Genotype-Molecular Phenotype Correlation using Lymphoblastoid Cell Lines from Patients with Multiple Primary Neoplasms

by

TABISH

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As members of the Viva Voce Board, we recommend that the dissertation prepared by Ms. Tabish titled 'Genotype-Molecular Phenotype Correlation using Lymphoblastoid Cell Lines from Patients with Multiple Primary Neoplasms' be accepted as fulfilling the requirements for the Degree of Doctor of Philosophy.

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Declaration

I declare that the thesis titled 'Genotype-Molecular Phenotype Correlation using Lymphoblastoid Cell Lines from Patients with Multiple Primary Neoplasms' is a record of the work carried out by me during the period September 2006 to February 2012 under the supervision of Dr. R. Mulherkar. This work is original and it has not been submitted earlier as a whole or in part for a degree, diploma, associateship or fellowship at this or any other institute or university.

Navi Mumbai,
September, 2012.

Tabish

Certificate

I certify that the thesis titled 'Genotype-Molecular Phenotype Correlation using Lymphoblastoid Cell Lines from Patients with Multiple Primary Neoplasms' submitted for the degree of Doctor of Philosophy by Tabish is a record of the research carried out by her during the period September 2006 to February 2012 under my supervision. This work has not formed the basis for the award of any degree, diploma, associateship or fellowship at this or any other institute or university.

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Synopsis

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Genotype-Molecular Phenotype Correlation using Lymphoblastoid Cell Lines from Patients with Multiple Primary Neoplasms

Introduction:

Squamous cell carcinomas of upper aero-digestive tract (UADT) are most prevalent cancers amongst Indian men and account for at least one third of total cancer deaths [1, 2]. Advancement in early detection and treatment has improved overall disease management, however, a 3-7% annual risk of development of a second/ multiple primary neoplasm(s) (MPN) among the survivors of early stage UADT cancer poses an additional threat in terms of morbidity and mortality [3-5]. Convincing evidence from case-control studies analyzing cumulative polymorphisms suggests aberrant gene-environment interactions to be an important etiological factor in the genesis of MPN [6, 7].

Collective effect of genetic polymorphisms and tobacco habit has been studied earlier in the lab and an association of polymorphisms in genes falling in major carcinogenesis pathways (SULT1A1-Arg^{213}His, hOGG1-Ser^{326}cys, BRCA2-Asn^{372}His, mEH-Try^{113}His, XRCC1-Arg^{280}His) was observed in MPN patients with tobacco habit [8]. Therefore it was considered important to validate the findings of genotyping study in at least a subset of the patients by phenotypic assays and develop a genotype-phenotype correlation. We hypothesized that MPN may be a manifestation of differences in the polygenic susceptibility to carcinogens between different individuals. Hence if genetic polymorphisms in important carcinogenesis pathways like DNA damage/repair, apoptosis, carcinogen metabolism and cell cycle regulation have functional significance then there may be a correlation between genotype and intermediate phenotypes like defect in DNA repair, apoptosis or cell cycle regulation.

One of the prime requisites for undertaking such studies is continuous supply of starting parent material. Being a spontaneous replicating source, Epstein Barr Virus (EBV) transformed Lymphoblastoid cell lines (LCLs) very well fulfil the requirement of constant supply of starting material for a variety of assays, sparing the need of re-sampling [9]. Using these LCLs as a model system we have compared the difference in the phenotypic response between MPN patient and control group in terms of percent cell death, DNA damage/repair, transcriptional response and cell cycle regulation after γ-radiation and Benzo[a]pyrene-diol-epoxide (BPDE, a tobacco specific carcinogen) exposure *in vitro*. In addition we have genotyped 22 candidate single nucleotide polymorphisms (SNPs) in candidate genes involved in DNA repair, apoptosis, cell

cycle regulation and carcinogen metabolism and created a Genotype Score (G Score) from the number of variant alleles.

Intermediate phenotypes including transient arrest in cell cycle, proper function of DNA damage repair machinery and activation of cell death program upon exposure to genotoxic agents are known to be disrupted in variety of cancers and are associated with neoplastic evolution [10-14]. Hence we assumed that individuals with inherited defects in cell cycle control, apoptosis, and/or DNA repair, owing to inter-individual difference at the genetic level, might be susceptible to UADT MPN development. Hence we have correlated these genotypic and phenotypic measurements to investigate their potential relationships to have a better understanding of pathogenesis of UADT MPN.

Aim:

To develop a correlation between genotype (polymorphisms in candidate genes involved in DNA repair, apoptosis, cell cycle regulation and carcinogen metabolism) with the phenotype (defect in DNA repair, apoptosis or cell cycle regulation) using lymphoblastoid cell lines established from MPN patients and healthy controls following genotoxic exposure *in vitro*.

Objectives:

1. To generate EBV LCLs from peripheral blood lymphocytes (PBLs) isolated from MPN patients and healthy controls-an established *in vitro* model for genetic studies.
2. To compare the response of MPN patients with tobacco habits and appropriate controls *in vitro*, to the exposure to DNA damaging agents such as γ-radiations and Benzo[a]pyrene-diol-epoxide (BPDE, a tobacco specific carcinogen), by assessing DNA damage and repair, cell cycle profiling, apoptosis and global gene expression profiling.
3. To genotype selected candidate genes involved in DNA repair, carcinogen metabolism, apoptosis and cell cycle regulation.
4. To establish a correlation between the phenotype (e.g., poor DNA repair capacity, apoptosis) with the genotype (e.g., polymorphisms in the genes).

Materials and Methods:

1. Collection of samples: After obtaining IRB approval and patient informed consent, 3 ml whole blood was collected in an EDTA vacutainer from patients with MPN and cancer free healthy individuals by venipuncture. Patients with MPN were accrued from the Cancer Genetics Clinic in Tata Memorial Hospital (TMH) and samples from healthy control individuals were obtained from Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Mumbai, India. The study was approved by the Hospital Ethics Committee, TMH. UADT MPN patients were recruited on the basis of criteria given by Hong et al. where they met one of the following standards[15]: (i) The second primary tumour (SPT) should not be the recurrence and regional or distant metastasis of the first primary tumour, (ii) the histology of SPT should be different from the first primary tumour, (iii) if the histology is same the SPT should be separated from first primary by more than 2 cm of normal epithelium, (iv) or has to occur at least three years after the diagnosis of the first primary tumour. Individuals were accrued as controls if they had no personal history of cancer and consented to participate in the study.

2. Generation of viable EBV stock: EBV-transformed B95-8 marmoset cell line was procured from National Centre for Cell Science (NCCS), India and the EBV stock prepared. Briefly, $0.5x10^6$ cells/ml were seeded in RPMI-1640, 15% FBS, 200 mM glutamine and 1X PenStrep. After 7 days confluent cultures of B95-8 appearing straw yellow in colour were lysed by freeze thawing at -80 °C and 37 °C and filtered to obtain EBV crude stock. The filtrate was stored at 4 °C for short term or -80 °C for long-term storage.

3. Lymphoblastoid cell line preparation: For separation of PBLs blood was separated on a Ficoll-Hypaque gradient. PBLs were seeded in a 24 well plate in complete medium (DMEM containing 15% FBS, 200 mM glutamine and 1X PenStrep). Crude stock of EBV at 1:1 ratio was added to the PBLs and incubated at 37 °C with 5% CO_2. After 24 h, medium containing viral supernatant was aspirated without disturbing the cells and fresh complete DMEM was added. After 3-4 weeks of incubation, rosette morphology of cells ascertained the transformed phenotype of PBL. Cells were mixed thoroughly to break clumps before splitting to ensure multiclonal population.

Important note: *For all cell characterisation and phenotypic assays freshly grown LCLs within 60 population doubling and with >90% viability were used. All biological waste was considered hazardous and discarded in 5% hypochlorite solution followed by safe disposal as per ACTREC bio-safety guidelines.*

4. Characterization of LCL: Characterization of LCLs was done using standard techniques as described below.

4.1 Immunophenotyping: Cells ($1x10^6$) from LCLs were incubated separately with PE labeled primary mouse anti-CD3 (T cell marker), anti-CD19 (pan-B cell marker) and anti-CD56 (NK cell marker) (antibodies used in this experiment were kind gift from Dr. Shubhada Chiplunkar and Dr. Naren Joshi, Immunology department, ACTREC). The cells were washed with FACS buffer and were passed through syringe to break any cell aggregates or clumps and analyzed on Flow Cytometer. A minimum of 10,000 events were analyzed for each sample. Cellular debris was removed by gating on Forward vs. Side Scatter. Statistical analysis was done using CellQuest Pro software.

4.2 Ploidy analysis of LCLs: DNA ploidy is defined as diploid DNA represented as single G0/G1 peak on a histogram corresponding to the same DNA content represented as single G0/G1 at the same position in the histogram of the control. Ploidy was measured by calculating DNA index (DI) which is the ratio between the channel number of G0/G1 peak on histogram of the cell line to the channel number of G0/G1 peak of control PBLs. For ploidy analysis cells from LCLs and control PBLs from healthy volunteer were fixed in 70% ethanol. The cells were washed twice in PBS followed by incubation with Propidium Iodide and RNaseA. Cells were passed through syringe before acquisition to break any cell aggregates or clumps and fluorescence was acquired on flow Cytometer at 488nm excitation. Data were analyzed using ModFit LT V 2.0 software.

4.3 Expression and activity of ATM gene: RNA was isolated from cell lines and lymphocytes isolated from the same subjects by TRIzol extraction method. cDNA was synthesized from total RNA using Superscript First-Strand synthesis system by RT-PCR according to manufacturer's instructions. Expression of ATM gene was measured semi-quantitatively by RT-PCR using gene specific primers and β actin was used as

loading control. PCR products were run on 2% agarose gel and stained with ethidium bromide.

For one sample, activity of ATM gene was compared between the PBL and its corresponding cell line, by immunostaining for pATM. Both the cell line and isolated lymphocytes were irradiated with 4 Gy radiation dose so as to activate ATM in phosphorylated form. Unirradiated cell line and lymphocytes treated in the same way served as control. Following 30 min incubation cells were attached on the surface of Poly-L-lysine coated cover slips and fixed with 4% paraformaldehyde. Permeabilization was performed by addition of 0.3% TritonX-100 in PBS, followed by blocking in 3% BSA in PBST (PBS containing 0.1% Tween-20). Next the cells were incubated with pATM primary antibody at 1:150 dilution in PBST overnight at 4 ^{o}C. pATM was visualized as foci by using Alexa-546 secondary antibody at 1:200 dilution in PBST and nuclei were counterstained with DAPI. pATM appeared as red foci on DAPI stained nuclei.

***4.4 Cell population doubling*:** 5x10^4 cells from LCLs were seeded for each time point in a 24 well plate with 1.5 ml of complete medium. Viable cell count was taken using Trypan blue dye exclusion method at different time points including 0, 12, 24, 36, 48, 72 and 96 h. For each time point four readings were taken.

5. Measurement of DNA damage and repair: LCLs were treated with γ-radiation to assess difference in the DNA repair capacity between MPN patient and control groups.

5.1 Immunofluorescence foci formation assay for γ-H2AX after γ-radiation exposure: 0.3x10^6 cells were irradiated with 2 Gy and 5 Gy γ-radiation in two sets using a Cobalt-60 isotope source (Bhabhatron-II) at room temperature. Unirradiated cells treated in the same way served as control. Following 30 min incubation (maximum γ-H2AX are seen after 30 min) from one set and 4 h incubation (time taken to repair the DNA) from the other set, cells were collected. Cells were attached, fixed, permeabilized and treated with blocking solution in the same way as described in 4.3. Cells were incubated with γ-H2AX primary antibody at 1:150 dilution in PBST overnight at 4 ^{o}C. γ-H2AX was visualized as foci by using Alexa-546 secondary antibody at 1:200 dilution in PBST and nuclei were counterstained with DAPI. γ-H2AX appeared as red foci on DAPI stained nuclei.

5.2 Measurement of γ-H2AX by flow cytometry after γ-radiation exposure: $0.5x10^6$ cells were irradiated with 2 Gy and 5 Gy γ-radiation in two sets. Unirradiated cell treated in the same way served as control. Following 30 min incubation from one set and 4 h incubation from the other set, cells were collected and washed with PBS and fixed with paraformaldehyde. Permeabilization was performed by addition of 0.3% TritonX-100 in FACS buffer (2% FBS and 0.02% sodium azide in 1X PBS) followed by blocking in 3% BSA in FACS buffer. Cells were incubated with γ-H2AX primary antibody at 1:250 dilution overnight at 4 ^oC and next day the cells were incubated with Alexa-488 secondary antibody at 1:300 dilution. Cells were further diluted with FACS buffer and acquired by flow cytometry. Data analysis was done using CellQuest Pro software.

6. Effect of genotoxic exposure on cell cycle profile: In order to assess the effect of genotoxic exposure on cell cycle profile the following experiments were done.

6.1 Effect of γ-radiation on cell cycle profile: $1x10^6$ cells were irradiated with 5 Gy and 10 Gy radiation dose and allowed to grow for 10 h. Cells were collected, fixed and stained as described in 4.2. Cell cycle phase distribution was obtained after analysing data using ModFit LT V 2.0 software. The differences in G2 cell percentages between treated cells and untreated cells were recorded as the G2 cell cycle delay percentage.

6.2 Effect of BPDE exposure on cell cycle profile: $1x10^6$ cells were treated with 5 µM and 8 µM BPDE dissolved in Tetrahydrofuran (THF) and incubated for 6 h. Untreated cells and cells treated with THF alone were taken as controls. After 6 h half of the cells were collected and fixed with 70% alcohol. Rest half were further allowed to grow for 18 h, after removing BPDE containing medium and adding fresh complete medium, so as to complete a 24 h cell cycle. After the 24 h rest half of the cells were collected and fixed with 70% alcohol. The cells for both BPDE concentration and both time points were stained as described in 4.2. Cell cycle phase distribution was obtained after analysing data using ModFit LT V 2.0 software.

7. Measurement of percent cell death after genotoxic exposure: LCLs were treated with γ-radiation and BPDE to assess difference in percent cell death between MPN patient and control groups.

7.1 Percent cell death after γ-radiation exposure: $0.5x10^6$ cells irradiated with 5 Gy and 10 Gy γ-radiation and were allowed to grow for 48 h. Unirradiated cells treated in the same way were taken as a control. To quantitate percent cell death cells were stained with Annexin-V-FLUOS antibody and Propidium iodide (PI) where Annexin-V-FLUOS stains apoptotic cells and PI stains the necrotic cells. Cells were incubated with Annexin-V-FLUOS antibody at 1:100 dilution and 1 µg PI made in incubation buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) and analyze by flow cytometry. Data analysis was done using CellQuest Pro software.

7.2 Percent cell death after BPDE exposure: $0.5x10^6$ cells were treated with 5 µM and 8 µM BPDE dissolved in tetrahydrofuran (THF) and incubated for 6 h. Untreated cells and cells treated with THF alone were taken as controls. After 6 h cells were collected and washed with PBS. To quantitate percent cell death Annexin-V- PI staining was performed as described in 7.1.

8. Genotyping of genes: Genotyping of genes was done using standard techniques as described below.

8.1 DNA isolation: RBCs were lysed using sucrose lysis buffer (0.32 M sucrose, 10 mM Tris pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100). Lysis buffer was added to blood in the ratio of 10:1 and centrifuged to get the nucleated blood cell pellet. Genomic DNA from the pellet was extracted by the lysis of cell membrane with STE buffer (0.1 M NaCl, 0.05 M Tris pH 7.5, 1 mM EDTA pH 8.0, 1% SDS) and proteinase K digestion over night at 37 °C. DNA was isolated by following standard phenol-chloroform extraction method and an aliquot was run on 0.8% agarose gel to check DNA integrity, quality and yield. The samples were stored at 0 °C till further use.

8.2 Polymerase chain reaction: PCR reactions were carried using gene specific primers in sterile thin walled microfuge tubes. The reactions were carried out in 25 µl volumes containing 100 ng of DNA template where reaction mixture without DNA served as negative control and the product was analysed by agarose gel electrophoresis. The remaining product was used for either restriction fragment length polymorphism or for SNaPshot reaction.

8.3 Restriction fragment length polymorphism (RFLP): Restriction digestion of the PCR product with appropriate restriction enzyme was done to analyse the genotype. PCR product from gene specific PCR reactions was incubated with specific restriction enzyme with appropriate buffer. Restriction digestion products were analysed by agarose gel electrophoresis along with DNA ladder.

8.4 SNaPshot reaction: SNaPshot genotyping is based on the dideoxy single-base extension of an unlabeled oligonucleotide primer which binds to a complementary template in the presence of fluorescently labelled ddNTPs and Taq DNA polymerase. The polymerase extends the primer by just one nucleotide, adding a single ddNTP to its 3' end. First the desired gene was amplified by PCR using gene specific primers. SNaPshot assay was performed according to manufacturer's protocol which consists of three steps: (1) EXO-SAP purification (remove unused dNTPs, primer and primer-dimers), (2) SNaPshot reaction (single base extension of the primer) and (3) Post SNaPshot purification (remove unused ddNTPs from SNaPshot reaction. The processed samples were then sequenced in Genetic analyzer for interpretation of SNaPshot results.

8.5 Genotype Score: Genotyping of 22 candidate single nucleotide polymorphisms (SNPs) in 17 candidate genes involved in DNA repair, apoptosis, cell cycle regulation and carcinogen metabolism was done and a Genotype Score (G Score) was created from the number of variant alleles. A value of 2, 1 and 0 was allotted to homozygous variant, heterozygous and homozygous wild type alleles respectively, and a consolidated G Score was calculated taking values of all genes together.

9. Microarray: Whole genome expression profiling was done for 5 MPN and 5 Control cell lines after 5 Gy γ-radiation exposure and 24 h time point using Toronto 27 K slides (University Health Network Microarray Centre, Toronto). RNA was isolated using TRIzol protocol and was used for first strand cDNA synthesis using Superscript II reverse transcriptase for each slide. Indirect labelling of test and reference cDNA samples was done using Cy5 and Cy3 dyes (Amersham), respectively. Labelled samples were added to microarray slides and were kept in hybridization chambers at 42 ^{o}C in water bath overnight. After hybridization slides were sequentially washed using reducing concentrations of saline sodium-citrate (SSC) buffer containing SDS. The

slides were then dried and the fluorescent images of hybridized microarrays were obtained using a GenePix 4200A microarray scanner. Primary data analysis for determining differential expression of genes between was carried out using the Genepix Pro software version 5.0.

10. Quantitative RT-PCR: Differential expression of genes was validated by Quantitative RT-PCR. Invitrogen RT-PCR kit was used to reverse transcribe total RNA using both oligo dT and random primers. The synthesized cDNA was diluted to a final concentration of 10 ng/µl with nuclease free H_2O to be used as a template for real time reactions. All PCR reactions were performed in duplicate using Power SYBR Green and analysed on ABI Prism 7700 Sequence Detection System. GAPDH was used as endogenous RNA control and each sample was normalized on the basis of its endogenous gene expression.

11. Statistical methods: Phenotypic assays were performed thrice wherever a statistical analysis was done. The differences between MPN patient and control groups, for DNA damage repair assay, cell cycle profile and percent cell death response, were examined by standard unpaired Student's t-test or Mann-Whitney U test depending on whether the data followed normal distribution or not, respectively. For statistical analysis of genotyping data G Score was compared between the two groups by unpaired Student's t-test. All analyses were performed using the GraphPad Prism V 5.0 and a p-value of $\leq$ 0.05 was considered significant. For genotype phenotype correlation, statistical dependence between two variables (G Score and phenotypic response) was calculated by applying standard Pearson or Spearman rank correlation. The negative or positive value of correlation coefficient indicated the negative or positive correlation between the variables. A p-value of ≤ 0.05 was taken to be significant.

Results:

1. Preparation and characterization of EBV cell line: A total of 34 LCLs from UADT MPN patients (n=24) and healthy controls (n=10) were prepared from PBLs isolated from blood samples. Sixteen UADT MPN patient (n=8) and healthy control cell lines (n=8) were prepared as a part of earlier study in the lab; eighteen MPN patient (n=16) and control cell lines (n=2) have been prepared in the present study. The average population doubling (PD) time of representative LCLs was found to be 24 h.

Immunophenotyping revealed that cells from representative randomly selected LCLs showed expression of typical B cell surface marker (CD19) while markers for T cell (CD3) and NK cells (CD56) were absent. DNA ploidy status of the LCLs was assessed immediately after cell line preparation at low population doubling (<5 PD) and at higher population doublings (>100 PD). All the cell lines had a DI ratio ranging between 0.9-1.3 and were considered to be diploid. Also there was apparently no change at the expression level of ATM gene between the LCL and respective RNA isolated from the subject. The activity of ATM as assessed by immunofluorescence foci formation assay was comparable between LCLs and PBLs. Out of 34 cell lines 4 MPN patient cell lines could not be grown properly in the continuous culture hence were not undertaken for any phenotypic assay.

2. DNA damage and repair after γ-radiation exposure: DNA damage and repair after γ-radiation exposure was measured by Immunofluorescence foci formation assay for γ-H2AX and measurement of γ-H2AX positive cells by flow cytometry.

2.1 Immunofluorescence foci formation assay for γ-H2AX: After DNA damage appearance and the disappearance of phosphorylated H2AX (γ-H2AX) can be used as a parameter to measure DNA damage and repair. In MPN patient and control cell lines number of γ-H2AX foci at 30 min time point were approximately same indicating that the extent of DNA damage was almost equal. But at 4 h time point most of the γ-H2AX foci disappeared in LCLs from healthy controls (n=4) indicating that DNA repair is active while in case of the MPN patient cell lines (n=4) approximately half of the foci were still present at 4 h indicating that DNA repair is either delayed or impaired in these cells.

2.2 Assessment of γ-H2AX by flow cytometry: This experiment was done on 20 MPN and 10 control cell lines using flow cytometry by analyzing percent positive cells for γ-H2AX at 30 min and 4 h time points.

2.2.1 Measurement of DNA damage and repair at 30 min time point: Percent γ-H2AX positive cells at 30 min time point were approximately same in both MPN patient (**42.14%**, range 25.08% - 69.38% at 2 Gy; and **76.31%**, range 52.06% - 97.69% at 5 Gy) and control cell lines (**36.44%**, range 14.33% - 55.25% at 2 Gy; and **76.08%**,

range 41.77% - 90.95% at 5 Gy) indicating that the extent of DNA damage was almost equal.

2.2.2 Measurement of DNA damage and repair at 4 h time point: At 4 h time point the number of percent positive cells were more in MPN patient cell lines (**29.98%**, range 2.80% - 80.2% at 2 Gy; and **43.69%**, range 5.82% - 96.89% at 5 Gy) as compared to control cell lines (**12.17%**, range 4.05% - 21.91% at 2 Gy; and **20.56%**, range 0.37% - 43.09% at 5 Gy).

The mean difference in percent positive cells at 4 h time point between MPN patient and control cell lines was **17.28%** at 2 Gy and **23.13%** at 5 Gy which was statistically significant ($p=0.0083$ at 2 Gy and $p=0.0109$ at 5 Gy), indicating that DNA repair is either delayed or impaired in MPN patient cell lines which was in concert with immunofluorescence data.

3. Effect of γ-radiation and BPDE exposure on cell cycle profile: After genotoxic exposure cell cycle arrest provides time for the cell to repair damaged DNA before entering into the next phase of the cycle. This experiment was done on 20 MPN and 10 control cell lines using flow cytometry by analysing percent G2 delay after radiation exposure and S and G2/M phase arrest after BPDE exposure.

3.1 Percent G2 delay after γ-radiation exposure: A considerable G2 phase delay was observed in control cell lines (**19.18%**, range -1.95% - 29.65% at 5 Gy; and **15.83%**, range -2.38% - 23.10% at 10 Gy) while the extent of G2 phase arrest was lower in MPN patient cell lines (**5.83%**, range -1.53% - 14.41% at 5 Gy; and **3.86%**, range -1.59% - 16.98% at 10 Gy) indicating that MPN cell lines bypass the cell cycle arrest. The mean difference of percent G2 delay between the two groups was **13.35%** at 5 Gy and **11.97%** at 10 Gy which was statistically significant ($p=0.0001$ at 5 Gy and $p=0.0017$ at 10 Gy).

3.2 Cell cycle arrest after BPDE exposure: When the cells were treated with BPDE there was no considerable difference in the mean percent cell arrested in S and G2M phase at both 6 h and 24 h time point between MPN patient and control cell lines.

