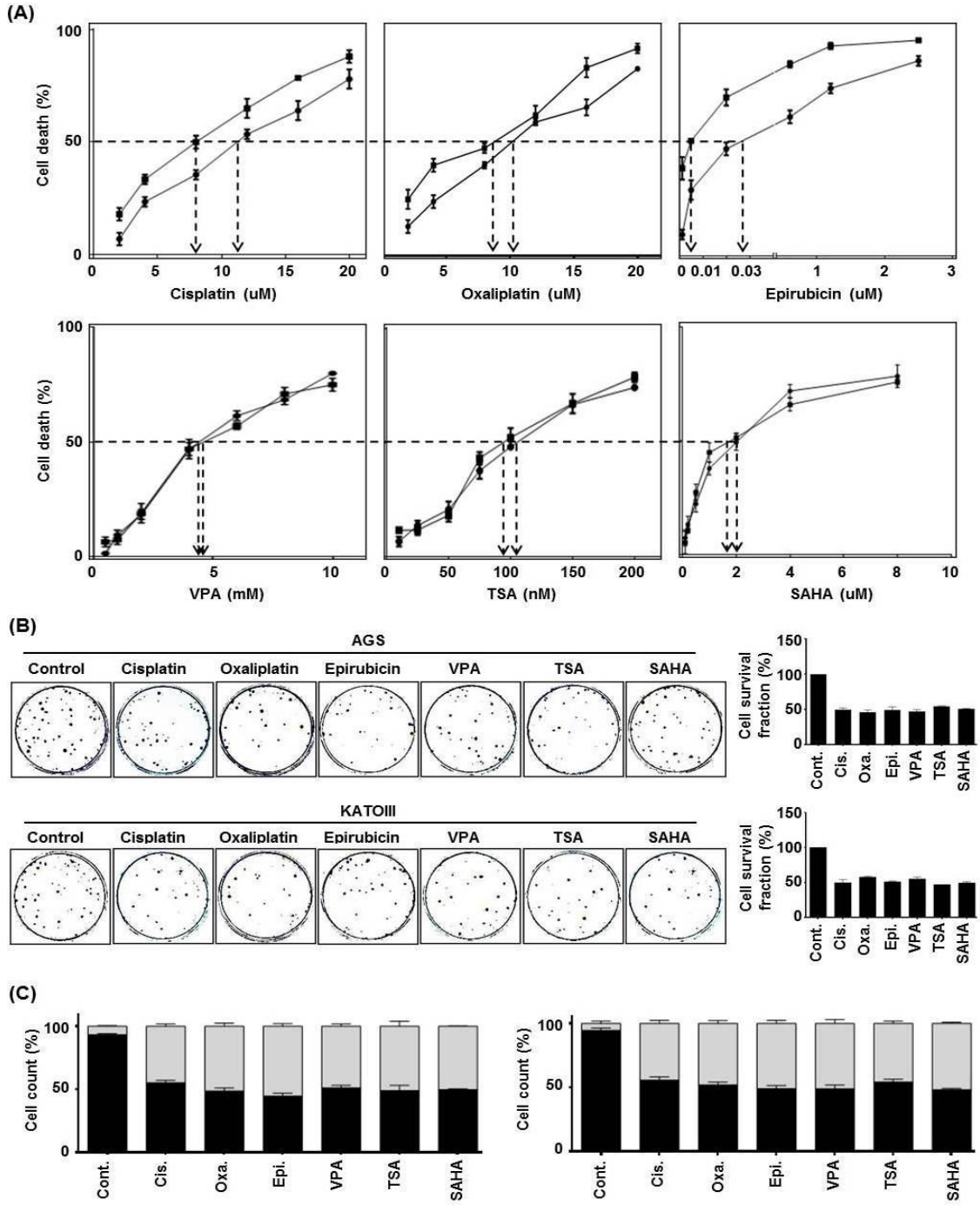

Figure 7.2: Dose response of chemotherapy drugs and HDACi on GC cells. (A) *MTT assay based dose response curve for AGS and KATOII GC cells on treatment of chemotherapy drugs Cisplatin (upper left panel), Oxaliplatin (upper middle panel) and Epirubicin (upper right panel) and HDAC inhibitors VPA (lower left panel), TSA (lower middle panel) and SAHA (lower right panel).* ***(B)*** *Colony formation assay at IC_{50} concentrations of chemotherapy drugs and HDAC inhibitors for AGS (upper left panel) and KATOIII (lower left panel) cells. Experiment was done in triplicates and mean survival fraction was expressed in terms of bar graph for both AGS (upper right panel) and KATOIII (lower right panel) cells. (C) Trypan blue dye exclusion assay at IC_{50} concentrations of chemotherapy drugs and HDAC inhibitors for AGS (left panel) and KATOIII (right panel) cells. Experiment was done in triplicates and mean percentage of live and dead cells are expressed in the form of bar graph.*

On the other hand, both AGS and KATOIII cells showed similar dose response curve upon treatment of VPA, TSA and SAHA with similar IC_{50} concentration as 4000µM (4mM), 2µM and 0.01µM (10nM) respectively (Figure 7.2A bottom panel). IC_{50} concentrations of chemotherapy drugs and HDAC inhibitors were further tested for their effect on proliferation and viability of AGS and KATOIII cells using clonogenic/ colony formation and trypan blue exclusion assay respectively (Figure. 7.2B and C). Comparison of mean of cell survival fraction from three independent clonogenic assay experiments showed IC_{50} concentration of chemotherapy drugs and HDAC inhibitors effectively inhibits approximately 50% proliferation ability of both AGS (Figure. 7.2B, upper panel) and KATOIII cells (Figure. 7.2B, lower panel). Further, trypan blue exclusion assay showed approximately equal mean percentage of live and dead cells after treatment of chemotherapy drugs. HDAC inhibitors at their respective IC_{50} concentrations confirms the results of MTT assay and clonogenic assays on AGS (Figure 7.2C, left panel) and KATOII (Figure. 7.2C, right panel) cells.

7.2.3 HDAC inhibitor mediated hyper-acetylation of histones and cell cycle of GC cells

HDAC inhibitors induce hyper-acetylation. Histone acetylation is closely associated with transcription activation, chromatin relaxation and phases of cell cycle. We assessed the effect of HDAC inhibitors VPA, TSA and SAHA treatment after 24hours on HDAC activity, histone acetylation levels, cell-cycle profile in same population of AGS cells (Figure. 7.3). Calorimetric assay, using NCF showed marked decrease in HDAC activity on treatment of HDAC inhibitors (Figure. 7.3A). Immunoblot data also showed hyper-acetylation of histone H3 and H4 on treatment of HDAC inhibitors (Figure. 7.3B). Moreover, no marked difference in the percentage of cell in G0-G1, G2-M, S phases of

cell cycle was observed among control and cells treated with VPA, TSA or SAHA (Figure. 7.3C).

Taken together, our data confirms that the HDAC inhibitors used in our study are functionally active and their effect on HDAC activity, histone acetylation is not due the change in cell cycle phases.

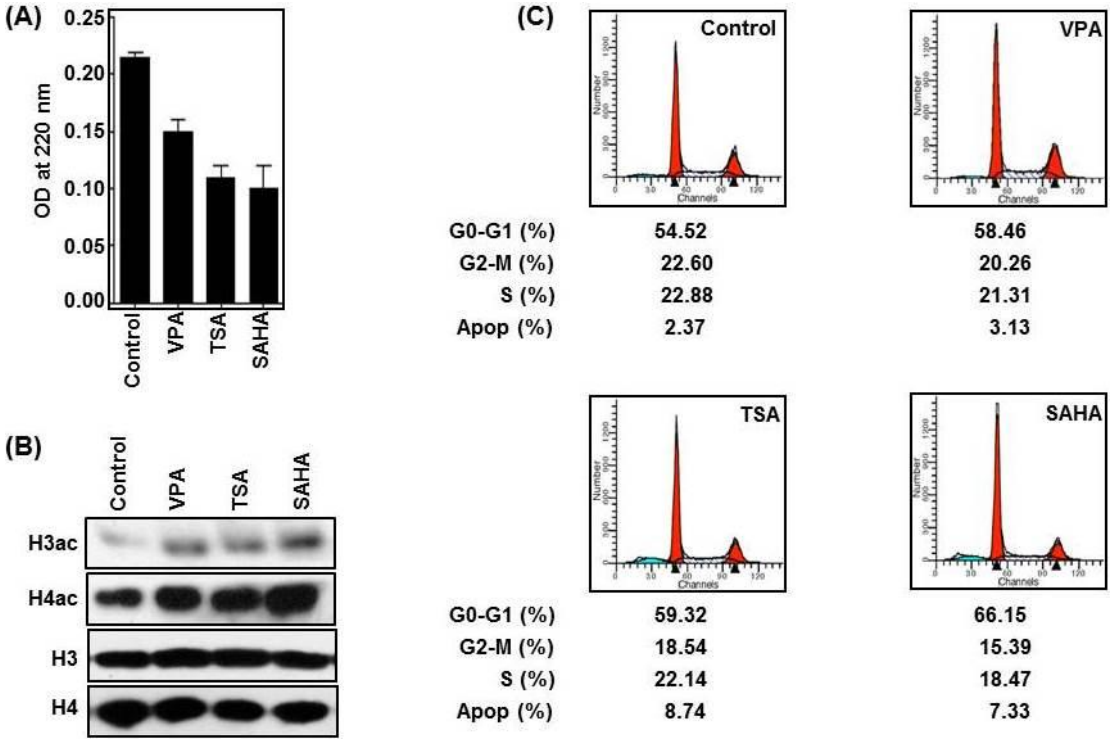

Figure 7.3: Effect of HDAC inhibitors on HDAC activity, histone acetylation and cell cycle. *AGS cells were treated at IC_{50} concentration of HDAC inhibitors (VPA, TSA and SAHA) for 24 hours.* ***(A)*** *Calorimetry based analysis of the effect of HDAC inhibitors on the HDAC activity. Experiment was done in triplicates and mean absorbance is expressed as bar graph.* ***(B)*** *Immunoblot analysis of histone H3 and H4 acetylation levels after the treatment of HDAC inhibitors. (C) FACS based cell cycle analysis of AGS cell after the treatment of HDAC inhibitors.*

7.2.4 Sequence specific effect of HDAC inhibitor treatment on the amount of chemotherapeutic drugs bound to DNA

It has been hypothesized that HDAC inhibitor mediates chromatin relaxation which may enhance the amount of chemotherapy drugs bound to DNA. To test whether this holds true, AGS cells were treated with HDAC inhibitors (VPA, TSA and SAHA) and with chemotherapy drugs (Cisplatin, Oxaliplatin and Epirubicin). Treatment was given at IC_{50}

values (12µM, 10µM and 0.2µM for cisplatin, oxaliplatin and epirubicin; 4000µM, 2µM and 0.01µM for VPA, TSA and SAHA) in three different combinations: *Concurrent* (24 hours HDACi and chemotherapy drug together), *Pre* (24 hours HDACi treatment followed by 24hours chemotherapy drug treatment), and *Post* (24 hours chemotherapy drug treatment followed by 24 hours HDACi treatment).

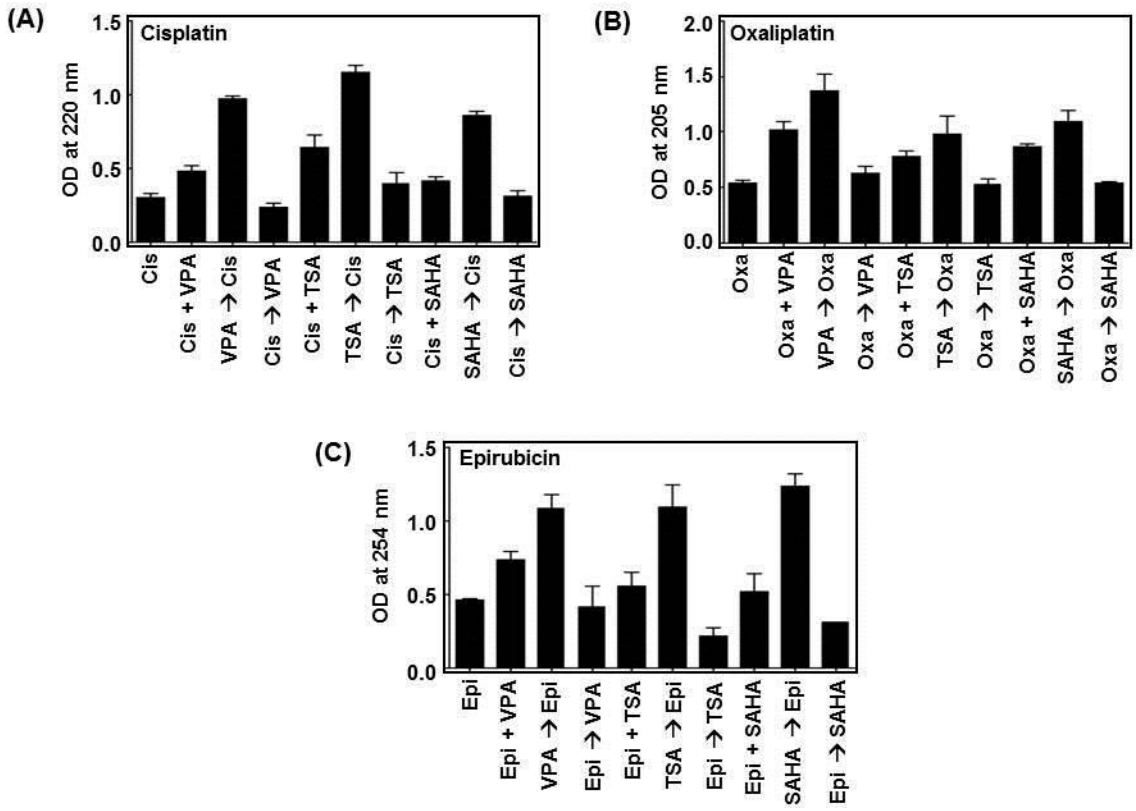

Figure 7.4: Effect of sequence specific HDACi treatment on the amount of DNA bound chemotherapy drugs. *AGS cells were treated with chemotherapy drugs and HDACi at their IC_{50} concentration for 24 hours in three different combinations- concurrent (HDACi+Drug), pre (HDACi→Drug) and post (Drug→HDACi). Experiment was done in triplicate, absorbance was taken for Cisplatin **(A)**, Oxaliplatin **(B)** and Epirubicin **(C)**, normalized with blank and mean absorbance is expressed in the form of bar graph.*

After the said treatments chromatin and nuclear fraction was prepared and amount of DNA bound chemotherapy drug measured using spectrophotometry. Mean absorbance of three independent experiments were plotted for cisplatin (Figure. 7.4A), oxaliplatin (Figure. 7.4B) and epirubicin (Figure. 7.4C). In all the case amount of DNA bound chemotherapy drugs increased in case of concurrent and pre-treatment combination of HDAC inhibitors, whereas, post-treatment combination did not show any difference

compared to control (only drug treatment). Moreover, maximum increase in DNA bound cisplatin, oxaliplatin and epirubicin was observed in case of pre-treatment combination of all HDAC inhibitors VPA, TSA and SAHA.

7.2.5 Sequence specific effect of HDAC inhibitor and chemotherapy drug treatment on dose response curve

Effect of sequence specific HDAC inhibitor treatment on chemotherapy drug mediated cell death was studied using Fraction affected (FA) plot analysis (Figure 7.5). MTT assay was done using seven concentration of chemotherapy drugs (Cisplatin, Oxaliplatin and Epirubicin) and HDAC inhibitor (VPA, TSA and SAHA) calculated on the principle of fixed constant ration(Table. 7.1); in three different combinations- concurrent, pre and post.

Table 7.1: Dose for combinatorial treatment of chemotherapy drugs and HDAC inhibitors in fixed constant ratio

Dilution factor →		**1/8 x IC50**	**1/4 x IC50**	**1/2 x IC50**	**IC50**	**2 x IC50**	**4 x IC50**	**8 x IC50**
Dose of single agent (µM)	**Cisplatin (Cis)**	1.5	3	6	12	24	48	96
	Oxaliplatin (Oxa)	1.25	2.5	5	10	20	40	80
	Epirubicin (Epi)	0.025	0.05	0.1	0.2	0.4	0.8	1.6
	VPA	500	1000	2000	4000	8000	16000	32000
	TSA	0/25	0.5	1	2	4	6	16
	SAHA	0.00125	0.0025	0.005	0.01	0.02	0.04	0.08
Dose of combined agents (µM)	**Cis. and VPA**	501.5	1003	2006	4012	8024	16048	32096
	Cis. and TSA	1.75	3.5	7	14	28	54	112
	Cis. and SAHA	1.50125	3.0025	6.005	12.01	24.02	48.04	112
	Oxa. and VPA	501.25	1002.5	2005	4010	8020	16040	32080
	Oxa. and TSA	1.5	3		12	24	46	96
	Oxa. and SAHA	1.25125	2.5025	5.005	10.01	20.02	40.04	80.08
	Epi and VPA	500.025	1000.05	2000.1	4000.2	8000.4	16000.8	32001.6
	Epi and TSA	0.275	0.55	1.1	2.2	4.4	6.8	17.6
	Epi and SAHA	0.02625	0.0525	0.105	0.21	0.42	0.84	1.68

Experiment was done in triplicates and average readings were used for FA plot analysis. Analysis of FA plot (Figure 7.5) showed pre- treatment of all three HDAC inhibitors VPA, TSA and SAHA leads to more cell death compared to concurrent or post-treatment combinations with Cisplatin (Figure 7.5A), Oxaliplatin (Figure 7.5B) and Epirubicin (Figure 7.5C).