3.2.1 S phase arrest at 6 h time point: Mean percent S phase arrest in MPN patient cell lines was **18.54%** (range 8.84% - 31.06%) at 5 µM; and **19.14%** (range 10.49% - 35.52%) at 8 µM and in control cell lines it was **13.85%** (range 4.77% - 22.09%) at 5 µM; and **13.58%** (range 7.5% - 16.2%) at 8 µM.

3.2.2 S phase arrest at 24 h time point: Mean percent S phase arrest in MPN patient cell lines was **19.53%** (range 3.26% - 31.33%) at 5 µM; and **20.09%** (range 3.73% - 30.72%) at 8 µM and in control cell lines it was **17.68%** (range 8.10% - 27.24%) at 5 µM; and **16.67%** (range 6.28% - 28.09%) at 8 µM.

3.2.3 G2/M phase arrest at 6 h time point: Mean percent G2/M arrest in MPN patient cell lines was **4.86%** (range 0 - 9.25%) at 5 µM; and **4.87%** (range 0 - 9.48%) at 8 µM and in control cell lines it was **9.57%** (range 5.30% - 16.82%) at 5 µM; and **9.55%** (range 4.03% - 17.05%) at 8 µM.

3.2.4 G2/M phase arrest at 24 h time point: Mean percent G2/M arrest in MPN patient cell lines was **3.65%** (range 0 - 9.9%) at 5 µM; and **3.39%** (range 0 - 8.8%) at 8 µM and control cell lines it was **5.19%** (range 0.01% - 17.01%) at 5 µM; and **4.83%** (range 0.01% - 15.9%) at 8 µM.

4. Apoptotic response in MPN and control samples in vitro: This experiment was done on 20 MPN and 10 control cell lines.

4.1 Percent cell death after γ-radiation exposure: Percent cell death after radiation exposure was higher in control cell lines (**19.22%**, range 9.05% - 42.82% at 5 Gy; and **22.09%**, range 12.04% - 42.4% at 10 Gy) as compared to MPN patient cell lines (**9.84%**, range 8.3% - 12.7% at 5 Gy; and **10.84%**, range 8.3% - 12.7% at 10 Gy). The mean difference between the two groups was **9.38%** at 5 Gy and **11.25%** at 10 Gy which was statistically significant (p=0.0003 at 5 Gy; p=0.0002 at 10 Gy).

4.2 Percent cell death after BPDE exposure: Similarly percent cell death after BPDE exposure was higher in control cell lines (**23.25%**, range 10.49% - 41.79% at 5 µM; and **24.98%**, range 10.23% - 51.76% at 8 µM) as compared to MPN patient cell lines (**13.57%**, range 8.02% - 21.77% at 5 µM and **14.02%**, range 8.46% - 24.69% at 8 µM). The mean difference between the two groups was **9.68%** at 5 µM and **10.98%** at 8 µM which was statistically significant (p=0.0009 at 5 µM and 8 µM BPDE concentration).

***5. Genotyping of genes*:** Genotyping of 22 candidate single nucleotide polymorphisms (SNPs) in 17 candidate genes involved in DNA repair, apoptosis, cell cycle regulation and carcinogen metabolism was done and G Score was calculated. For all samples (n=30) value of G Score ranged from **10-22**. G Score of only DNA repair genes (XRCC1-Arg^{194}Trp, XRCC1-Arg^{280}His, XRCC1-Arg^{399}Trp, XRCC3-Thr^{241}Met, XPD-Lys^{751}Gln, hOGG1-Ser^{326}cys, BRCA2-5'UTR, BRCA2-Asn^{372}His) ranged from **3-9** and MPN risk association signature (SULT1A1-Arg^{213}His, hOGG1-Ser^{326}cys, BRCA2-Asn^{372}His, mEH-Try^{113}His, XRCC1-Arg^{280}His) ranged from **0-6**. G Score for DNA repair genes and MPN risk association signature could significantly differentiate between MPN patient and control group with a p=0.012 and p=0.039 respectively.

6. Global gene expression profiling: Data obtained after microarray experiment was analysed using GeneSpring GX 10.0 software. A differential expression of few genes associated with various cancers or involved in signal transduction or transcriptional regulation was observed. Genes with differential expression of more than 2 fold (ARHGAP25, MAFB, AREG, ZNF429 and TMEFF) were selected and validated using real time PCR. The difference in the expression of genes that was observed in microarray experiment was also observed in the real time validation.

7. Genotype phenotype correlation: Phenotypic responses after radiation and BPDE exposure were either correlated with total G Score or G Score of DNA repair genes or MPN risk association signature only. Either Pearson or Spearman correlation was used to calculate the relationship between phenotype and genotype on the basis of whether the data followed normal distribution respectively. A comparison between G Score and phenotypic response between most groups revealed statistically significant correlation.

Discussion:

In this study we investigated the combined effect of multiple genetic variations and phenotypic effects *in vitro* in order to assess UADT MPN risk, by developing a genotype-phenotype correlation. The advantage of undertaking multigenic pathway based approach in studying complex diseases like cancer is well evident from various association studies [16]. Use of patient derived cell lines is of immense practical value for such long term genotype-phenotype correlation studies involving execution of a number of experiments on single sample. In order to have a continuous supply of

starting material we successfully generated continuously growing LCLs following infection of isolated PBLs with EBV-containing supernatants. LCLs showed expression of B cell surface marker (CD19) while markers for T cell (CD3) and NK cells (CD56) were absent. DNA ploidy status of the LCLs demonstrated that they were diploid in nature. Generation of EBV transformed cell lines has proven to be cost effective, rapid and reliable method. A comparison with normal PBLs revealed that these cell lines exhibit minimal deviation from normal phenotype and genotype and can be used as an ideal surrogate of lymphocytes.

It is apparent from our results using LCLs that there is a marked difference in γ-radiation and BPDE induced phenotypic responses between MPN patient and healthy controls. Exposure to ionising radiations results in DNA double strand breaks (DSB) and phosphorylation of histone H2AX at ser139 (γH2AX), which is a sensitive marker for measuring DSB. We observed that after radiation exposure the level of DSB between the two groups was almost similar as the number of γ-H2AX foci and percent γ-H2AX positive cells at 30 min time point were approximately same. But the extent of DNA repair at 4 h time point was significantly different between the two groups as revealed by residual γ-H2AX foci and percent positive cells at both the radiation doses. This indicates that DNA repair is compromised in UADT MPN patients resulting in accumulation of more DNA damage than healthy individuals after genotoxic exposure and suggests that DNA repair capacity might be a risk factor for UADT MPN development.

It is well proven that when a cell is exposed to DNA damaging agents it results in cell cycle arrest and activation of cell cycle check points which play an essential role in DNA repair [10]. The checkpoints provide time for DNA repair before entering into the next phase of cell cycle and prevent cell multiplication with damaged DNA. A disruption in any of the cell cycle check points may result in accumulation of gene mutations and chromosomal aberrations by reducing the efficiency of DNA repair leading to genetic instability that may drive neoplastic evolution. Tobacco specific carcinogen BPDE forms adducts with genomic DNA resulting in replication errors that can lead to formation of replicative gaps. These replicative gaps can be repaired during S phase by post-replicative repair pathways [17]. If these gaps remain in the genome due to inefficient DNA repair then they may get converted into DSB in G2 phase [17]. Hence BPDE exposure in normal cells results in S or G2/M phase arrest. Similarly G2 phase cells are extremely sensitive to ionising radiation induced DNA damage resulting in

G2/M arrest and delaying entry into mitosis until the damage has been repaired [17]. Our results demonstrated that after exposure to γ-radiation G2 delay was more pronounced and significantly higher in control cell lines as compared to MPN patients' cell lines. Defects in cell cycle G2 delay are strongly associated to human carcinogenesis. Consequently we speculate that individuals with inherited defects in the G2 checkpoint may be predisposed to MPN development. Contrary to the observations following radiation exposure, there was no marked difference in S or G2/M arrest between patient and control group after BPDE exposure.

Under normal circumstances when DNA damage is severe and cannot be repaired, an apoptotic response is elicited to eliminate the damaged cell. A failure in proper apoptotic response after extreme genotoxic exposure may also contribute to MPN development. There are reports available where disruption of apoptosis is associated with cancer [11, 14, 18]. We found that after radiation as well as BPDE exposure there was remarkable difference in the apoptotic response between MPN patient and control cell lines which was statistically significant. We believe that low apoptotic response in MPN cases could enable cells with DNA damage to accumulate, possibly conserving mutations in critical genes and inducing carcinogenesis.

Differences in cellular responses can be traced back to differential gene expression. In order to find any association between varied phenotypic response after genotoxic exposure and gene expression in our cell lines we carried out expression profiling. Although there was no marked difference in expression of genes falling in important pathways related to cancer development but we indeed observed a variation in expression of genes like MAFB, ZNF429 and HMGA2 that are involved in transcription regulation, along with MAPK10, ARHGAP25, and GNG12 signal transduction genes which may in turn have an effect on downstream molecules. While few other differentially expressed genes like TMEFF1, AREG and SMAD6 are known to be associated with cancers. It is unlikely that such microarray studies will identify the etiology of UADT MPN but we expect that it may help in highlighting pathway defects playing crucial role related to pathogenesis.

The differences that were observed in phenotypic response after genotoxic exposure between patient and controls can be attributed to differences in the polygenic susceptibility to genotoxins between the two groups. Genetic variations present in patients may give rise to phenotypes leading to multiple cancers on genotoxic exposure. Therefore we considered it important to compare phenotypic findings with the genotype

and develop a correlation. We genotyped 22 SNPs in 17 genes falling in important carcinogenesis pathways viz. carcinogen metabolism, DNA damage/repair, cell cycle regulation and apoptosis. To understand genotyping data we calculated a consolidated G Score on the basis of whether the subject had homozygous variant, heterozygous or homozygous wildtype form of a gene. A high G Score designated more number of risk alleles in an individual. Our assumption was that individuals with a high G Score might have a higher probability of MPN development as compared to those with the low G Score. Hence a higher G Score indicated magnitude of cancer predisposing genotype.

A total G Score with all 22 SNPs, G Score of only DNA repair genes and G Score of MPN risk association signature, which included SNPs in genes that could predict the outcome of tobacco related MPN in our previous study [8], was calculated and was found to be higher in MPN patient group as compared to control group. Although total G Score was not statistically significant between the two groups at the 5% level ($p=0.12$), G Score of DNA repair genes and MPN risk association signature was statistically significant, indicating that indeed there existed inter-individual difference at genetic level between MPN patients and controls at least in a subset of important genes falling in carcinogenesis pathways. A comparison of total G Score in all 22 SNPs, G Score of DNA repair genes and MPN risk association signature showed a negative correlation with percent cell death and percent G2 delay. This correlation was statistically significant for most of the groups. While a positive correlation was observed between G Score (all three groups) and percent H2AX positive cells at 4 h time point, although it was not significant.

Our findings emphasize the importance of assessing the collective effects of a panel of polymorphisms in modulating phenotypic effects after genotoxic exposure. The genotype-phenotype correlation observed in this study supports our hypothesis that variations in important genes may alter phenotypic response and may contribute to UADT MPN risk. In the present state for understanding UADT MPN pathogenesis it is important to find association between multiple genetic combinations and cancer risks, which may otherwise remain undetectable in single SNP analysis.

In summary our results clearly suggest that extent of DNA repair, percent cell death and cell cycle delay might be potentially useful in identifying susceptibility to UADT MPN. It also demonstrates that identifying distinctive polymorphism based G Score signature can differentiate the study participants into two separate subsets, and its correlation with various phenotypic effects (indicating a gene-environment interaction)

may have an important bearing on predisposing an individual to UADT MPN development. The significance of this study lies in the fact that it guides us in identifying high risk individuals thus increasing the possibility of identifying cohort of patients of clinical relevance which can be undertaken for chemo preventive studies although larger prospective studies are needed to verify these findings.

Publications:

1. **Hussain T**, Kotnis A, Sarin R and Mulherkar R. Establishment and characterization of lymphoblastoid cell lines from patients with Multiple Primary Neoplasms in the upper aero-digestive tract and healthy individuals. Indian J Med Res. 2012 Jun; 135(6):820-9.
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4. **Hussain T**, Kotnis A, Sarin R and Mulherkar R. Genetic Susceptibility to Multiple Primary Neoplasms in the Upper Aero-Digestive Tract: Genotype Score and Phenotype Correlation. (communicated to *Cancer letters*)

Poster/oral Presentations:

1. **2008:** *13th Human Genome Meeting "Genomics and the future of medicine", India* ***(poster presentation)****.*
2. **2010:** *29th Annual Convention of Indian Association for Cancer Research, India* ***(oral presentation)****.*
3. **2010:** *6th Graduate Students Meet, ACTREC, Navi Mumbai, India* ***(Award for best poster presentation)***.
4. **2011:** *30th Annual Convention of Indian Association for Cancer Research and International Symposium on "Signaling Network and Cancer", CSIR-IICB, Kolkata India* ***(Award for best poster presentation)***.
5. **2011:** *American Association of Cancer Research (AACR) international conference on "New Horizons in Cancer Research: Biology to prevention to Therapy"* (***poster presentation***).

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List of abbreviations

AREG	: Amphiregulin
ARHGAP25	: Rho GTPase activating protein 25
ATM	: Ataxia Telangiectasia Mutated
BM	: Buccal mucosa
BPDE	: Benzo[a]pyrene diol epoxide
BRCA1	: Breast cancer 1
BRCA2	: Breast cancer 2
BSA	: Bovine serum albumin
CCND1	: Cyclin D1
CDC25C	: Cell division cycle 25
CDKN1A	: Cyclin dependent kinase inhibitor 1A
CIA	: Chloroform and iso-amyl alcohol
CR2	: Complement receptor 2
DAPI	: 4',6-diamidino-2-phenylindole
DEPC	: Diethylpyrocarbonate
DI	: DNA Index
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl Sulfoxide
DTT	: Dithiothreitol
EBNA	: Epstein- Barr Virus Nuclear Antigen
EBV	: Epstein Barr Virus
EDTA	: Ethylenediaminetetraacetic acid
EPHX1	: Epoxide hydrolase 1
ERCC6	: Excision repair cross-complementing rodent repair deficiency, complementation group 6
EtBr	: Ethidium Bromide
EXO	: Exonuclease 1
Fas	: TNF receptor superfamily, member 6
FBS	: Foetal Bovine Serum
G Score	: Genotype Score
GAPDH	: Glyceraldehyde-3-Phosphate Dehydrogenase
GBS	: Gingivo buccal Sulcus
GNG12	: guanine nucleotide binding protein Gamma 12
GPX1	: Glutathione peroxidase 1
GSTM1	: Glutathione S transferase Mu 1
GSTP1	: Glutathione S transferase Pi 1
GSTT1	: Glutathione S transferase Theta 1
HLA	: Human Leukocyte Antigen
HMGA2	: High-mobility group AT-hook 2
HNSCC	: Head and Neck Squamous Cell Carcinoma
hOGG1	: Human 8-oxoguanine DNA N-glycosylase 1
HPV	: Human Papilloma Virus
ICAM1	: Intercellular Adhesion molecule 1
IFN	: Interferon
LCL	: Lymphoblastoid Cell Line
LFA1	: Lymphocyte Function Associated Antigen 1
MAFB	: v-maf musculoaponeurotic fibrosarcoma oncogene homolog B
MAPK10	: Mitogen-activated protein kinase

MOPS	: 3-(N-morpholino)propanesulfonic acid
MPN	: Multiple Primary Neoplasma
MPO	: Myeloperoxidase
p21	: Cyclin dependent kinase inhibitor 1A
pATM	: Phosphorylated Ataxia Telangiectasia Mutated
PBL	: Peripheral Blood Lymphocytes
PBMC	: Peripheral Blood Mononuclear Cells
PBS	: Phosphate buffer saline
PBST	: Phosphate buffer saline -Tween
PCR	: Polymerase chain reaction
PD	: Population Doubling
PE	: Phycoerythrin
PHA	: Phytohaemagglutinin
PI	: Phoshpotidyl Inositol
RFLP	: Restriction Fragment Length Polymorphism
RMT	: Retro Molar Trigone
RPMI	: Roswell Park Memorial Institute Medium
RT	: Room Temperature
RT-PCR	: Reverse transcription polymerase chain reaction
S phase	: Synthetic Phase
SAP	: Shrimp Alkaline Phosphatase
SDS	: Sodium Dodecyl Sulphate
SMAD6	: SMAD family member 6
SNP	: Single Nucleotide Polymorphism
SPT	: Second Primary Tumour
SSC	: Saline-Sodium Citrate
SS II	: Superscript II
SULT1	: Sulpho Transferase I
THF	: Tetra Hydro Furan
TMEFF1	: Transmembrane protein with EGF-like and two follistatin-like domains 1
TP53	: Tumour protein 53
TP53BP1	: Tumour protein 53 binding protein 1
UADT	: Upper Aero Digestive Tract
UTR	: Untranslated region
XP	: Xeroderma Pigmentosum
XPA	: Xeroderma Pigmentosum Complimentation Group A
XPD	: Xeroderma Pigmentosum D
XRCC1	: X-ray repair cross-complementing protein 1
XRCC3	: X-ray repair cross-complementing protein 3
ZNF429	: Zinc finger protein 429

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Chapter 1

Introduction

1.1 Introduction

Cancer has been rightfully crowned the 'Emperor of all Maladies' owing to its increasing incidence, diverse forms and ability to affect various organs across different age groups. Even worse is its intractability to treatment and its irrepressible property to recur. With such dynamic efficacies this sovereign has evaded human understanding despite decades of research and ample funds being invested. This explains why cancer continues to be one of the principal causes of deaths worldwide and also in India, where it accounts for approximately 6% of total human deaths [1, 2].

One of the major cancers prevalent in the country is tobacco-related, Head and Neck Squamous Cell Carcinoma (HNSCC), of which, squamous cell carcinoma of Upper Aero-Digestive Tract (UADT) is the commonest cancer amongst Indian men [2, 3]. Early detection and advancement in diagnostic and treatment modalities has lead to improved disease management and increased survival of patients. However this improvement has not been able to mitigate the development of second/multiple primary neoplasm(s) (MPN) which now looms large as one of the major threats in early stage UADT cancer survivors [4-6].

In recent past increasing number of MPN cases in UADT cancers has become an important therapeutic concern. Survivors of early stage HNSCC have a 10-30% risk of developing second primary tumour in the same or distant region which is one of the prime reasons of increased morbidity and mortality [7, 8]. The most important risk factors for developing such cancers are tobacco habit and alcohol consumption, which have a synergistic effect. These genotoxic agents act by disrupting the genomic integrity leading to malignant transformation and subsequent cancer development. However it is well established that there is a considerable difference between individuals at the genetic level that affect their response to genotoxic insult and thus susceptibility to cancer, which is reflected by the fact that not all individuals exposed to similar type and dose of carcinogen develop cancer. There is a possibility of a different host environment interaction in individuals who develop UADT MPN. This difference could arise due to genetic variations in genes that are involved in carcinogen detoxification, repair of damaged DNA, cell cycle and cell death regulation and thereby, their ability to handle carcinogens. Convincing evidence from case-control studies analyzing cumulative polymorphisms suggests aberrant gene-environment interactions to be an important etiological factor in the genesis of MPN [7, 9-11].

In previous studies from this laboratory, the ability of key single nucleotide polymorphisms (SNPs) in genes falling in major carcinogenesis pathways, combined with tobacco usage, in predicting the incidence of tobacco related MPN was demonstrated [11, 12]. However, in order to establish involvement of genetic variations, phenotypic analysis is necessary. If genetic polymorphisms in important carcinogenesis pathways like DNA damage/repair, apoptosis, carcinogen metabolism and cell cycle regulation have functional significance then there may be a correlation between genotype and intermediate phenotypes like defect in DNA repair, apoptosis or cell cycle regulation. We hypothesized that MPN is a genetically enriched source and is possibly a manifestation of differences in the polygenic susceptibility to carcinogens. This hypothesis was tested by establishing a correlation between genotype with the molecular phenotype, following *in vitro* genotoxic exposure.

1.2 Rationale of the study

One of the keystones of epidemiological studies is to attempt to classify individuals into high or low risk groups in the context of disease onset and outcomes. This disease risk assessment becomes even more important when early detection of the disease is one the major factors for successful outcomes. Therefore identifying genetic risk factors to understand complex sporadic cancers like UADT cancers is of immense practical relevance as UADT cancers are a major cause of mortality in our country and often patients detected at an early stage return with a second primary cancer.

Etiological role of genetic susceptibility in UADT squamous cell carcinomas has been supported by data from recent case control studies based on genotypic and phenotypic assays. The rationale behind gene-cancer risk association studies is that variations at genetic level may result in alterations in intermediate phenotypes leading to inefficient DNA repair or improper cell cycle or cell death control and thereby cancer development. Therefore inter-individual difference in their ability to deal with genotoxic agents can be an important determinant of UADT MPN. This study deals with correlating various gene polymorphisms with intermediate phenotypes and establishing a genotype-phenotype correlation. This may allow us to understand the genetic predisposition of these patients to cancer and understanding gene-environment interactions better at the functional level.

Intriguingly, previous studies emphasizing role of SNPs in gene(s) involved in a single pathway with complex disease like cancer show limited predictive value as

most of the genetic variants contribute to relatively small risk. Therefore studies investigating genotypic effect of a panel of polymorphisms in multiple pathways regulating critical cellular functions are of immense importance. The unique feature of our study is that potential susceptibility of 22 SNPs, together or in combinations, has been identified. Genotyping involved documentation of 22 SNPs in candidate genes and calculating a Genotype Score (G Score) from the number of variant alleles. Subsequently, the G Score was correlated with phenotypic effects using Epstein Barr Virus (EBV) transformed lymphoblastoid cell lines (LCLs) generated from UADT MPN patients (n=20) and healthy individuals (n=10) as controls.

Using these LCLs, the effect to exposure to γ-radiation or Benzo[a]pyrene-diol-epoxide (BPDE) was studied. Intermediate phenotypes including transient arrest in cell cycle, proper function of DNA damage repair machinery and activation of cell death program upon exposure to genotoxic agents are known to be disrupted in variety of cancers and are associated with neoplastic evolution. We assumed that individuals with inherited defects in cell cycle control, apoptosis, and/or DNA repair, owing to inter-individual difference at the genetic level, might be susceptible to UADT MPN development. We have correlated these genotypic and phenotypic measurements to investigate their potential relationships to have a better understanding of pathogenesis of UADT MPN.

Thus the study has the potential to identify a signature of polymorphisms and their probable phenotypic effects associated with UADT MPN etiology. The potential susceptibility markers could assist in early detection of patients who are genetically predisposed to develop second primary neoplasia. These susceptibility markers could increase the possibility of identifying cohort of clinically relevant patients that can be accrued for chemo prevention studies.

Hypothesis

Our hypothesis is that UADT MPN is a manifestation of inter individual difference in the susceptibility to carcinogen exposure. Individuals carrying multiple, less efficient variant forms of gene products involved in DNA damage/repair, apoptosis, carcinogen metabolism and cell cycle regulation, are possibly at a greater risk of having compromised/dysfunctional intermediate phenotypes like DNA damage/repair, apoptosis or cell cycle regulation after genotoxic exposure, and hence an increased UADT MPN risk. Therefore, if genetic polymorphisms in genes falling in

important carcinogenesis pathways have functional significance, then a correlation between genotype and intermediate phenotypes needs to be unraveled.

1.3 Aim and objectives

The aim of present study was to develop a correlation between ***genotype*** - performed by evaluating SNPs in candidate genes involved in DNA repair, apoptosis, cell cycle regulation and carcinogen metabolism, with the ***phenotype*** - measured as defect in DNA repair, apoptosis or cell cycle regulation, using lymphoblastoid cell lines established from MPN patients and healthy controls following genotoxic exposure *in vitro*.

The objectives of the study were as follows:

1. To generate EBV LCLs from peripheral blood lymphocytes (PBLs) isolated from MPN patients and healthy controls - an established *in vitro* model for genetic studies.
2. To compare the response of MPN patients with tobacco habits and appropriate controls *in vitro*, to the exposure to DNA damaging agents such as γ-radiations and Benzo[a]pyrene-diol-epoxide (BPDE, a tobacco specific carcinogen), by assessing DNA damage and repair, cell cycle profiling, apoptosis and global gene expression profiling.
3. To genotype selected candidate genes involved in DNA repair, carcinogen metabolism, apoptosis and cell cycle regulation.
4. To establish a correlation between the phenotype (e.g., poor DNA repair capacity, apoptosis) with the genotype (e.g., polymorphisms in the genes).

Chapter 2

Review of Literature

The yet undeciphered extraordinary complexity of the disease cancer has remained the "Achilles' heel" of scientific progress for many decades. This disease with countable names, but uncountable causes, has been intensely studied with inquisitive ideas using advanced technologies. Although these advances made in prevention, diagnosis and treatment of the ailment have undoubtedly brought us a long way in understanding cancer, but the outcomes have not enabled us to conquer the disease yet, thereby leaving us still in the state of learning. The disease has even tighter clasp on developing countries and India is no exception where one of the leading causes of death is cancer. According to recent statistics the estimated number of cancer incidences in India is approximately 9 lakh which claims over 6 lakh lives annually [1, 2].

2.1 Upper aero-digestive tract cancer

Amongst all cancers, HNSCC including squamous cell carcinomas of UADT are the most common cancers found in India with approximately 25% incidence rate [2]. These cancers are also most prevalent amidst Indian men and are a significant problem accounting approximately 40% of total male cancer burden (Fig. 1, Fig. 2) [2]. These include cancer of the lip, tongue, major salivary glands, gums and adjacent oral cavity tissues, floor of the mouth, tonsils, pharynx, larynx and other oral regions, nasal cavity, trachea, bronchus and oesophagus (Fig. 3) [13, 14]. Mainly alcohol and tobacco habit, alone or in combination, are associated with an increased risk of upper aero-digestive tract cancers and their combined use has a multiplicative effect on risk [15, 16].

Over the past decades a significant improvement has been observed in control of UADT cancers owing to the introduction of improved diagnostic, surgical and radiotherapy techniques and use of chemotherapy. However the achievement has not been able to improve the overall survival owing to development of second/multiple primary neoplasm(s) that occur more often in head and neck cancer patients than in patients with cancers of other sites [5, 17]. There is around a 10-30% risk of development of a second/ multiple primary neoplasm (s) or recurrence among the survivors of early stage HNSCC during the first 5 years after initial diagnosis which poses an additional threat in terms of morbidity and mortality [10, 18, 19], with a relatively constant rate of about 3-7% yearly [7, 8, 20].

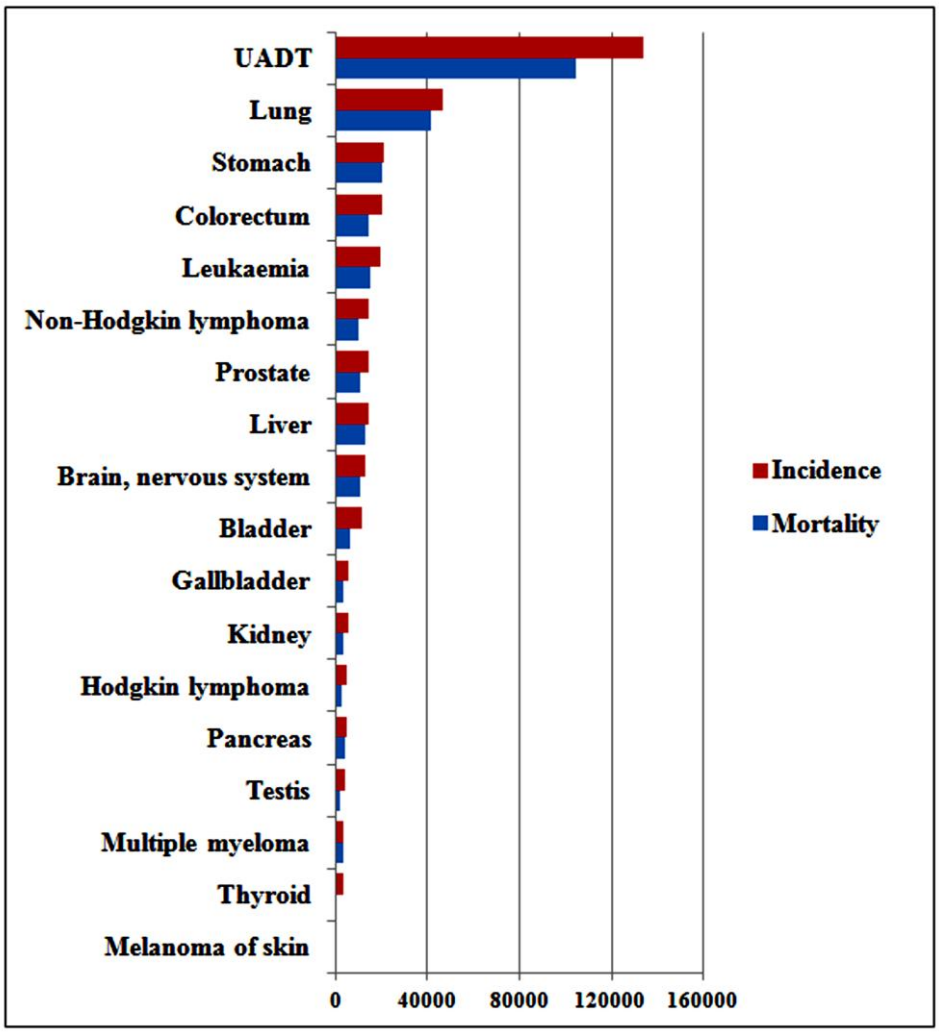


Fig. 1: Estimated incidence and mortality rates of all male cancers (excluding non melanoma skin cancer) in India. UADT cancers are most common cancers with highest incidence and mortality (GLOBOCAN, 2008)

2.2 Multiple primary neoplasms

Multiple primary neoplasm is the occurrence of two or more primary cancers in an individual. Survivors of early stage cancers have an approximately 14% chance of developing second primary cancer and this risk is even higher for the survivors of early stage HNSCC as described above [5, 21]. In 1889, Billroth first described the occurrence of second primary cancer of stomach in a patient after treatment of epithelioma of external ear [22]. However multiple primary malignant tumours remained medical curiosities until 1932 when Warren and Gates identified around 1000 cases of MPN in literature or encountered them in post-mortem examinations [23].

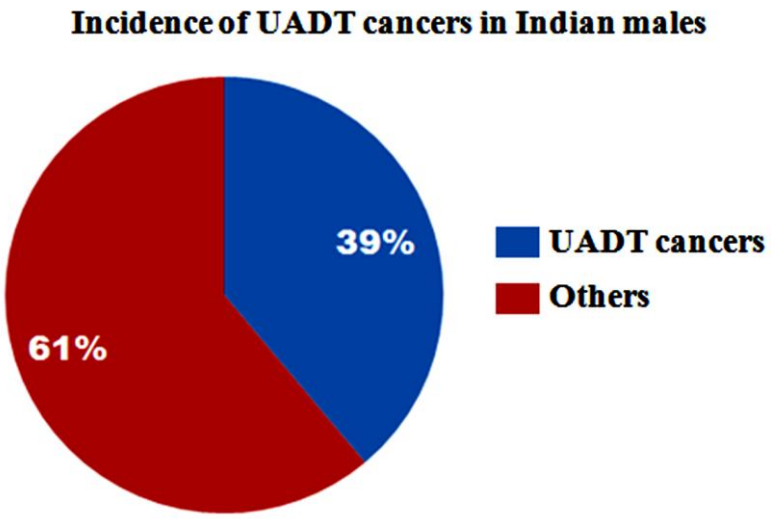


Fig. 2: Incidence of UADT cancer in Indian males. These cancers account approximately 40% of total male cancer burden with highest incidence and mortality (GLOBOCAN, 2008)

Later in 1992, Day and Blot observed a 3.7% yearly rate for the onset of a second primary tumour in approximately 20,000 patients diagnosed with oral and pharyngeal cancer from nine population based registries from United States of America. Oral, pharyngeal and esophagous were the most common sites of second primary tumours followed by nose, larynx and lung and remaining were found in the lower digestive tract, the prostate, the urinary tract, the breast, and the female genital tract [24]. Other then head and neck cancers, MPN is a common feature of various inherited syndromes like, Li-Fraumani syndrome, hereditary breast ovarian cancer syndrome, Fanconi anaemia, Xeroderma pigmentosum and Blooms syndrome. While the cause for occurrence of MPN in above mentioned medical states is almost known the pathogenesis of UADT MPN is not completely understood making it one of the worst problems to deal with [25-28].

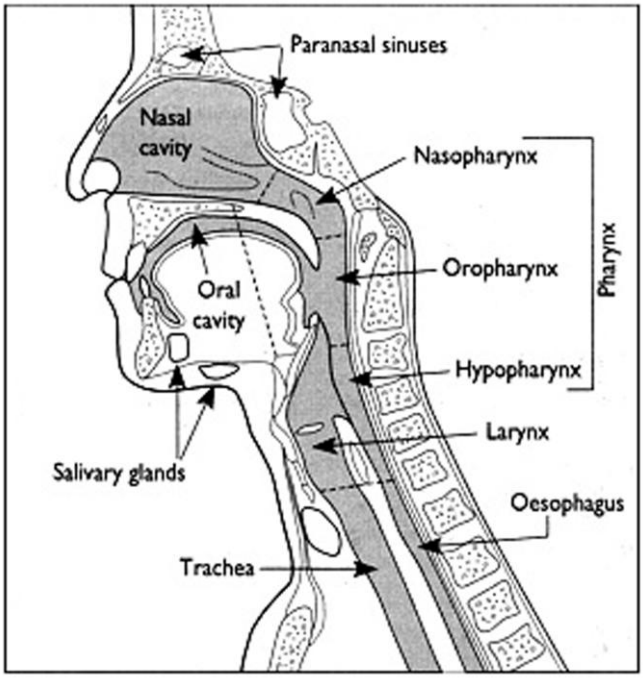


Fig. 3: Sagittal section of Upper aero digestive tract showing various cancer sites in humans. (Tobias, 1994)

2.3 Understanding the pathogenesis of UADT MPN

Various theories have been put forward to understand etiology of UADT MPN, the earliest being the concept of 'Field cancerization' described by Slaughter and Southwick in 1953. They demonstrated that second primary tumours develop independently from the first primary tumour because of the widespread exposure to carcinogens in the upper aero-digestive tract mucosa. They found that total epithelium beyond the boundaries of the tumour showed histological changes, suggesting that due to carcinogen exposure mucosa of the head and neck undergoes a change and becomes more susceptible to the development of many foci of malignant transformation [29].

Later Braakhuis et al. provided a genetic explanation of field cancerization and described a 'progression model' according to which a cell acquires one (or more) genetic alterations and forms a patch with genetically altered daughter cells. This genetically altered patch expands into a field of epithelial lesion with cancer related genetic alterations and later develops into multiple primary tumours [30]. It is now well established that cancers develop through a multistep process of genetic alterations mainly to oncogenes and tumour-suppressor genes. These genetic alterations disrupt several molecular pathways and the cell acquire sustained proliferative signalling, insensitivity to growth repressors, resistance to cell death, replicative immortality,

sustained angiogenesis, invasion, metastasis, reprogrammed energy metabolism and immune evasion [31]. Therefore UADT MPN may also arise due to genetic susceptibility.

The reason for acquisition of genetic alterations in head and neck cancers can be mainly attributed to carcinogenic exposure. Tobacco and alcohol are considered to be the major risk determinant for these cancers [15, 32]. Exposure to tobacco alone or in combination with heavy alcohol consumption can disrupt genomic integrity which contributes to malignant transformation and subsequent cancer development [15, 33, 34]. Since only a fraction of exposed individuals develop the disease therefore an intrinsic susceptibility to environmental genotoxic exposures has also been suggested as playing an important role. Consequently there is a possibility of a different host environment interaction in individuals who develop such MPN.

2.4 Gene environment interaction

Cancer is a multi-factorial disease that arises due to aberrations in many cellular functions that are involved in the normal cellular homeostasis [31]. Although, the exact origin or cause of UADT MPN is still not well understood, but it appears to develop in a susceptible host where both genetic and environmental factors play a significant role in initiation of such sporadic cancers [35]. The involvement of environmental factors in cancer predisposition is known since 18th century when Percival Pott identified soot as a possible agent causing scrotal cancer in chimney sweepers [36]. Since then it has been established that environmental factors play an important role in the etiology of sporadic human cancers [37].

However it is well accepted that all individuals exposed to same type and dose of carcinogens do not develop cancer and there is a considerable inter-individual variations that influences the way an individual handles the genotoxic insult and thus susceptibility to cancer. This difference could arise due to varied interaction of the carcinogens with genes that are involved in carcinogen detoxification, repair of damaged DNA, cell cycle and cell death regulation. The variation in gene-carcinogen interaction can be a consequence of cumulative effect of mutations and polymorphisms in these genes that fall in important carcinogenesis pathways. Each variant allele confers a small genotyping risk that combines additively or multiplicatively to confer range of cancer susceptibilities [7, 12, 38-40]. Therefore genetic predisposition alone may not be responsible for causing cancer but a combination of susceptibility genes and

exposures including environmental factors could contribute to the development of UADT MPN.

2.5 Single nucleotide polymorphism and genetic susceptibility to UADT MPN

Presently much emphasis has been laid on understanding effect of gene polymorphisms in terms of their associations with risk of various cancers. The genetic component influencing susceptibility to cancer includes gene falling mainly in important carcinogenesis pathways of DNA repair, carcinogen metabolism, cell cycle regulation and cell death control. Variation in any of these mechanisms may result in accumulation of cell with genetic alteration in critical genes leading to tumourigenesis [9]. So far no single gene mutation/ variation has been attributed to be the cause of UADT MPN. Hence cumulative effects of low penetrance genes are likely to contribute susceptibility to such cancers. Evidences from various studies analyzing cumulative polymorphisms suggest aberrant gene-environment interactions to be an important etiological factor in carcinogenesis. A large number of case control studies have attempted to establish the role of polymorphisms in critical regulatory pathway genes with susceptibility to development of second primary tumours [7, 12, 40-44].

Studies from our own group describe the involvement of single nucleotide polymorphisms and genetic susceptibility in etiology of UADT MPN in Indian population. In studies done by Jhavar et al., GSTM1 and GSTT1 gene null genotype has been found to confer risk for developing UADT MPN in Indian males using tobacco and females with paired occurrence of cancers in UADT and genital region [7, 45]. In another case-control study and meta-analysis of SULT1A1 $Arg^{213}His$ polymorphism from our group, UADT MPN patients showed significant risk association with SULT1A1 gene polymorphism [12]. As risk assessment for cancer risk is moving toward a multigenic pathway-based approach in a recent report from our lab in an attempt to understand collective effect of a panel of SNPs, an association of polymorphisms in genes falling in major carcinogenesis pathways was observed in MPN patients with tobacco habit [40]. These studies emphasise the role of genetic predisposition in UADT MPN and importance of undertaking studies evaluating a cumulative effect of panel of important gene SNPs.

2.6 Genotype phenotype correlation studies

Since last few decades a number of studies have reported association of gene polymorphisms with cancer risk. However the real implication of these gene polymorphisms with increased cancer risk can only be interpreted if they have any functional significance. Therefore undertaking genotype-phenotype correlation studies is extremely important to understand the functional significance of the gene polymorphisms. Numerous studies have been conducted during last one decade to evaluate the functional significance of gene polymorphisms in normal individuals [46-48]. This approach of understanding effect of gene polymorphisms at functional level has been phenomenal and has been applied to deduce the functional significance of genetic predisposition in cancer risk [49-52].

One of such reports describes alteration in the apoptotic capacity to be a risk factor for lung cancer development and the risk was observed to be modulated by the Fas -A^{670}G polymorphism [51]. Similarly, variations in p53 gene have been associated with apoptosis and DNA repair and in turn with lung cancer risk [53]. On the contrary XPA gene polymorphism, a DNA binding protein in the nucleotide excision repair pathway, has been observed to modulate repair capacity and is associated with decreased lung cancer risk, especially in the presence of exposure to tobacco carcinogens [52]. Other than lung cancer, analogous reports are available for breast cancer also where polymorphisms in nucleotide excision repair genes and DNA repair capacity phenotype has been with breast cancer risk [54, 55]. In another report by Minard et al. evaluation of glutathione S-transferase polymorphisms and mutagen sensitivity as risk factors for the development of second primary tumours in 303 patients previously diagnosed with early-stage head and neck cancer has been done [49]. These studies demonstrate the functional significance of genetic variation in different individuals and then their probable implication on various phenotypes and eventually cancer risk, hence emphasizing the importance of undertaking genotype-phenotype correlation studies in understanding UADT MPN pathogenesis.

2.7 Model system: Epstein Barr virus transformed lymphoblastoid cell lines

In order to understand the etiology of UADT MPN there is a need to undertake genotype phenotype correlation studies. However, one of the prime requisites for performing long term genotype-phenotype correlation studies is the continuous supply of starting parent material. This can be overcome to some extent by establishing

lymphoblastoid cell lines by infecting peripheral blood lymphocytes with EBV which is known to immortalize human resting B cells *in vitro* giving rise to actively proliferating B-lymphoblastoid cell lines [56]. Being a spontaneous replicating source, and exhibiting close resemblance to the parent lymphocytes, EBV transformed LCLs very well fulfil the requirement of constant supply of patient derived cells for a variety of assays sparing the need of re-sampling. LCLs, which carry the complete set of germ line genetic material, have been instrumental in general as an unlimited source of biomolecules like DNA, RNA or proteins and are a promising *in vitro* model system for genetic screening studies, genotype-phenotype correlation studies, a variety of molecular and functional assays related to immunology and cellular biology studies [57-59]. Utility of LCLs in *in vitro* carcinogen sensitivity and DNA damage/repair studies accounts for major segment of such studies and has been very frequently documented [60-62]. This proves the usefulness of LCLs in various genetic and functional studies.

2.7.1 Generation of lymphoblastoid cell lines

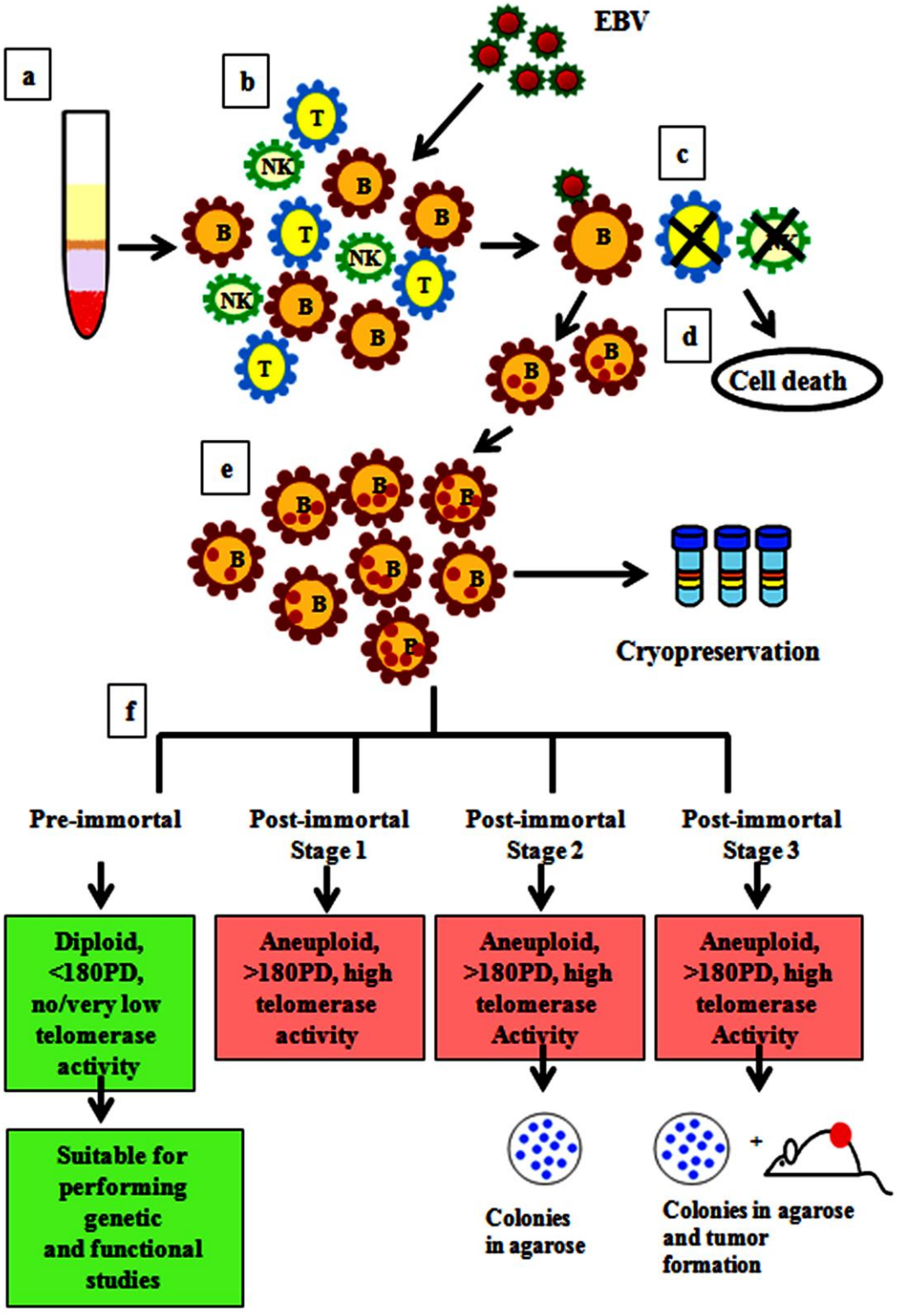
LCLs are developed by infecting peripheral blood lymphocytes (PBLs) with EBV which has been shown to immortalize human resting B cells *in-vitro* giving rise to an actively proliferating cell population with minimum genotypic and phenotypic alterations (Table 1, Fig. 4) [56, 63]. EBV is a lymphotropic DNA virus of γ-herpes virus family. Marmoset lymphoblastoid cell line B95-8, which was established by infecting marmoset B lymphocytes with EBV isolated from human patient with infectious mononucleosis, is a constant source for producing transforming virus [56]. LCLs display ease of preparation as well as effortless maintenance and exhibit minimum somatic mutation rate in continuous culture [64]. Presence of complement receptor type 2, commonly known as CR2 (CD21) on B cells creates a route for virus entry into the cell [65]. Viral envelope glycoprotein gp350 binds to CR2 and triggers endocytosis [66]. In addition a second glycoprotein gp42 binds to human leukocyte antigen HLA class II molecule as co-receptor [66]. For B cell immortalization, EBV establishes latent infection mainly existing as covalently closed circular episome with 5-800 copies per cell [56]. This latent infection is characterized by expression of limited number of viral genes. EBV encoded nuclear antigenic protein EBNA2 and latent infection membrane protein LMP1 play crucial role in cell immortalization along with other latent phase proteins, EBNA-1, EBNA-LP, EBNA-3A, EBNA-3C [66, 67].

2.7.2 Biological characteristics of LCLs

Immunophenotyping of LCLs confirms that the cells are positive for B cell marker CD19 and negative for T cell marker CD3 as well as for NK cell marker CD56 [63]. Average population doubling time of LCLs is 24 h; they grow as clusters and exhibit typical rosette morphology due to the expression of adhesion molecules leukocyte function antigen 1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) [56, 63, 68]. LCLs are commonly considered as 'immortal cell lines' that can grow for a large number of population doublings retaining a diploid karyotype without becoming tumourigenic, however, this classification of LCLs is slightly ambiguous since most of the LCLs are mortal, named as 'preimmortal' LCLs because of low level of telomerase activity and shortening of telomeres with each cell division. Although few LCLs truly become immortalized, named as 'post immortal' LCLs by development of strong telomerase activity and aneuploidy accompanied with other changes including down regulation and mutation of certain genes [69]. Hence it is recommended that LCLs be used within 2-3 months, which is far less than 180 population doublings assuming cell doubling time as 24 h, so as to minimise the undesirable and questionable effects of post immortalization [57].

2.7.3 Utility of lymphoblastoid cell lines for genetic and functional studies

LCLs have been used as a source of basic biomolecules like, DNA, including mitochondrial DNA, RNA and protein [57, 70]. DNA isolated from LCLs has been widely used for mutation analysis [70, 71], while RNA isolated from these cell lines has been commonly used for cDNA library preparation and to assess transcriptional response to genotoxins using high throughput technologies including cDNA microarray [72, 73] together with this LCL have as well been used for proteomic studies [74, 75]. In addition, in a number of other reports, LCL have been extensively used to measure DNA damage and repair as well as apoptosis [60-62]. To a great extent LCLs have also been used to assess inter-individual variation in response to DNA-damaging agents and to develop correlation between DNA repair genes and repair capacity and analysing the relationship with cancer risk [54, 76, 77] (Fig. 5).