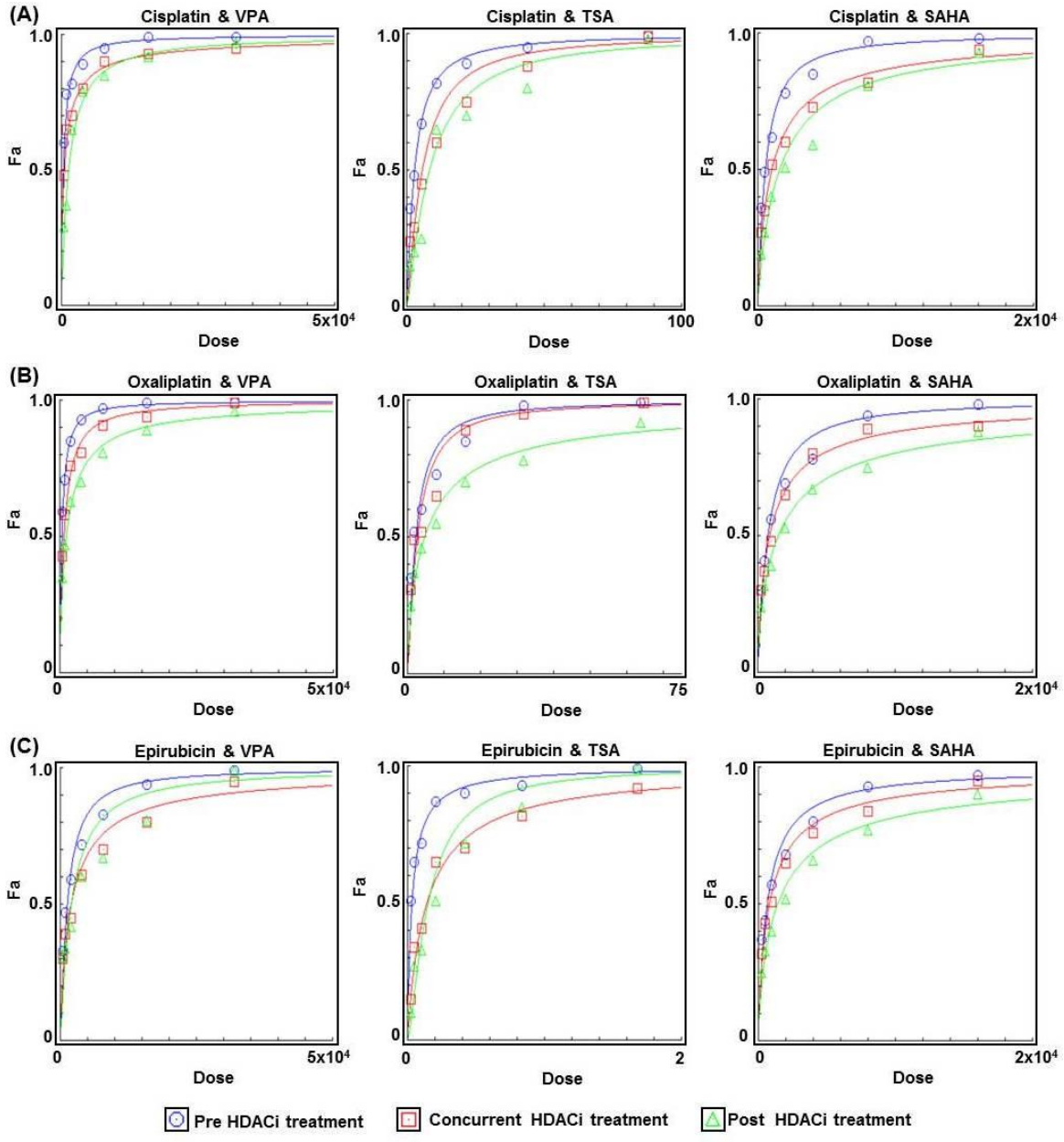

Figure 7.5: Fraction affected (FA) plot analysis*. AGS cells were treated for Chemotherapy drugs (Cisplatin, Oxaliplatin and Epirubicin) and HDAC inhibitors (VPA, TSA and SAHA) for 24 hours each in three different combinations- concurrent (HDACi+Drug), pre (HDACi→Drug) and post (Drug→HDACi) at the combined dose as mentioned in Table 7.1 and MTT assay was performed.* ***(A)****,* ***(B)*** *and* ***(C)*** *Dose response cure of Cisplatin, Oxlaiplatin and Epirubicin in dfferent combination with VPA (left panel), TSA (middle panel) and SAHA (right panel).*

Further, combined doses of chemotherapy drugs and HDAC inhibitors required to achieve FA 0.5, 0.75 and 0.95 was analyzed (Appendix, Table A2.1). Pre-treatment combination of TSA and Cisplatin required lesser combined dose to achieve FA 0.5, 0.75 and 0.95 compared to both concurrent and post-treatment combinations (Figure 7.5A, middle panel). However, pre-treatment combination of VPA or SAHA and Cisplatin could achieve only FA 0.5 and 0.75 at a lower combined dose than concurrent or post-treatment combinations (Figure 7.5A, left and right panels). In case of Oxaliplatin, pre-treatment combination of all HDAC inhibitors VPA, TSA or SAHA achieved FA 0.5, 0.75 and 0.95 at lower combined dose than concurrent or post-treatment combinations (Figure 7.5B, left, middle and right panel respectively). In case of Epirubicin, pre-treatment combination of VPA, TSA or SAHA required lesser dose at FA 0.5, 0.75 and 0.95 than concurrent or post-treatment combinations (Fig. 7.5C, left, middle and right panel). Hence, the data suggest that pre-treatment combination of HDAC inhibitors is most effective in cell death when combined with chemotherapy drugs.

7.2.6 Sequence specific synergistic effect of HDAC inhibitors and chemotherapeutic drug on GC cell line

In order to assess which combination (concurrent, pre or post) of chemotherapy drugs (Cisplatin, Oxaliplatin and Epirubicin) and HDAC inhibitors (VPA, TSA and SAHA) have a synergistic effect, combined dose of the drugs (chemotherapy drugs and HDAC inhibitors) and FA values obtained in the experiment of previous section through MTT assay on AGS cell were used. Median effect plot was generated and data were quantitatively analyzed using a combination index (CI) based on the Chou-Talalay method[163] by the software compusyn (Figure. 7.6). Further, CI values at FA levels 0.5, 0.75 and 0.95 were analyzed (Figure 7.6 and Appendix Table. A2.1). At FA value 0.5, concurrent and pre-treatment combination of VPA and Cisplatin or Oxaliplatin (Figure.

7.6A and B, left panel) showed synergistic effect, pre-treatment combination of TSA or SAHA and Cisplatin (Figure. 7.6A, middle and right panel) showed synergistic effect and concurrent and pre-treatment combinations of TSA and Epirubicin showed synergistic effect, all other combinations showed antagonistic effect.

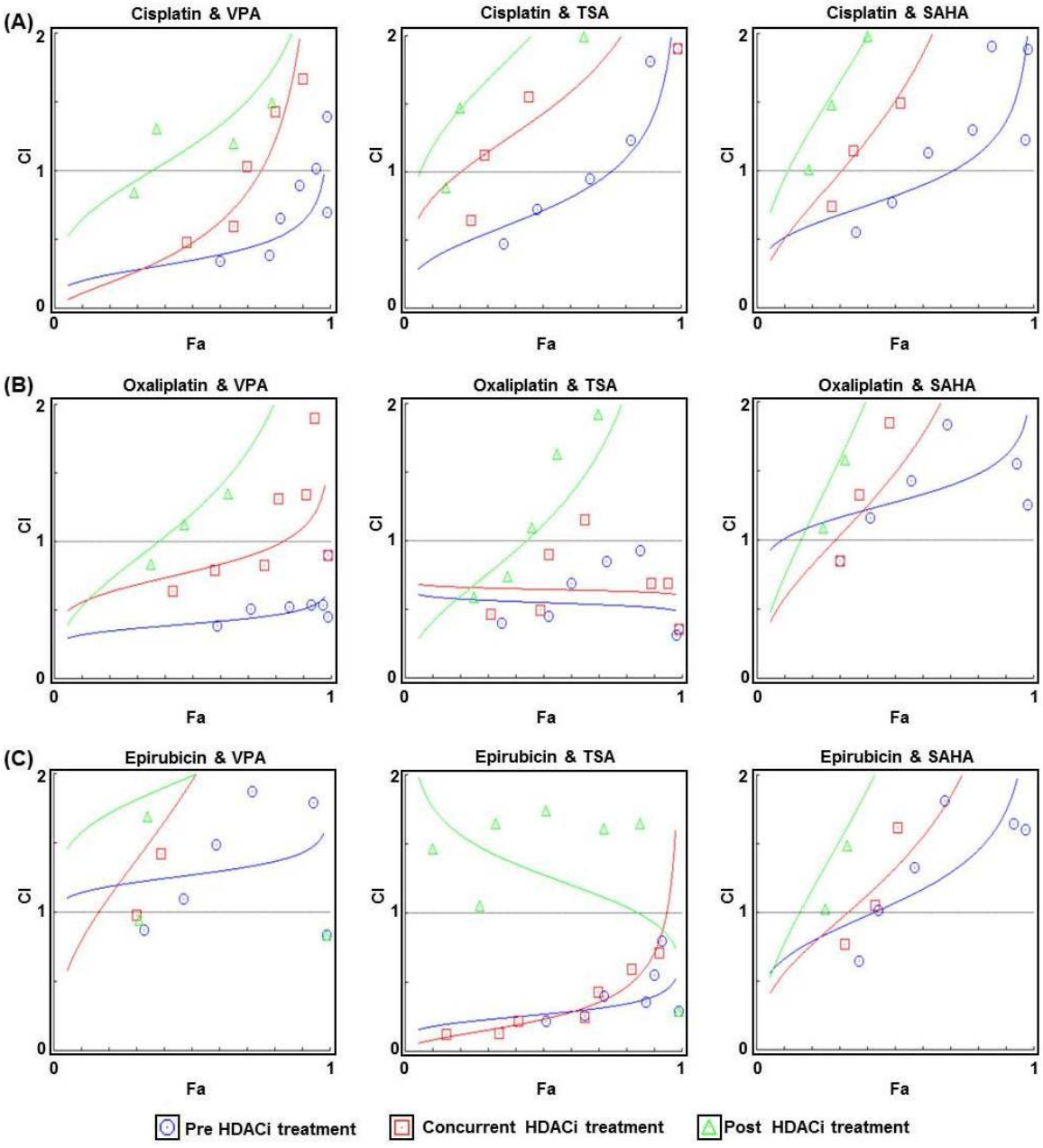

Figure 7.6: Median effect plot analysis. *AGS cells were treated for Chemotherapy drugs (Cisplatin, Oxaliplatin and Epirubicin) and HDAC inhibitors (VPA, TSA and SAHA) for 24 hours each in three different combinations- concurrent (HDACi+Drug), pre (HDACi→Drug) and post (Drug→HDACi) at the combined dose as mentioned in Table 7.1 and MTT assay was performed.* ***(A)****,* ***(B)*** *and* ***(C)*** *median effect plot of Cisplatin, Oxlaiplatin and Epirubicin respective in dfferent combination with VPA (left panel), TSA (middle panel) and SAHA (right panel).*

At FA value 0.75, pre-treatment combination of VPA and Cisplatin or Oxaliplatin (Figure. 7.6A and B, left panel), concurrent and pre-combination of TSA and Oxaliplatin or Epirubicin (Figure. 7.6B and C, middle panel) showed synergistic effect; all other combinations showed additive or antagonistic effect. At FA level 0.95, pre-treatment combination of VPA and Oxaliplatin (Fig. 7.6B, left panel), concurrent and pre-tretment combinations of TSA and Oxaliplatin (Figure. 7.6B, middle panel) and pre-treatment combination of TSA and Epirubicin (Figure. 7.5C, middle panel) showed synergistic effect; all other combinations showed antagonistic effect.

Taken together, the data shows that post-treatment combination of VAP, TSA or SAHA did not have any synergistic effect when combined with Cisplatin, Oxaliplatin or Epirubicin. VPA was found to have more synergistic effect in combination with Cisplatin and Oxaliplatin; however, TSA showed more synergistic active in combination with Oxaliplatin and Epirubicin.

7.3 Discussion

In last decade, the discovery of several histone post-translational modifications (PTMs) and histone modifying enzymes has undoubtedly added to our understanding of epigenetic aspect of cancer biology. Among all histone PTMs, acetylation marks are most studied in cancer for their diagnostic, prognostic and therapeutic potential[68]. Histone acetylations are regulated through the balancing act of histone acetyl-transferases (HAT) and histone deacetylases (HDAC) and have significant effect on modulating chromatin architecture and transcription[240]. Therefore, several HAT and HDAC inhibitors have been identified and a large amount of preclinical in vivo and in vitro data has been gathered on their antitumor properties, opened a new area of cancer epigenetic therapy. As epigenetic therapy, these inhibitors are used to reactivate tumour-suppressor genes restoring the normal function of cells; and, combined with other drugs to increase the efficacy of existing therapies.

In human gastric cancer, we observed the global loss of site specific acetylations (Appendix, Figure A3.1) and pan-acetylation (Figure. 7.6A) of histone H3 and H4 and high level of HDAC activity in tumor compared to normal adjacent mucosa. Our observation corroborates with earlier findings where global loss of histone acetylations such as H4K16ac, H3K9ac, H3K14ac and H3K18ac has been reported in several cancers including prostate, pancreatic, lung, breast and kidney cancers. Earlier studies have also showed high levels of HDACs in number of cancers including gastric, prostate, colorectal, lung, lever, breast and nuroblastoma[68]. Taken together, our and previous studies explain the exponential growth in the area of histone deacetylase inhibitors (HDACi) research for their therapeutic potential. Based on promising preclinical data several HDACi are now being investigated in early phase clinical trials, both as single

agents and in combination with other cytotoxic therapies, showing activity against several malignancies[244].

In solid tumors, studies of HDAC inhibitors as an agent in combination chemotherapy are very limited. Therefore, we investigated the effect of three HDACi- Valproic acid (VPA), Trichostatic A (TSA) and Vorinostat or Suberoylanilide hydroxamic acid (SAHA) when combined with chemotherapy drugs- Cisplatin, Oxaliplatin and Epirubicin on GC cells. Mechanism of action HDAC inhibitors are not very well understood but VPA is class I HDAC inhibitor; whereas, TSA and SAHA are pan-HDAC inhibitors[245, 246]. Chemotherapy drugs used in the study exert their effect mainly by binding or intercalating with DNA, which in turn induces DNA damage and halts DNA replication and transcription[234]. Pre-treatment combination of HDACi and chemotherapy drugs increased the amount of DNA bound cisplatin, oxaliplatin and epirubicin compared to concurrent and post-treatment combinations in AGS cells (Figure 7.4). Moreover, fraction affected (FA) plot analysis also showed low amount of combined dose of HDACi and chemotherapy drug is required to achieve same level of cell death in case of pre-treatment combination (Figure 7.5). Thus, our results suggest that pre-treatment of HDAC inhibitors could be more potent in combinatorial chemotherapy than concurrent or post-treatment combinations. Further, we also showed histone hyper-acetylation of histones without any change in the cell cycle profile on HDACi treatment (Figure 7.3) on AGS cell. Taken together, our data confirm the hypothesis that histone hyper-acetylation associated relaxation of chromatin on HDAC inhibitor treatment facilitates the binding of chemotherapy drugs to DNA. This action of HDAC inhibitors have been thought to enable a reduction in the dose of the chemotherapy drug without compromising cancer cell death. This could also offer the potential for reducing chemotherapy-associated toxicity in gastric cancer.

Increase in cell death on combination of two or more drugs does not form the basis of pre-clinical or clinical studies which can only be taken-up if the combination shows synergistic effect. Encouraged by our results we did median effect plot analysis for concurrent, pre and post combinations of HDAC inhibitors and chemotherapy drugs to most synergistic combination. Any of the post-treatment combinations did not show synergistic effect. All pre-treatment combinations of HDAC inhibitors and chemotherapy drugs showed higher percentage of cell death at low combined doses; however, only VPA-Oxaliplatin and TSA-Epirubicin are found to be best due to their synergistic effect throughout FA values from 0.5 to 0.95. Pre-treatment combination of VPA-Cisplatin also showed synergistic effect but till FA value 0.75; however, TSA-Oxaliplatin showed synergy at higher FA values 0.75 to 0.95. Therefore, our findings suggest pre-treatment of HDAC inhibitors acts more synergistically than concurrent-treatment combinations. This could be explained based on the extra time provided to induce histone acetylation by HDAC inhibitors in pre-treatment combination than concurrent. This notion has been further strengthened by our observation of no synergistic effect of post-treatment combinations and synergistic effect of concurrent-treatment combination (VPA-Cisplatin/ Oxaliplatin and TSA-Epirubicin) at low FA values (0.5 to 0.55). Despite of low percentage of cell death compared to pre-treatment combinations, synergistic effect of concurrent-treatment combinations further establishes the fact that only enhanced cell death in combinatorial chemotherapy cannot guarantee synergistic effect. Taken together, our results establishes VPA as a most potent HDAC inhibitor when combined with platinum based chemotherapy drugs like Cisplatin and Oxaliplatin, whereas, TSA shows more synergistic activity in combination with athracyclin based drugs like Epirubicin.

In conclusion, our results offer a firm rationale for exploring HDAC inhibitors as an epigenetic therapy for gastric as well as other solid cancers in pre-clinical and clinical

settings. Variation in the HDAC and HAT activity in tumor tissues among GC patients as observed (Figure 7.1B and C) suggest the possibility of the failure of HDAC inhibitors in solid tumor chemotherapy as in earlier studies their levels were not checked in cancer patients. Thus, first identifying the levels of HAT/ HDAC in cancer patients and then deciding on the drug accordingly will help us in personalizing the chemotherapy in future. Further, apart from as single agent, HDAC inhibitors can be of immense therapeutic use as part of a combination with other therapeutic modalities, such as chemotherapy, immunotherapy or radiotherapy. Epigenetic therapy might also be useful as a chemopreventive approach, especially for individuals diagnosed with aberrant epigenetic alterations but have not yet acquired neoplastic lesions. Furthermore, with the comprehensive knowledge of mechanistic aspect of HDACs and HDAC inhibitors development of more specific epigenetic drugs are anticipated in the near future.

Chapter 8

Summary and Conclusion

8.1 Summary and Conclusion

Epigenetic mechanisms are essential for normal development and differentiation, but also act in adult organisms, either by patho-physiological state of the cell or under the influence of the environment. Further, it became increasingly evident that epigenetic disruption underlies the development of several human diseases, including cancer. In gastric cancer, with the exception of DNA promoter hypermethylation studies, no other epigenetic mechanism, such as histone post-translational modifications and miRNA have been explored in-depth as a determinant of etiology of disease, clinical implication and regarding their potential importance in therapy. The present study investigated the differential pattern of site-specific histone PTMs with their regulatory mechanism, sequence specific time-dependent potential use of HDACi in combinatorial chemotherapy, and as an offshoot studied in detail an interesting finding on the expression of housekeeping gene, β-actin in gastric cancer.

8.1.1 Salient findings:

1. Histone H3 Serine 10 phosphorylation: Regulation and its correlation with clinico-pathological parameters in gastric cancer.

(i) The significantly ($p < 0.01$) higher level of H3S10ph is observed in tumor tissues compared to histopathologically confirmed R0 resection margins.

(ii) H3S10ph levels of tumor tissues showed a significant positive correlation with World Health Organization (WHO) classification ($p = 0.0001$), T stage ($p = 0.005$), pTNM stage ($p = 0.016$) and recurrence ($p = 0.034$).

(iii) The higher level of H3S10ph in tumor tissues is correlated with poor prognosis of gastric cancer.

(iv) The distance of resection margin is an important factor in GC prognosis and H3S10ph could be a potential biomarker in predicting the association between distance of resection margin and clinical parameters.

(v) p38 MAPK cascade is responsible for MSK1 mediated H3S10 phosphorylation in gastric cancer.

2. β-actin expression and its clinicopathological correlation in gastric adenocarcinoma

(i) Tissue disruptive techniques revealed significant overexpression of β-actin level, at both mRNA and protein level in tumor tissues compared to histopathologically confirmed R0 resection margins.

(ii) Immunostaining studies revealed that β-actin expression is majorly distributed between epithelial and inflammatory cells of the tissues. However, comparative analysis between normal and tumor tissues revealed that both epithelial and inflammatory cells overexpress β-actin in tumor tissues, however, significant difference was observed only in inflammatory cells.

(iii) A positive correlation of β-actin level of inflammatory cells is observed with tumor grade, while epithelial cells exhibited negative correlation.

3. Global hypo-acetylation of histones: Combinatorial effect of HDAC inhibitors with DNA-targeted chemotherapeutic drugs on gastric cancer cell lines

(i) Global loss of acetylation is observed at histone H3 and H4 in tumour tissues compared to R0 resection margins in gastric cancer.

(ii) In gastric cancer tissues, HDAC's are significantly up-regulated, whereas the level of HAT did not show significant alteration suggesting that the observed hypoacetylation is associated with the increase of HDAC.

(iii) The 'pre' treatment of HDAC inhibitors on gastric cancer cell line show maximum cell death, and is associated with significant increases in the binding/intercalation of chemotherapy drugs to DNA.

(iv) The combination index analysis shows that 'pre' treatment synergic effect at the fraction effect (Fa) levels 0.5, 0.75 and 0.9 compared to 'concurrent' or 'post' HDACi treatment.

(v) Dose reduction index analysis also showed the reduction in dose of chemotherapy drugs in combination with HDACi may lead to decreasing the toxicity associated with chemotherapy.

In conclusion, our study has revealed histone hypo-acetylation and hyper-phosphorylation across a large cohort of gastric tumor samples. The identified hyper-phosphorylation of H3S10 correlates with different tumor grades, morphologic types, and phenotypic classes of gastric tumors. Additionally, hyper-phosphorylated H3S10 correlates with distance of resection margins, prognosis and clinical outcome. Further, association of histone hypo-acetylation with overexpression of HDAC enzymes lead to the use of small-molecule, HDACi as epigenetic modulators acting synergistically in a sequence specific pattern along with chemotherapeutic drugs for better management of gastric cancer.