Key: EBV-Epstein-Barr virus, PD-population doubling, B-B cell, T-T cell, NK-Natural killer cells, RBC-Red blood cells

Fig. 4: Schematic representation of B lymphocytes transformation with EBV. Detail of all the steps involved is explained in Table 1.

Table 1: Steps involved in establishment of EBV transformed LCLs

a. Isolation of peripheral blood lymphocytes	To obtain peripheral blood lymphocytes approximately 3 ml of blood is taken and mixed with equal amount of PBS and subjected to density gradient separation using Ficoll-hypaque.
b. Infection of Peripheral blood lymphocytes with EBV	Separated PBLs are a mixture of B cells, T cells and Natural Killer (NK) cells which are suspended in complete medium and are treated with EBV isolated form B95-8 cell line in 1:1 ratio. After 24 h the EBV containing medium is aspirated and cells are allowed to grow undisturbed with regular medium change as and when required.
c. Specific infection of B cells	EBV specifically infects B cells only owing to the presence of EBV receptor CR2 on B cell surface resulting in transformation while other cells (T cells and NK cells) die gradually during ongoing culture.
d. B cell transformation	EBV genome remains in the episomal form inside B cells in multiple copies and only a few viral genes are transcribed resulting in unlimited proliferation of cells.
e. Expansion of transformed cells	There is a considerable cell death after EBV transformation however infected cells repopulate the culture within 3-4 weeks giving rise to continuously proliferating lymphoblastoid cell lines.
f. Subdivisions in transformed LCLs	i) Pre-immortal cell lines grow for <180 population doublings show normal karyotype. They have either very low or negative telomerase activity and are suitable for performing various genetic and functional studies. ii) Post-immortal cell line stage-1 grow for >180 population doublings, have high telomerase activity and show aneuploidy. iii) Post-immortal cell line stage-2 grow for >180 population doublings, have high telomerase activity, show aneuploidy and have ability to grow in soft agar. iv) Post-immortal cell line stage-3 or tumourigenic stage grow for >180 population doublings, have high telomerase activity, show aneuploidy, have ability to grow in soft agar and form tumour in mice.

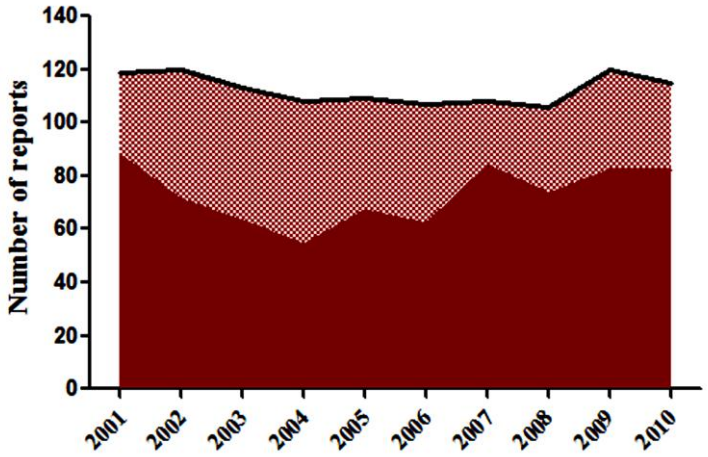


Fig. 5: Trend of usage of human EBV LCLs in last one decade. Usage of EBV LCLs for carcinogen sensitivity, DNA damage/ repair and other studies has been consistent throughout the years. Solid region in the graph represents reports where EBV LCLs are used as a model system. Shaded region represents other EBV LCL reports. Black Line above shaded region represents total available EBV LCL reports in each year

2.7.4 EBV LCLs are an ideal surrogate of isolated lymphocytes

Most of the studies show LCLs as a good surrogate to study effect of genotoxic exposure. However few reports have contradictory observation where utility of LCLs as isolated lymphocyte surrogate has been questioned. It is observed that LCLs do not show similar and reproducible results as compared to lymphocytes with certain experiments like micronucleus test or comet assay and pulsed field gel electrophoresis [78-81]. One of the noticeable reasons for occurrence of such variation in observations between LCLs and lymphocytes could be the use of stimulated lymphocytes which is mainly T cell population, as this is the only subpopulation reactive to PHA, whereas LCLs are derived specifically from B lymphocytes. More over differences in the mutagenic response can occur as LCLs are exposed in the G1/S/G2 phase of the cell cycle as they grow and undergo continuous cell division whereas stimulated T lymphocytes remain only in G0 phase and cells are known to have different sensitivity to DNA damaging agents in different phases of the cell cycle. Furthermore the type of assay used to compare the sensitivity of LCLs with lymphocytes can also give rise to variation due to varied sensitivity of the assays. Therefore care must be taken to ascertain the suitability of using LCLs in certain experiments and selecting the type of assay. With information from available reports LCLs present themselves as a valuable, cost effective, *in vitro* model system ensuring adequate starting material for current and

future analysis. They are being in use from last few decades and their utility is increasingly being recognised as a surrogate for isolated lymphocytes.

2.8 Molecular/ intermediate phenotype associated with cancer risk

The intermediate/molecular phenotypes that are frequently been reported to be altered in cancers include highly preserved and well-regulated DNA repair, cell cycle checkpoints and apoptosis. As it is reported, that environmental exposure has an important role along with genetic makeup of an individual in cancer susceptibility; therefore it is important to correlate the genotype of affected individuals with their phenotypic response after exposure to genotoxic agents which will help in understanding the interplay of gene and environment in MPN predisposition.

2.8.1 Altered DNA damage/repair and cancer

It is widely recognized that in cancer initiation both genetic and environmental factors play a significant role. Aberrations in many cellular functions are involved in the etiology of cancer, among which DNA repair is of fundamental importance. Inter-individual differences in DNA repair capacity have been suggested to be an important source of variability in cancer risk [82]. Deficiency in DNA repair capacity contributes to the accumulation of DNA damage and accelerates genetic variations involved in human carcinogenesis. A number of epidemiologic studies, primarily of done on lung, breast and head and neck cancer suggest association of variation in extent of DNA damage and repair with cancer risk [60, 83-86].

There are various assays by which DNA damage and repair can be measured in a cell after genotoxic exposure including mutagen sensitivity assay, comet assay, host cell reactivation assay and measurement of γ-H2AX activation. Mutagen sensitivity which has been used as an indirect assessment of DNA repair capacity is measured by quantifying number of chromatid breaks induced by mutagens [87]. The theoretical basis for mutagen sensitivity assay is that, in individuals with suboptimal DNA repair capacity than in normal individuals, in response to mutagen exposure, higher levels of genetic damage accumulate. Therefore, individual's ability to repair DNA damage is reflected by the level of chromatid breaks induced by a mutagen challenge. There are various reports in which the assay has been used to measure cancer susceptibility especially head and neck cancer, breast cancer and lung cancer [88-90]

Comet assay or single cell gel electrophoresis assay is a sensitive technique for the detection of DNA damage at the level of the individual cell. The length of comet tail relative to head is the measure of number of DNA breaks [91, 92]. Comet assay has been a method of choice for undertaking various DNA damage and repair studies deciphering cancer risk association [89, 93]. In Host-Cell Reactivation Assay the host cell is transfected with a damaged plasmid containing reporter gene usually luciferase which has been deactivated due to the damage. The ability of the cell to repair the damage in the plasmid after it is introduced to the cell allows the reporter gene to be reactivated leading it to produce its reporting product and indirectly measuring the ability of the cell to repair DNA damage. This assay has also been frequently reported in assessing altered DNA repair capacity and cancer risk association [94, 95].

Another sensitive assay to measure DNA double strand breaks (DSB) and repair is measurement of activated γH2AX. An early cellular response to DSBs is the rapid phosphorylation of H2AX (histone H2A variant) at Ser-139 to produce γH2AX. Immunofluorescence based assays that allows visualization of discrete nuclear foci formed as a result of H2AX phosphorylation is a very sensitive and reliable methods of detecting DSBs [96]. Activation of H2AX plays an important role in signalling and initiating the DNA repair by facilitating downstream repair proteins to reach the site of damage [96, 97]. Hence after DNA damage, appearance and disappearance of γ-H2AX can be used as a parameter to measure DNA damage and repair. Foci of γ-H2AX can be quantified by immunofluorescence microscopy or flow cytometry. Measurement of association of cancer risk with DNA damage and repair is often done by measuring γ-H2AX [98-100]. In present study we have selected γ-H2AX foci formation assay as a measure of DNA damage and repair.

2.8.2 Cell cycle regulation and cancer

After DNA damage cell cycle check points provide time for the cell to repair possible defects upon exposure to DNA damaging agents before entering into the next phase of cell cycle [101, 102]. There are two important check point for cell cycle regulation G1/S and G2/M. The G1/S checkpoint prevents the cell from replicating damaged DNA and G2/M checkpoint is activated by DNA damage and by incompletely replicated DNA. Considerable experimental evidence support the view that alteration in these cell cycle check points can lead to genomic instability and inappropriate survival of genetically damaged cells and contribute to the evolution of cells to malignancy [103].

Inefficient cell cycle regulation with loss of cell cycle arrest after genotoxic exposure is associated with various cancers mainly lung cancer, oral cancer [93, 103-106]. Therefore assessment of cell cycle regulation is important intermediated phenotype to be measured to completely understand cancer predisposition. This is frequently done by staining cells with DNA binding dyes like, Propidium Iodide, Ethidium Bromide and DAPI.

2.8.3 Apoptotic response and cancer

Under normal circumstances when a cell is exposed to a genotoxic agent it elicits highly preserved and well-regulated responses including cell cycle check point which allows cell to repair the damaged DNA. However in cases where the damage is severe and cannot be repaired, the cells will go into apoptosis or programmed cell death which is a safe way to inhibit risk acquiring neoplastic autonomy [107]. Studies in transgenic and knockout mice provide direct evidence that disruption of apoptosis can promote tumour development [108]

A failure in proper apoptotic response after extreme genotoxic exposure may also contribute to cancer development. There are few reports available where disruption of apoptosis is associated with cancer [53, 104, 105]. In a report by Zheng et al, γ-radiation induced apoptosis has been observed as a biomarker of genetic susceptibility to salivary and thyroid carcinoma along with defects in cell cycle regulation [104]. Similarly defective apoptotic response has also been associated with Lung cancer risk and is suggested to be used a susceptibility marker [53, 105]. Therefore apoptotic response after genotoxic exposure is also an important phenotype to be assessed when developing an association of cancer risk.

2.8.4 Altered gene expression and cancer

Differences in cellular responses after genotoxic exposure can be attributed differential gene expression. The tool of microarray analysis is an efficient means to study the differential expression of many genes simultaneously [109, 110]. Differential expression of genes in important carcinogenesis pathways is frequently reported which can be considered as driving malignant changes [111, 112]. Differential expression of genes in DNA repair pathways like nucleotide excision repair genes are found to be associated with in lung cancer [111]. A more than 2 fold increased of risk of head and neck cancer has been observed in individuals expressing lower levels of nucleotide

excision repair genes [112]. Similarly in study done on Indian population reduced expression of DNA repair genes was observed in head and neck cancers [113].

Apart from this in various studies differential expression of genes is monitored after genotoxic exposure in order to obtain biomarkers for separating study population in high and low risk groups for better risk assessment. This includes study done by Kote-Jarai et al where they observed that normal cells from heterozygous BRCA1 mutation carriers display a different gene expression profile from controls in response to DNA damage which can help in development of a functional assay for BRCA1 mutation status [114]. In a similar study done by same group a distinctive expression phenotype was observed after irradiation-induced DNA damage in BRCA1 and BRCA2 mutation carrier cells showed and was suggested to help in genotype prediction, with application to clinical detection and classification of mutations [115]. This implies the importance gene expression profiling after genotoxic exposure in cancer risk assessment at whole genome level.

2.9 Gene polymorphisms associated with cancer risk

As described above association of various gene polymorphisms with cancer is very frequently reported. The choice of polymorphisms to be genotyped for genotype-phenotype correlation studies is extremely crucial. For present study we selected gene polymorphisms that are part of major carcinogenesis pathways and are already reported to be associated with cancer. They mainly belonged to the following categories:

1. Genes involved in DNA repair
2. Gene involved in detoxification of xenobiotics (tobacco related carcinogens)-carcinogen metabolising enzymes
3. Genes involved in cell cycle regulation
4. Genes involved in apoptosis

2.9.1 DNA repair genes

Maintenance of genomic integrity is of immense importance to a cell. Endogenous and exogenous agents constantly inflict DNA damage in the cell. Uncorrected DNA damage can lead to mutations which if persists may lead to further genomic aberrations altering cellular functions and subsequently resulting in cancer. The genes that are undertaken for genotyping in present study are described below:

XRCC1 (X-ray repair cross-complementing protein 1)

Gene encodes an enzyme involved in the repair of DNA single-strand breaks formed by exposure to ionizing radiation and alkylating agents by base excision repair [116]. There are three polymorphic sites known for human XRCC1 gene that are frequently reported to be associated with head and neck squamous cell carcinomas viz. XRCC1-exon6 $Arg^{194}Trp$, XRCC1-exon9 $Arg^{280}His$, XRCC1-exon10 $Arg^{399}Gln$) [117-120]. XRCC1-exon6 Trp/Trp^{194} polymorphism is found to be associated with oral and pharyngeal cancer [119]. XRCC1-exon9 $Arg^{280}His$ variant polymorphism is associated with high risk of head and neck cancers in heavy smokers [121]. In a recent study from the lab XRCC1-exon9 variant is found to be associated with risk for tobacco related MPN. A meta-analysis has shown association of XRCC1-exon10 Gln/Gln^{399} with increased risk of tobacco-related cancers among light smokers but decreased risk among heavy smokers [122]. Where as in other study XRCC1-exon10 variant has been associated with increased risk of head and neck cancer [119]. Polymorphisms in XRCC1 gene therefore represent excellent candidates suitable for undertaking in genotype-phenotype correlation study.

XRCC3 (X-ray repair cross-complementing protein 3)

Gene encodes enzyme required for efficient repair of DNA double strand breaks through homologous recombination repair pathway, DNA cross linking and for chromosomal segregation. XRCC3 gene has a polymorphism in codon 241 which results in $Thr^{241}Met$ substitution. This polymorphism is frequently reported to be associated with head and neck cancers [123-125]. Met/Met^{241} variant of XRCC3 gene is known to be associated with increased risk of second primary neoplasms and mortality in oral cancer patients [126]. In a meta-analysis of 48 case-control studies the variant allele has been found to confer small risk to lung cancer, significant risk to breast cancer and elevated risk to head and neck and bladder cancer [127]. Hence XRCC3-exon7 polymorphism was also selected for genotypic study.

XPD (Xeroderma pigmentosum group D)

Gene encodes nucleotide excision repair enzyme involved in transcription-coupled nucleotide excision repair. Germ line XPD mutations can result in three different disorders, the cancer-prone syndrome xeroderma pigmentosum, trichothiodystrophy, and Cockayne syndrome [128]. It repairs DNA damage caused by

genotoxic agents including tobacco carcinogens. Several XPD polymorphisms have been identified in the coding regions with a relatively high frequency [129]. The XPD-exon23 Lys^{751}Gln is one of the very well studied polymorphism and is implicated in Lung cancer and head and neck cancers [118, 130, 131]. In a study performed on Indian population presence of the polymorphic variant of XPD was associated with increased risk of oral cancer compared to the wild genotype [132]. In a recent meta analysis that comprised 15 published case-control studies examining the association of head and neck cancer risk with XPD Lys^{751}Gln polymorphism in different populations a significantly elevated cancer risk was associated with the polymorphism and it was considered to be a prediction marker for risk of head and neck cancer [133].

hOGG1 (Human 8-oxoguanine DNA glycosylase)

Gene encodes the enzyme responsible for the excision of 8-oxoguanine, a mutagenic base by product which occurs as a result of exposure to reactive oxygen by base excision repair. 8-oxoguanine adducts are produced during oxidative DNA damage by carcinogens including tobacco. A nonsynonymous hOGG1-exon7 Ser^{326}Cys polymorphism has frequently been associated with head and neck cancer in various population based studies [134-136]. In a recent study done on 92 patients with primary HNSCC an association between the Cys/Cys^{326} genotype and HNSCC with cigarette smoking was observed [134]. In another report Ser^{326}Cys variant genotype was found to be associated with an increased risk of HNSCC in north Indian population and was claimed to serve as a biomarker for early diagnosis of HNSCC [135].

BRCA2 (Breast cancer 2)

BRCA2 is a tumour suppressor gene and the protein encoded by this gene is involved in the repair of chromosomal damage with an important role in the error free repair of DNA double strand breaks. This gene is present in the region of chromosome 13 which has been implicated to harbour alterations in HNSCC [137, 138]. In present study we are undertaking two BRCA2 gene polymorphisms viz. BRCA2 -26 G/A 5'UTR polymorphism and BRCA2-exon10 Asn^{372}His polymorphism. As this gene has an important role in DNA damage repair therefore polymorphisms in the gene may have an association with cancer risk. In our earlier study, increased risk association was observed for MPN patients with at least one tobacco related cancer in the upper aero digestive tract with BRCA2-exon10 Asn^{372}His polymorphism [40].

2.9.2 Gene involved in detoxification of carcinogens

The metabolism of carcinogens is often divided into three phases: modification, conjugation, and excretion. These reactions act in concert to detoxify carcinogens and remove them from system. The phase I enzymes activate the carcinogens by oxidation, reduction, hydrolysis and/or hydration. In subsequent phase II reactions, these activated carcinogen metabolites are conjugated with charged species such as glutathione (GSH), sulfate, glycine, or glucuronic acid. After phase II reactions the carcinogen conjugates can be excreted from cells in bile or urine. Together these enzymes are known as carcinogen metabolizing enzymes. Polymorphisms in genes encoding these enzymes can modify an individual's response to carcinogen exposure. The genes that are undertaken for genotyping in present study are described below:

GSTM1 (Glutathione-S-Transferase-Mu 1)

Glutathione-S-transferases are phase II detoxification enzymes that catalyses the conjugation of sulfhydryl group of glutathione (GSH) to a wide variety of carcinogens hence making them water soluble for excretion mainly through urine. GSTM1 is one of the enzymes of mu class [139]. Loss of GSTM1 enzyme function is due to homozygous deletion resulting in the GSTM1 null genotype [140]. GSTM1 null genotype is very well studied in terms of having an association with increased risk of head neck cancer in population based studies [141-143]. The risk conferred by GSTM1 null genotype was observed in Asians with head and neck cancer in meta analysis of 22 case control studies [144]. GSTM1 null genotype is also found to be a risk factor for developing multiple primary neoplasms in the upper aero-digestive tract in Indian males using tobacco [7]. In a similar study done on MPN patients, subjects with a single tumour of head and neck and cancer-free male volunteer, GSTM1 null polymorphism was found to be associated with MPN [10]. In few studies GSTM1 null genotype is shown to have an additive interaction with smoking on head and neck cancer risk [145].

GSTT1 (Glutathione-S-Transferase-Theta 1)

The enzyme encoded by the gene is one of the members of theta class of GSTs. GSTT1 null genotype is found to be associated with risk for head and neck cancer [146]. More often GSTT1 polymorphism is evaluated with GSTM1 polymorphism and represents association with cancers of head and neck [141, 147, 148]. GSTT1 null genotype confers risk to males with MPN in the head and neck [7]. In the meta-analysis

of 31 published case-control associations of GSTT1 null genotype along with GSTM1 null genotype with head and neck cancer risk was observed [149]. Apart from this, in Indian population as well GSTT1 null genotype has emerged as associated with risk of upper aero digestive tract cancers [143, 150]. On the contrary a large Indian study has shown GSTT1 to be significantly protective in oral cancer patients with tobacco habit [151].

GSTP1 (Glutathione-S-Transferase-Pi 1)

The gene encodes an enzyme belonging to GST pi class. GSTP1-exon5 $Ile^{105}Val$ polymorphism is extensively studied in terms of association with cancer [152]. This polymorphism is reported to confer risk of upper aero digestive tract cancers among Indians along with other GSTT1 and GSTM1 polymorphism [153]. In addition GST profile may have a prognostic value since it is associated with the response to therapy in human malignancies [148, 154, 155]. GSTP1 $Ile^{105}Val$ polymorphism is found to be associated with modestly increased risk of second primary malignancy after index head and neck squamous cell carcinomas [44].

SULT1A1 (Sulfotransferase 1A1)

The gene encodes sulfotransferase enzyme that belongs to phase II carcinogen detoxifying enzyme category. It catalyzes the sulfate conjugation of many hormones, neurotransmitters, drugs, and carcinogenic compounds resulting in increased polarity and solubility of the compound that facilitates excretion of carcinogens through bile or urine. SULT1A1-exon7 $Arg^{213}His$ polymorphism is very extensively studied and is found to be associated with head and neck cancer and lung cancer risk [156, 157]. Our study of meta-analysis of 33 case control studies showed His/His^{213} genotype to confer significant risk UADT cancers [12].

MPO (Myeloperoxidase)

It activates wide range of tobacco smoke procarcinogens such as benzo[a]pyrene and aromatic amines into activated carcinogens. The -463G>A SNP occurs in the MPO promoter region leading to decreased expression and activity [158]. The variant allele of MPO is shown to have mixed effect on cancer risk. It is reported that subjects bearing the A/G or A/A allele have a reduced risk to suffer from advanced lung cancer and esophageal cancer [159, 160]. Where as in other reports genetic effect of

MPO gene polymorphism is shown to slightly modify the risk of oral cavity cancer development [161, 162].

NAT2 (N-acetyltransferase 2)

This gene encodes an enzyme that transfers acetyl group from acetyl coenzyme-A to the N-terminal group of carcinogenic compounds. It functions to both activate and deactivate aryl-amine, hydrazine drugs and carcinogens. Polymorphisms in this gene are associated with higher incidences of cancer and drug toxicity. There are three polymorphic sites in NAT2 gene that are very frequently reported and are found to be associated with conferring risk to cancer viz., NAT2-exon2 $Ile^{114}Thr$, NAT2-exon2 $Arg^{197}Gln$ and NAT2-exon2 $Gly^{286}Glu$. The NAT gene polymorphisms are associated with increased risk of oral and pharyngeal squamous cell carcinoma [163, 164]. These polymorphisms have been implicated as risk in case control study of oral and oropharyngeal cancer in whites [162]. These polymorphisms along with cigarette smoking and alcohol consumption confer oral squamous cell cancer risk [165], A meta-analysis showed NAT2 slow acetylation may contribute to a risk factor for laryngeal cancer in Asians [166]. NAT2 polymorphism is also associated with development of second primary cancer in head and neck cancer individual [10]. Slow NAT2 activity is a risk factor possibly leading to the development of head and neck cancer in response to tobacco carcinogens [167].

EPHX1 (mEH, Microsomal epoxide hydrolase)

This gene encodes a carcinogen metabolizing enzyme mEH (microsomal epoxide hydrolase) that converts epoxides from the degradation of aromatic compounds to trans-dihydrodiols which can be conjugated and excreted from the body. mEH-exon3 $Try^{113}His$ polymorphism was selected for present study as increased risk for head and neck cancer is associated with EPHX1-exon3 Tyr/His^{113} and His/His^{113} genotypes [141, 168]. In a recent comprehensive meta-analysis of 84 studies, EPHX1 enzyme activity is related with risk of lung and upper aerodigestive tract cancers. It is described that low EPHX1 enzyme activity may have a potential protective effect on tobacco-related carcinogenesis of lung and UADT cancers, whereas high EPHX1 activity may have a harmful effect [169].

GPX1 (Glutathione peroxidise 1)

This gene encodes a member of the glutathione peroxidase family. Glutathione peroxidise 1 is an intracellular selenium-dependent enzyme that is ubiquitously expressed and detoxifies hydrogen and lipid peroxides [170]. GPX1-exon2 $Pro^{198}Leu$ polymorphism is often associated with lung cancer risk [171]. The GPX gene polymorphism along with polymorphisms in MPO -463G>A is implicated in eliciting the risk of oral cavity cancers [161]. This polymorphism has also been associated with second primary tumour development after a primary head and neck cancer [172].

2.9.3 Genes involved in cell cycle regulation and apoptosis

Genes in this pathway are critical for normal growth and differentiation. Thus disruption of these mechanisms can lead to tumour growth and progression. Cyclins and their partners are important candidates involved in cell cycle regulation. Cells with deregulated cell cycle normally undergo apoptosis to prevent malignancy. Therefore defective apoptosis also has an important role in tumourigenesis. The candidate SNPs in the genes involved in cell cycle regulation and apoptosis that are undertaken in present study are as follows:

CCND1 (Cyclin D1)

This gene encodes a protein that has a crucial role in G1/S cell cycle phase transition and for transcriptional regulation, cell proliferation and differentiation [173]. Deregulated CCND1 resulting due to gene amplification or translocation results in *in vitro* genomic instability and tumourigenesis [174, 175]. The exon4 $A^{870}G$ is a well studied SNP of CCND1 that results in alternate splicing of a stable mRNA variant, which may result in the bypass of the G1/S cell cycle checkpoint [131]. The CCND1 GG^{870} genotype is associated with increased susceptibility to oral cancers as well as prognostic indicator of disease-free interval in non-small cell lung cancer [176, 177]. In a recent meta-analysis study where data from sixty studies were combined it was observed that an increased cancer risk associated with CCND1-$A^{870}G$ polymorphism in the human population [178].

Fas (TNF receptor superfamily, member 6)

The protein encoded by this gene is a member of the TNF-receptor superfamily. FAS is a cell surface receptor that can interact with the FAS ligand (FASLG) to trigger apoptosis [153]. Therefore, the FAS/FASLG pathway plays an

important role in regulation of apoptosis and maintenance of cellular homeostasis, and genetic alteration of the pathway may result in altered cell death mechanism and thus tumourigenesis [179, 180]. The FAS A>G base change at nucleotide -670 in enhancer region is well studied polymorphism and seem to contribute to risk of developing head and neck cancer particularly the pharyngeal cancer in non-Hispanic Whites [180]. FAS-670 A>G polymorphism along with FAS ligand polymorphism has also been shown recently to confer a greater risk for second primary malignancy in head and neck cancer patients [179].

TP53 (Tumour protein p53)

This gene encodes tumour protein p53, which responds to diverse cellular stresses to regulate target genes that induce cell cycle arrest, apoptosis, senescence, or DNA repair. Along with a range of p53 gene mutations, p53-exon4 $Pro^{72}Arg$ polymorphism is frequently studied for association with various diseases. This variation is frequently being associated with risk for head and neck cancers [181, 182]. Polymorphism in p53 may influence individual responsiveness to cancer chemotherapy [183]. This polymorphism is often associated with risk of HPV associated cancers of head and neck [184]. It has been also shown to be associated with risk of second primary malignancy in patients with squamous cell carcinoma of head and neck [185].

p21 (CDKN1A, cyclin-dependent kinase inhibitor 1A)

This gene encodes a cyclin-dependent kinase inhibitor. The encoded protein binds to and inhibits the activity of cyclin-CDK2 or -CDK4 complexes, and thus functions as a regulator of cell cycle progression at G1. The expression of this gene is tightly controlled by the tumour suppressor protein p53, through which this protein mediates the p53-dependent cell cycle G1 phase arrest in response to a variety of stress stimuli. The most commonly studies polymorphism of p21 is exon2 $Ser^{31}Arg$ polymorphism. p21 gene variant may play a role in increased susceptibility for the development of squamous cell carcinoma of the head and neck [186, 187]. This polymorphism along with the other polymorphism of p21 gene is also associated with increased of second primary malignancy after index HNSCC [42].

Chapter 3

Materials and Methods

Source of chemicals, reagents and consumables

1. ***Common salts, buffers, detergents, organic reagents:*** USB corp./ Sigma Chemical Co./ Sigma Aldrich Inc./ Invitrogen, USA; Jebsen & Jessen, GmbH & Co., Germany; BDH AnalaR, UK; Qualigens fine chemicals/ Thomas Baker Chemical Ltd./ SD Fine-Chem. Ltd./Merck India Ltd., India.
2. ***DNA/ RNA detection:*** USB corp./ Sigma Chemical Co./ Sigma Aldrich Inc., USA; Genei, India.
3. ***Antibodies:*** Millipore/ Cell Signalling Tech./ Molecular Probes-Invitrogen/ BD Parmingen, USA; Roche Diagnostics GmbH, Germany.
4. ***PCR/ Quantitative Real-Time PCR/ cDNA synthesis/ Restriction and other enzymes/ Snapshot reagents/ DNA free kit:*** MBI Fermentas Canada Inc., Canada; Applied Biosystems/ USB corp./ Axygen/ Sigma Aldrich Inc., St. Louis/ Invitrogen, USA; New England Biolabs Inc. (UK) Ltd., UK.
5. ***Reagents for mammalian cell culture****:* Gibco-Invitrogen/ Sigma Aldrich Inc., USA.
6. ***B95-8 cell line:*** National Centre for Cell Science, Pune, India.
7. ***Tissue culture plastic wares, filters & centrifuge tubes:*** Nunc, Denmark; Corning/ BD biosciences/ Falcon/ Millipore/ Nalgene/Thermo-Fisher, USA.

3.1 Accrual of UADT MPN patients and healthy matched controls

After obtaining IRB approval and patient informed consent, 3 ml whole blood was collected in an EDTA vacutainer from patients with MPN and cancer free healthy individuals by venipuncture. Patients with MPN were accrued from the Cancer Genetics Clinic in Tata Memorial Hospital (TMH) and samples from healthy control individuals were obtained from Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Mumbai, India. The study was approved by the Hospital Ethics Committee, TMH. Purpose of study, method of sample collection, and other details were explained to patients and their relatives. Comprehensive questionnaire including tobacco and alcohol habit, medical and family history and ethnicity were recorded on ethical committee approved questionnaire.

UADT MPN patients were recruited when they were diagnosed by the oncologist for histologically confirmed multiple primary cancers on the basis of criteria given by Hong et al. [188] and as described earlier [11, 12] where they met one of the following standards: (i) The second primary tumour (SPT) should not be the recurrence, regional metastasis and distant metastasis of the first primary tumour, (ii) the histology of SPT should be different from the first primary tumour, (iii) if the histology is same the SPT should be separated from first primary by more than 2 cm of normal epithelium, (iv) or has to occur at least three years after the diagnosis of the first primary tumour, (v) lung as second primary if present should be solitary and histologically distinct from the first primary or has to occur three or more years later. Bilateral cancers in paired organs such as breast, ovaries or kidneys were not classified as MPN hence were not included. While individuals were accrued as controls if they had no personal history of cancer or premalignant or malignant lesion on clinical examination of the oral cavity and consented to participate in the study.

3.2 Generation of viable EBV stock

Cell transforming EBV is obtained from lymphoblastoid marmoset cell line B95-8 which was established by infecting marmoset B lymphocytes with EBV from a human patient with infectious mononucleosis [56].

Materials:

1. B95-8 marmoset cell line
2. RPMI-1640 (containing 15% FBS, 200 mM Glutamine, 1x PenStrep)
3. T25 flask

Method:

EBV-transformed B95-8 marmoset cell line was procured from National Centre for Cell Science (NCCS) India to prepare EBV crude stock. B95-8 cell line was revived and $0.5x10^6$ cells/ml were seeded in complete RPMI-1640 in T25 flask. After 7 days confluent cultures of B95-8 appearing straw yellow in colour were lysed by freeze thawing 3-4 times at -80 °C and 37 °C alternately. The lysed suspension was then filtered through 0.22 μM filter to obtain EBV crude stock. The filtrate was aliquoted in sterile 15 ml tubes and kept at 4 °C for short term or -80 °C for long-term storage.

3.3 Lymphoblastoid cell line preparation

LCLs are routinely developed by infecting PBLs with EBV which is known to immortalize human resting B cells *in-vitro* giving rise to an actively proliferating cell population.

Materials:

1. Ficoll-Hypaque
2. 24 well plate
3. EBV crude stock
4. DMEM (containing 15% FBS, 200 mM Glutamine, 1x PenStrep)

Method:

Ficoll-Hypaque: 250 ml, 9% w/v Ficoll was prepared in sterile distilled water. Solution was heated at 45 °C to dissolve Ficoll completely. Simultaneously 100 ml, 33.3% w/v Hypaque (Sodium diatrizoate) was also prepared in sterile distilled water and both the solutions were mixed in the ratio of 24:10 i.e. 240 ml of Ficoll and 100 ml Hypaque, to obtain final Ficoll-Hypaque gradient. Specific gravity of this gradient ranges from 1.076-1.078, which is ideal for separating PBLs from whole blood by centrifugation. This is a simple and rapid method of purifying PBLs that takes advantage of the density differences between mononuclear cells and other elements found in the blood sample.

Lymphoblastoid cell line preparation: For separation of PBLs approximately 3 ml of blood was separated on a Ficoll-Hypaque gradient. Briefly, 3 ml of blood was mixed with 3 ml PBS and carefully layered over 2.5 ml Ficoll-Hypaque in a 15 ml centrifuge tube. Cells were centrifuged at 1500 rpm/ 20 min so as to obtain a ring of PBLs at serum-Ficoll-Hypaque interface, while rest of the cells were collected at the bottom. These PBLs were collected, washed with PBS and seeded in a sterile 24 well plate at a density of $1.5\text{-}2x10^6$ cells/ml in complete DMEM. EBV crude stock at 1:1 ratio was

added to the cells and placed in an incubator maintained at 37 °C with 5% CO_2. After 24 h medium containing viral supernatant was aspirated without disturbing the cells and fresh complete DMEM was added**.** Medium was changes as and when required by carefully aspirating the old medium and adding new medium without disturbing growing clumps of transformed cells. After 3-4 weeks of incubation rosette morphology of cells ascertained the transformed phenotype of PBLs. Cells were mixed thoroughly to break clumps before splitting to ensure multiclonal population.

3.4 Characterization of LCL

Characterization of LCLs was done using standard techniques as described below. Cell lines were grown in complete DMEM (10% FBS, 200 mM Glutamine, 1x PenStrep) in T25 flasks routinely. For all genotypic and phenotypic characterization assays freshly grown LCLs within 60 population doubling and with >90% cell viability were used. Viability count was done by Trypan Blue Exclusion method where 10 µl of cell suspension was mixed with 10 µl of Trypan blue (Sigma). 10 µl of this mixture was loaded on Neubauer counting chamber and cell count was taken. Trypan blue selectively colour dead cells blue while live cells with intact membrane remain colourless.

3.4.1 Immunophenotyping:

Materials:

1. Anti-CD3, anti-CD19 and anti-CD56 antibodies
2. FACS buffer (PBS containing 2% FBS and 0.02% Na-azide)
3. 1ml 26G ½ syringe
4. FACS tubes

Method:

Flow cytometry: Cell surface markers, microscopic particles inside the cells and DNA content can be efficiently analysed by suspending them in a stream of fluid passing through an electronic laser detection apparatus by a technique called flow cytometry. This technique allows studying the physical and chemical characteristics of thousands of cells in a very short span of time. Cellular components to be analysed are labelled with fluorescent conjugated antibodies or fluorescent dyes that are excited by a laser and produce signal that can be captured and evaluated to interpret the results.

Immunophenotyping: $1x10^6$ cells from LCLs were incubated separately with PE labeled primary mouse anti-CD3 (T cell marker), anti-CD19 (pan-B cell marker) and anti-CD56 (NK cell marker) antibodies for 1 h on ice (antibodies used in this experiment were kind gift from Dr. Shubhada Chiplunkar, Immunology department, ACTREC). The cells were washed and suspended in 500 µl FACS buffer. Further cells were passed through BD™ 1 ml 26G ½ syringe to break any cell aggregates or clumps and analyzed on Flow Cytometer (FACS Calibur, BD Biosciences, USA) at 488 nm excitation. A minimum of 10,000 events were analyzed for each sample. Cellular debris was removed by gating on Forward vs. Side Scatter. Data analysis was done using CellQuest Pro software (BD Biosciences, USA).

3.4.2 Ploidy analysis of LCLs:

DNA ploidy is defined as diploid DNA represented as single G0/G1 peak on a histogram of test sample corresponding to the same DNA content represented as single G0/G1 at the same position in the histogram of control sample. DNA ploidy was measured by calculating DNA index (DI) which is the ratio between the channel number of G0/G1 peak on histogram of the cell line to the channel number of G0/G1 peak of control PBLs. DNA content of the cell is measured by labeling the cells with Propidium Iodide (PI). PI is a fluorescent intercalating agent which is when excited at 488 nm fluoresces red. It is routinely used to stain DNA to evaluate cell cycle analysis and cell viability.

Materials:

1. 70% ethanol
2. Propidium Iodide (0.4 mg/ml)
3. RNase A (1 mg/ml)
4. 1ml 26G ½ syringe
5. FACS tubes

Method:

$1x10^6$ cells from LCLs and control PBLs were washed and suspended in 500 µl PBS. Equal amount of 70% alcohol was added to the cells very slowly and drop wise. The cells were then mixed gently and were incubated for 10 min/ RT. Further Cells were centrifuged at 1500 rpm/ 10 min/ RT and were fixed in 500 µl 70% ethanol at 4 °C for 1 h. These fixed cells can be stored in 70% alcohol for a long time at 4 °C till further use. At the time of ploidy analyses cells were centrifuged at 1500 rpm/ 10

min/ RT and all alcohol was carefully removed. Cells were then washed with PBS followed by incubation with 40 µg Propidium Iodide (100 µl of stock solution) and 25 µg of RNaseA (25 µl of stock solution) in a final volume of 500 µl PBS for 30 min at 37 °C. Cells were passed through 1 ml 26G ½ syringe before acquisition to break any cell aggregates or clumps. Fluorescence was acquired on Flow Cytometer at 488 nm excitation and a minimum of 10,000 events were analyzed for each sample. Data were analyzed using ModFit LT V 2.0 software.

3.4.3 Expression of ATM gene:

3.4.3.1 RNA extraction from cell lines and PBLs:

RNA extraction is carried out using reagent such as TRIzol. It is a monophasic solution of acidic phenol (partitioning of RNA into aqueous supernatant for separation) and guanidine isothiocyanate (powerful protein denaturant). TRIzol maintains the integrity of RNA while disrupting cells and dissolving cell components. Addition of chloroform separates the solution into aqueous and organic phase. The RNA is recovered by addition of isopropanol to the aqueous phase.

Materials:

1. TRIzol reagent
2. Chloroform
3. Isopropanol
4. 75% ethanol in DEPC water/ nuclease free water

Method:

For RNA extraction $5x10^6$ cells from LCLs and PBLs isolated from the same subjects were collected and washed with PBS. After a single wash with PBS cells were suspended in 1 ml TRIzol in 1.5 ml eppendorf tubes and the sample was either processed immediately or stored at -80 °C till further use. Whenever required cells were thawed at RT and the pellet was dissolved completely by vortex mixing and repeated pipetting and incubated at RT for 5 min. For phase separation 200 µl of chloroform was added, the mixture was vortex mixed and kept on the bench top till two phases could be distinguished. To separate the phases completely tubes were centrifuged at 13500 rpm/ 15 min/ 4 °C. Following centrifugation the upper aqueous phase was collected in another eppendorf tube and 500 µl isopropanol was added for RNA precipitation. Content of the tubes was mixed gently, incubated at 10 min/ RT and was centrifuged at 13500 rpm/ 15 min/ 4 °C. Isopropanol was carefully removed and pellet was washed

with 500 µl 75% ethanol. Pellet was semi dried and dissolved in nuclease free water and kept at 65 °C for 7 min in water bath. Quantity and quality of RNA was assessed by measuring concentration and OD_{260}/OD_{280}; OD_{260}/OD_{230} ratio on NanoDrop UV-Vis spectrophotometer. The integrity of RNA was ascertained by reducing agarose gel electrophoresis.

Important note: *RNA is highly unstable molecule hence to prevent any degradation always keep the sample on ice and wear gloves while handling it.*

3.4.3.2 Formaldehyde agarose gel electrophoresis of RNA:

Agarose gel electrophoresis is a routinely used method for separation of nucleic acids. The method uses naturally occurring polymer obtained from an Alga in order to achieve a semi solid gel conformation on which nucleic acids are separated based on their size and visualized as fluorescent bands by intercalating dyes like EtBr on exposure to UV. Formaldehyde agarose gels allow separation and identification of RNA. Unlike DNA, RNA has a high degree of secondary structure, making it necessary to use a denaturing gel. Formaldehyde in the gel disrupts secondary RNA structure so that RNA molecules can be separated by their charge migration.

Materials:

1. Formamide
2. Formaldehyde
3. 10x MOPS: Sodium salt of 3-[N-Morpholino] propane sulphonic acid

MOPS pH 7.0	0.2 M
Sodium acetate	50m M
EDTA pH 8.0	5 mM

The solution was prepared in DEPC water, filtered through 0.45 µ Millipore filter and stored in brown coloured bottle.

4. RNA loading buffer (one sample)

10x MOPS	1 µl
Formaldehyde	1.75 µl
Formamide	5 µl
DEPC water	1.25 µl

5. Agarose
6. 6x Loading dye (0.25% Bromophenol blue, 0.25% xylene cyanol and 30% glecerol in distilled water)

7. Ethidium bromide (10mg/ml)

Method:

A 1.2% reducing agarose gel was prepared by melting 0.36 gm agarose in 25.5 ml DEPC water and cooled down to 55 °C. To this 3 ml 10x MOPS, 1.5 ml formaldehyde was added and the gel was allowed to set at RT. 1µl of RNA was mixed with above mentioned RNA loading buffer and heated at 65 °C for 7 min. sample was allowed to cool and after adding 2 µl of loading dye and 0.2 µl EtBr samples were loaded on the gel and run at a constant voltage of 50 V for 1.5 h. RNA bands were visualized and captured on UV exposure on a Gel documentation system.

3.4.3.3 DNase treatment of RNA:

Any downstream application of RNA requires it to be free of DNA contamination hence β-actin PCR was performed on isolated RNA to ensure any DNA content and samples were treated with DNase using DNA-free kit wherever required.

Materials:

1. DNA free kit (Ambion)
2. Total RNA
3. Water bath

Method:

For DNase treatment RNA concentration should be ≤200 ng/µl. Total RNA with ≈200 ng/µl concentration was taken in thin walled 0.6 ml eppendorf tube. Depending upon the amount of RNA being treated with the enzyme, per 10 µl reaction, 1 µl 10x DNase Buffer and 1 U γ DNase I enzyme was added and the reaction was incubated at 37 °C for 30 min. To stop the enzyme activity 1 µl DNase inactivator slurry was added and the reaction was incubated for 2 min at RT with intermittent mixing so that inactivator can interact with total enzyme in the reaction mixture. The contents were centrifuged at 10,000 rpm/ RT/ 1.5 min and RNA was transferred to fresh tube taking care not to pick up the slurry.

3.4.3.4 Preparation of cDNA from total RNA:

cDNA is synthesized *in vitro* from a RNA template using reverse transcriptase. This process is called reverse transcription (RT) or first strand cDNA synthesis. The purpose of converting mRNA to cDNA is mainly for the analysis of the template mRNA because DNA is much stable than RNA. Once mRNA is converted to

cDNA it can be used for a variety of assays like QRT-PCR and as probe for expression analysis etc.

Materials:

1. Superscript^{TM} first stand synthesis for RT-PCR (Invitrogen)
2. Total RNA
3. Water bath at various temperatures

Method:

3 µg of DNA free total RNA was taken in 0.6 ml eppendorf tube. 1 µl random primers, 1 µl oligo dT primers and 1 µl dNTPs were added and total volume was made 10 µl with nuclease free water and the tubes were incubated at 65 °C for 5 min followed by snap cooling on ice for 5 min.

A reaction mixture containing:

10x buffer	2 µl
$MgCl_2$	4 µl
0.1M DTT	2 µl
RNase out	1 µl
SS II	1 µl

was added to each sample after denaturation of the RNA in the same reaction tube. Contents of the tube were mixed gently and were incubated for 10 min at RT followed by 42 °C incubation for 1 h. The reaction was terminated by placing the tubes at 85 °C for 5 min. 1 µl RNase was added to each tube and incubated at 37 °C for 20 min. This cDNA was used as a template for PCR using gene specific primers.

3.4.3.5 Polymerase chain reaction:

PCR is an *in vitro* method of enzymatic synthesis and amplification of specific DNA sequences. It uses two oligonucleotide primers that hybridize to opposite DNA strands flanking the region of interest in the target DNA. Repeated cycles of heat denaturation of DNA, primer annealing, extension of the annealed primer using DNA polymerase and dNTP's results in an exponential accumulation of specific DNA sequences. Expression of ATM gene was measured semi-quantitatively by RT-PCR using gene specific primers and β actin was used as loading control. PCR products were run on 2% agarose gel and stained with ethidium bromide.

Materials:

1. 10X Taq. Buffer (+KCl, -$MgCl_2$)

2. 25 mM $MgCl_2$
3. 10 mM dNTPs
4. 10 pM forward primer
5. 10 pM reverse primer
6. Taq. polymerase
7. Filter sterilized water
8. DNA sample dissolved in TE (template)
9. Thermal cycler (Tetrad2-Peltier thermal cycler from Bio-Rad)

Method:

PCR reactions were carried out in sterile thin walled 0.6 ml capacity eppendorf tubes. PCR reactions were carried out in 25 µl reaction volumes containing 100 ng of DNA template, 10 µM of each forward and reverse gene specific primers (Table 2), 500 µM each of dNTPs, 2 U of Taq polymerase and 1.5-2.5 mM MgCl2 and PCR buffer containing 10 mM Tris HCl pH 8.3 and 50 mM KCl. Reaction mixture without DNA served as negative control. Thermocycling was performed using DNA thermal cycler. PCR products were visualised on 1.6% agaorose gel as described in section 8.2.

3.4.4 Activity of ATM gene:

For one sample activity of ATM gene was compared between cell line and PBL by immunostaining for pATM.

Materials:

1. Cobalt-60 isotope source (Bhabhatron-II)
2. 0.1% lysine (Sigma) coated cover slips
3. 4% paraformaldehyde (PFA)
4. 0.3% TritonX-100 in PBS
5. BSA
6. Anti pATM antibody
7. Anti mouse Alexa-546 secondary antibody
8. DABCO (1,4-diazabicyclo[2.2.2]octane) (4% w/v DABCO in 80% v/v glycerol in PBS)

Method:

$0.3x10^6$ cells from both the cell line and respective isolated lymphocytes were seeded in 1ml complete medium and irradiated with 4 Gy radiation dose using a

Cobalt-60 isotope source (Bhabhatron-II) at RT so as to activate ATM in phosphorylated form. Unirradiated cell line and lymphocytes treated in the same way served as control. Following 30 min incubation cells were collected in 1.5 ml eppendorf tubes. Cells were centrifuged at 1500 rpm to obtain a pellet and washed with PBS. After PBS wash the pellet was resuspended in 200 µl PBS and carefully layered on 0.1% lysine coated coverslips. These cells were allowed to attach on coverslip for 5 min. After 5 min the remaining suspension was carefully pipetted back leaving the cells attached on the coverslip. Cells were air dried to get rid of excess liquid and were then fixed with 4% PFA for 15 min at RT. Cells were washed with PBS twice and permeabilized by addition of 0.3% TritonX-100 in PBS for 15 min. Cells were again washed with PBS twice followed by blocking with 3% BSA in PBST (0.1% Tween-20 in PBS) for 1 h at RT. Next the cells were incubated with pATM primary antibody at 1:150 dilution in blocking buffer overnight at 4 ^{o}C. Next day cells were washed with PBS and PBST alternately thrice to get rid of any non specific binding. Cells were further incubated with Alexa-546 secondary antibody at 1:200 dilution in blocking buffer for 1 h at RT. Cells were washed for the second time with PBS and PBST thrice alternately to get rid of nonspecific binding. Nuclei were counterstained with DAPI and to remove excess DAPI cells were washed twice with PBS. Coverslips were mounted on glass slide using Dabco as anti-quenching agent. Edges of the coverslip were sealed with nail paint. pATM was visualized as red foci on DAPI stained nuclei and acquired on confocal microscope.