8.2 Future Perspectives:

1. Histone H3 Serine 10 phosphorylation: Regulation and its correlation with clinico-pathological parameters in gastric cancer

   While screening for differential patterns of histone PTMs between tumor and negative resection margin tissue samples from GC patients, significant increase in H3S10ph and decrease in total histone acetylation levels were observed. In future, investigations in three different directions will give further insights to the finds presented in this thesis. First, identification of the genomic regions/ genes which are enriched in H3S10ph using ChIP-seq (Tumor vs RM) and their further validation the GC carcinogenesis. Second, as cross-talk among histone PTMs are at the core of their effect on pahto-physiological characteristics; therefore, in-depth investigation of other histone PTMs, especially acetylation along with H3S10ph is required with respect to carcinogenesis. Such efforts may result in the finding regulatory switch of histone PTMs involved in GC. Third, the regulatory pathway identified for H3S10ph in GC should be explored in future to identify novel targets for cancer therepy.

2. β-actin expression and its clinicopathological correlation in gastric adenocarcinoma

   The findings of the presented study strengthens the area of actin biology and emphasize on the fact that conventional housekeeping genes should not be chosen as internal loading control without validation. This work provides impetus to further study of β-actin expression in different cancers and implicate the findings to understand the role of β-actin in carcinogenesis. It also laid the foundation to find prognostic and diagnostic value of β-actin in cancer along with as a direct or

indirect target for chemotherapeutic intervention similarly as other cytoskeletal element such as microtubules.

3. Global hypo-acetylation of histones: Combinatorial effect of HDAC inhibitors with DNA-targeted chemotherapeutic drugs on gastric cancer cell lines

This part of the study presents encouraging results by in vitro experiments for further detailed study to test the potential of HDAC inhibitors pre and. Or concurrent-treatment combination chemotherapy. In future, animal model based xenograft studies will validate our findings that HDAC inhibitors, specifically VPA and TSA could work in synergy when combine with DNA based chemotherapy drugs. Such investigations will also be helpful in assessing the enhanced cytotoxic effect, reduction in the dose of chemotherapy drugs and associated side effects. Thus, forming a firm rational for investigation of HAT/ HDAC level in GC patients, group them and conduct a clinical trial to test the efficacy of HDACi in GC chemotherapy.

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Appendix

Appendix 1: Informed Consent Form

Informed Consent Form

"Identification of post-translational modifications/ variants of histones for exploitation as biomarker in patients with gastric cancer"

i) Principal Investigator: Dr. Shailesh V. Shrikhande, TMH

Dr. Sanjay Gupta, CRI – ACTREC

ii) Co-Investigator(s) Dr. Parul J. Shukla, Dr. KM Mohandas, TMH
Dr. Shaesta Mehta, Dr. Mukta Ramadwar, TMH

Introduction: You are invited to participate in a study/research/experiment. The purpose of this study is to find the defects that occur in proteins and genes that cause cancer of the stomach. We request you to give consent to use tissue that we are going to remove from your body at the time of your surgical procedure, which will be carried out on you as a part of your treatment. This tissue will be made available for biomedical research to find more about cause of cancer and how to better diagnose, treat, or even cure it in the future. This will not be of any risk to you.

If you agree, a piece of the tumour and the surrounding area along with normal mucosa of the stomach would be resected. The removal of the small portion of normal mucosa may cause inflammation that will heal readily and rapidly.

- The tissue will be used for research at this institution.
- You will not be given the results of any research performed on the tissue.
- The research will not benefit you directly, but may benefit someone like you in the future.
- The researchers who use the tissue may need to know some things about your health before and after surgery (for example; your age, sex, ethnic group, dietary habits, do you smoke, alcohol intake, what is your diagnosis, how have you been medically treated for your condition, what is your family history?).
- Use of your tissue sample and information does not create any right, title, or interest in the tissue or products that may be developed as a result of the research.
- Your participation is voluntary.

You are free to decline or withdraw from participation without giving any reasons and this will not affect your care or your relation with the treating doctor.

Information

A small portion of your tumour that is removed at the time of surgery will be sent for biopsy testing. A small portion of this tumour will be used to obtain proteins. These proteins will be studied in the laboratory.

No extra time will be required to be spent by you in the hospital. You will not suffer any extra pain for giving the tissue as it will be removed from the tissue taken out during your operation.

If applicable to your study, list:

All patients who are being operated for stomach cancer will be asked to take part in the study.

Nobody else will know that you are participating in the study

The portion of the tissue that we are taking from you will be broken down in the laboratory at ACTREC into small pieces and the protein will be removed for study

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Risks

There are no risks to your health by this study as you do not have to undergo any extra procedure for the removal of the tissue other than the operation. You will have no side effects as a result of this other than the normal changes that occur after surgery.

Costs

You will not have to pay any extra money for taking part in the study other than the amount that is paid for the surgery. This study is funded by the Tata Memorial Centre.

Reimbursement for Participation

We will not be reimbursing any money as you are not undergoing any extra procedure for taking part in the study.

Emergency Medical Treatment (If applicable, add here)

Not applicable

Benefits

The results of this study will help us to understand the changes that occur in stomach cancer, a disease that is so common in India.

The results of this study may not directly benefit you, but will help us understand how cancer is caused which may finally help us to find better treatments for stomach cancer in the future.

We may be able to find out whether your disease is a good disease that will allow you a longer life, or otherwise.

We may be able to find better agents to treat stomach cancer in the future

The most effective treatments used today are the result of clinical trials done in the past.

Confidentiality

The information in the study records will be kept confidential and the clinical charts will be housed in the TMH/CRS. Data will be stored securely and will be made available only to persons conducting the study unless you specifically give permission in writing to do otherwise. No reference will be made in oral or written reports that could link you to the study. Result of the project will not be communicated to the subject unless deemed necessary.

Compensation for protocol Related Injury

Not applicable

Contact

If you have questions at any time about the study or the procedures, you may contact the researcher,

Dr. Shailesh Shrikhande

Office number 21, Department of GI Surgical Oncology

Tata Memorial Hospital, Parel – Mumbai 400 012

Ph No. (022) 27147173

If you have questions about your rights as a participant, contact the member secretary, HEC

Dr. Medha Joshi, Secretary – HEC-II

Digital Lib. Sciences, Tata Memorial Hospital, Parel – Mumbai

Ph No. (022) 2417 7000

Participation

Your participation in this study is voluntary; you may decline to participate at anytime without penalty and without loss of benefits to which you are otherwise entitled.

If you withdraw from the study prior to its completion, you will receive the usual standard of care for your disease, and your non participation will not have any adverse effects on your subsequent medical treatment or relationship with the treating physician

If you withdraw from the study before data collection is completed, your data will not be entered in the project report.

Consent

I have read the above information and agree to participate in this study. I have received a copy of this form.

Participant's name

Participant's signature

Address (capital letters)

Tel No. Date

Witness's name (print)

Witness's signature

Tel No Date

PI or the person administering the consent: Name (Print) & signature

Participant Information Sheet/Glossary

- Gastric cancer is a common cancer in India.
- It is diagnosed by barium meal and endoscopy
- The treatment of gastric cancer is surgery
- Chemotherapy is used in patients with advanced cancer and when surgery cannot be done
- In this study, you will be given the treatment that is given to all patients with gastric cancer as per the current standards of care
- Your treatment and follow up will be at Tata Memorial Hospital
- No extra time and cost will be involved

Questionnaire to be given to the participant before administration of

the Informed Consent Form

(This is to ensure that the Participant is now ready for Informed Decision Making)

1. What is the purpose of this study?
2. Who is doing it?
3. How long will the study last?
4. How many other people are included?
5. Do you know why you are chosen to be part of the study?
6. Do you know what tests are going to be done? Are they over and above the usual tests?
7. What do you have to do?
8. What are the possible side effects?
9. Who will you contact if you face any problem?
10. How will the study affect your daily life?
11. Does the study involve extra time, costs and/or follow up visits?
12. Do you know that the information collected about yourself will be kept confidential?
13. What will happen if you do not agree to participate?

Appendix 2: Tables

Table A2.1: Combination sequence specific synergistic, additive or antagonistic effect of Chemotherapy drugs and HDAC inhibitor

Drugs	Treatment Sequence AND result of FA and median effect plot analysis		Fraction affected (FA)		
			0.5	0.75	0.95
Cisplatin and VPA	Concurrent	Combined Dose (µg)	527.879±46.1	2330.46±36.4	28247.6±612.5
		Combination index (CI)	0.48056	1.01035	3.59544
	Pre	Combined Dose (µg)	387.325±4.4	1087.36±12.3	6159.97±69.8
		Combination index (CI)	0.3526	0.47142	0.78406
	Post	Combined Dose (µg)	1305.16±47.2	3754.52±47.8	22159.4±282.3
		Combination index (CI)	1.18816	1.62774	2.82052
Cisplatin and TSA	Concurrent	Combined Dose (µg)	5.65044±0.3	13.579±0.6	59.2432±2.5
		Combination index (CI)	1.43174	1.43174	3.09615
	Pre	Combined Dose (µg)	3.09615±0.02	7.19817±0.08	34.3041±0.4
		Combination index (CI)	0.7201	1.0106	1.79279
	Post	Combined Dose (µg)	8.25348±0.3	19.7974±1.1	86.1022±5.5
		Combination index (CI)	2.09132	2.77949	4.49984
Cisplatin and SAHA	Concurrent	Combined Dose (µg)	1008.18±46.7	3681.16±154.3	32.43±1359.2
		Combination index (CI)	1.51412	2.65574	2.65574
	Pre	Combined Dose (µg)	546.892±6.8	1460.1±9.3	7.60±48.8
		Combination index (CI)	0.82134	1.05338	1.63974
	Post	Combined Dose (µg)	1568.23±58.5	5206.54±154.6	39.16±1161.4
		Combination index (CI)	2.35522	3.75622	8.43298
Oxaliplatin and VPA	Concurrent	Combined Dose (µg)	768.166±25	2157.23±27.3	12227.6±263.8
		Combination index (CI)	0.78612	0.93329	1.24977
	Pre	Combined Dose (µg)	395.046±6.6	1048.94±12.7	5411.31±119.4
		Combination index (CI)	0.40428	0.45381	0.55309
	Post	Combined Dose (µg)	1183.83±23.7	4234.09±64.7	36030.5±541.09
		Combination index (CI)	1.2115	1.83182	3.68265
Oxaliplatin and TSA	Concurrent	Combined Dose (µg)	2.69697±0.1	6.52483±0.3	28.7891±1.3
		Combination index (CI)	0.64865	0.6366	0.61875
	Pre	Combined Dose (µg)	2.29643±0.06	5.46451±0.2	23.4489±1.4
		Combination index (CI)	0.55232	0.53315	0.50397
	Post	Combined Dose (µg)	4.66842±3.9	18.9379±16.3	199.126±171
		Combination index (CI)	1.12281	1.84768	4.2797

Oxaliplatin and SAHA	Concurrent	Combined Dose (µg)	861.884±21.4	3310.03±51.6	31.74±478
		Combination index (CI)	1.48934	2.38296	5.26134
	Pre	Combined Dose (µg)	739.242±10.8	1995.4±52.6	10.58±278.7
		Combination index (CI)	1.27741	1.43653	1.75387
	Post	Combined Dose (µg)	1482.15±74.6	6586.37±302.2	80.71±1682.3
		Combination index (CI)	2.56116	4.74167	13.3777
Epirubicin and VPA	Concurrent	Combined Dose (µg)	1963.4±193.6	7387.71±462.4	68459.5±3997.3
		Combination index (CI)	1.95315	3.06639	6.59033
	Pre	Combined Dose (µg)	1278.26±38	3246.62±104.6	15544.4±265
		Combination index (CI)	1.27159	1.34756	1.4964
	Post	Combined Dose (µg)	1997.06±48.9	5397.95±129.7	28693.2±689.6
		Combination index (CI)	1.98663	2.24051	2.76218
Epirubicin and TSA	Concurrent	Combined Dose (µg)	0.13966±0.02	0.45355±0.3	3.28193±0.3
		Combination index (CI)	0.2358	0.4065	1.06633
	Pre	Combined Dose (µg)	0.03158±0.004	0.09785±0.02	0.65427±0.05
		Combination index (CI)	0.27129	0.32981	0.45981
	Post	Combined Dose (µg)	0.14882±0.03	0.32325±0.04	1.18996±0.26
		Combination index (CI)	0.2358	0.4065	1.06633
Epirubicin and SAHA	Concurrent	Combined Dose (µg)	791.556±25.2	2979.11±115.2	27.61±1902
		Combination index (CI)	1.32472	2.04339	4.25583
	Pre	Combined Dose (µg)	640.884±18.9	1994±66.1	13.42±485.5
		Combination index (CI)	1.07256	1.3677	2.06891
	Post	Combined Dose (µg)	1391.23±82.9	5882.06±214.2	66.30±1016
		Combination index (CI)	2.3283	4.03454	10.2169

Table A2.2: Antibodies used for western blotting

S. No	Antibody (Ab)	1º Ab condition	Blocking	2º Ab condition
1	H3 Upstate	5% BSA- TBST 60min RT	1:2000 1%BSA -TBST O/N 4℃	Anti-Mouse 1:5000 5% BSA-TBST 60min RT
2	H3S10P Millipore 06-570	5% BSA- TBST 60min RT	1:5000 1%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
3	H3ac Upstate	5% Milk- TBST 60min RT	1:3000 1%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
4	H3K9ac	1% BSA- TBST 60min RT	1:1500 1%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
5	H3K14ac Abcam 52946	1% BSA -TBST 60min RT	1:2000 1%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
6	H3K18 Millipore 07-354	5% BSA- TBST 60min RT	1:2000 1%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
7	H3K23ac Millipore 07-335	5% BSA -TBST 60min RT	1:10000 1%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
8	H3K27ac Abcam 4729	1% BSA -TBST 60min RT	1:3000 1%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
9	H3K56ac Abcam 76309	1% BSA -TBST 60min RT	1:2000 1%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT

10	H3K4me Ab-8895	5% BSA -TBST 60min RT	1:5000 5%BSA-TBST 90min RT	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
11	H3K4me2 Abcam-32356	1% BSA -TBST 60min RT	1:2000 1%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
12	H4 Millipore 07-108	5% BSA- TBST 60min RT	1:4000 1%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
13	H4K5ac Millipore 06-729	5% BSA- TBST 60min RT	1:10000 1%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
14	H4K8ac Abcam 45166	5% BSA- TBST 60min RT	1:4000 1%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
15	H4K12ac Upstate 06-761	5% BSA- TBST 60min RT	1:5000 1%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
16	H4K16ac Millipore 07-329	5% BSA- TBST 60min RT	1:8000 5%BSA-TBST O/N 4℃	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
17	H4K20me Ab 9051	5% BSA- TBST 60min RT	1:4000 5%BSA-TBST 90min RT	Anti-Rabbit 1:8000 5%BSA-TBST 60min RT
18	H4K20me3 Ab 9053	5% BSA- TBST 60min RT	1:4000 5%BSA-TBST 90min RT	Anti -Rabbit 1:8000 5%BSA-TBST 60min RT
19	Msk1 Santacruz	5% Milk -TBST 60min RT	1:2000 1%BSA-TBST O/N 4℃	Anti –Rabbit 1:8000 5%BSA-TBST

				60min RT
20	pMsk1 Abcam	5% Milk -TBST 60min RT	1:2000 1%BSA-TBST O/N 4℃	Anti -Rabbit 1:8000 5%BSA-TBST 60min RT
21	P38 Santacruz 728	5% Milk -TBST 60min RT	1:2000 1%BSA-TBST O/N 4℃	Anti -Rabbit 1:8000 5%BSA-TBST 60min RT
22	Phospho p38 Cell signaling	5% Milk -TBST 60min RT	1:2000 1%BSA-TBST O/N 4℃	Anti -Rabbit 1:8000 5%BSA-TBST 60min RT
23	ERK1/2 santacruz	5% Milk -TBST 60min RT	1:2000 1%BSA-TBST O/N 4℃	Anti –Rabbit 1:8000 5%BSA-TBST 60min RT
24	Phospho ERK1/2 Cell signaling	5% Milk -TBST 60min RT	1:2000 1%BSA-TBST O/N 4℃	Anti –Rabbit 1:8000 5%BSA-TBST 60min RT
25	JNK Santacurz	5% Milk -TBST 60min RT	1:2000 1%BSA-TBST O/N 4℃	Anti -Rabbit 1:8000 5%BSA-TBST 60min RT
26	Phospho JNK Cell signaling	5% Milk -TBST 60min RT	1:2000 1%BSA-TBST O/N 4℃	Anti -Rabbit 1:8000 5%BSA-TBST 60min RT
27	Beta actin Sigma	5% Milk -TBST 60min RT	1:10000 1%BSA-TBST O/N 4℃	Anti –Mouse 1:5000 5%BSA-TBST 60min RT

Table A2.3: Clinicopathological characteristics of gastric cancer patients included in the study

S. No.	Sample code	Type of Surgery	Sex	Age (years)	WHO Classification	T stage	N stage	M stage	NACT	OS (months)	DFS (months)	Status at last follow-up (Dead/ Alive)	Recurrence (Yes/ No)	PRM distance in cm	DRM distance in cm
1	7	Distal gastrectomy	M	53	PD	T2	N2	M0	Yes	6	6	Dead	No	5	4.5
2	8	Subtotal gastrectomy	M	40	PD	T3	N1	M0	No	60	60	Alive	No	9	2.3
3	9	Total gastrectomy	F	54	SRC	T2	N2	M0	No	12	5	Dead	Yes	2.8	4
4	10	Subtotal gastrectomy	M	67	PD	T1	N0	M0	Yes	24	24	Dead	Yes	3.5	1
5	11	Distal gastrectomy	M	62	PD	T3	N2	M0	No	59	59	Alive	No	5.8	4.5
6	13	Proximal gastrectomy	F	44	MD	T2	N1	M0	Yes	23	23	Dead	Yes	1	6
7	14	Distal gastrectomy	F	36	PD	T1	N2	M0	Yes	3	3	Dead	No	8	1
8	17	Distal gastrectomy	M	47	SRC	T4	N2	M0	No	6	5	Dead	yes	3.5	1
9	21	Distal gastrectomy	M	71	PD	T2	N1	M0	No	34	34	Alive	No	4	3.5
10	22	Distal gastrectomy	M	63	PD	T3	N3	M0	No	59	59	Alive	No	8	2.5
11	34	Distal gastrectomy	M	66	PD	T4	N0	M0	No	41	41	Alive	No	3	3.5
12	36	Subtotal gastrectomy	F	25	PD	T2	N0	M0	No	12	6	Dead	Yes	4.5	1.2
13	37	Subtotal gastrectomy	M	46	MD	T3	N2	M0	No	53	53	Alive	No	2	6.5
14	39	Distal gastrectomy	M	56	PD	T3	N1	M0	No	54	40	Alive	yes	6	1.5
15	40	Distal gastrectomy	F	76	WD	T1	N0	M0	No	22	22	Alive	No	11	7