3.4.5 Cell population doubling:

Materials:

1. 24 well plate
2. Trypan blue dye (Sigma)
3. Neubauer counting chamber

Method:

$5x10^4$ cells from LCLs were seeded for each time point in a 24 well plate with 1.5 ml of complete medium. Viable cell count was taken using Trypan blue dye exclusion method at different time points including 0, 12, 24, 36, 48, 72 and 96 h as described in section 4. For each time point four readings were taken.

3.5 Measurement of DNA damage and repair

Formation of DNA double strand breaks triggers phosphorylation of the histone variant H2AX, producing γH2AX. Phosphorylation of H2AX plays a key role in damage repair and is required for the assembly of DNA repair proteins at the sites of DNA damaged as well as for activation of checkpoints proteins required for cell cycle arrest. Analysis of γ-H2AX expression can be used to detect the genotoxic effect of various toxic agents. To assess DNA repair capacity between MPN patient and control groups, LCLs were treated with γ-radiation. For qualitative analysis the experiment was performed by immunofluorescence foci formation assay for γ-H2AX by confocal microscopy and for quantitative measurement the experiment was done by flow cytometry for γ-H2AX positive cells.

3.5.1 Immunofluorescence foci formation assay for γ-H2AX after γ-radiation exposure:

Materials:

1. Cobalt-60 isotope source (Bhabhatron-II)
2. 0.1% lysine (Sigma) coated cover slips
3. 4% paraformaldehyde
4. 0.3% TritonX-100 in PBS
5. BSA
6. Anti γ-H2AX primary antibody
7. Anti mouse Alexa-546 secondary antibody
8. DABCO

Method:

$0.3x10^6$ cells were seeded in 1ml complete DMEM in 35 mm culture plates and irradiated with 2 Gy and 5 Gy γ-radiation dose in two sets using a Cobalt-60 isotope source (Bhabhatron-II) at RT. Unirradiated cells treated in the same way served as control. Cells were collected following 30 min incubation (maximum γ-H2AX are seen after 30 min) from one set and 4 h incubation (time taken to repair the DNA) from the other set. Cells were attached, fixed, permeabilized and treated with blocking solution in the same way as described in section 4.4. Cells were incubated with γ-H2AX primary antibody at 1:150 dilution in blocking buffer overnight at 4 ^oC. Next day cells were washed with PBS and PBST alternately thrice to get rid of any non

specific binding. Cells were further incubated with Alexa-546 secondary antibody at 1:200 dilution in blocking buffer for 1 h at RT. Cells were washed for the second time with PBS and PBST thrice alternately to get rid of nonspecific antibody binding. Nuclei were counterstained with DAPI and to remove excess DAPI cells were washed twice with PBS. Coverslips were mounted on glass slide using Dabco as anti-quenching agent. Edges of the coverslip were sealed with nail paint. γ-H2AX was visualized as red foci on DAPI stained nuclei and acquired on confocal microscope.

3.5.2 Measurement of γ-H2AX by flow cytometry after γ-radiation exposure:

Materials:

1. Cobalt-60 isotope source (Bhabhatron-II)
2. 1% PFA
3. FACS buffer (PBS containing 2% FBS and 0.02% Na-azide)
4. 0.3% TritonX-100 in FACS buffer
5. BSA
6. Anti γ-H2AX primary antibody
7. Anti mouse Alexa-488 secondary antibody

Method:

$0.5x10^6$ cells were seeded in 1ml complete DMEM in 35 mm culture plates and irradiated with 2 Gy and 5 Gy γ-radiation in two sets. Unirradiated cells treated in the same way served as control. Following 30 min incubation from one set and 4 h incubation from the other set, cells were collected and washed with FACS buffer and fixed with 1% PFA for 15 min at RT. Cells were washed with PBS and permeabilized by addition of 0.3% TritonX-100 in FACS buffer for 15 min at RT. Cells were again washed with FACS buffer followed by blocking in 3% BSA in FACS buffer for 1 h. Next the cells were collected by centrifuging at 1500 rpm/ 10 min/ RT and incubated with 50 µl γ-H2AX primary antibody at 1:250 dilution in blocking buffer overnight at 4 ^oC. Next day the cells were washed with FACS buffer and incubated with 50 µl Alexa-488 secondary antibody at 1:300 dilution in blocking buffer. Cells were once again washed with FACS buffer to get rid of any unbound antibody and further diluted with 200 µl FACS buffer and acquired on flow. Data analysis was done using CellQuest Pro software.

3.6 Effect of genotoxic exposure on cell cycle profile

After genotoxic exposure cell cycle arrest provides time for the cell to repair damaged DNA before entering into the next phase of the cycle. This experiment was done on 20 MPN and 10 control cell lines using flow cytometry by analysing percent G2 delay after radiation exposure and S and G2/M phase arrest after BPDE exposure. In order to assess the effect of genotoxic exposure on cell cycle profile the following experiments were done:

3.6.1 Effect of γ-radiation on cell cycle profile:

Materials:

1. Cobalt-60 isotope source (Bhabhatron-II)
2. 70% ethanol
3. Propidium Iodide (0.4 mg/ml)
4. RNase A (1 mg/ml)

Method:

$1x10^6$ cells were seeded in 1ml complete DMEM in 35 mm culture plates, were irradiated with 5 Gy and 10 Gy radiation dose and allowed to grow for 10 h. Cells were collected, fixed and stained as described in section 4.2. Cell cycle phase distribution was obtained after analysing data using ModFit LT V 2.0 software. The differences in G2 cell percentages between treated cells and untreated cells were recorded as the G2 cell cycle delay percentage using the following formula [105].

%G2 delay = % cells in G2/M in irradiated cells - % cells in G2/M in control

3.6.2 Effect of BPDE exposure on cell cycle profile:

Materials:

1. BPDE
2. THF
3. 70% ethanol
4. Propidium Iodide (0.4 mg/ml)
5. RNase A (1 mg/ml)

Method:

$1x10^6$ cells were seeded in 1ml complete medium in 35 mm culture plates and treated with 5 µM and 8 µM BPDE dissolved in tetrahydrofuran (THF) and incubated for 6 h. Untreated cells and cells treated with THF alone were taken as controls. After 6

h half of the cells were collected and fixed as described in section 4.2. Rest half were further allowed to grow for 18 h, after removing BPDE containing medium and adding fresh complete medium, so as to complete a 24 h cell cycle. After the 24 h rest half of the cells were collected and fixed. The cells for both BPDE concentration and both time points were stained as described in section 4.2. Cell cycle phase distribution was obtained after analysing data using ModFit LT V 2.0 software.

3.7 Measurement of percent cell death after genotoxic exposure

Under normal circumstances when cells are exposed to genotoxic agents and DNA damage is so severe and that it cannot be repaired, an apoptotic response is elicited to eliminate the damaged cell. LCLs were treated with γ-radiation and BPDE to assess difference in percent cell death between MPN patient and control groups.

3.7.1 Percent cell death after γ-radiation exposure:

Materials:

1. Cobalt-60 isotope source (Bhabhatron-II)
2. Annexin-V-FLOUS antibody
3. Propidium Iodide (0.4 mg/ml)
4. Incubation buffer (10 mM Hepes/NaOH, pH7.4, 140 mM NaCl, 5 mM $CaCl_2$)

Method:

$0.5x10^6$ Cells were seeded in 1.5 ml complete DMEM in 35 mm culture plates. Cells were irradiated with 5 Gy and 10 Gy γ-radiation and were allowed to grow for 48 h. Unirradiated cells treated in the same way were taken as a control. To quantitate percent cell death cells were stained with Annexin-V-FLUOS antibody and Propidium iodide (PI) where Annexin-V-FLUOS stains apoptotic cells and PI stains the necrotic cells. Cells were incubated with 100 µl Annexin-V-FLUOS antibody at 1:100 dilution and 1 µg PI made in incubation buffer for 15 min at RT. Cells were further diluted with 200 µl of incubation buffer in each tube and analyze by flow cytometry within 1 h. Data analysis was done using CellQuest Pro software.

3.7.2 Percent cell death after BPDE exposure:

Materials:

1. BPDE
2. THF

3. Complete DMEM
4. 35 mm culture plates
5. Annexin-V-FLOUS antibody
6. Propidium Iodide (0.4 mg/ml)
7. Incubation buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$)

Method:

$0.5x10^6$ Cells were seeded in 1 ml complete DMEM in 35 mm culture dish. Cells were treated with 5 µM and 8 µM BPDE dissolved in tetrahydrofuran (THF) and incubated for 6 h. Untreated cells and cells treated with THF alone were taken as controls. After 6 h cells were collected in 1.5 ml eppendorf tubes and washed with PBS twice. To quantitate percent cell death Annexin-V- PI staining was performed as described in section 7.1.

3.8 Genotyping of genes

Genotyping of genes was done using standard techniques as described below.

3.8.1 DNA isolation

Materials:

1. Sucrose lysis buffer (0.32 M sucrose, 10 mM Tris pH 7.5, 5 mM $MgCl_2$, 1% Triton X-100)
2. STE buffer (0.1 M NaCl, 0.05 M Tris pH 7.5, 1 mM EDTA pH 8.0, 1% SDS)
3. Proteinase K (20 mg/ml)
4. Tris equilibrated Phenol
5. Chloroform and isoamylalcohol in a ratio of 24:1 v/v (CIA)
6. 100% ethanol and 70% ethanol
7. 3 M sodium acetate pH 7.0

Method:

Tris equilibrated phenol: Distilled phenol was melted at 55 °C, to it 0.1% w/v hydroxyquinoline and equal volume of Milli Q water was added and the mixture was stirred on magnetic stirrer for 5-6 h. The two phases were allowed to separate and the aqueous phase was aspirated. After this phenol was equilibrated with 1M Tris-HCl pH 8.0, where equal volume of Tris was added to phenol and stirred overnight followed by aspiration of aqueous phase. Next equal volume of 0.5M Tris-HCl pH 8.0 was added to

phenol and the mixture was stirred for 5-6 h. Again the phases were allowed to separate and aqueous phase was aspirated. Finally phenol was treated with 0.1 M Tris-HCl pH 8.0. After equilibration pH of phenol was ascertained using pH strips. The process of equilibration was continued till the pH of 8.0 was achieved. Equilibrated phenol was stored under 0.1 M Tris-HCl in a dark bottle at 4 °C.

Important note: *Phenol is irritating and corrosive to the skin; it can pose severe health hazards and should be handled with extreme caution. All phenol work should be performed in a chemical fume hood. Any skin contact should be avoided by wearing gloves and laboratory coat.*

DNA isolation: Genomic DNA from mammalian cells is usually isolated by using a hypotonic lysis buffer containing a detergent (SDS), EDTA and proteinase K, where SDS and proteinase K helps in lysing the cell membrane while EDTA inhibits the action of any contaminating DNase by chelating divalent cations like Mg^{2+} required for the activity of enzymes. This is followed by extraction with phenol chloroform and alcohol precipitation. This method yields genomic DNA suitable as a source for amplification of required DNA fragments. During phenol chloroform extraction, protein contaminants are denatured and partition either with the organic phase or at the interface between organic and aqueous phases, whereas chloroform helps in removing any lipid components from the lysate leaving nucleic acids in the aqueous phase. If there is a problem of excessive foaming during the extraction, isoamyl alcohol can be added to obtain an organic composition of chloroform/ isoamyl alcohol (24:1). Finally 100% alcohol leads to precipitation of DNA in the aqueous phase due to salting out.

For genomic DNA extraction 3 ml of peripheral blood was collected in sterile EDTA vacutainer. RBC present in whole blood or the pellet obtained after isolating PBLs for cell line preparation were lysed using sucrose lysis buffer. Lysis buffer was added to blood in the ratio of 10:1 and centrifuged at 4000 rpm/ 20 min to get the nucleated blood cell pellet. Genomic DNA from the pellet was extracted by the lysis of cell membrane with 500 µl STE buffer and 20 mg/ml proteinase K over night at 37 °C. 500 µl Tris equilibrated phenol was added to the lysed cell suspension, mixed vigorously for 10 min, centrifuged at 12000 rpm/ 10 min/ RT and the upper aqueous phase was carefully collected in a fresh tube. Equal volume of phenol:CIA mix was added to the aqueous phase, mixed vigorously for 10 min and centrifuged at 12000

rpm/ 10 min/ RT. The upper aqueous phase was collected in a fresh tube and extraction was repeated with CIA alone. Phases were separated by centrifugation as mentioned earlier.

Aqueous phase obtained was collected and DNA was precipitated by adding 1ml chilled absolute ethanol at -20 °C for 20 min. DNA was pelleted by centrifugation at 7000 rpm/ 10 min/ RT, washed with 500 µl of 70% ethanol, DNA pellet obtained after decantation of ethanol was semi air dried and resuspended in ~200 µl TE (pH 8.0). Quantity and quality of DNA was assessed by measuring concentration and OD_{260}/OD_{280}; OD_{260}/OD_{230} ratio on NanoDrop UV-Vis spectrophotometer. The integrity of DNA was ascertained by agarose gel electrophoresis.

3.8.2 Agarose gel electrophoresis

Materials:

1. Agarose
2. 1xTAE (0.484% w/v Tris, 0.15% v/v glacial acetic acid and 10% v/v 0.5M EDTA in distilled water)
3. 6x Loading dye

Method:

DNA samples or PCR products were analyzed on agarose gel (made in TAE) by mixing 1 µg DNA or 10 µl of PCR reaction product with 2 µl of 6x loading dye and then loading in to the well. The agarose gel percentage varied (0.8% – 2%) according to the size of the DNA to be resolved. Standard DNA markers of known fragment sizes were run in parallel to the samples in order to have standard reference. The gel was run at a constant voltage not exceeding 100 V, EtBr stained DNA bands were visualized and captured on a Gel documentation system.

3.8.3 Polymerase chain reaction:

Materials:

1. 10X Taq. Buffer (+KCl, -$MgCl_2$)
2. 25 mM $MgCl_2$
3. 10 mM dNTPs
4. 10 pM forward primer
5. 10 pM reverse primer
6. Taq. polymerase

7. Filter sterilized water
8. DNA sample dissolved in TE (template)
9. Thermal cycler (Tetrad2-Peltier thermal cycler from Bio-Rad)

Method:

PCR reactions were carried using gene specific primers (Table 2) out in sterile thin walled 0.6 ml capacity microfuge tubes. PCR reactions were carried out in 25 µl reaction volumes containing 100 ng of DNA template, 10 µM of each forward and reverse gene specific primers (Table 2), 500 µM each of dNTPs, 2 U of Taq polymerase and 1.5-2.5 mM MgCl2 and PCR buffer containing 10 mM Tris HCl pH 8.3 and 50 mM KCl. Reaction mixture without DNA served as negative control. The cycling profile was standardized according the target DNA to be amplified (Table 3). Thermocycling was performed using DNA thermal cycler. Reaction mixture without DNA served as negative control All the PCR reagents were handled in a dedicated PCR work station and the template was added separately to avoid any cross contamination. 8 µl reaction product was analysed on 1.8% agarose gel electrophoresis. The remaining product was used for either restriction fragment length polymorphism (RFLP) or for SNaPshot reaction.

3.8.4 Restriction fragment length polymorphism (RFLP):

RFLP analysis is one of the first DNA based techniques to be widely used for detecting variations at the DNA sequence level. The principle behind the technique rests on the possibility of comparing band profiles generated after restriction enzyme digestion in DNA molecules of different individuals. Variations present in the DNA may give rise to generation or loss of restriction sites hence producing fragments of variable length after enzyme digestion. This difference in fragment length of digested products can be visualised after gel electrophoresis and is a direct measure of variations in the DNA. Extracted DNA is digested with specific restriction enzymes. Each restriction enzyme, under appropriate conditions, will recognise and cut DNA in a predictable way resulting in set of DNA fragments of different lengths.

Materials:

1. Amplified PCR product
2. Appropriate restriction enzymes
3. Buffer specific to the enzyme
4. 0.6ml PCR tubes

Method:

Restriction digestion of the PCR product with appropriate restriction enzyme (Table 2) was done to analyse the genotype. 10 µl of PCR product out of 25 µl reaction mixture was incubated with restriction enzyme at a concentration of 2 U/10 µl and $1/10^{th}$ volume of appropriate buffer in the final volume of 20 µl at 37 °C overnight. Restriction digestion products were analysed on 2% agarose gel electrophoresis described in section 8.2 along with 100 bp DNA ladder.

3.8.5 SNaPshot reaction:

SNaPshot assay developed on the principle of single base extension of primer serves as a simple, sensitive, robust, time saving and specific approach to detect multiple known SNPs in a pooled PCR product containing more than one gene. A primer which anneals immediately adjacent to the SNP is extended by one base using a fluorescently labelled ddNTP. Unlike PCR-RFLP, in SNaPshot assay the presence of any restriction site for the detection of polymorphism is not mandatory. This method further offers a direct visualization of the genotype of the SNP present in the expected region on a DNA fragment by its novel four different fluorescently labeled ddNTPs which give a coloured peak when incorporated in the SNP region on a electropherogram after capillary electrophoresis. Thus, allowing SNP genotyping on a large scale and in a short time.

Materials:

1. Amplified PCR product
2. PCR products
3. EXO-1 (10 U/µl) (USB)
4. SAP (1 U/µl) (USB)
5. SNaPshot ready reaction mix (provided in the kit) (Applied Biosystems)
6. Primers (Sigma-aldrich)
7. PCR buffer (10X)
8. Thermalcycler (Tetrad2 by BIO-RAD)
9. Distilled water
10. Genetic analyzer (Applied Biosystems- Avant 3100/3500)

Method:

First the desired gene was amplified by PCR using gene specific primers as described in section 8.3. SNaPshot assay was performed which consists of three steps

viz. EXO-SAP purification, SNaPshot reaction and Post SNaPshot purification. For PCR product purification EXOI and SAP enzymes are required. EXO-I (Exonuclease-I) is an enzyme with 5' to 3' exonuclease activity which helps clearing off the short single-stranded primers. SAP (Shrimp alkaline phosphatase) is a hydrolase enzyme responsible for dephosphorylation of nucleotides thereby hampering their migration and preventing their competition with ddNTPs in the SNaPshot reaction.

EXO-SAP purification: This step deals with the purifications of PCR products as it contains free, unused dNTPs, primer and primer-dimers. It is important to get rid of these unwanted materials to avoid non-specific addition of ddNTPs resulting in false positive peaks in the SNaPshot electropherogram and these may also result in quenching of the signals.

Reaction mixture for EXO-SAP purification

Component	Vol. (µl)	Final concentration
EXO-I (1 U/µl)	0.1	0.01 U
SAP (1 U/µl)	1	0.1 U
PCR-buffer (10X)	1	1
D/W	1.9	-
Product	6	-
Total	10	-

Thermal-cycler program for EXO-SAP purification

Step	Temperature (°C)	Duration
Enzyme activity	37	2 h
Enzyme deactivation	72	15 min
Reaction end	4	30 min

SNaPshot reaction: In this step, single base extension of the primer takes place. Purified PCR product was added in a reaction mixture containing ready reaction mix (contains fluorescently labelled ddNTPs with a different label for each of the 4 ddNTPs, DNA polymerase and essential buffers) and SNaPshot primers.

Reaction mixture for SNaPshot reaction

Reagents	Vol. (µl)	Final concentration
Ready reaction mix	2.5	-
Primer (10 pM)	1	10 µM
D/W	3.5	-
Product	3	-
Total	10	-

Thermal-cycler program for SNaPshot reaction

Reaction steps	Temperature (°C)	Duration
Denaturation	96	10 sec
Primer annealing	50	5 sec
Single base extension	60	30 sec
25 cycles from step 1 to step 3		
Reaction end	4	30 min

Post SNaPshot purification: This step is essential to remove the unused ddNTPs from SNaPshot reaction. If not removed properly, they can result in non-specific peaks in the electropherogram. This step is essential to remove the unused ddNTPs from SNaPshot reaction. If not removed properly, they can result in non-specific peaks in the electropherogram. In the final 10 µl product SAP was added and the reaction was carried out at 37 °C for 1 h for the activity of enzyme followed by 15 min at 75 °C for enzyme deactivation.

Reaction mixture for post-SNaPshot purification

Reagents	Vol. (µl)	Final concentration
SAP (1 U/ µl)	2	~0.153
Deionized water	1	-
Total	3	-

Thermal-cycler program for post-SNaPshot purification

Step	Temperature (°C)	Duration
Enzyme activity	37	1 h
Enzyme deactivation	75	15 min
Reaction end	4	30 min

Sequencing: After the SNaPshot reaction and post SNaPshot cleaning steps the vials were stored at -20 °C (if stored for long) or 4 °C (if for 24 h). These processed samples were then sequenced (fragment analysis) in Genetic analyzer for interpretation of SNaPshot reaction. The details of expected results for 7 gene polymorphisms are described in Table 4.

3.8.6 Genotype Score:

Genotyping of 22 candidate single nucleotide polymorphisms (SNPs) in 17 candidate genes involved in DNA repair, apoptosis, cell cycle regulation and carcinogen metabolism was done and a G Score was created from the number of variant alleles. A value of 2, 1 and 0 was allotted to homozygous variant, heterozygous and homozygous wild type alleles respectively, and a consolidated G Score was calculated taking values of all genes together.