16	42	Distal gastrectomy	M	51	PD	T4	N0	M1	No	12	12	Dead	Yes	6	1
17	45	Distal gastrectomy	M	46	PD	T2	N0	M0	Yes	25	23	Dead	Yes	2.8	6
18	46	Distal gastrectomy	F	61	PD	T4	N2	M0	No	29	10	Dead	Yes	6	2
19	47	Total gasterectomy	M	55	PD	T4	N0	M1	No	12	12	Dead	No	10.5	9.5
20	48	Distal gastrectomy	F	50	PD	T1	N0	M0	No	43	43	Alive	No	3	1.5
21	49	Distal gastrectomy	F	29	PD	T4	N3	M0	No	1	1	Dead	No	14	2.8
22	50	Distal gastrectomy	M	36	PD	T4	N0	M0	Yes	22	17	Dead	yes	6.5	1.5
23	51	Distal gastrectomy	M	48	PD	T3	N1	M0	Yes	9	6	Dead	yes	9	3
24	52	Proximal gastrectomy	M	74	MD	T3	N0	M0	Yes	59	59	Alive	No	1.4	6
25	53	Distal gastrectomy	M	41	PD	T3	N0	M0	No	45	45	Alive	No	11	10
26	54	Oesophago-gastrectomy	M	60	MD	T3	N0	M0	Yes	50	50	Alive	No	0.5	7
27	55	Distal gastrectomy	M	60	MD	T1	N0	M0	No	30	30	Alive	No	1	7.5
28	56	Distal radical gastrectomy	M	79	PD	T4	N2	M0	No	1	1	Dead	No	3.5	3
29	57	Wedge resection	F	50	PD	T2	N0	M0	No	46	46	Alive	No	1.5	1.5
30	58	Distal gastrectomy	M	58	MD	T1	N1	M0	No	1	1	Dead	No	10	2.8
31	59	Total gasterectomy	F	57	PD	T3	N1	M0	No	6	6	Dead	No	2	3
32	60	Distal gastrectomy	M	55	PD	T4	N3	M0	No	14	14	Alive	No	6.5	1.5
33	61	Distal gastrectomy	F	36	PD	T3	N0	M0	No	43	43	Alive	No	12	2.5
34	62	Total gasterectomy	M	72	MD	T4	N1	M0	No	11	11	Dead	Yes	9	16
35	70	Distal gastrectomy	F	38	SRC	T2	N0	M0	No	58	58	Alive	No	12.5	1

36	71	Subtotal radical gastrectomy	M	73	MD	T2	N1	M0	No	56	56	Alive	No	4	4
37	73	Subtotal gastrectomy	M	40	PD	T3	N1	M0	No	60	60	Alive	No	9	2.3
38	74	Subtotal proximal gastrectomy	M	53	SRC	T1	N0	M0	No	60	60	Alive	No	12	7
39	75	Subtotal gastrectomy	M	52	PD	T3	N0	M0	No	1	1	Dead	No	3.5	1.5
40	76	Distal gastrectomy	M	51	PD	T3	N1	M0	No	1	1	Alive	No	6	2
41	77	Distal gastrectomy	F	67	MD	T4	N1	M0	No	57	57	Alive	No	3	4
42	78	Radical gastrec	M	46	SRC	T4	N2	M0	No	31	31	Dead	Yes	6	1.5
43	79	Subtotal gastrectomy	M	53	PD	T2	N2	M0	Yes	56	56	Alive	No	10	2.5
44	80	Distal or subtotal gastrectomy	M	66	MD	T3	N1	M0	Yes	6	6	Alive	No	2.5	5.5
45	81	Distal gastrectomy	M	47	SRC	T3	N0	M0	Yes	28	28	Alive	No	2.5	0.7
46	82	Distal gastrectomy	M	37	WD	T1	N0	M0	No	53	53	Alive	No	4	3.8
47	83	Oesophago-gastrectomy	M	57	PD	T3	N1	M0	Yes	50	6	Alive	yes	0.5	6
48	84	Distal Gastrectomy	M	34	PD	T1	N1	M0	No	49	49	Alive	No	1.5	7.5
49	85	Distal gastrectomy	M	72	PD	T4	N1	M0	No	5	5	Dead	No	4.5	3
50	86	Subtotal gastrectomy	M	60	PD	T4	N0	M0	No	15	15	Alive	No	7	1.2
51	87	Distal gastrectomy	M	75	MD	T2	N2	M0	No	2	2	Dead	No	6	4.5
52	88	Total gastrectomy	F	47	PD	T3	N3	M0	Yes	2	2	Dead	No	0.5	4
53	90	Subtotal gastrectomy	M	75	PD	T4	N3	M0	No	1	1	Dead	No	0.5	5
54	91	Distal gastrectomy	F	62	SRC	T3	N1	M0	No	47	47	Alive	No	6	9

55	92	Distal gastrectomy	M	52	PD	T4	N0	M0	No	49	49	Alive	No	2	4.8
56	93	Distal gastrectomy	M	66	SRC	T4	N3	M0	No	12	6	Dead	yes	3	1.3
57	94	Proximal gastrectomy	M	55	MD	T3	N1	M0	Yes	16	16	Alive	No	5.5	7
58	95	Oesophago-gastrectomy	M	29	PD	T4	N1	M0	Yes	34	34	Alive	No	1	3.5
59	96	Distal gastrectomy	F	52	MD	T3	N0	M0	Yes	1	1	Dead	No	7	1.4
60	97	Distal gastrectomy	M	74	MD	T2	N0	M0	Yes	1	1	Dead	No	3	8
61	98	Distal gastrectomy	F	37	PD	T2	N3	M0	Yes	9	9	Alive	No	7	3
62	99	Distal gastrectomy	M	61	PD	T2	N1	M0	No	36	36	Alive	No	2	2
63	100	Distal gastrectomy	M	61	PD	T3	N0	M0	Yes	28	25	Dead	yes	6	1.2
64	102	Distal gastrectomy	M	64	SRC	T4	N2	M0	No	3	3	Dead	No	3.5	1.5
65	103	Distal gastrectomy	M	63	MD	T1	N2	M0	No	5	5	Dead	No	7.5	3.3
66	104	Distal gastrectomy	M	52	PD	T3	N0	M0	No	27	27	Dead	yes	10	0.7
67	105	Distal gastrectomy	M	62	PD	T3	N0	M0	No	26	26	Alive	No	6	5
68	106	Subtotal gastrectomy	F	62	PD	T3	N0	M0	Yes	19	19	Alive	Yes	5.5	5.5
69	107	Distal gastrectomy	M	68	MD	T3	N1	M0	No	47	47	Alive	No	4	6
70	108	Distal gastrectomy	M	49	PD	T1	N0	M0	Yes	46	46	Alive	No	4	10
71	109	Distal gastrectomy	M	56	PD	T3	N3	M0	Yes	13	9	Alive	Yes	3.8	0.5
72	110	Distal gastrectomy	M	42	MD	T2	N0	M0	Yes	44	44	Alive	No	7	2
73	111	Total gastrectomy	M	77	PD	T4	N3	M0	Yes	1	1	Dead	No	7.5	4.5
74	112	Distal gastrectomy	F	63	PD	T4	N1	M0	No	11	11	Dead	yes	4	6.5

75	113	Proximal gastrectomy	M	57	PD	T3	N1	M0	Yes	30	30	Dead	No	1.1	4.8
76	114	Distal gastrectomy	M	50	MD	T3	N2	M0	Yes	8	8	Alive	No	4.5	1.5
77	115	Subtotal gastrectomy	F	36	SRC	T1	N0	M0	No	41	41	Alive	No	6.5	2
78	116	Distal gastrectomy	F	45	PD	T3	N2	M0	Yes	5	4	Dead	Yes	5.5	3
79	117	Proximal gastrectomy	M	53	PD	T3	N0	M0	Yes	14	4	Dead	yes	1	5
80	118	Total gastrectomy	M	42	PD	T3	N3	M0	No	38	38	Dead	No	1	3
81	119	Distal gastrectomy	M	33	PD	T2	N0	M0	Yes	43	43	Alive	No	2	9
82	120	Distal gastrectomy	M	65	PD	T2	N0	M0	Yes	12	12	Alive	No	11	1.5
83	121	Subtotal gastrectomy	F	41	PD	T3	N3	M0	Yes	9	9	Dead	No	10	1.5
84	122	Total gastrectomy	M	60	MD	T1	N0	M0	No	42	42	Alive	No	8.5	16
85	123	Distal gastrectomy	F	62	PD	T3	N0	M0	Yes	42	42	Alive	No	6	3.2
86	124	Distal gastrectomy	F	61	MD	T2	N0	M0	Yes	5	5	Dead	No	2.5	7.5
87	125	Subtotal gastrectomy	F	40	MD	T4	N3	M0	Yes	14	14	Alive	No	0.9	2.5
88	126	Total gastrectomy	M	86	PD	T3	N0	M0	No	1	1	Dead	No	1	1.5
89	127	Distal gastrectomy	M	23	PD	T2	N0	M0	Yes	37	36	Alive	Yes	7	6.5
90	128	Subtotal gastrectomy	F	52	PD	T3	N2	M0	Yes	10	10	Alive	Yes	2.2	5.5
91	129	Distal gastrectomy	F	8	PD	T4	No	M0	No	19	19	Alive	Yes	1.5	5
92	130	Distal gastrectomy	F	46	PD	T4	N3	M0	Yes	6	5	Alive	Yes	7.5	2
93	131	Proximal gastrectomy	M	61	SRC	T2	N1	M0	No	4	4	Dead	Yes	7	10
94	132	Distal gastrectomy	F	57	MD	T1	N0	M0	Yes	38	38	Alive	No	1.5	3

95	133	Distal gastrectomy	M	32	PD	T2	N2	M0	Yes	13	7	Dead	Yes	5	1.3
96	134	Proximal gastrectomy	M	43	MD	T2	N1	M0	No	10	10	Dead	Yes	0.3	3.5
97	135	Distal gastrectomy	M	79	PD	T2	N0	M0	No	56	56	Alive	No	10	1.5
98	136	Distal gastrectomy	M	29	PD	T4	N3	M0	Yes	12	6	Dead	yes	5.5	2
99	137	Distal gastrectomy	F	51	MD	T3	N3	M0	Yes	6	1	Dead	yes	5.5	2
100	138	Subtotal gastrectomy	F	44	PD	T2	N0	M0	Yes	26	5	Dead	yes	0.5	7
101	139	Distal gastrectomy	M	66	MD	T3	N0	M0	Yes	31	31	Alive	No	3	8.5

Table A2.4: Score for Immunohistochemistry analysis

S. No.	Sample code	Tumor				Proximal resection margin (PRM)				Distal resection margin (DRM)			
		H3S10ph IHC H-score	phMSK1 IHC H-score	β-actin IHC score		H3S10ph IHC H-score	phMSK1 IHC H-score	β-actin IHC score		H3S10ph IHC H-score	phMSK1 IHC H-score	β-actin IHC score	
				Epithelial cells	Inflammatory cells			Epithelial cells	Inflammatory cells			Epithelial cells	Inflammatory cells
1	7	120				80				80			
2	8	155	170	3	6	40	40	2	5	120			
3	9	230	100			200	60			149			
4	10	110		4	7	90				120		2	5
5	11	90				30				60			
6	13	50				55				30			
7	14	250	200			35	100			44			
8	17	80		2	7	53		2	6	70			
9	21	150		2	7	90		2	6	130			
10	22	90		2	7	30		3	7	80			
11	34	190				180				190			
12	36	160		2	7	90		3	7	155			
13	37	60		2	7	62				10		2	5
14	39	54		4	7	40				60		4	7
15	40	8				2	85			5			
16	42	250	135	2	7	115		2	7	110			
17	45	115		2	7	110				50		2	7

18	46	200		2	7	70		3	7	185			
19	47	200	120	2	7	67	65	3	7	70			
20	48	80		2	7	100		2	4	60			
21	49	185	230	5	7	40	155	3	7	160			
22	50	210		4	7	30		2	5	220			
23	51	155	115	2	7	100	100	2	5	145			
24	52	55		5	6	73				40		2	6
25	53	170	270	5	7	70	45			40		2	4
26	54	45		2	6	50				60		2	7
27	55	25		2	6	20				10		4	5
28	56	195		2	6	175		2	5	175			
29	57	85				70				110			
30	58	150		5	7	60		3	4	130			
31	59	170				135				160			
32	60	210		2	7	80		3	7	170			
33	61	60		2	7	10		2	7	50			
34	62	80				45				30			
35	70	215				90				150			
36	71	40				20				35			
37	73	180				60				140			
38	74	95				5				35			
39	75	160				120				120			
40	76	140				30				160			
41	77	80				100				50			
42	78	260				110				260			
43	79	135				100				120			

44	80	120				110				75			
45	81	280				220				200			
46	82	20				18				20			
47	83	60				60				30			
48	84	110				105				20			
49	85	100				50				80			
50	86	185				70				160			
51	87	140				65				130			
52	88	165				150				140			
53	90	220				300				80			
54	91	220				71				110			
55	92	55				40				20			
56	93	300				230				285			
57	94	90				40				100			
58	95	200				250				200			
59	96	60				25				54			
60	97	25				10				5			
61	98	120				45				100			
62	99	60				70				70			
63	100	170				115				200			
64	102	285				250				210			
65	103	40				20				100			
66	104	185				95				170			
67	105	190				75				120			
68	106	160				140				70			
69	107	35				30				24			

70	108	100				100				20			
71	109	160				100				140			
72	110	50				35				100			
73	111	190				70				55			
74	112	210				170				150			
75	113	180				210				90			
76	114	50				15				20			
77	115	195				30				110			
78	116	170				120				145			
79	117	140				140				40			
80	118	70				100				70			
81	119	65				35				10			
82	120	130				55				135			
83	121	110				20				100			
84	122	40				14				10			
85	123	75				15				40			
86	124	30				12				2			
87	125	90				72				30			
88	126	150				150				150			
89	127	120				40				50			
90	128	190				180				105			
91	129	205				130				120			
92	130	190				70				190			
93	131	300				75				160			
94	132	50				60				40			
95	133	140				50				110			

96	134	60				100				70			
97	135	63				30				50			
98	136	160				95				140			
99	137	70				60				80			
100	138	100				80				20			
101	139	30				35				10			

Table A2.5: Global post-translational modifications of histones in cancer diagnosis, prognosis and treatment				
Histone PTM	**Writer**	**Eraser**	**Function**	**Cancer Diagnosis/ Prognosis/ Treatment**
H3K9ac	GCN-5	SIRT-1; SIRT-6	Transcription initiation	*Diagnosis:* ? *Prognosis:* Lung, Breast, Ovarian *Treatment:* ?
H3K18ac	CBP/p300	?	Transcription initiation and repression	*Diagnosis:*? *Prognosis:* Lung, Prostate, Breast, Esophagus *Treatment:*?
H4K5ac	CBP/P300; HAT1; TIP60; HB01	?	Transcription activation	*Diagnosis:* ? *Prognosis:* Lung *Treatment:* ?
H4K8ac	TIP60; HB01	?	Transcription activation	*Diagnosis:* ? *Prognosis:* Lung, *Treatment:* ?
H4K16ac	TIP60; hMOF	SIRT-1; SIRT-2	Transcription activation	*Diagnosis:* Colorectal *Prognosis:* Lung, Breast *Treatment:* ?
H3K4me	SETD1A; SETD1B; ASH1L; MLL; MLL2; MLL3: MLL4; SETD7	KDM1A; KDM1B; KDM5B; NO66	Transcription activation	*Diagnosis:* ? *Prognosis:* Prostate, Kidney *Treatment:* ?
H3K4me2	SETD1A; SETD1B; MLL; MLL2; MLL3; MLL4; SMYD3	KDM1A; KDM1B; KDM5A; KDM5B; KDM5C; KDM5D; NO66	Transcription activation	*Diagnosis:* ? *Prognosis:* Prostate, Lung, Kidney, Breast, Pancreatic, Liver, *Treatment:* ?
H3K4me3	SETD1A; SETD1B; ASH1L; MLL; MLL2; MLL3; MLL4; SMYD3; PRMD9	KDM2B; KDM5A; KDM5B; KDM5C; KDM5D; NO66	Transcription elongation	*Diagnosis:* ? *Prognosis:* Kidney, Liver, Prostate *Treatment:* ?
H3K9me	SETDB1; G9a; EHMT1; PRDM2	KDM3A; KDM3B§; PHF8; JHDM1D	Transcription initiation	*Diagnosis:* Myeloma *Prognosis:* Kidney, Pancreas, Prostate *Treatment:* ?
H3K9me2	SUV39H1; SUV39H2; SETDB1; G9a; EHMT1; PRDM2	KDM3A; KDM3B§; KDM4A; KDM4B; KDM4C; KDM4D; PHF8; KDM1A; JHDM1D	Transcription initiation and repression	*Diagnosis:* ? *Prognosis:* Prostate, Pancreas *Treatment:* ?
H3K9me3	SUV39H1; SUV39H2;	KDM3B§; KDM4A;	Transcription initiation and	*Diagnosis:* Colorectal, Myeloma, Prostate, Breast and

	SETDB1; PRDM2	KDM4B; KDM4C; KDM4D	repression	lung. *Prognosis:* Lung, Prostate, Breast, Leukemia, Stomach *Treatment:* ?
H3K27me	EZH2; EZH1	JHDM1D	Transcription activation	*Diagnosis:* ? *Prognosis:* Kidney *Treatment:* ?
H3K27me3	EZH2; EZH1	KDM6A; KDM6B;	Transcription repression	*Diagnosis:* ? *Prognosis:* Breast, Pancreatic, Ovarian, Prostate, Stomach, Esophagus, Liver *Treatment:* ?
H4K20me3	SUV420H1; SUV420H2	?	Transcription repression	*Diagnosis:* Colorectal, Myeloma, Prostate, Breast and lung. *Prognosis:* Breast, Lymphoma, Colon, Ovarian *Treatment:* ?

Appendix 3: Figures

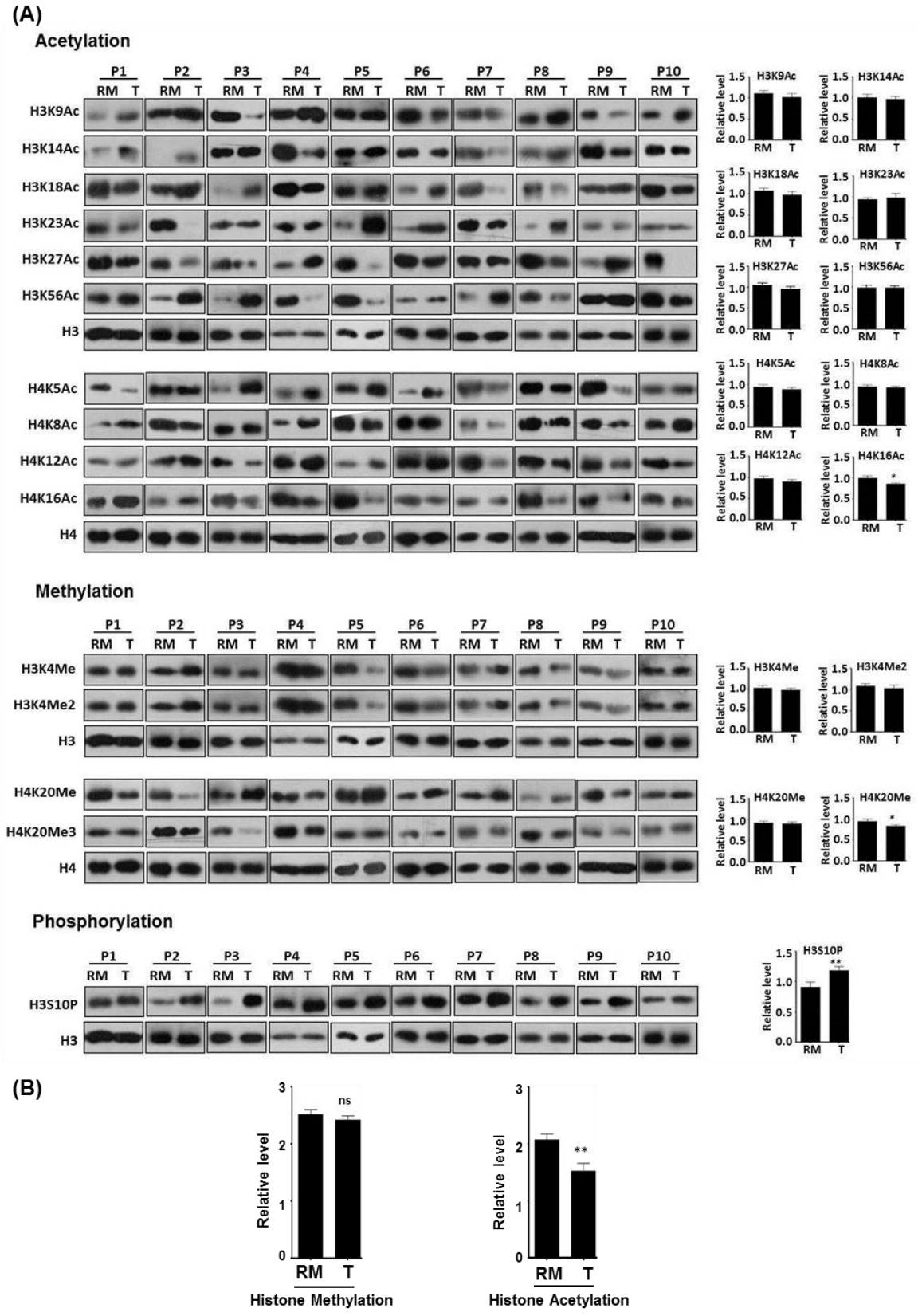

Figure A3.1: Immunoblot based screening of global histone PTMs.