Table 2: List of primers used for PCR and QRT-PCR

Gene-exon/region, SNP, RE	Forward and reverse primer sequences (5'-3')	PCR product (bp)
GSTM1 (Deletion)	F-CTGCCCTACTTGATTGATGGG	217 bp
	R-CTGGATTGTAGCAGATCATGC	
GSTT1 (Deletion)	F-TTCCTTACTGGTCCTCACATCTC	459 bp
	R-TCACCGGATCATGGCCAGCA	
GSTP1-exon5 $Val^{105}Ile$, *Alw261*	F-ACCCCAGGGCTCTATGGGAA	176 bp
	R-TGAGGGCACAAGAAGCCCCT	
MPO-upstream $G^{-463}A$, *AciI*	F-CGGTATAGGCACACAATGGTAG	350 bp
	R-GCAATGGTTCAAGCGATTCTTC	
SULT1A1-exon7 $Arg^{213}His$, *HaeII*	F-GTTGGCTCTGCAGGGTTTCTAGGA	331 bp
	R-CCCAAACCCCCTGCTGGCCAGCACCC	
Fas-Enhancer $A^{-670}G$, *MvaI*	F-CTACCTAAGAGCTATCTACCGTTC	331 bp
	R-GGCTGTCCATGTTGTGGCTGC	
CyclinD1-splice site , $A^{870}G$, *ScrfI*	F-GTGAAGTTCATTTCCATTCCGC	167 bp
	R-GGGACATCACCCTCACTTAC	
XRCC1-exon9 $Arg^{280}His$, *RsaI*	F-CCAGTGGTACTAACCTAATC	200 bp
	R-TCAGCACCAGTACCACA	
XRCC1-exon6 $Arg^{194}Trp$, *PvuII*	F-GCCCCGTCCCAGGTA	485 bp
	R-AGCCCCAAGACCCTTT	
hOGG1-exon7 $Ser^{326}Cys$, *Fnu4H1*	F-ACTGTCACTAGTCTCACCAG	207 bp
	R-TGAATTCGGAAGGTGCTTGGGG	
XPD-exon23 $Lys^{751}Gln$, *Pst1*	F-CTATCATCTCCTGGCCCCC	436 bp
	R-GCCCGCTCTGGATTATACG	
p21 $Ser^{31}Arg$, *BlpI*	F-GTCAGAACCGGCTGGGGATG	300 bp
	R-CTCCTCCCAACTCATCCCGG	
XRCC3-exon7 $Thr^{241}Met$, *NcoI*	F-GCCTGGTGGTCATCGACTC	136 bp
	R-ACAGGGCTCTGGAAGGCACTGCTCAG CTCACGCACC	
XRCC1-exon10 $Arg^{399}Trp$, *MspI*	F-CCCCAAGTACACCCAGGTC	242 bp
	R-TGTCCCGCTCCTCTCTCAGTAG	

p53-exon4 Pro^{72}Arg, *BstUI*	F-GACCTGGTCCTCTGACTGCT	380 bp
	R-GCATTGAGTCTCATGGAAG	
NAT2-exon2 Ile^{114}Thr	F-CAAATACAGCACTGGCATGG	139 bp
	R-GGCTGATCCTTCCCAGAAAT	
NAT2-exon2 Arg^{197}Gln	F-CCTGCCAAAGAAGAAACACC	143 bp
	R-GGGTCTGCAAGGAACAAAAT	
NAT2-exon2 Gly^{286}Gln	F-TCCTTGGGGAGAAATCTCGT	92 bp
	R-GGGTGATACATACACAAGGGTTT	
BRCA2-5'UTR G^{-26}A	F-AAATTTTCCAGCGCTTCTGA	158 bp
	R-AATGTTGGCCTCTCTTTGGA	
BRCA2-exon10 Asn^{372}His	F-AGCAAACGCTGATGAATGTG	150 bp
	R-GTGGAAGTGACAAAATCTCCAA	
mEH-exon4 Tyr^{51}His	F-CTCTCAACTTGGGGTCCTGA	231 bp
	R-GGCGTTTTGCAAACATACCT	
GPX-exon1 Pro^{198}Leu	F-ACTGGGATCAACAGGACCAG	213 bp
	R-TTGACATCGAGCCTGACATC	
β-actin	F-ACACTGTGCCCATCTACGAGGG	621 bp
	R-AGGGGCCGGACTCGTCATACT	
IFN	F-GGCACAACAGGTAGTAGGCG	173 bp
	R-GCCACAGGACGTTCTGACAC	
MAFB	F- GACGCAGCTCATTCAGCAG	88 bp
	R- CTCGCACTTGACCTTGTAGGC	
MAPK10	F-GCGAAACCCAGCGTTCTTCAGG	119 bp
	R- GGCTTGGATTCTCTCCCTTGCTG	
GNG12	F- AGCAAGCACCAACAATATAGCC	113 bp
	R-AGTAGGACATGAGGTCCGCT	
ZNF429	F-TGCAAGATGAAGCGACATGAA	240 bp
	R-GCTCTGGGTAAGTGTCAAACAT	
ARHGAP29	F-TAAAGCTCTACCTCCGAGACC	63 bp
	R- GGAACCCTTCGTACTGGCTC	
TMEFF1	F-TGTGAGGGAGTCTGACGTAAG	149 bp
	R-AAGTGTCCCCATTTGATCCAC	

SMAD6	F-TCGCGACGAGTACAAGCCACTG	109 bp
	R-GCTGGCGTCTGAGAATTCACCC	
AREG	F-CTGTCGCTCTTGATACTCGGC	49 bp
	R-GGTCCAATCCAGCAGCATAATG	
HMGA2	F-GCCAACGTTCGATTTCTACCTCAGC	217 bp
	R-AACCTGGGACTGTGAAGGGATTACA	

Table 3: PCR programs for different genes

Gene of interest	Denaturation	Annealing	Elongation
GSTM1	94 ºC 1 min	55 ºC 1 min	72 ºC 1 min
GSTT1	94 ºC 1 min	66 ºC 1 min	72 ºC 1 min
GSTP1	94 ºC 1 min	52 ºC 1 min	72 ºC 1 min
MPO	94 ºC 30 sec	60 ºC 30 sec	72 ºC 30 sec
SULT1A1	95 ºC 1 min	62 ºC 30 sec	72 ºC 45 sec
Fas	95 ºC 30 sec	61 ºC 30 sec	72 ºC 45 sec
CyclinD1	95 ºC 30 sec	56 ºC 30 sec	72 ºC 1 min
XRCC1-exon6	94 ºC 30 sec	57 ºC 45 sec	72 ºC 45 sec
XRCC1-exon9	94 ºC 30 sec	53 ºC 1 min	72 ºC 30 sec
XRCC1-exon10	94 ºC 45 sec	59 ºC 1 min	72 ºC 30 sec
XRCC3-exon7	94 ºC 30 sec	60 ºC 1 min	72 ºC 30 sec
hOGG1	95 ºC 30 sec	60 ºC 1 min	72 ºC 30 sec
XPD	94 ºC 30 sec	64 ºC 45 sec	72 ºC 1 min
p21	94 ºC 30 sec	55 ºC 30 sec	72 ºC 30 sec
p53	94 ºC 1 min	55 ºC 1 min	72 ºC 1 min
NAT2-1	94 ºC 30 sec	57 ºC 30 sec	72 ºC 30 sec
NAT2-2	94 ºC 30 sec	55 ºC 30 sec	72 ºC 30 sec
NAT2-3	94 ºC 30 sec	54 ºC 30 sec	72 ºC 30 sec
BRCA2-1	94 ºC 30 sec	58 ºC 30 sec	72 ºC 30 sec
BRCA2-2	94 ºC 30 sec	54 ºC 30 sec	72 ºC 30 sec
GPX	94 ºC 30 sec	60 ºC 30 sec	72 ºC 30 sec
mEH	94 ºC 30 sec	55 ºC 30 sec	72 ºC 30 sec
β-actin	94 ºC 1 min	55 ºC 1 min	72 ºC 1 min 30 sec

For all PCR reactions initial denaturation was done at 95 °C/10 min, final elongation was done at 72 ºC/ 10 min and number of cycles ranged between 30-35.

Table 4: SNaPshot primers

Gene	Mutation	SNaPshot Primer (5'-3')	Size	Wildtype*	Variant*
NAT2-1	T→C	(36)-X-TTCACCTTCTCCTGCAGGTGACCA	60	**T**	**C**
NAT2-2	G→A	(6)-X-AAAAAATATACTTATTTACGCTTGAACCTC	36	**G**	**A**
NAT2-3	G→A	(47)-X-GCCCAAACCTGGTGATG	64	**G**	**A**
BRCA2-1	G→A	(27)-X-GGTCTTCTGTTTTGCAGACTTATTTACCAA	57	**G**	**A**
BRCA2-2	A→C	(19)-X-AAATGATACTGATCCATTAGATTCAAATGTAGCA	53	**A**	**C**
GPX	G→A	(19)-X-GGCGCCCTAGGCACAGCTG	38	**G**	**A**
mEH	T→C	(16)-X-TGGAAGAAGCAGGTGGAGATTCTCAACAGA	46	**T**	**C**

X is the number of uncomplimentary bases added at the 5' end for tailing the primers. These bases are added to the primers so that there is difference in lengths of different fragments on the SNaPshot electropherogram during multiplex reactions.

*Indicates the nucleotide added by extension of the primer and the colour of the corresponding peak in the wild type and variant DNA sample.

3.9 Effect of γ-radiation on gene expression profiling

Materials:

1. Cobalt-60 isotope source (Bhabhatron-II)
2. TRIzol
3. Total RNA
4. Superscript II reverse transcriptase
5. dNTP mix (dATP, dCTP, dGTP and dTTP + amino allyl dUPP)
6. Microcon YM30 columns
7. Sodium bicarbonate
8. Cy3 & Cy5 dyes
9. PCR purification kit
10. Human Cot 1 DNA and yeast tRNA
11. Toronto microarray slides (19K+8K, total 27K genes)
12. Heating block
13. Hybridization chambers
14. Centrifuge
15. GenePix 4200A microarray scanner

Method:

Whole genome expression profiling was done for 5 MPN and 5 Control cell lines where $10x10^6$ cells were seeded in 100 mm plates in 5 ml complete DMEM and irradiated with 5 Gy γ-radiation dose. Cells were allowed to grow for 24 h and were collected by centrifuging at 1500 rpm/ 10 min/ RT. Cells were washed twice with PBS, RNA was isolated using TRIzol protocol as described in section 4.3.1 and RNA integrity was checked on 1.2% reducing agarose gel as described in section 4.3.2. After isolating RNA the following steps were followed to perform whole genome expression profiling.

3.9.1 Microcon concentration of the sample:

25 µg of RNA in 10 µl volume (concentration =2.5 µg/µl) was used for first strand cDNA synthesis using Superscript II reverse transcriptase for each slide. If the concentration of sample is less than 2.5 µg/µl, then it is subjected to Microcon column concentration. For sample concentration volume of the sample was made upto100 µl with nuclease free water and it was loaded on Microcon YM30 column. Sample was centrifuged at 10000 rpm/ 3 min/ RT. The sample was centrifuged till the volume of the

sample was reduced to 10 µl or less. Later the filter containing the sample was inverted into a new tube and centrifuged at 10000 rpm/ 3 min/ RT. Volume of the sample collected was measured and made up to 10 µl.

3.9.2 Preparation of cDNA:

Total RNA from test as well as control samples was treated with DNase wherever required as described in section 4.3.3 and later subjected to cDNA synthesis as described below.

Reaction mixture-1

RNA (2.5 µg/µl)	10 µl
Oligo dT (Invitrogen; 1 µg/µl)	6 µl
Random Primer (Invitrogen; 0.5 µg/µl)	2 µl

A mixture of Oligo dT and random primers was made and 8 µl was dispensed on each tube containing RNA. The tubes were incubated at 65 °C for 10 min and immediately transferred on ice and incubated for 10 min.

Reaction mixture-2

5X FS Buffer	6 µl
0.1M DTT	3 µl
50X aadUTP.dNTP mix	0.6 µl
RNase IN	0.5 µl
Superscript SSII	1.5 µl
D/W	0.4 µl

12 µl of this reaction mixture was added to each tube and was incubated at 42 °C for 1 h in water bath. The reaction was terminated by adding 10 µl 0.5M EDTA. 10 µl of freshly prepared 1 N NaOH was added to degrade RNA the reaction mixture was incubated at 65 °C for 10 min and cooled at RT.

3.9.3 Purification of cDNA

To the newly synthesized cDNA 25 µl 1 M HEPES buffer pH 7.0 was added. Reaction volume was made 500 µl with nuclease free water and contents were transferred to microcon column. Samples were centrifuged at 10000 rpm/ 10 min/ RT. Flow through was discarded and again 450 µl nuclease free water was added to the column. Samples were again centrifuged at 10000 rpm/ 10 min/ RT and continued to be centrifuged for small intervals so as to reduce the volume <5µl. cDNA was eluted in s

fresh eppendorf tube by inverting the column and centrifuging at 10000 rpm/ 3 min/ RT. Sample was subjected to speed vac to completely dry the cDNA.

3.9.4 Labelling:

Speed vacced cDNA was dissolved in 18 µl freshly prepared 1 M sodium bircoarbonate pH 9.0. Cy5 (blue) and Cy3 (pink) dyes were suspended in anhydrous DMSO and 2 µl each was added in test and reference samples respectively. Samples were incubated in dark for 1 h followed by addition of 9 µl 4 M hydroxylamine. Samples were further incubated for 15 min in dark followed by addition of 21 µl nuclease free water to make up the reaction volume to 50 µl.

3.9.5 Purification of labelled probes:

Purification of labelled probes was done using PCR purification kit (QIAGEN). To 50 µl reaction mixture 250 µl PB buffer was added and test and reference samples were mixed together. This mixture was transferred to QIAGEN columns and centrifuged at 13000 rpm/ 1 min/ RT. Flow through was discarded and 500 µl PE buffer was added to the column and centrifuged at 13000 rpm/ 1 min/ RT. Flow through was discarded again and columns were centrifuged again at 13000 rpm/ 1 min/ RT. Columns were transferred to the collection tubes and 30 µl elution buffer was added. Samples were incubated for 5 min at RT and collected by centrifuging at 13000 rpm/ 1 min/ RT.

3.9.6 Concentration of the probes:

To the purified labelled probes 20 µl Human Cot-1 DNA and 2 µl Yeast tRNA was added and volume was made up 100 µl using nuclease free water. Mixture was transfered to microcon column and centrifuged at 10000 rpm/ 4 min/ RT. Spin was repeated for small intervals till the volume was reduced to 25 µl or less. Columns were inverted to new tubes and eluted at 10000 rpm/ 3 min/ RT. Volume was made upto 25 µl. Probes were incubate at 100 °C for 3 min cooled at RT and added to 25 µl pre-warmed hybridisation buffer (100 µl 20X SSC, 100 µl formamide and 2 µl 20% SDS).

3.9.7 Hybridization:

Hybridisation chambers were cleaned and microarray slides were placed inside. Probes were added in the centre of the slide and spread using coverslip taking

care that there were no air bubbles. 40 µl 3X SSC buffer was put in the grooves of the chamber. Chamber was screwed tight and placed in the water bath at 42 ^{o}C overnight.

3.9.8 Washing:

Washing Solution

	20x SSC	SDS	Water
2X SSC	50 ml	2.5 ml	500 ml
1X SSC	25 ml	2.5 ml	500 ml
0.2X SSC	5 ml	2.5 ml	500 ml
0.2X SSC	5 ml	-	500 ml

The hybridisation chambers were removed from 42 °C water bath and whipped carefully with tissue paper. The slides were directly opened in 2x SSC with SDS and coverslips were removed carefully. The slides were kept in slide rack and washed in 2x, 1x and 0.2x SSC with SDS and 0.2x SSC without SDS for 3, 5, 5, and 3 minutes respectively. After washing the slides were dried by centrifugation at 1500 rpm /5 min/ RT and the fluorescent images of hybridized microarrays were obtained using a GenePix 4200A microarray scanner (Axon Instruments) within a couple of hours. Primary data analysis for determining differential expression of genes between was carried out using the Genepix Pro software version 5.0 (Axon Instruments).

3.10 Quantitative RT-PCR

Materials:

1. Power SYBR Green
2. cDNA
3. gene specific primers
4. Distilled sterile water

Method:

Differential expression of genes was validated by Quantitative RT-PCR. Invitrogen RT-PCR kit was used to reverse transcribe 3 µg of total RNA using both oligo dT and random primers in 30 µl reaction volume as described in section 4.3.4. The synthesized cDNA was diluted to a final concentration of 10 ng/µl with nuclease free H_2O and 4 µl was used as a template for real time reactions. A reaction mixture containing 40 ng cDNA, 2.5 µM of each forward and reverse primers and 5 µl of 2x

Power SYBR Green was made in 10 µl volume. All PCR reactions were performed in duplicate with an ABI Prism 7700 Sequence Detection System. GAPDH was used as endogenous RNA control and each sample was normalized on the basis of its endogenous gene expression.

3.11 Genotype phenotype correlation

Phenotypic responses after radiation and BPDE exposure were either correlated with total G Score or G Score of DNA repair genes or MPN risk association signature only. Either Pearson or Spearman correlation was used to calculate the relationship between phenotype and genotype on the basis of whether the data followed normal distribution respectively.

3.12 Statistical methods

Phenotypic assays were performed trice wherever a statistical analysis was done. The statistical analysis between the MPN patient and control groups for DNA damage repair assay, cell cycle profile and percent cell death assays were made by standard unpaired Student '*t'* test or Mann-Whitney U test depending on whether the data followed normal distribution or not, respectively. For genotype phenotype correlation statistical significance was calculated by applying Pearson correlation when values followed normal distribution and Spearman correlation when the values did not follow normal distribution.

Chapter 4

Results

Objective 1

To generate EBV LCLs from peripheral blood lymphocytes (PBLs) isolated from MPN patients and healthy controls - an established, in vitro model for genetic studies

Long term genotype-phenotype correlation studies necessitate the availability of patient DNA sample and a continuous source of patient derived cells to avoid repeated blood sampling for multiple phenotypic assays. A major drawback for such studies is the lack of representative human cell lines providing a continuous source of basic biomolecules and a system to carry out various experimental investigations. This can be overcome to some extent by establishing LCLs by infecting peripheral blood lymphocytes with EBV which is known to immortalize human resting B cells *in vitro* giving rise to actively proliferating B-lymphoblastoid cell lines. Therefore blood samples were collected from patients with multiple primary cancers (MPN) of upper aero digestive tract (UADT) and healthy controls, to develop lymphoblastoid cell lines and isolate genomic DNA.

4.1.1 Accrual and demographic characterization of study samples

UADT MPN patients were accrued from the Cancer Genetics Clinic in Tata Memorial Hospital (TMH) where patients with familial history of cancers, rare genetic disorders and multiple primary cancers frequently visit for counselling. For this study blood sample from patients with at least one cancer in UADT were collected following Hong's criteria described in material and methods section-3.1. A total of 24 patients were taken for the study and preliminary analysis showed that majority of them had tobacco habits (n=20). The incidence of UADT MPN was almost equal in both the sexes with a male to female ratio of 6:4 in the study subjects with median age 53 years (age range 32-75 years). Tobacco habit varied between individuals in terms of type and amount consumed. Habit included smoking, tobacco chewing, tobacco with pan (betel quid) and areca nut chewing. Four out of 20 patients had a habit of alcohol consumption along with tobacco habit (Table 5, Fig. 6).

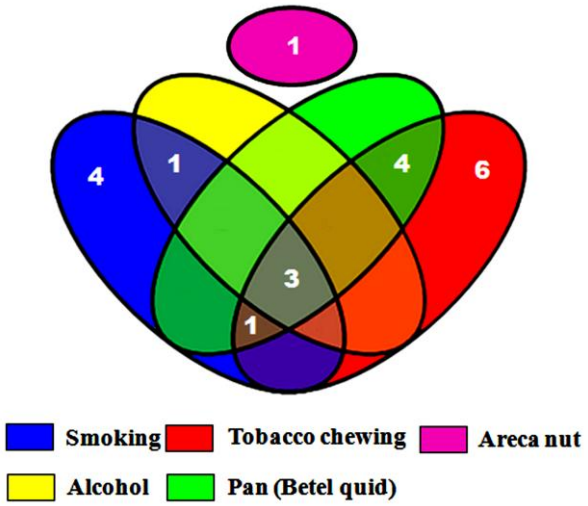


Fig. 6: Frequency of tobacco and alcohol habit in MPN patient samples. The subjects had mixed type of tobacco habit including smoking, tobacco chewing, pan (betel quid). One subject had a habit of areca nut chewing and four subjects had a habit of alcohol along with tobacco.

The patients accrued were highly heterogeneous with respect to tumour sites. Major sites of first and second primary cancer included various regions of buccal mucosa (lip, upper and lower alveolus, floor of mouth, retromolar trigone (RMT), gingivobuccal sulcus (GBS) and cheeks), hard palate, tongue, tonsil, larynx, oesophagus, parapharyngeal carcinoma and nasal columella (Fig. 7, Fig. 8).

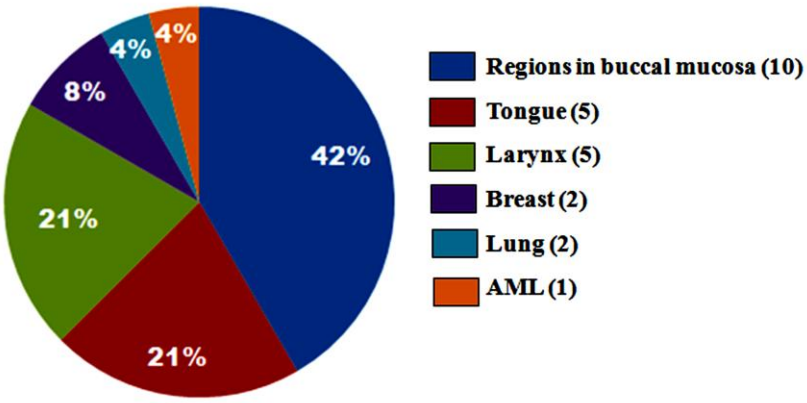


Fig. 7: Frequency of first primary cancers in study subjects (n=24). Values in parenthesis denote the actual number out of 24 subjects in each category.

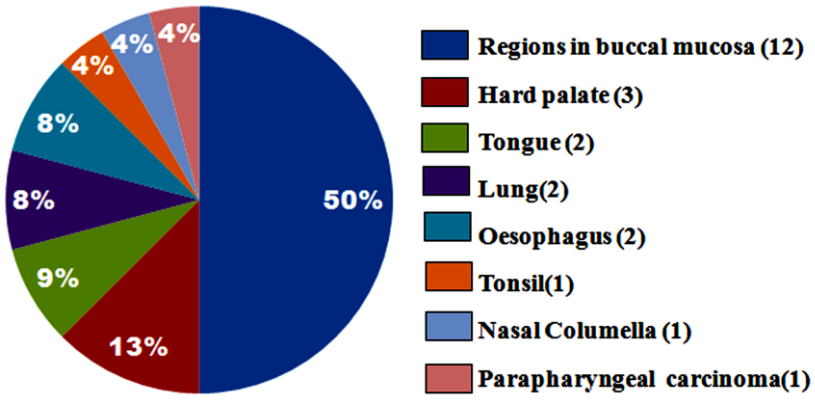


Fig. 8: Frequency of second primary cancers in study subjects (n=24). Values in parenthesis denote the actual number out of 24 subjects in each category.

All patients had both cancers in UADT except 4 patients who had one cancer in UADT and the second cancer in lung, breast or acute myeloid leukaemia. All UADT cancers were histologically squamous cell carcinomas. Various regions in buccal mucosa dominated in first primary cancer (42%) as well as site of second primary cancer (50%). Tongue (21%) and larynx (21%) were also frequent sites of first primary cancer while cancer of hard palate (13%) and tongue (9%) were next most frequent cancers as second primaries (Fig. 7, Fig. 8). Detailed demographics of MPN patients accrued for the study is described in Table 5.

Blood sample from healthy controls that were cancer free, and were habit and age matched, were collected mainly from Advanced Centre for Treatment Research and Education in Cancer (ACTREC), while two samples were taken from healthy subjects belonging to Shendurjane Village, Wai, Satra, India. While collecting sample subjects were analysed with respect to gender, age, location, habit and family history of cancer, based on questionnaire similar to cases. Detailed demographic of control samples is described in Table 6.

Table 5: Demographics of MPN patients

LCL	Type	Age	Sex	Habit	Cancer	
					I Site	II Site
E195*	MPN	48	F	Areca nut chewing	Breast	Hard palate
E242*	MPN	45	M	Pan+Smoking+Tobacco chewing+Alcohol	Tongue	Hard palate
E252*	MPN	38	M	Tobacco chewing	Buccal mucosa	Buccal mucosa
E253*	MPN	65	F	Tobacco chewing	Buccal mucosa	Alveolus
E254*	MPN	71	F	Nil	Tongue	Oesophagus
E264*	MPN	71	M	Tobacco chewing	Lip	Mouth
E265*	MPN	48	M	Nil	Larynx	Tonsil
E270*	MPN	60	M	Tobacco chewing	Alveolus	Tongue
E305	MPN	64	F	Pan+Tobacco chewing	RMT	Upper alveolus
E306	MPN	55	M	Smoking+Pan+Tobacco chewing	Larynx	Hard palate
E307	MPN	49	M	Smoking	Larynx	Nasal coolumella
E308	MPN	53	M	Smoking	Tongue	Tongue
E311	MPN	63	F	Nil	Lat. Border tongue	Buccal mucosa
E319	MPN	59	M	Smoking+Alcohol	Supraglottic Larynx	Lung+ Oesophagus
E320	MPN	45	F	Pan+Tobacco chewing	Right Cheek	Left Cheek
E321	MPN	49	F	Tobacco chewing	Left tongue	Left bronchus
E322	MPN	50	M	Smoking	Buccal mucosa	Lower alveolus
E325	MPN	43	F	Tobacco chewing	Buccal mucosa	Lower alveolus
E326	MPN	75	M	Smoking	Supraglottis	Oesophagus
E329	MPN	47	M	Pan+Tobacco chewing	GBS	Lower alveolus+ buccal mucosa
E330	MPN	63	F	Nil	Breast	Floor of mouth
E331	MPN	32	M	Pan+Smoking+Tobacco chewing+Alcohol	AML	Alveolus
E332	MPN	51	M	Pan+Smoking+Tobacco chewing+Alcohol	Lung	Parapharyngeal carcinoma
E333	MPN	55	F	Pan+ Tobacco chewing	Buccal mucosa	GBS+RMT+ lower alveolus

Key: * cell lines prepared by Ashwin Kotnis, MPN-multiple primary neoplasms, RMT-Retromolar Trigone, GBS- Gingivobuccal sulcus, AML-Acute myeloid leukaemia

Table 6: Demographics of healthy control subjects

LCL	Type	Age	Sex	Habit
E245*	Control	52	M	Smoking
E246*	Control	62	M	Smoking
E247*	Control	52	M	Smoking
E248*	Control	58	M	Smoking+Tobacco chewing
E249*	Control	46	M	Smoking+Tobacco chewing
E250*	Control	41	M	Tobacco chewing
E313	Control	49	M	Pan+Smoking+Tobacco chewing+Alcohol
E314	Control	61	M	Pan+Smoking+Tobacco chewing+Alcohol
E334*	Control	45	F	Masheri (pyriform tobacco)
E335*	Control	65	F	Masheri (pyriform tobacco)

Key: * cell lines prepared by Ashwin Kotnis

A total of 10 control cell lines were used for the study in which 8 samples were obtained from males, while 2 were from females. The age range for healthy controls was from 41-65 years with a median age of 52 years. In the control group as well, tobacco and alcohol habit varied between different individuals in terms of type and amount (Fig. 9). A comparison of demographics of cases and controls showed that the controls were fairly matched for age and habit. The patients and controls were mainly residents of Indian states of Maharashtra, Assam, Mizoram, Bihar, Uttar Pradesh, West Bengal, Gujarat, Karnataka and the families belonged to upper middle to lower income groups.