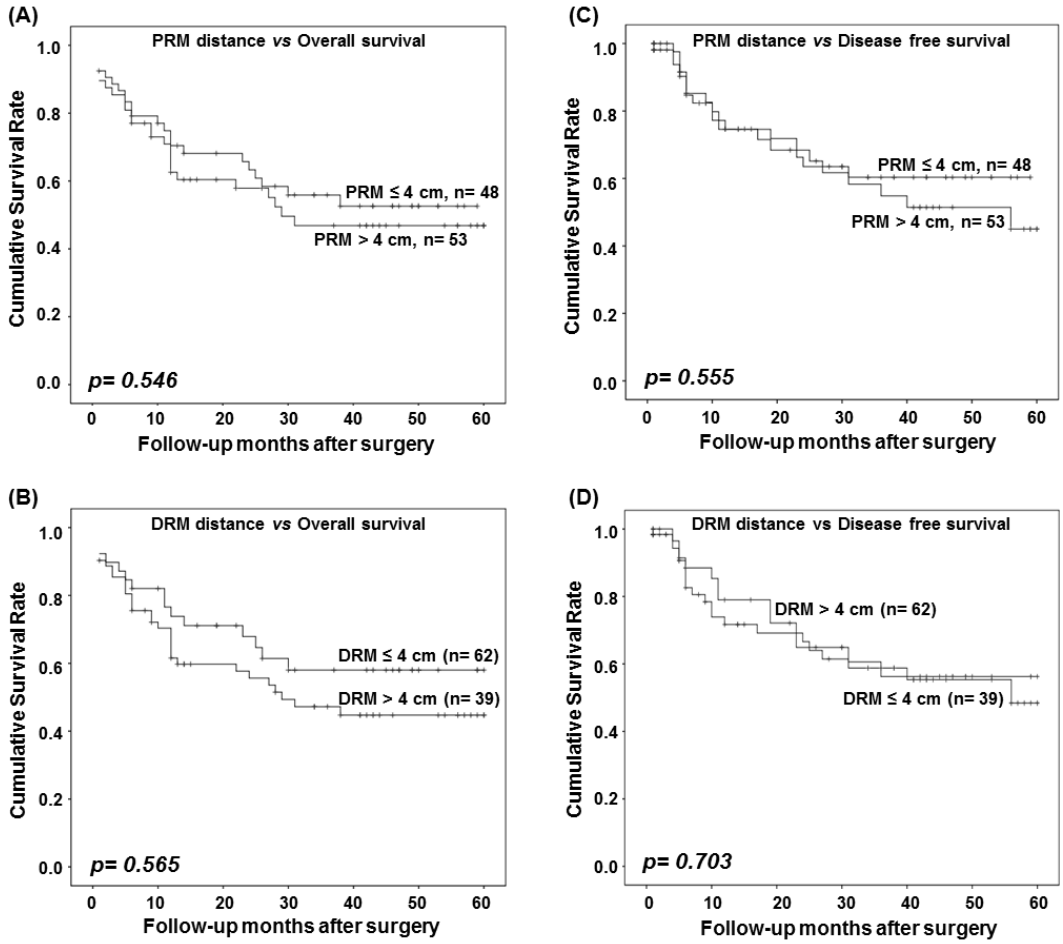

Figure A3.2: Resection margin distance dependent survival analysis of GC patients.

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Cell-type specificity of β-actin expression and its clinicopathological correlation in gastric adenocarcinoma

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Abstract

AIM: To investigate cell type specific distribution of β-actin expression in gastric adenocarcinoma and its correlation with clinicopathological parameters.

METHODS: β-actin is a housekeeping gene, frequently used as loading control, but, differentially expresses in cancer. In gastric cancer, an overall increased expression of β-actin has been reported using tissue disruptive techniques. At present, no histological data is available to indicate its cell type-specific expression and distribution pattern. In the present study, we analyzed β-actin expression and distribution in paired normal and tumor tissue samples of gastric adenocarcinoma patients using immunohistochemistry (IHC), a tissue non-disruptive technique as well as tissue disruptive techniques like reverse transcriptase-polymerase chain reaction (RT-PCR) and western blotting. Correlation of β-actin level with clinicopathological parameters was done using univariate analysis.

RESULTS: The results of this study showed significant overexpression, at both mRNA and protein level in tumor tissues as confirmed by RT-PCR (1.47 ± 0.13 *vs* 2.36 ± 0.16; $P < 0.001$) and western blotting (1.92 ± 0.26 *vs* 2.88 ± 0.32; $P < 0.01$). IHC revealed that β-actin expression is majorly distributed between epithelial and inflammatory cells of the tissues. Inflammatory cells showed a significantly higher expression compared to epithelial cells in normal (2.46 ± 0.13 *vs* 5.92 ± 0.23, $P < 0.001$), as well as, in tumor tissues (2.79 ± 0.24 *vs* 6.71 ± 0.14, $P < 0.001$). Further, comparison of immunostaining between normal and tumor tissues revealed that both epithelial and inflammatory cells overexpress β-actin in tumor tissues, however, significant difference was observed only in inflammatory cells (5.92 ± 0.23 *vs* 6.71 ± 0.14, $P < 0.01$). Moreover, combined expression in epithelial and inflammatory cells also showed significant increase (4.19 ± 0.15 *vs* 4.75 ± 0.14, $P < 0.05$) in tumor tissues. In addition, univariate analysis showed a positive correlation of β-actin level of inflammatory cells with tumor grade ($P < 0.05$) while epithelial cells exhibited negative correlation ($P > 0.05$).

CONCLUSION: In gastric cancer, β-actin showed an overall higher expression predominantly contributed by inflammatory or tumor infiltrating immune cells of the tissue microenvironment and correlates with tumor grade.

Key words: Gastric cancer; β-actin; Immunohistochemistry; Epithelial cells; Inflammatory cells; Tumor infiltrating immune cells; Adjacent mucosa; Resection margin

Core tip: Clinical implications of β-actin have been ignored despite the reports of its differential expression in cancer. The present study provides first histological evidence of an overall increase in β-actin expression in gastric cancer compared to histologically normal adjacent mucosa. Inflammatory and epithelial cells of tumor tissues showed differential pattern of β-actin expression and correlated with tumor grade. Further, overexpression of β-actin was predominantly contributed by inflammatory cells, suggesting further extensive studies to use β-actin as a diagnostic and prognostic biomarker and target of direct or indirect chemotherapeutic intervention.

Khan SA, Tyagi M, Sharma AK, Barreto SG, Sirohi B, Ramadwar M, Shrikhande SV, Gupta S. Cell-type specificity of β-actin expression and its clinicopathological correlation in gastric adenocarcinoma. *World J Gastroenterol* 2014; 20(34): 12202-12211 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i34/12202.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i34.12202

INTRODUCTION

Gastric cancer (GC) incidence and mortality is decreasing over several decades, however, it still remains the fourth most common type of cancer and the second leading cause of cancer related deaths worldwide[1]. In India, there are limited epidemiological studies on gastric cancer which also suffers from the juvenile state of cancer registries and under-reporting of cases. However, similar to global trend, Indian registries have also observed statistically significant reducing trend in stomach cancer cases in last 20-years with approximately 35675 estimated case in 2001; about 3.91% of global incidence[2,3]. A radical D2 gastrectomy and more recently radical surgery along with perioperative chemotherapy holds the best prospect of a cure in gastric cancer[4,5]. However, delayed presentation and thus diagnosis owing to the non-specific symptoms often preclude the possibility of a curative surgical resection making palliative chemotherapy and other measures as the treatment mainstay in these patients. The development of chemoresistance[6] is also an increasingly appreciated phenomenon contributing to the poor outcomes in the disease. Therefore, an improved understanding of GC molecular biology to ascertain new potential tumor biomarkers useful to guide patient management and develop new therapeutic options is essential.

β-actin is a housekeeping gene and an obligatory part of the cell cytoskeleton. It expresses in almost all eukaryotic cells and is involved in controlling basic housekeeping functions such as development and maintenance of cell shape, cell migration, cell division, growth and signaling. It also plays a critical role in transcriptional regulation, mRNA transport, mRNA processing and chromatin remodeling[7,8]. Further, β-actin is also one of the most commonly used endogenous reference loading controls in laboratory techniques to normalize gene and protein expressions as it is believed to have constant expression levels in different cellular, experimental and physiological conditions. However, growing evidences have demonstrated its differential expression in certain situations like growth, ageing, differentiation, developmental stages and diseases like asthma, Alzheimer's disease, congenital heart disease and cancer[9].

In comparison to normal, an overall differential expression of β-actin is reported in multiple cancers[10-16]. The methodologies used in earlier tissue based studies make it difficult to answer, which specific cell type out of the heterogeneous population of cells in a tissue, is responsible for altered expression of β-actin in cancer. To date, no histological studies have been conducted to provide informations about the pattern of β-actin expression and distribution in different cell types of the normal and tumor tissues. Such information of β-actin expression in a tissue will provide a better understanding of its role in carcinogenesis, its correlation with clinicopathological parameters and its potential to be used as a tumor biomarker or therapeutic target. β-actin polymerization or remodeling plays a crucial role in a cell's physiology and drugs altering the dynamics of β-actin have been studied as potential chemotherapeutic agent, however, clinical implications of these drugs are yet to be established[17-19]. The present study aimed to provide histological evidence of β-actin expression and distribution in specific cell types of gastric adenocarcinoma and its correlation with clinicopathological parameters. A total 31 paired (from the same patient) tumor and corresponding adjacent histopathologically normal mucosa tissue samples were analyzed using reverse transcription polymerase chain reaction (RT-PCR), western blotting and immunohistochemistry (IHC). We report, an overall higher expression of β-actin in gastric cancer at both mRNA and protein level. Further, as per the best of our knowledge, IHC analysis revealed it for the first time that overall higher expression of β-actin in gastric cancer is majorly contributed by tumor inflammatory cells (5.92 ± 0.23 *vs* 6.71 ± 0.14, $P < 0.01$), though, tumor epithelial cells (2.46 ± 0.13 *vs* 2.79 ± 0.24, $P > 0.05$) also showed overexpression. Moreover, univariate analysis showed a positive correlation between β-actin levels of inflammatory cells and tumor grade ($P < 0.05$) while epithelial cells exhibited a negative correlation ($P > 0.05$).

Table 1 Scoring system for β-actin immune-staining

Percent positivity of stained cells	IHC score	Staining intensity	IHC score
0%	0	None	0
< 25%	1	Weak	1
25%-50%	2	Moderate	2
50%-75%	3	Strong	3
75%-100%	4		

Total IHC score = IHC score of percent positivity + IHC score of staining intensity
Average total IHC score = (Total IHC score of EC + Total IHC score of IC)/2

EC: Epithelial cells; IC: Inflammatory cells; IHC: Immunohistochemistry.

MATERIALS AND METHODS

Tissue samples and histopathological analysis

Surgically resected fresh tissues of 5 and formalin-fixed paraffin-embedded tissue blocks of 26 gastric adenocarcinoma patients were collected from ICMR-tumor tissue repository of Tata Memorial Hospital, Mumbai, India. Surgically resected tissues were frozen immediately in liquid nitrogen, and then stored at -80 ℃ until required for experimental use. Form each patient, tumor and apparently normal adjacent gastric mucosa proximal and distal to the tumor was collected, however, only either one of the mucosa was used in the study depending upon their maximum resection-margin distance from the tumor site. All tumor samples had more than 60% tumor content, as confirmed by a blinded specialist gastrointestinal pathologist. The adjacent mucosa was confirmed to be free of tumor for all surgically resected fresh tissues and 24 (out of 26) formalin-fixed paraffin-embedded tissues on histopathological analysis. Surgically resected fresh tissues (n = 5) were used for RT-PCR and western blot analysis while formalin-fixed paraffin-embedded tissues were used for IHC analysis and correlational study. The protocol was reviewed and approved by institutional review board and ethics committee. All patients provided a written informed consent.

Cell lines and culture conditions

Gastric cancer cell lines AGS (ATCC® Number: CRL-1739™; moderately differentiated) and KATO Ⅲ (ATCC® Number: HTB-103™; signet ring cell carcinoma) was used. AGS and KATO Ⅲ cells were cultured in RPMI1640 (Invitrogen) and F12K (Himedia) media respectively at 37 ℃ with 5% CO_2 supplemented with 10% FBS, 100U/ml penicillin, 100 mg/mL streptomycin (Sigma). For trypsinization, 0.05% trypsin-EDTA (Sigma) was used for both the cell lines.

Total RNA isolation and RT-PCR

Total RNA from 25 mg of tissues was extracted (Thermo scientific, 0731) and 10 μg of which was used for cDNA synthesis (Fermentas life sciences, K1632). RT-PCR amplification was done using specific primers for β-actin (F: 5' AGAAAATCTGGCACCACACC 3' and R: 5' CCATCTCTTGCTCGAAGTCC 3') and 18S rRNA (F: 5' AAACGGCTACCACATCCAAG 3' and R: 5' CCTCCAATGGATCCTCGTTA 3') with an initial denaturation step at 95 ℃ for 2 min, followed by 20 cycles of denaturation at 95 ℃ for 45 min, primer annealing at 55 ℃ for 30 s, primer extension at 72 ℃ for 30 s and a final extension at 72 ℃ for 10 min. Each reaction was performed in triplicate. Amplified products were resolved on 1% agarose gels and visualized by Ethidium bromide staining.

Total protein lysate preparation and western blotting

Total cell lysate was prepared from 100 mg of tissue using Lysis buffer (20 mmol/L Tris-Cl pH 8, 2 mmol/L EDTA pH 8, 10 mmol/L EGTA, 5 mmol/L $MgCl_2$, 0.1% Triton X-100, 1 mmol/L Sodium orthovandate, 1 mmol/L Sodium fluoride, 20 mmol/L β-Glycerophosphate, 1 mmol/L DTT, 1 mmol/L PMSF, 10 ug/mL Leupeptin, 10 ug/mL Aprotinin). Tissues were powdered in liquid nitrogen, homogenized in 2 mL of lysis buffer and then kept at 4 ℃ for 30 min with intermittent mixing. Further, the total cell lysate from gastric cancer cell lines AGS and KATO Ⅲ was prepared using MKK lysis buffer[20]. The homogenate was then centrifuged at 100000 xg and supernatant was collected as total cell lysate and stored at -20 ℃. For western blotting, total cell lysate was first estimated using Bradford method and then 75 μg of protein was loaded on 10% SDS-PAGE and transferred to PVDF membrane. Anti-β-actin antibody (Sigma, A5316) was used at the dilution of 1:10000.

Immunohistochemistry

Immunohistochemical staining using VECTASTAIN® ABC kit (Vector Lab, P6200) was performed. Formalin-fixed paraffin-embedded tissue blocks were sectioned at a thickness of 5 μm and mounted on poly-L-lysine coated glass slides. The sections were deparaffinized through a graded series of xylene and rehydrated through a graded series of absolute alcohol to distilled water. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol at room temperature for 30 min in dark. Microwave antigen retrieval was carried out with 0.01 mol/L Sodium citrate buffer (pH 6.0). Anti-β-actin monoclonal antibody (Sigma, A5316) was applied for 16 h at 4 ℃ at the dilution of 1:1000. Immunoreactive proteins were chromogenically detected with Diaminobenzidine (DAB) (Sigma, D5537). The sections were counterstained with Harris's hematoxylene and then dehydrated and mounted. In parallel, control staining was performed without adding primary antibody.

Evaluation of Immunohistochemistry

The cytoplasmic immunohistochemical staining of β-actin was scored semi-quantitavely for epithelial and inflammatory cells as described in a previous study by Yip *et al*[21]. "IHC score", "Total IHC score" and "Average Total IHC score" were calculated by taking the account into

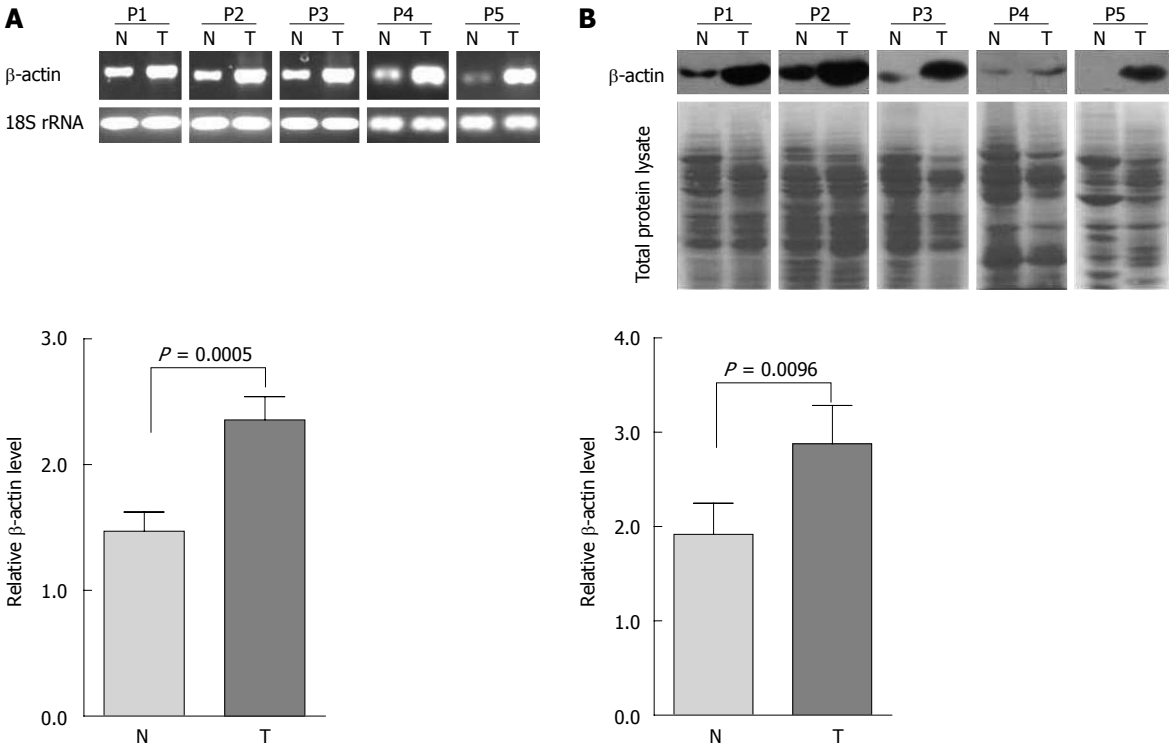

Figure 1 Comparison of overall β-actin level in gastric normal and tumor tissue (*n* = 5). A: Reverse transcription polymerase chain reaction analysis of β-actin and 18S rRNA was used as an internal loading control (upper panel). Band intensities of β-actin mRNA were normalized with 18S rRNA band intensity of respective lanes and obtained values were plotted (lower panel); B: Western blot analysis of β-actin (upper panel). Band intensity of blot was normalized with the total protein lysate intensity of respective lanes and obtained values were plotted (lower panel). Statistical significance was tested using "paired *t*-test". N: Normal; T: Tumor.

percentage of immunostained cells and staining intensity (Table 1). Total IHC score of 2 and above was considered as positive immunoreactivity. Total IHC score ranges from 2 to 7 and further grouped into: low (score 2 and 3), intermediate (score 4 and 5) and high (score 6 and 7). The immunohistochemical staining was examined by two independent researchers one of whom is a senior consultant pathologist to ensure the evaluations were performed properly and accurately. Both the researchers were blinded to all clinicopathological and outcome variables.

Statistical analysis

To test the statistical significance of β-actin differential expression between normal and tumor paired tissue samples by RT-PCR or western blotting and IHC, paired t-test with one-tailed *P*-value and Wilcoxon matched pair test with two-tailed *P*-value was applied respectively. To establish statistical correlation between clinicopathological parameters and β-actin expression level Mann-whitney and Krukal-wallis test with two-tailed *P*-value was applied. Wherever applicable, data is presented as mean ± SE and $P < 0.05$ was considered as statistically significant.

RESULTS

Overexpression of β-actin in tumor compared to normal gastric tissue

To detect an overall relative mRNA and protein expression of β-actin between gastric normal and tumor tissues, RT-PCR and western blot was performed on curatively resected fresh tissues from 5 randomly selected gastric cancer patients. Relative β-actin mRNA and protein levels were expressed after normalizing their intensities with the intensity of 18S rRNA and total protein respectively. Intensities were calculated by using ImageJ software[22]. Compared to normal, RT-PCR and western blot analysis showed a significant higher expression of β-actin level in tumor tissues both at mRNA (1.47 ± 0.13 *vs* 2.36 ± 0.16; $P < 0.001$) and protein level (1.92 ± 0.26 *vs* 2.88 ± 0.32; $P < 0.01$) as confirmed by paired *t*-test (Figure 1A and B).

Overexpression of β-actin in tumor tissue is predominantly contributed by inflammatory cells

After confirming an overall higher expression of β-actin in tumor compared normal gastric tissues, distribution of β-acting expression was studied in different cell types of the tissues on formalin-fixed paraffin-embedded tissue blocks using IHC. Study was carried out in paired normal and tumor tissues from 24 gastric adenocarcinoma patients. Analysis of immunostained tissue sections revealed that the β-actin immunostaining was majorly distributed between epithelial and inflammatory cells (Figure 2A). "Total IHC score" for β-actin immunostaining was calculated for both epithelial and inflammatory cells as mentioned in Table 1 and frequency of tissue sample for a particular total IHC score was determined (Table 2). For both normal and tumor tissues, analysis of frequency table showed that the most of the samples scored low to

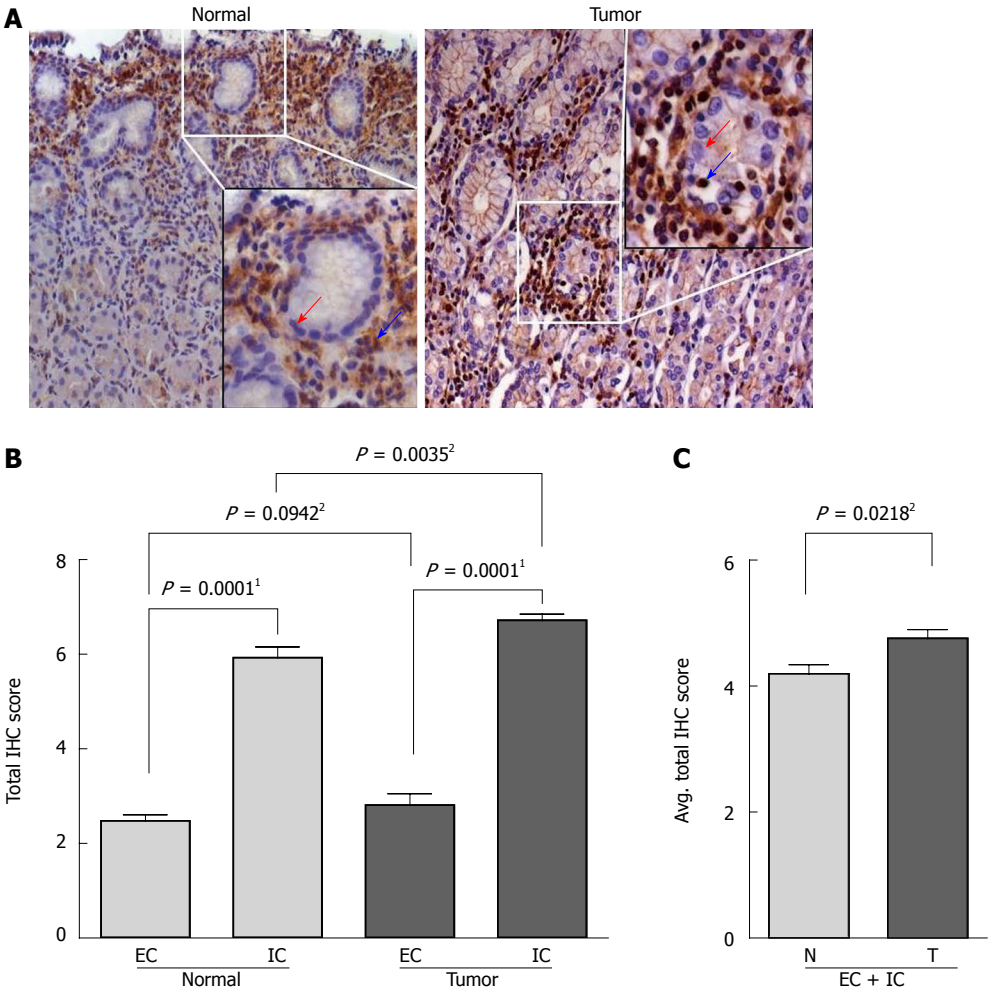

Figure 2 Histological analysis of β-actin in gastric normal and tumor tissues (*n* = 24). "Total IHC score" and "Average total IHC score" were calculated as described in Table 1. A: Representative pictures of β-actin immuno-staining of normal (left panel) and tumor (right panel) tissues showed β-actin expression is majorly distributed between epithelial (red arrow) and inflammatory (blue arrow) cells. The image is taken at 20 × magnification; B: "Total IHC score" of EC and IC of normal (N) and tumor (T) tissues were plotted; C: "Average total IHC score" for normal and tumor tissues were plotted. [1]Mann-Whitney test; [2]Wilcoxon matched pair test. IHC: Immunohistochemistry; EC: Epithelial cells; IC: Inflammatory cells.

Table 2 Frequency of samples with respect to total immunohistochemistry score of β-actin *n* (%)

β-actin immune-positive cells in tissues		Total IHC score (*n* = 24)					
		Low		Intermediate		High	
		2	3	4	5	6	7
Epithelial cells	Normal tissue	15 (63)	7 (29)	2 (8)	0 (0)	0 (0)	0 (0)
	Tumor tissue	16 (67)	2 (8)	3 (13)	3 (13)	0 (0)	0 (0)
Inflammatory cells	Normal tissue	0 (0)	0 (0)	3 (13)	7 (29)	3 (13)	11 (46)
	Tumor tissue	0 (0)	0 (0)	1 (4)	0 (0)	4 (17)	19 (79)

IHC: Immunohistochemistry.

intermediate "total IHC score" for β-actin immunostaining of epithelial cells while in case of inflammatory cells most of the samples scored Intermediate to high "total IHC score".

Comparison of "total IHC scores" showed that inflammatory cells express significantly higher level of β-actin compared to the epithelial cells in both normal (2.46 ± 0.13 *vs* 5.29 ± 0.23, $P < 0.001$) and tumor (2.76 ± 0.24 *vs* 6.70 ± 0.14, $P < 0.001$) tissues as confirmed by Mann-whitney test (Figure 2B). Furthermore, tumor tissues express relatively higher level of β-actin compared to normal in both epithelial and inflammatory cells, however, difference between epithelial cells was not significant (2.46 ± 0.13 *vs* 2.79 ± 0.24, $P > 0.05$) whereas inflammatory cells differed significantly (5.92 ± 0.23 *vs* 6.71 ± 0.14, $P < 0.01$) as confirmed by Wilcoxon matched-pair test (Figure 2B).

As overall β-actin level in a tissue will be a combined

Table 3 Univariate analysis of β-actin immunostaining with clinicopathological parameters *n* (%)

Clinicopathological parameters	Groups		Epithelial cells (total IHC score) *P* value	Inflammatory cells (total IHC score) *P* value	Epithelial + Inflammatory cells (avg. total IHC score) *P* value
Age (yr)					
	≤ 50	11 (42)	0.4933[1]	0.2724[1]	0.2941[1]
	> 50	15 (58)			
Sex					
	Male	20 (77)	0.9721[1]	0.2724[1]	0.5275[1]
	Female	6 (23)			
Tumor grade					
	WD	0 (0)	0.6089[2]	0.0168[2]	0.8393[2]
	MD	4 (15)			
	PD	14 (54)			
	Mucinous	0 (0)			
	SRC	8 (31)			
Depth of invasion[3]					
	T1	2 (8)	0.5446[2]	0.6618[2]	0.8804[2]
	T2	2 (8)			
	T3	13 (52)			
	T4	8 (32)			
Lymph Node status[3]					
	N0	6 (24)	0.7510[2]	0.6293[2]	0.5426[2]
	N1	8 (32)			
	N2	8 (32)			
	N3	3 (12)			
Treatment Modality[3]					
	Surgery	14 (56)	0.3542[1]	0.8135[1]	0.2910[1]
	NACT + surgery	11 (44)			

[1]Mann Whitney Test; [2]Kruskal Wallis Test; [3]TNM staging and Treatment modality information was available for only 25 (out of 26) patients. $P < 0.05$ indicates statistically significant difference. IHC: Immunohistochemistry; MD: Moderately differentiated; PD: Poorly differentiated; SRC: Signet ring cell carcinoma.

result of its expression in all cell types of the tissue, therefore, we asked, whether our IHC analysis corroborates with our RT-PCR and western blot data showing an overall higher expression of β-actin in tumor tissues? To answer this, we compared "average total IHC score" (average of "total IHC scores" of epithelial and inflammatory cells) of normal and tumor tissue. IHC analysis supports the results of RT-PCR and western blotting and also showed a significant increase of β-actin expression in tumor tissues (4.19 ± 0.15 *vs* 4.75 ± 0.14, $P < 0.05$) compared to normal (Figure 2C).

Correlation of β-actin expression with clinicopathological parameters

A total 26 non-metastatic gastric adenocarcinoma cases were examined and analyzed. Although, only inflammatory cells showed significant increase in β-actin level of tumor tissues; for correlational studies, epithelial cells were also considered because they have also shown an increase in tumor compared to normal tissues (Figure 2B). Univariate analysis was performed to correlate "total IHC score" and "average total IHC score" of epithelial and inflammatory cells for β-actin immunostaining with clinicopathological parameters like age, sex, tumor grade, depth of invasion, lymph node status and mode of treatment. The associations between β-actin expression and clinicopathological parameters are shown in Table 3. Epithelial and overall level of β-actin did not show any significant correlation with any of the clinicopathological parameters while β-actin level of inflammatory cells showed significant correlation with tumor grade or WHO classification ($P < 0.05$). Further, identification of pattern and statistical significance of β-actin level in inflammatory cells of tumor tissues of different tumor grades: moderately differentiated (MD), poorly differentiated (PD) and signet ring cell carcinoma (SRC) was carried out. The results showed a positive correlation of β-actin level with tumor grade (Figure 3A) with significantly higher level in PD (6.25 ± 0.22 *vs* 6.79 ± 0.21, $P < 0.05$) and SRC (6.25 ± 0.22 *vs* 6.88 ± 0.14, $P < 0.05$) compared to MD; however, PD to SRC difference was not significant (6.79 ± 0.21 *vs* 6.88 ± 0.14, $P > 0.05$). In addition, low level of β-actin in signet ring cell carcinoma (a type of poorly differentiated cell) cell line KATO Ⅲ compared to moderately differentiate gastric adenocarcinoma cell line AGS (Figure 3B) attracted us to look for the pattern of β-actin expression of tissue epithelial cells with tumor grade. β-actin level in tissue epithelial cells followed a similar pattern of cell lines and decreases from MD to PD and to SRC (Figure 3C), a negative correlation with tumor grade, though insignificant.

The SRC is a type of poorly differentiated adenocarcinoma, therefore, SRC and PD was combined together and analyzed for their β-actin expression in epithelial and inflammatory cells compared to MD (Figure 3A and C). The significance of differential expression of β-actin in-

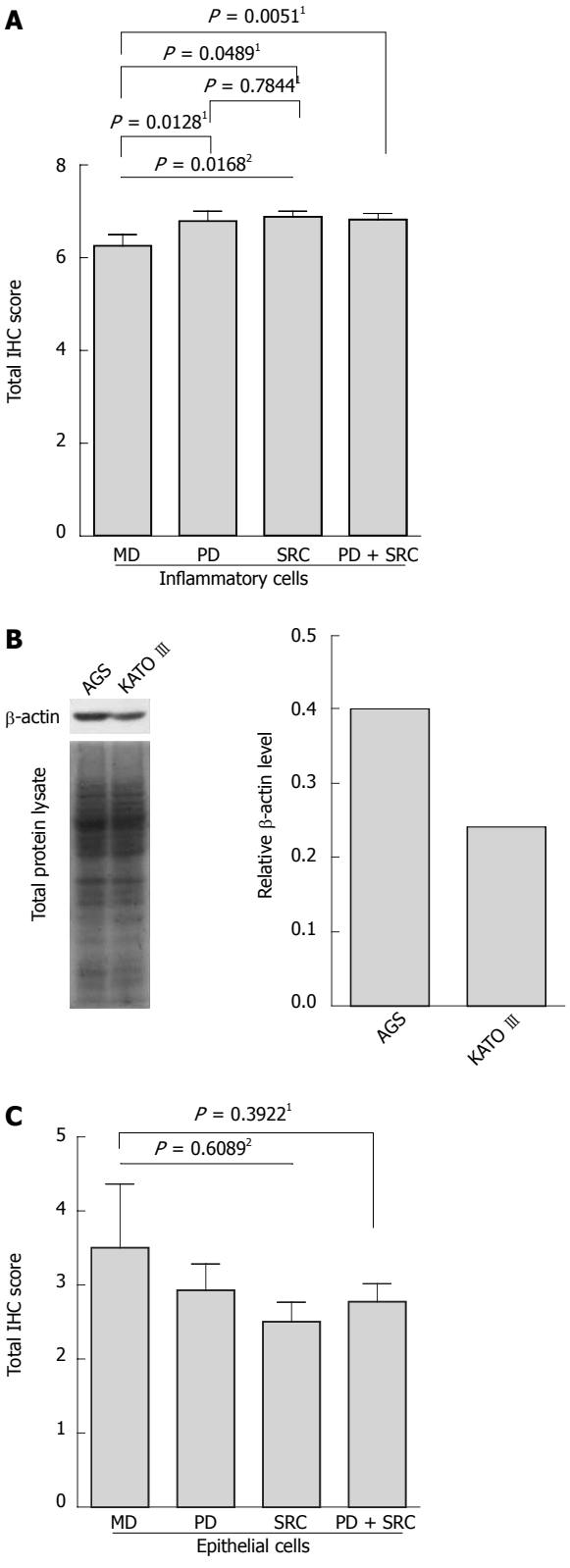

Figure 3 Correlation of β-actin expression with tumor grade. A: "Total IHC scores" of β-actin immunostaining in inflammatory cells were correlated with tumor grade; B: β-actin expression between gastric cancer cell lines AGS and KATO Ⅲ was analyzed using western blotting (right panel). Blot intensities were normalized with the intensity of total protein lysate of respective lanes and obtained values from three independent experiments were plotted (left panel); C: "Total IHC scores" of β-actin immunostaining in epithelial cells were correlated with tumor grade. [1]Mann-Whitney test; [2]Kruskal-Wallis test.

creased both in case of inflammatory cells ($P = 0.0168$ to $P = 0.0051$) and epithelial cells ($P = 0.6089$ to $P = 0.3922$), further confirming the association of β-actin expression with tumor grade in gastric adenocarcinoma.

DISCUSSION

β-actin has been reported to be differentially expressed in multiple cancers[10-16] and suggested as a possible target for chemotherapy[17-19]. These studies signify the potential of β-actin to be considered as a tumor biomarker. Till date, only overall level of varying expression of β-actin in cancer has been reported at the mRNA and protein level by "tissue disruptive techniques", where whole tissue with heterogeneous population of cells crushed and lysed, therefore, observed differential level of β-actin can not be attributed to a specific cell type. The present study, along with tissue disruptive techniques (RT-PCR and western blotting) provides histological evidences (IHC) of differential expression and distribution of β-actin in different cell types of gastric adenocarcinoma.

β-actin overexpression in tumor compared to normal tissues at mRNA level was most consistent and significant as evident by comparing *P*-values of RT-PCR (1.47 ± 0.13 *vs* 2.36 ± 0.16; $P < 0.001$) and western blot (1.92 ± 0.26 *vs* 2.88 ± 0.32; $P < 0.01$) analysis (Figure 1A and B). Therefore, the significant overexpression of β-actin at mRNA level in gastric cancer suggests its deregulation at the level of transcription or mRNA turnover. Earlier reports have also shown β-actin overexpression in colorectal, pancreatic, esophageal, hepatic and gastric cancers patients using tissue disruptive techniques. Molecular mechanism of β-actin transcription control is still unclear, however, CpG island hypermethylation of β-actin promoter has been found to be a negative regulator of expression[23]. Further, rapid upregulation in β-actin transcription in response to mitogenic stimuli including epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and platelet derived growth factor[24-26] have also been reported. In addition, miR-145, miR-206 and miR-466a are known to target and degrade β-actin mRNA, therefore, playing a critical role in altering its mRNA turnover[27-30]. Functionally, β-actin plays a predominant role in cell migration as its overexpression is observed in cells with metastatic potential compared to non-metastatic or cells with less metastatic potential; for example, metastatic variants of human colon adenocarcinoma cell line LS180[15], hepatoma morris 5123[31] and human invasive melanoma cells[32] overexpress β-actin. Collectively, our results along with the existing literature suggest, β-actin transcription is tightly regulated in a normal cell, required for its diverse and critical functions in cell's physiology and its deregulation may have an important role in carcinogenesis.

Immunohistochemistry analysis ($n = 24$) shows an overall increase (4.19 ± 0.15 *vs* 4.75 ± 0.14, $P < 0.05$) in β-actin expression in tumor compared to normal gastric

adenocarcinoma tissues (Figure 2C), this is in conjunction with β-actin profile observed by western blotting (Figure 1B). Further, the expression of the β-actin is mainly distributed between epithelial and inflammatory cells of the tissues with significantly higher level in inflammatory cells than their corresponding epithelial cells both in normal (2.46 ± 0.13 *vs* 5.92 ± 0.23, P <0.001) and tumor tissues (2.79 ± 0.24 *vs* 6.71 ± 0.14, $P < 0.001$) (Figure 2A and B). Both epithelial and inflammatory cells of tumor overexpressed β-actin compared to normal tissues, however, only inflammatory cells showed significant increase (5.92 ± 0.23 *vs* 6.71 ± 0.14, $P < 0.01$). The increased expression of β-actin of inflammatory cells is in strong correlation with chronic inflammation in gastric cancer[33] which leads to the homing of large number of inflammatory cells with higher level of β-actin required for immediate cytoskeleton rearrangement for the formation of membrane protrusions at the time of their migration[34-36]. This observation is important as inflammation is a key component of the tumor microenvironment, promotes tumor development and being considered as a hallmark of cancer[37,38].

Further, univariate analysis showed β-actin level of tumor inflammatory cells positively correlates ($P < 0.05$) with tumor grade or poorer differentiation of gastric cancer while epithelial cells showed an inverse correlation ($P > 0.05$) (Figure 3A and C). The insignificant correlation of epithelial cells can be attributed to low number of moderately differentiated gastric adenocarcinoma tissue samples ($n = 4$) with high range of "total IHC score" (3.5 ± 1.5). This correlation indicates toward an important role of β-actin in tumor dedifferentiation. The chronic inflammation in gastric cancer, predominantly caused by *Helicobacter pylori* infection, is known to promote poorer tumor differentiation and CpG-island hypermethylation[33,39,40] and β-actin promoter hypermethylation downregulates the gene expression[23]. Therefore, the positive correlation of β-actin level of tumor inflammatory cells with tumor grade may be due to the persistent inflammation in tumor micro-environment. On the other hand, hypermethylation of β-actin promoter may be a cause of negative correlation of β-actin level of tumor epithelial with tumor grade. Low level of β-actin in gastric adenocarcinoma cell line KATO Ⅲ (signet ring cell carcinoma, a type of poorly differentiated cell) compared to AGS (moderately differentiated) (Figure 3B), further strengthens the observation that β-actin level of tumor epithelial cells negatively correlates with poorer tumor differentiation.

In summary, to the best of our knowledge, present study provides first histological evidence of cell type specific distribution of β-actin in normal and tumor gastric tissues. The significant increase in β-actin expression in tumor tissues is due to inflammation, an initial characteristic in the stage of gastric cancer progression and positively correlates with tumor grade. Therefore, β-actin may represent a promising biomarker in early diagnosis and prognosis of gastric cancer. However, further studies are needed to explore the relationship of cell type specific differential expression of β-actin with its functional implications in carcinogenesis and to be used as a chemotherapeutic target.

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COMMENTS

Background

On one side β-actin has been a renowned internal equal loading control for RNA and protein expression studies, on the other side reports of its differential expression in growth, ageing, differentiation, development as well as diseases like asthma, Alzheimer's disease, congenital heart disease and cancer is increasing progressively. Further, there is an emerging view of the use of β-actin as a potential direct or indirect target for chemotherapy. Therefore, the study of this "so called" housekeeping gene in cancer becomes as important as any other molecule involved in this critical disease.

Research frontiers

Validation of housekeeping genes as an internal loading control, role of actin in biological process important in carcinogenesis, investigation of actin binding proteins specifying its function and identifying new chemotherapy targets affecting actin cytoskeleton directly or indirectly are the major research areas which is related to the article.

Innovations and breakthroughs

Differential expression of β-actin has been reported in a number of physiological conditions along with its overexpression in multiple cancers. Now days, oncology research is emphasizing on tumor microenvironment, the present study provides first histological proof of β-actin overexpression but differentially in different cell types in gastric cancer. The histology based investigation provides evidence that β-actin overexpression in gastric cancer is predominantly contributed by the infiltrating inflammatory cells in between tumor epithelial cells. In addition, a significant correlation was observed between β-actin expression and tumor grade which emphasizes the role of β-actin in carcinogenesis.

Applications

The findings of the present study strengthen the area of actin biology and emphasize on the fact that conventional housekeeping genes should not be chosen as internal loading control without validation. This article provides impetus to further study of β-actin expression in different cancers and implicate the findings to understand the role of β-actin in carcinogenesis. It also encourages us to find prognostic and diagnostic value of β-actin in cancer along with as a direct or indirect target for chemotherapeutic intervention similarly as other cytoskeletal element such as microtubules.

Terminology

Tissue disruptive and non-disruptive techniques: A tumor tissue is comprised of heterogeneous population of cells. Therefore, crush and/or homogenizing a tissue for genomics, proteomic and expression studies is defined as tissue disruptive technique. This technique does not give specific information about the type of cells contributing to the results and therefore can be misleading. On the other hand, tissue non-disruptive techniques like histology based immunohistochemistry provide information at the level of specific cell type.

Peer review

In the present study, the authors revealed an overall increase in β-actin expression in gastric cancer compared to histologically normal adjacent mucosa. They revealed that inflammatory and epithelial cells of tumor tissues showed differential pattern of β-actin expression and correlated with tumor grade. Overexpression of β-actin was predominantly contributed by inflammatory cells. According to the results, they concluded that β-actin might be a promising biomarker of gastric cancer and chemotherapeutic target. They showed interesting and valu-

able data in this paper.

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REVIEW

Global histone post-translational modifications and cancer: Biomarkers for diagnosis, prognosis and treatment?

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Abstract

Global alterations in epigenetic landscape are now recognized as a hallmark of cancer. Epigenetic mechanisms such as DNA methylation, histone modifications, nucleosome positioning and non-coding RNAs are proven to have strong association with cancer. In particular, covalent post-translational modifications of histone proteins are known to play an important role in chromatin remodeling and thereby in regulation of gene expression. Further, histone modifications have also been associated with different aspects of carcinogenesis and have been studied for their role in the better management of cancer patients. In this review, we will explore and discuss how histone modifications are involved in cancer diagnosis, prognosis and treatment.

Key words: Epigenetics; Cancer; Diagnosis; Prognosis; Histone post-translational modifications; Treatment

Core tip: The purpose of the review is to describe the potential of histone post-translational modifications in the field of cancer.

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INTRODUCTION

Cancer is a manifestation of both genetic and epigenetic alterations leading to the genomic instability and thus affecting several classes of genes, such as oncogenes, tumor suppressor genes, apoptotic genes and DNA repair genes. The field of cancer genetics which include the study of point mutation, deletion, insertion, gene amplification, chromosomal deletion/inversion/translocation, and allelic

loss/gain has got the attention of most cancer researchers in the last few decades. However, the appreciation of cancer epigenetics is more recent as several studies have now shown that in addition to numerous genetic alterations human cancers also harbor global epigenetic abnormalities[1,2].

Epigenetics, was initially defined by C. H. Waddington as "the causal interactions between genes and their products, which bring the phenotype into being"[3]. With time, the definition of epigenetics has evolved and is implicated in a wide variety of biological processes. The current definition is "the study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence". Epigenetic mechanisms include DNA methylation[4], noncoding RNA[5,6], histone variants[7] and histone post translational modifications (PTMs). These mechanisms together alter the local structural dynamics of chromatin to regulate the functioning of the genome, mostly by regulating its accessibility and compactness. All together, these mechanisms govern the chromatin architecture and gene function in various cell types, developmental and disease states[2,8-12]. Disruption in the proper maintenance of these heritable epigenetic mechanisms can result in activation or inhibition of various critical cell signaling pathways thus leading to disease states such as cancer[1,13]. Epigenetic mechanisms also cooperate with genetic alteration and work together at all stages of cancer development from initiation to progression[14]. Unlike genetic alterations, epigenetic changes are reversible in nature and can be potentially restored back to their original state by epigenetic therapy. These findings have inspired many studies aimed to understand the role of epigenetics in tumorigenesis and further explore its utility in cancer diagnosis, prognosis and therapy[15]. In recent years, research focus has been shifted to understand various post translational modifications for gaining deeper insights in to the functioning of histone/chromatin associated proteins. Information about the PTMs and the related modifying enzymes is available in the database HIstome: The Histone Infobase (http://www.actrec.gov.in/histome/)[16]. This review will discuss the role of histone post-translational modifications and its utility in cancer diagnosis, prognosis and treatment.

HISTONE PTMS: A DYNAMIC PROCESS

Histones are highly conserved and basic proteins with a globular C-terminal domain and an unstructured N-terminal tail[17]. They are also the most important proteins for converting a linear naked genome in to physiologically sensible architecture, chromatin. Nucleosomes are fundamental units of chromatin, consisting an octamer of H2A, H2B, H3 and H4 (two each) around which 146 base pairs of DNA is wrapped-. There are sequence variants of these histones which are expressed and incorporated into chromatin in a context dependent manner in normal and disease related processes. In cancer, histone H2A variants, H2A.1, H2A.Z and macroH2A have also been reported to express aberrantly[18-20]. Also, histones proteins can undergo a variety of PTMs some of which are methylation (me), acetylation (ac), ubiquitylation (ub), sumoylation (su) and phosphorylation (ph) on specific amino acid (Figure 1)[10]. Apart from these modifications, histones are also known to undergo homocysteinylation, crotonylation and glucosylation amongst others[21]. These histone modifications occur at several degrees, for example, methylation can be of monomethyl (me), dimethyl (me2) and trimethyl (me3).

Histone PTMs are added and removed from histones by enzymes called "writers" and "erasers" respectively. Histone acetyltransferases (HATs), histone methyltransferases (HMTs) and histone kinases are the examples of "writers" which add acetyl, methyl and phosphoryl groups, whereas histone deacetylases (HDACs), histone demethylases (HDMs) and histone phosphatases are examples of "erasers" which remove acetyl, methyl and phosphoryl groups, respectively (Figure 2)[22-24]. Histone-modifying enzymes are also known to interact with each other as well as other chromatin related proteins thus influencing key cellular processes such as transcription, replication and repair[10].

The mechanism behind the regulation of key cellular processes by histone post-translational modifications is not fully understood; however, it can be generalized into two categories. First, the addition of any PTM on histone protein affects inter/intra-nucleosomal interactions and their binding to DNA by steric hindrance or charge interactions. Second, addition of these PTMs to histone proteins inhibits or facilitates the binding of various proteins to chromatin[10]. These mechanisms allow a vast range of flexibility in regulating chromatin dynamics and signaling transmission and thereby regulating the gene expression. As an example of first mechanism, histone acetylation is proposed to be associated with chromatin relaxation and transcription activation, H4K16ac inhibits the formation of compact 30 nm fibers and higher order chromatin structures[25,26]. As an example of second mechanism, evolutionarily conserved specialized proteins, termed "histone readers," possess the ability to specifically bind certain histone modifications and affects a defined nuclear process such as transcription, DNA repair and replication, *etc.* (Figure 2). For example, through its evolutionary conserved chromodomain heterochromatin protein 1 recognize and gets recruited to H3K9me3 and leads to the formation of compact chromatin which in turn inhibits the access of the transcriptional machinery[27,28]. Moreover, the fact that there are different variants of each histone protein differing from few to many amino acids adds another level of complexity in functional aspects of histone PTMs. Such complicated and multilayered regulatory mechanisms of cellular processes through histone modifications have led to the hypothesis of "histone code" where a set of histone variants and modifications together perform a specific function[29]. However, due to its complexity histone code is still not fully understood[30]. Further, the status of one histone modification also regulates that of another by

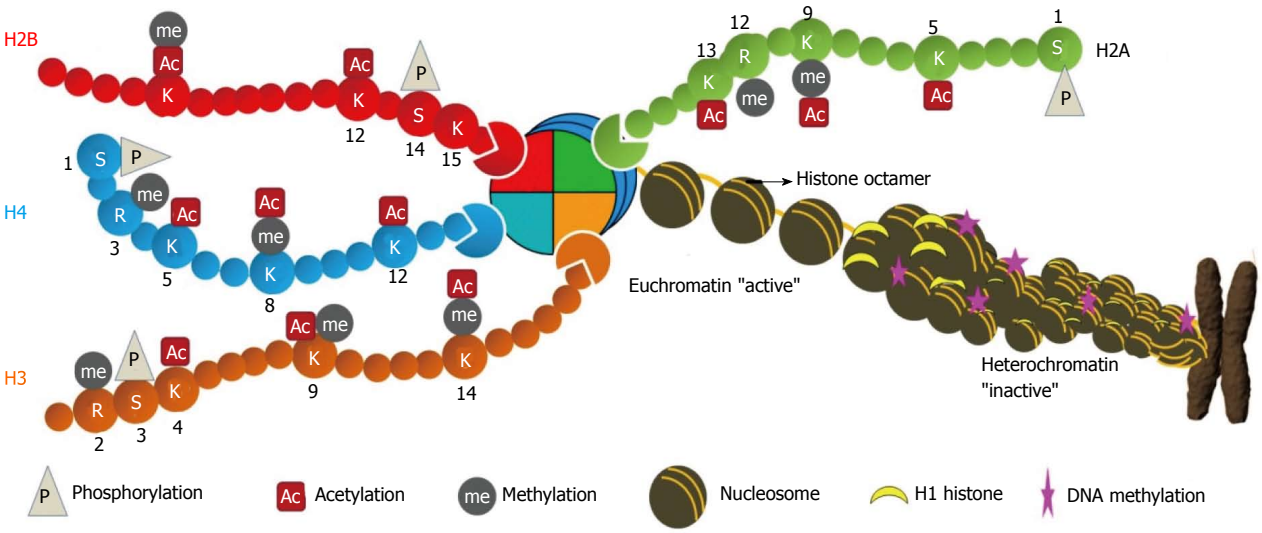

Figure 1 Chromatin architecture. The DNA is wrapped in two turns around histone octamers (nucleosomes) at intervals of about 200 bp along the DNA. Histones within the nucleosome (two each of H2A, H2B, H3 and H4) undergo numerous post-translational modifications at their N-terminal tail which protrudes from the nucleosome. Further folding of nucleosome with linker histone H1 creates a spiral structure, the heterochromatin leading to metaphase chromosome. These modifications directly regulate the chromatin structure and thus DNA-mediated cellular processes. The diagram indicates some modifications at specific residues: M: Methylation; A: Acetylation; P: Phosphorylation.

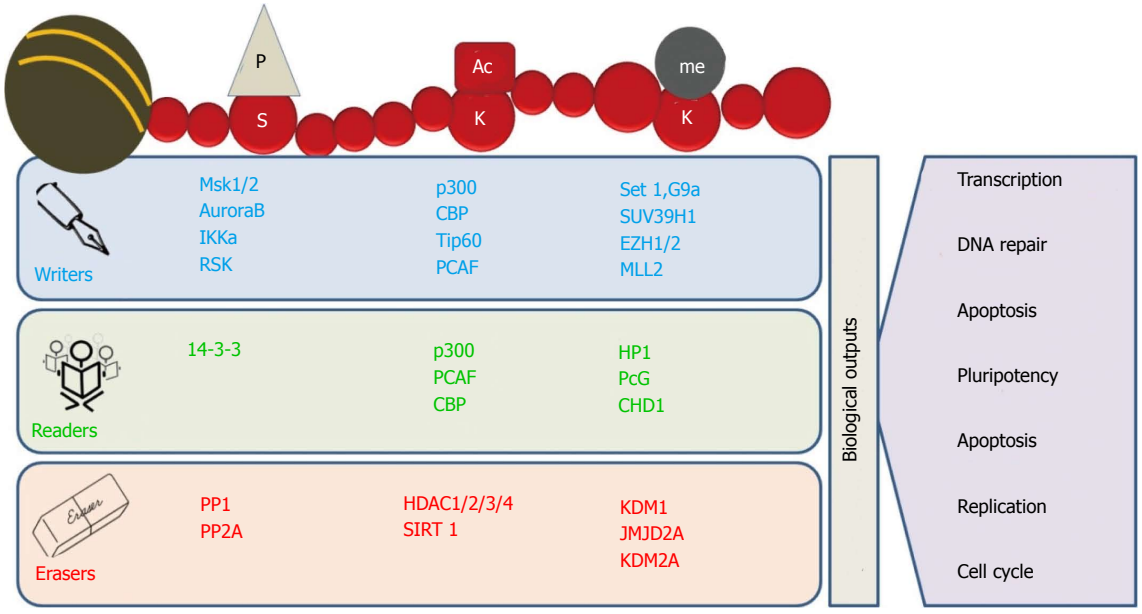

Figure 2 Readers, writers and erasers of chromatin marks. Histone modifications are highly dynamic in nature. The "writers" like histone acetyltransferases (HATs), histone methyltransferases (HMTs) and kinases add specific marks on specific amino acid residues on histone tails. These marks are identified by various proteins containing specific domains such as bromodomains, chromodomains and Tudor domain containing proteins called "readers". The written marks are removed by "erasers" like histone deacetylases (HDACs), lysine demethylases (KDMs) and phosphatases. In addition, removal and identification of these post-translational modifications on histone tails regulate various biological processes, including transcription, DNA replication and DNA repair.

cross-talk and affects chromatin remodeling and gene expression. Cross-talk between H3S10ph and H3K14ac, H2Bub and H3K4me and H3K4ac and H3K4me3 and H3K14ac are few prominent examples regulating gene expression[31]. For example, acetylation of H3K18 and H3K23 by CBP (CREB binding protein) can promote the methylation of H3R17 by Coactivator-Associated Arginine Methyltransferase 1 (CARM1), resulting in activation of estrogene-responsive genes[32].

HISTONE PTMS IN CANCER

In cancer, several histone PTMs have been reported to be misregulated; however, their involvement in cancer pathophysiological characteristics like cellular transformation, angiogenesis and metastasis *etc.*, is not well understood. Moreover, there are very few studies commenting on the cancer specific regulatory mechanism behind the alteration of histone PTMs. It has been a

decade when global loss of H4K16ac and H4K20me3 was reported for their association with cancer and considered as a common hallmark of tumor cells[33]. However, still there are no reports of their direct involvement in cellular transformation or any other cancer characteristics. Despite of the awareness of hMOF (human Male absent Of First) and HDAC4 as writer and eraser of H4K16ac, it is a recent development that low expression of hMOF has been implicated for its loss in gastric cancer[34]. Moving on to histone methylation, Lin *et al*[35] showed histone lysine demethylase KDM1A mediated loss of H3K4me2 is associated with epithelial to mesenchymal transition (EMT) in human breast cancer cells. Loss of H3ac, H3K9me3 and H3S10ph is observed at the promoters of Sfrp2, Sfrp5 and Wnt5a during genistein induced development of colon cancer in the rat model system[36]. Alterations in methylation patterns of H3K9 and H3K27 are related to aberrant gene silencing in many cancers[37,38]. Tissue microarrays done to compare the levels of H2B ub1 levels in normal mammary epithelial tissue as well as benign, malignant, and metastatic breast cancer samples have clearly shown a sequential decrease in H2B monoubiquitination with breast cancer progression and metastasis in comparision with normal epithelia[39]. A very important discovery has been made in term of phosphorylation of H3S10 as the only histone marks directly associated with cellular transformation. The knockdown and mutant (S10A) of histone H3 suppressed LMP1-induced proliferation of nasopharyngeal carcinoma cell line CNE1[40]. H3S10P has been reported to increase and has been established as indispensable for cellular transformation[41,42]. Cellular transformation by v-src constitutively activated phosphorylation of histone H3 at Ser10 in a transformation-specific manner; while, non-transforming mutant of v-src did not activate H3 phosphorylation[43]. Further, Mitogen- and stress-activated kinase 1 (MSK1) has been shown to phosphorylate H3S10 in TPA and EGF mediated cellular transformation[44]. Unpublished data from our lab has also shown increase in H3S10ph in gastric cancer, which is regulated by p38-MAPK/MSK1 pathway.

It has now been clear that acetylation, methylation and phosphorylation of histones are the most studied histone marks. In cancer, most of the studies have been done for these modifications with respect to the identification of their enzymes, regulation, effect on cellular physiology and as well as molecular biological markers for the disease management. The National Institute of Health defines a biological marker (biomarker) as a biological molecule found in blood, other body fluids, or tissues that are an objective indicator of normal or abnormal process, or of a condition or disease[45]. From the next part of the review we will see how histone acetylation, methylation and phosphorylation can be exploited as biomarkers for cancer diagnosis, prognosis and treatment.

HISTONE PTMS IN CANCER DIAGNOSIS

Diagnosis of a disease majorly depends on the analysis of physical symptoms, body fluids and fecal samples. A sensitive and specific diagnostic marker is not only useful in early diagnosis, but also helps in assessing the risk of developing the disease. Advances in the technology have enabled investigators to isolate metabolites, proteins and DNA from body fluids and fecal material and correlate them with pathophysiological symptoms of diseases including cancer.

Decades of research have discovered a battery of markers for cancer diagnosis; however, only few could reach to clinics because of issues of sensitivity and specificity. Therefore, at one side there is a need to improve techniques and on the other hand discovery of new markers is of immense importance. The discovery of the presence of DNA in fecal and urine samples[46] and circulating nucleosomes in serum[47,48] has led to the foundation of identifying epigenetic markers such as DNA methylation and histone posttranslational modification for cancer diagnosis. Ahlquist *et al*[49] demonstrated the recovery of DNA from frozen fecal samples of colorectal cancer patients which was followed by other investigators showing matching DNA methylation patterns between DNA from tissue and fecal samples of gastric and colorectal cancer patients[50-52]. Methylation pattern of DNA isolated from urine samples was also used to diagnose bladder and prostate cancer[53-57]. All these methylation studies have successfully detected global hypomethylation and gene specific hypermethylation of DNA, as established from tissue based studies.

Presence of histone proteins is not known in fecal and urine samples; therefore, histone posttranslational modifications have been utilized as cancer diagnostic markers using circulating nucleosomes (cNUCs) in serum samples. Two histone methylation marks, H3K9me3 and H4K20me3, the hallmarks of pericentric heterochromatin[58], were investigated in circulating nucleosomes by subsequent studies. Gezer *et al*[59] investigated the correlation between the H3K9me3 and H4K20me3 of cNUCs in healthy subjects and patients with colorectal cancer (CRC) and multiple myeloma and found low levels of these PTMs in cancer. Sera of patients with malignant tumors including colorectal, lung, breast, ovarian, renal, prostate cancer, and lymphoma showed high level of nucleosome concentration compared with those of healthy persons and patients with benign diseases[60]. Further, the same group showed high level ALU115 DNA sequence associated H3K9Me in multiple myeloma patients compared to healthy individuals[61]. ChIP based analysis of circulating nucleosomes in serum samples by Gloria *et al* reported a low level of H3K9me3 and H4K20me3 in patients with colorectal, pancreatic, breast and lung cancer compared to healthy control[62,63]. Moreover, H3K9me3 and H4K20me3 have been found to be lower at the pericentromeric satellite Ⅱ repeat in patients with CRC when compared with healthy controls or patients with multiple myeloma. In summary, identification of histone PTMs from serum isolated circulating nucleosomes have open the doors of immense possibility that blood samples collected by

Table 1 Global post-translational modifications of histones in cancer

Histone PTM	Writer	Eraser	Function	Cancer Diagnosis/ Prognosis/ Treatment
H3K9ac	GCN-5	SIRT-1; SIRT-6	Transcription initiation	Diagnosis: ? Prognosis: Lung, breast, ovarian Treatment: ?
H3K18ac	CBP/p300	?	Transcription initiation and repression	Diagnosis: ? Prognosis: Lung, prostate, breast, esophagus Treatment: ?
H4K5ac	CBP/P300; HAT1; TIP60; HB01	?	Transcription activation	Diagnosis: ? Prognosis: Lung Treatment: ?
H4K8ac	TIP60; HB01	?	Transcription activation	Diagnosis: ? Prognosis: Lung, Treatment: ?
H4K16ac	TIP60; hMOF	SIRT-1; SIRT-2	Transcription activation	Diagnosis: Colorectal Prognosis: Lung, breast Treatment: ?
H3K4me	SETD1A; SETD1B; ASH1L; MLL; MLL2; MLL3: MLL4; SETD7	KDM1A; KDM1B; KDM5B; NO66	Transcription activation	Diagnosis: ? Prognosis: Prostate, kidney Treatment: ?
H3K4me2	SETD1A; SETD1B; MLL; MLL2; MLL3; MLL4; SMYD3	KDM1A; KDM1B; KDM5A; KDM5B; KDM5C; KDM5D; NO66	Transcription activation	Diagnosis: ? Prognosis: Prostate, lung, kidney, breast, pancreatic, liver, Treatment: ?
H3K4me3	SETD1A; SETD1B; ASH1L; MLL; MLL2; MLL3; MLL4; SMYD3; PRMD9	KDM2B; KDM5A; KDM5B; KDM5C; KDM5D; NO66	Transcription elongation	Diagnosis: ? Prognosis: Kidney, liver, prostate Treatment: ?
H3K9me	SETDB1; G9a; EHMT1; PRDM2	KDM3A; KDM3B§; PHF8; JHDM1D	Transcription initiation	Diagnosis: Myeloma Prognosis: Kidney, pancreas, prostate Treatment: ?
H3K9me2	SUV39H1; SUV39H2; SETDB1; G9a; EHMT1; PRDM2	KDM3A; KDM3B§; KDM4A; KDM4B; KDM4C; KDM4D; PHF8; KDM1A; JHDM1D	Transcription initiation and repression	Diagnosis: ? Prognosis: Prostate, pancreas Treatment: ?
H3K9me3	SUV39H1; SUV39H2; SETDB1; PRDM2	KDM3B§; KDM4A; KDM4B; KDM4C; KDM4D	Transcription initiation and repression	Diagnosis: Colorectal, myeloma, prostate, breast and lung Prognosis: Lung, prostate, breast, leukemia, stomach Treatment: ?
H3K27me	EZH2; EZH1	JHDM1D	Transcription activation	Diagnosis: ? Prognosis: Kidney Treatment: ?
H3K27me3	EZH2; EZH1	KDM6A; KDM6B;	Transcription repression	Diagnosis: ? Prognosis: Breast, pancreatic, ovarian, prostate, stomach, Esophagus, Liver Treatment: ?
H4K20me3	SUV420H1; SUV420H2	?	Transcription repression	Diagnosis: Colorectal, myeloma, prostate, breast and lung Prognosis: Breast, lymphoma, colon, ovarian Treatment: ?

PTM: Post translational modification.

cancer patients can also be used for histone PTM based cancer diagnosis.

HISTONE PTMS IN CANCER PROGNOSIS

In cancer, to date, histones PTMs have been mostly studied for their potential as prognostic marker (Table 1). The first report in this area strongly suggested the utility of histone PTMs in cancer diagnosis and showed loss of H4K16ac and H4K20me3 in several cancers and establish these two marks as a hallmark of tumor and establishes the correlation of H4K16ac with tumor progression[33]. Further, loss of H4K20me3 is as also detected in various cancer animal models[64,65]. A study on prostate cancer showed a positive correlation of H3K18ac, H4K12ac and H4R3me2 with increasing tumor grade[66]. Another study on prostate cancer showed independently of other clinical and pathologic parameters, high rate of tumor recurrence in low-grade prostate carcinoma patients with low level of H3K4me2[66]. Loss of H3K4me2/me3 is reported in various neoplastic tissues such as non-small cell lung cancer, breast cancer, renal cell carcinoma and pancreatic adenocarcinoma serving as a predictor of clinical outcomes[67-72].

Acetylation of histone H3K9 has shown ambiguous results with the increase in some and decrease in other cancers. Decrease of H3K9ac has been linked with tumor progression, histological grading and clinical stage in prostate and ovarian tumors, hence is coupled with a poor prognosis for these patients[66,73-75]. Patients with non-small cell lung adenocarcinoma exhibited better prognosis on the reduction of the H3K9ac expression

level[68,76]. In contrast, increase in H3K9ac levels was reported in liver cancer[73]. Methylation of the same residue K9 of histone H3 requires loss of H3K9ac and is also linked to number of cancers. An association with the increase in methylation of H3K9 and aberrant gene silencing, has been found in many cancers[37,77] and its high level is associated with poor prognosis in gastric adenocarcinoma patients[77]. However, in patients with acute myeloid leukemia decrease in H3K9me3 has been found to be associated with better prognosis[78]. Decrease in H3K18ac is correlated with poor prognosis in prostate, pancreatic, lung, breast and kidney cancers[66,69,71]. It has also shown a strong correlation with tumor grade, signifying its importance in tumor progression[69]. In this regard, Kurdistani laboratory has confirmed that oncogenic transformation by the adenovirus protein E1a is associated with drastic changes in the global H3K18 acetylation pattern[79,80]. In addition, H3K18 hypoacetylation has been associated with an high risk of tumor recurrence in low-grade prostate cancer patients[66]. However, in contrast to this, low expression of H3K18ac has been correlated with a better prognosis for esophageal squamous cell carcinoma and glioblastoma patients[76,81]. This suggests that a single histone modification could predict differential prognosis in different cancers depending on it tissue specificity.

Another histone mark, H3K27me3 has been evaluated as a prognostic factor in patients with prostate, breast, ovarian, pancreatic and esophageal cancer[81-84], however, some of the results are perplexing and need further investigation. High level of H3K27me3 correlates with poor prognosis in esophageal cancers[81,84]. On the other hand H3K27me3 showed a negative correlation with overall survival time in breast, prostate, ovarian and pancreatic cancer patients[83]. Zhang *et al*[85] have identified many genes like oncogenes, tumor suppressor genes, cell cycle regulators, and genes involved in cell adhesion with significant differences in H3K27me3 pattern in gastric cancer samples in comparison to adjacent non-neoplastic gastric tissues. Further they were able to correlate changes in H3K27me3 to gene expression pattern of MMP15, UNC5B, and SHH. In non-small cell lung cancer enhanced H3K27me3 was correlated with longer overall survival (OS) and better prognosis. Moreover, both univariate and multivariate analyses indicated that H3K27me3 level was a significant and independent predictor of better survival[86]. Recently, a study showed K27M mutations of histone H3.3 variants in 31% pediatric glioblastoma tumors suggesting another level of complexity in alteration of histone PTMs in cancer which is independent of histone modifying enzymes[87]. Mass spectrometry based analysis showed high level of H3K27ac in colorectal cancer than the corresponding normal mucosa[88]. Immunohistochemical analysis on metachronous liver metastasis of colorectal carcinomas by Tamagawa *et al*[89] has correlated H3K4me2 and H3K9ac with the tumor histological type. In addition, lower levels of H3K4me2 correlated with a poor survival rate and also found to be an independent prognostic factor.

Recently DNA damage mark γH2AX also have shown its prognostic value. In triple negative breast tumors, high level of γH2AX was associated poor overall survival[90] and which was further found to be associated with shorter telomere length[91]. In colorectal cancer a high γH2AX expression in CRC tissues was associated with tumor stage and perineurial invasion. Furthermore, a high γH2AX expression was associated with poor distant metastasis-free survival (DMFS) and OS. Cox regression analysis also revealed that γH2AX was an independent predictor of DMFS and OS. A high γH2AX expression in CRC tissues is associated with a more malignant cancer behavior, as well as poor patient survival[92]. ELISA based analysis in glioblastoma multiformes tumors showed the high level of H3T6ph,H3S10p and H3Y41ph as signatures associated with a poor overall survival[93]. Increase in H3S10ph has been associated with poor prognosis in several cancers including glioblastoma multiformes[93], cutaneous nodular melanoma[94], cutaneous melanoma[95], breast cancer[96,97], esophageal squamous cell carcinoma[98], gastric cancer[99,100], melanoma[101] and nasopharyngeal carcinoma[40].

HISTONE PTM'S IN CANCER TREATMENT

Reversible nature of epigenetic changes or mechanisms has drawn major attention of scientific community to study the molecular mechanism regulating the alteration in epigenetic marks, specifically the histone post-translational modifications. Such efforts have led to the discovery of several histone modifying enzymes[102] and their chemical inhibitors[103] which has emerged as an attractive strategy in cancer treatment. Targeting these enzymes can reactivate epigenetically silenced tumor-suppressor genes by modulating the levels of histone posttranslational modifications[104]. Further, these drugs have also given additional advantage in the area of combinatorial chemotherapy[105,106].

Histone acetyl-transferases and histone deacetylases as the targets

Loss of histone acetylation has a strong correlation with aberrant gene silencing in cancer. Treatment with HDAC inhibitors reactivate silenced tumor suppressor genes by increasing histone acetylation levels and act as anti-tumorigenic agent by promoting growth arrest, apoptosis and cell differentiation[107]. Additionally, HDACi have shown their potential in reversing chemoresistance and induce antiproliferative effects on a number of cancer cell lines[108-113]. However, the question still remains whether the promise shown in the above studies by HDAC inhibitors are mainly due to their potency to alter epigenetic mechanisms or mere its effect on key cellular growth regulatory pathways.

Initial results upon treatment with HDACi like valproic acid and phenylbutyrate, as a single agent against hematologic malignancies were not encouraging[81]. However, the field showed much promise with the development of more

potent HDACi such as the class-specific inhibitors (entinostat and romidepsin) and the pan HDAC inhibitors (vorinostat, belinostat and panobinostat). The field however gained boost when in a landmark Phase IIb multicenter trial, Yu *et al*[82] have shown vorinostat as effective treatment modality for refractory cutaneous T-cell lymphoma. Further, in Phase Ⅱ multi-institutional trial, romidepsin has also been shown to have significant and durable efficacy against cutaneous T-cell lymphoma[83]. Due to their great successes in many studies, HDACi romidepsin and vorinostat have been approved by FDA as the treatment regime of cutaneous T-cell lymphoma, and romidepsin also for the treatment of relapsed peripheral T-cell lymphoma[84]. Since then many other HDACi have been under study of phase Ⅰ and/or Ⅱ trials as monotherapy, including belinostat, panobinostat, entinostat, chidamide, SB939 and LAQ824 in various cancers like ovarian, lung, soft tissue carcinoma, non-small-cell lung and breast[114-121]. However, unlike that of earlier success in treatment of lymphomas the majority of the results among solid tumor patients have been disappointing. In spite of achieving only intermittent anecdotal clinical responses, HDACi been related with severe toxicities.

Interactions between different epigenetic mechanisms have led to the foundation of research on combinatorial approach of cancer treatment using epigenetic drugs. Indeed, combinations of DNA methyltransferase and histone deacetylase inhibitors appear to synergize effectively in the reactivation of epigenetically silenced genes[107,122-124]. Such combinatorial approaches of cancer treatment have been found to be more effective than treatment with a single therapeutic agent. For example, treatment with 5-Aza-CdR and trichostatin-A in combination led to the derepression of certain putative tumor suppressor genes unlike individual treatments[107]. Pre-treatment of HDAC inhibitor SAHA relaxes the chromatin sensitizes cells to DNA damage induced by Topoisomerase Ⅱ inhibitor[125]. Similarly pretreatment of valproic acid act in synergy with epirubicine and reduces the tumor volume in breast cancer mouse model[126].

Furthermore, synergistic activity of decitabine and HDACi sodium phenylbutyrate was shown to decrease the lung cancer formation by more than 50% in comparison with decitabine alone in a murine model based study by Belinsky *et al*[124]. The same group also reported that the combination of HDACi entinostat with the DNMTi azacitidine was able to decrease tumor size and reduce the growth of K-ras/p53 mutant lung adenocarcinomas orthotopic engrafted in immunocompromised nude rats[127]. In another case HDACi sodium butyrate reduces the cell proliferation of MCF-7 cell when combine with vitamin-A[128].

Histone methyl-transferases and histone demethylases as the targets

Studies on histone methylation and their modifiers have been slow. Only few histone methylases (HMT) and demethylases (HDM) and their inhibitors have been discovered. However, studies on histone methylation could be more fruitful for their therapeutic potential because the less redundancy in HMTs and HDM compared to HATs and HDACs in targeting specific amino acid residue of histone[129]. This property of HMTs and HDMs provides exciting opportunities with more tailored treatment, while potentially minimizing side effects.

LSD1/KDM1 was among the first identified histone demethylases selectively targeting H3K4me1 and H3K4me2[130] and mediate gene repression. LSD1 has been reported to be overexpressed in many cancers like brain, breast, and prostate, thus thought to be a promising target for drug therapy[130-132]. Small molecules such as SL11144 and tranylcypromine have been developed to inhibit LSD1[133,134], Since then have shown to restore expression many silenced tumor suppressors like secreted frizzled-related protein and GATA transcription factors in many cancer cell lines. They have also been shown to possess antitumor activity in a study involving neuroblastoma xenografts model[132]. However, similar to HDACi, HDM and HMT inhibitors also have off-target effects on H3K9me2 and DNMT1 thus limiting their use[135] and further in-depth studies are required. EZH2 is another methyltransferase responsible for H3K27me3 leads to gene silencing by promoting DNA methylation[136]. EZH2 is overexpressed in head and neck, breast, and prostate cancers[137] and can be targeted by a hydrolase inhibitor called 3-deazaneplanocin A (DZNep). It induces differentiation as well as apoptosis in cancer cell lines and xenografts by countering EZH2 and inhibiting H3K27 trimethylation[138,139], while sparing normal cells.

Histone kinases and phosphatases as the targets

Compared to histone acetylation and methylation, the effort of regulating histone phosphorylation by targeting kinases and phosphatases for therapeutic uses is new. High level of several histone H3 phosphorylations such as H3S10ph, H3T6ph has been reported in a number of cancers. Unpublished data from our lab shows increase of H3S10ph in cisplatin resistance gastric cancer cell lines AGS and KATOIII. Our observation further supported the finding that p38 MAPK pathway mediated increase in H3S10ph in response to cisplatin treatment[140] in HeLa and MCF7 cells. Pacaud *et al*[93] recently reported that the kinase inhibitors like Enzastaurin (PKC-beta inhibitor), AZD1152 (Aurora-B inhibitor) and AZD1480 (Jak2 inhibitor) increases the cell death of TMZ-Irrad resistant GBM and decreases H3T3ph, H3S10ph and H3Y41ph respectively. Further, H89 (MSK1 inhibitor) treatment reduces the TPA and EGF mediated cellular transformation and by decreasing H3S10ph[44]. All these studies represent the potential of regulating histone phosphorylation for therapeutic use in cancer; however, these observations need to be further explored.

Despite of all this progress in the utilization of histone PTMs in chemotherapeutic interventions, a very little is known about their utility in monitoring the response to chemotherapy. For this purpose, levels of cNUCs and their modifications can be utilized. Because, circulating nucleosomes in serum are a result of apoptosis of

actively dividing cells; therefore, after chemotherapy/radiotherapy increase in the circulating nucleosomes correlates with progressive disease and decrease was associated with disease regression. Increase in the concentration of serum nucleosomes has been shown at 24-72 h after the first application of chemotherapy and 6-24 h after the start of radiotherapy[60]. Thus, the concentration of nucleosomes in serum might be a useful tool for monitoring the biochemical responses during antitumor therapy, particularly for the early estimation of therapeutic efficacy. Histone modifications such as H4K16ac for example, can be utilized in this regard as its loss has been reported in several cancers and also chemosensitize cancer cells[33,69,141]. Histone modifications like H3K27me3 have indeed showed perplexing results when analyzed with respect to various cancers. This can be attributed to tissue type, and indeed histone PTMs are known to be showing their abundance in a tissue specific manner[142]. This might be as because many writers and erasers utilize co-factors or substrates like acetyl CoA, SAM, NAD^+, FAD^+ or ATP which are crucial metabolites in core pathways of intermediary metabolism[143]. The cellular concentrations of these metabolites fluctuate with the metabolic status of the cells and thus, the activity of these enzymes gets affected thus the histone PTMs.

CONCLUSION AND FUTURE DIRECTIONS

The role of histone modifications in governing cellular functions has been not yet fully understood. However, with increased research over the past decade, all the organisms studied so far (from yeast to man) have bought to light the importance of chromatin environment especially histone PTMs in development and disease. These observations have revolutionized the field of epigenetics and have challenged the old hypothesis of the genetic code being the sole determinant of the pathophysiology of any disease. In cancer, especially this is further established with the discovery of small molecule inhibitors targeting histone modifying enzymes, which can restore the expression of various genes to normal and can induce apoptosis of transformed cells. The best studied examples of these drugs are HDACi, which have proven to be highly effective anticancer drugs, thus are in clinics. Although the exact nature of the mechanism by which these drugs act is not understood yet, still these drugs are faring better against cancer. Future studies need to be directed more towards understanding these mechanisms and increasing the potency of these drugs. Though many histone PTMs are known to change during cancer, less is understood regarding the significance and mechanistic details of the change observed. Much of the work done in this direction has been hindered due to technical limitations. However with the advent of new technologies, and also decrease in the cost of high throughput technologies like ChIP-seq and TMA amongst other global approaches, it is a matter of time we have more knowledge of these mechanisms. Also, new targets for development of more potent drugs need to be explored by careful understanding of an already existing chromatin atlas of various cancer cell lines and tissues. Further work in the next decade may gain deeper understanding of the global patterns of histone posttranslational modifications and their corresponding changes which will hopefully reveal many molecular targets that can be employed as new weapons in long fought battle against cancer.

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