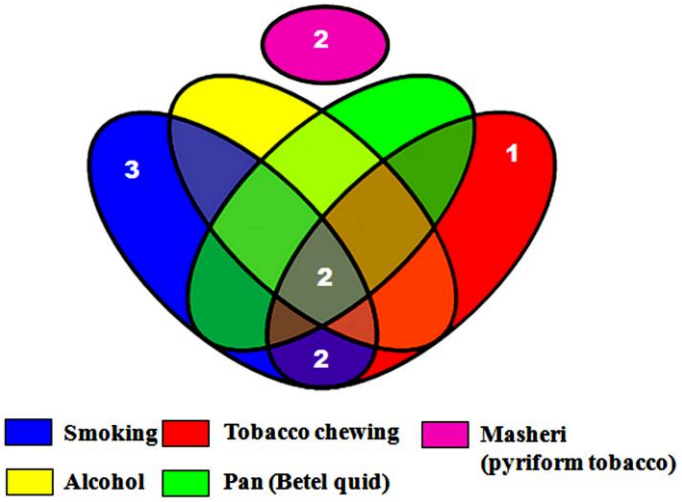


Fig. 9: Frequency of tobacco and alcohol habit in healthy control samples. Control subjects had mixed type of tobacco habit including smoking, tobacco chewing, pan (betel quid). Two controls had a habit of using Masheri and two had a habit of alcohol along with tobacco.

4.1.2 Establishment of LCLs and morphological analysis

A total of 34 LCLs from UADT MPN patients (n=24) and healthy controls (n=10) were prepared from PBLs isolated from blood samples. Sixteen UADT MPN patient (n=8) and healthy control cell lines (n=8) were prepared as a part of earlier study in the lab (marked with * in Table 5 and Table 6, prepared by Ashwin Kotnis); eighteen MPN patient (n=16) and control cell lines (n=2) have been prepared in the present study. Blood sample (3 ml) from MPN patients and cancer free control individuals was obtained and subjected to PBL isolation by ficoll-hypaque density gradient centrifugation. Approximately $4x10^6$ cells were obtained from 3 ml blood.

After lymphocytes isolation, LCLs were generated by infecting isolated lymphocytes with EBV which is known to infect only B cells in a mixed population of B, T and natural killer cells present in PBLs. Presence of complement receptor type 2, commonly known as CR2 (CD21) on B cells creates a route for virus entry into the cell. Considerable cell death of the PBLs was observed 24 h post EBV infection; however virus infection promoted B cells to re-populate the culture. The time taken for each LCL preparation varied, on an average culturing the cells 3-4 weeks post infection was sufficient to produce >1 million cells. Once the cell lines started growing, initial passage cells were cryopreserved for future use.

The LCLs in the present study were established as a model to study carcinogen sensitivity *in vitro* and to understand the aetiology of UADT MPN. Hence it was necessary to ensure that the process of EBV transformation did not affect the basic genotypic and phenotypic properties of the cells and LCLs were comparable to isolated lymphocytes. Therefore, random LCLs were undertaken for partial characterization with respect to cell population doubling time (PD), ploidy status, cell surface marker and expression and activity of ATM gene.

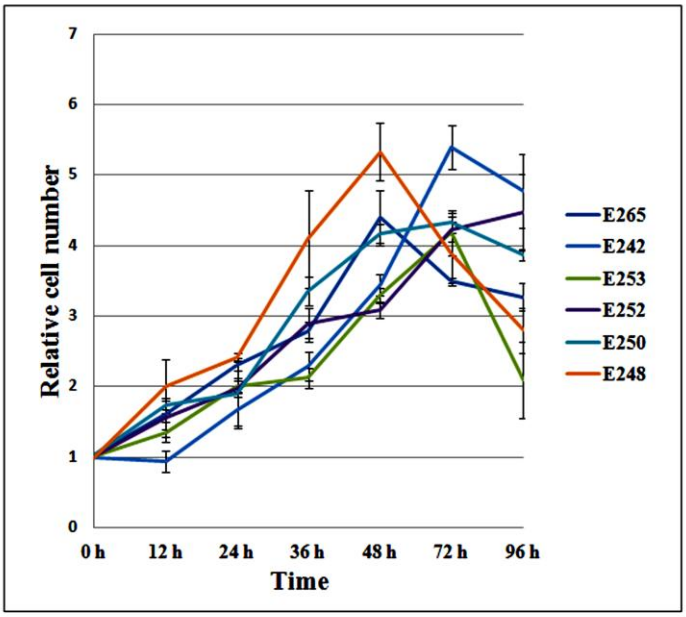


Fig. 10: Cell population doubling time for 6 representative cell lines at 6 time points (0-96 h). The doubling time ranged from 12 h to 36 h with an average of approximately 24 h. Each point represents mean value of four different readings. Bars indicate standard error of mean.

The average PD of few representative LCLs was found to be 24 h, ranging from 12 h to 36 h (Fig. 10). The LCLs grew as clusters exhibiting typical rosette morphology in suspension cultures, but single cells were also observed having big nucleus and numerous vacuoles (Fig.6). EBV is known to specifically infect B cells allowing their growth in culture hence the transformed phenotype is expected to have homogeneous cell population, however flow cytometric analysis revealed the presence of dual population (Fig. 11). On the basis of morphology and granularity it was revealed that lower (R1) population represents mono cell suspension of interest and the other (R2) population probably represents fraction of cells that have spontaneously differentiated into smaller lymphoid cells with shrunken nucleus that ultimately undergo apoptosis during conventional cell culture thus representing diverse size and granularity. Out of 34 cell lines 4 MPN patient cell lines did not grow efficiently in the continuous culture, hence were not undertaken for any phenotypic assays.

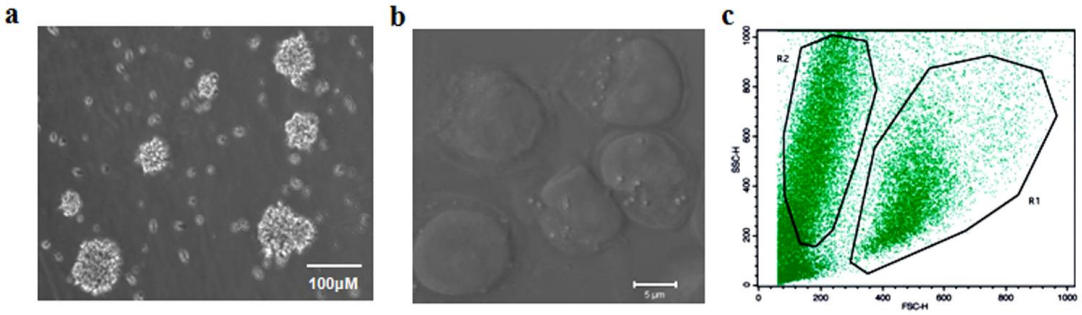


Fig. 11: Morphological analysis of established lymphoblastoid cell lines. Light microscopy image of LCL showing typical rosette morphology. Cells grow in clumps while single cells are also seen (a). Confocal microscopy image of cells in EBV transformed cell lines showing big nucleus and numerous vacuoles (b). Flow cytometry cluster plot showing two distinct populations in LCL where lower R1 population represents mono cell suspension and R2 population represents cell aggregates (c).

4.1.3 Cell surface marker

EBV specifically infects B cells hence immunophenotyping for B cell specific marker was done to ensure pure population. Antibodies against B (CD19), T (CD3) and NK (CD56) cell surface markers were used. Data confirmed that cells from representative randomly selected LCLs showed expression of typical B cell surface marker (CD19) while markers for T cell (CD3) and NK cells (CD56) were absent (Fig. 12), thus ascertaining the purity of growing cultures.

4.1.4 DNA ploidy analysis

DNA ploidy status of the LCLs was assessed immediately after cell line preparation at low population doubling (<5 PD) using PBLs from healthy individuals as diploid control. A shift in the position of the diploid peak of LCL away from the expected diploid position of the control can be taken as an evidence of DNA aneuploidy. Ratio between the channel number of G0/G1 peak on histogram of the cell line to the channel number of G0/G1 peak of control PBLs is called DNA Index (DI). All the cell lines studied had DI values ranging between 0.8-1.3 and were considered diploid (Table 7, Fig. 13). Cell lines in continuous culture can show aberrant DNA content hence ploidy was measured even at higher population doublings of 30, 45, 60, 120 and 150 in few cell lines. The DI values ranged between 0.93 and 1.18 hence were considered to be diploid in cell lines at higher population doublings as well (Table 8).

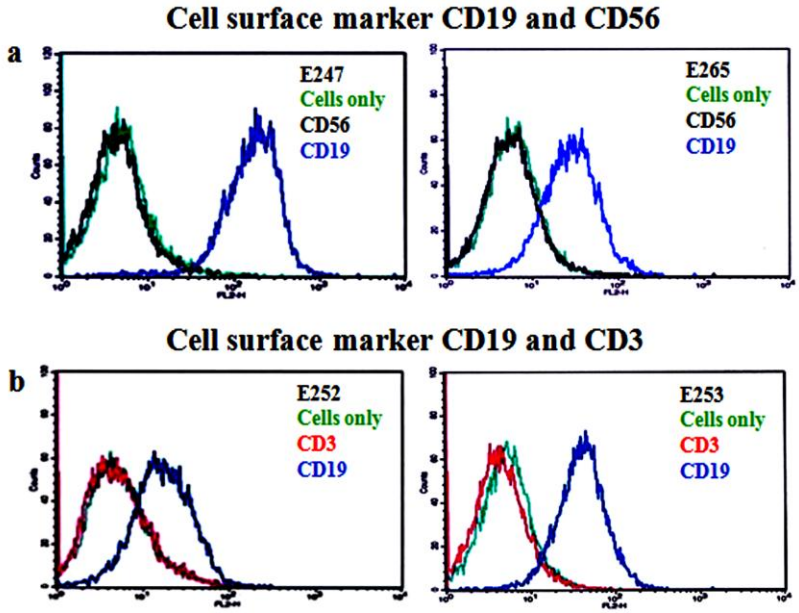


Fig. 12: Cell surface markers - CD3 (T cell), CD19 (B cell) and CD56 (NK cell) in representative cell lines. Unstained cells were used as internal control. X-axis represents the fluorescence intensity. A forward shift in the peak, caused by binding of fluorophore tagged antibody, as compared to unstained cell is considered positive (a) LCLs E242, E265, E245 and E247 showing positive staining for CD19 and negative for CD56 marker, (b) LCLs E252, E253, E246 and E249 showing positive staining for CD19 and negative for CD3 marker.

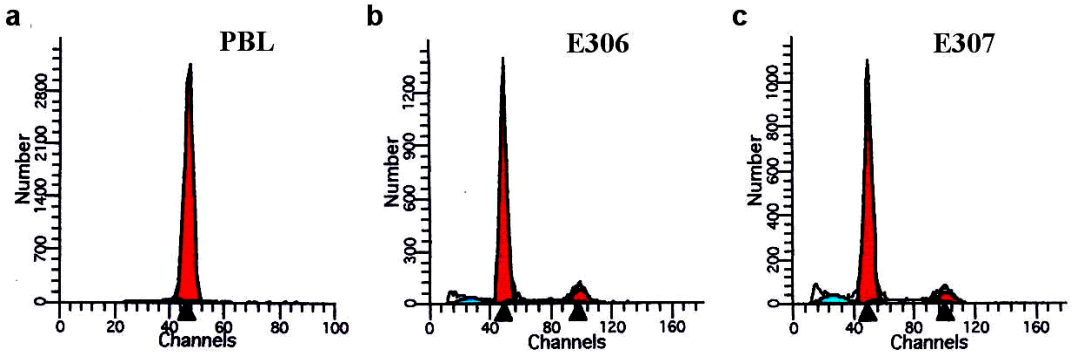


Fig. 13: Diploid status of PBLs isolated from healthy subject and two representative LCLs E306 and E307. The black arrows in the histogram X- axis represent the channel number of the respective cell cycle stage. Arrow at smaller channel number corresponds to G0/G1 stage of cell cycle while the other arrow corresponds to G2/M stage which is located at approximately double position.

Table 7: DNA ploidy status of MPN patient and Healthy control cell lines. PBLs from healthy individual were used as reference. Values in the range 0.9-1.3 are considered diploid

LCL	Channel Ratio (LCL/PBL)	DNA Index (DI)
E245	63.75/49.62	1.28
E246	53.83/49.62	1.08
E247	54.62/49.62	1.10
E248	55.16/50.40	1.09
E249	57.64/49.62	1.16
E250	52.55/49.62	1.06
E242	55.31/49.62	1.11
E252	53.98/49.62	1.09
E265	58.95/48.54	1.21
E267	60.93/48.54	1.26
E270	57.52/50.40	1.14
E304	47.23/46.58	1.01
E305	46.40/46.58	0.99
E306	49.37/46.58	1.05
E307	49.61/46.58	1.06

Table 8: DNA ploidy status of MPN patient and Healthy control cell lines at higher population doublings. PBLs from healthy individual were used as reference. Values in the range 0.9-1.3 are considered diploid

LCLs	PD	DNA Index
E304	30	0.97
	120	1.00
	150	0.93
E305	30	1.05
	120	1.18
	150	1.17
E311	45	0.99
	60	0.93
E313	30	0.97
	45	1.14

4.1.5 Expression of ATM gene

Lytic cycle of Epstein-Barr virus elicits a cellular DNA damage response resulting in activation of the ataxia telangiectasia-mutated (ATM) signal transduction pathway [189]. As described earlier these cell lines were prepared for undertaking *in vitro* DNA damage/repair studies, hence ATM expression and activity between the cell lines and their respective PBLs was studied. As revealed by semi-quantitative real time PCR there was apparently no change in the expression level of ATM gene between the LCL and respective PBLs from the subject (Fig. 14).

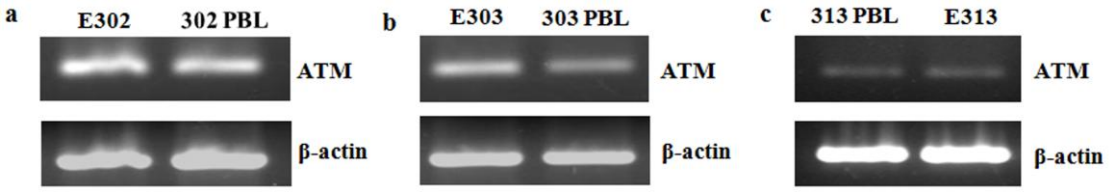


Fig. 14: Expression of ATM gene in different cell lines and their respective PBLs (a) E302 (b) E303 (c) E313. RT-PCR products were run in 2% agarose gel stained with ethidium bromide. β-actin gene was taken as loading control.

4.1.6 Activity of ATM gene

For one cell line the activity of ATM gene was compared between LCL and matched PBLs by immunofluorescence foci formation assay for phosphorylated (pATM Ser1981) after exposing cells to DNA damaging agents such as γ-radiation. The number of pATM Ser1981 foci seen as distinct nuclear foci in response to cellular DNA damage, were similar in both - the cell line as well as lymphocytes (Fig. 15).

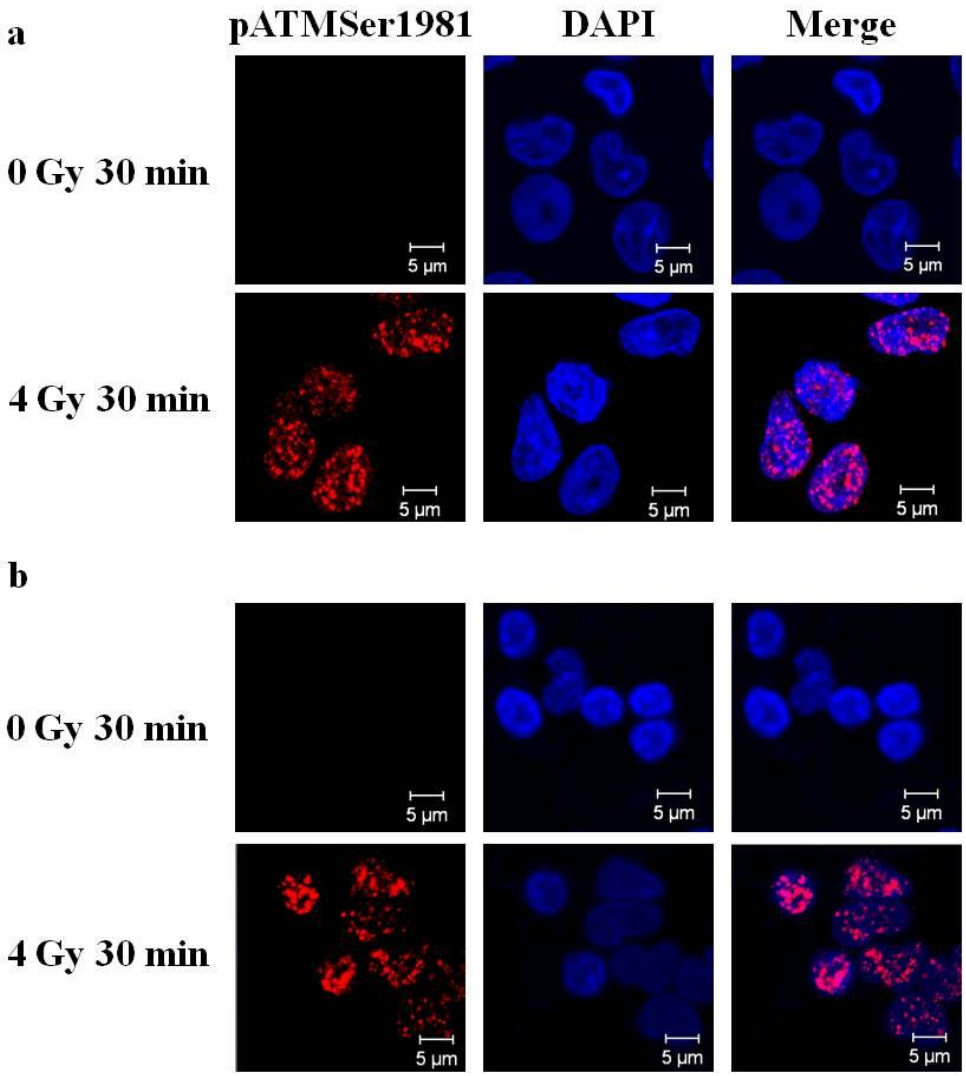


Fig. 15: Comparison of ATM activation between EBV LCL and PBL control. Activated ATM (pATM Ser1981, Alexa-546, red) and DNA (DAPI staining, blue) in EBV LCL (a) and peripheral blood lymphocytes (b) 30 min after 4 Gy radiation exposure.

This part of thesis work titled 'Establishment and characterization of lymphoblastoid cell lines from patients with Multiple Primary Neoplasms in the upper aero-digestive tract and healthy individuals' has been accepted for publication in Indian Journal of Medical Research (Manuscript number IJMR_449_11).

Objective 2

To compare the response of MPN patients with tobacco habits and appropriate controls in vitro, to exposure to DNA damaging agents such as γ-radiations and Benzo[a]pyrene-diol-epoxide (BPDE, a tobacco specific carcinogen), by assessing DNA damage and repair, cell cycle profiling, apoptosis and global gene expression profiling

Elevated levels of cellular damage caused by excessive exposure to genotoxic agents like γ-radiation and BPDE can elicit protective cellular responses like cell cycle regulation, DNA repair and apoptosis. Inter-individual difference leading to inherited deficiency in any of the above cellular mechanisms may increase an individual's risk to cancer. Using various *in vitro* phenotypic assays we have assessed the difference in the DNA repair capacity, cell cycle regulation and apoptotic response, after radiation and BPDE exposure, between MPN patients and controls. In addition whole genome expression profiling was also done to evaluate difference in the gene expression profile after γ-radiation exposure between the two groups.

4.2.1 Standardization of γ-radiation dose and BPDE concentration for phenotypic assays

For apoptotic response assay standardization of γ-radiation dose and time point was done by irradiating few randomly selected MPN and control cell lines with γ-radiation dose ranging from 2 Gy-10 Gy and measuring percent cell death using Annexin-V-FLUOS-PI staining by flow cytometry at 48 h time points (Fig. 16). Radiation doses of 5 Gy and 10 Gy were found to be optimum for comparing cell death between MPN and control cell lines. To further standardise the time, percent cell death was measured at 24 h and 48 h time points. The difference in cell death between MPN and control cell lines was more pronounced at 48 h. Hence 5 Gy and 10 Gy radiation dose and 48 h time point were selected to assess percent cell death after γ-radiation exposure (Fig. 16).

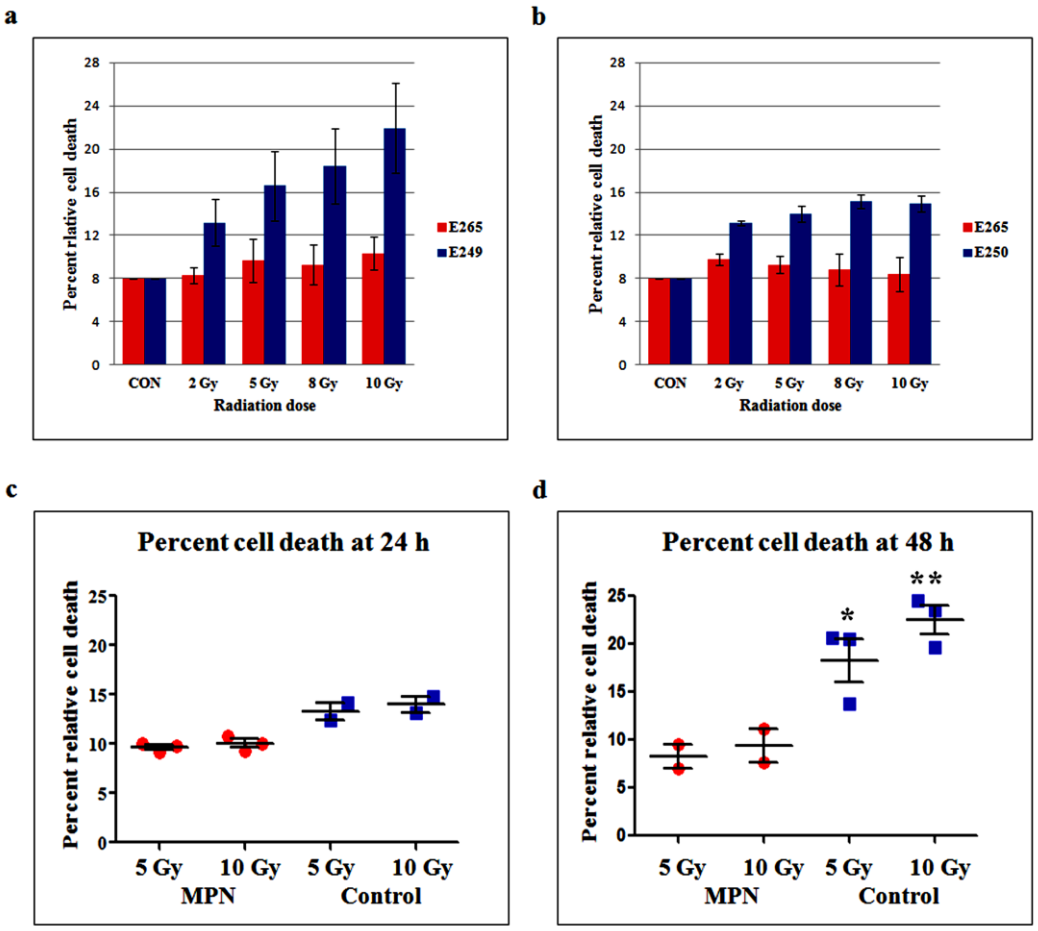


Fig. 16: Standardization of γ-radiation dose to measure percent cell death using Annexin-V-FLUOS-PI staining by flow cytometry. Standardization of radiation exposure using E264 (MPN) and E249 (Control) cell lines. A range of radiation doses was selected to assess cell death response (a). Standardization of radiation exposure using E265 (MPN) and E250 (Control) cell lines. Vertical line on each bar shows standard deviation (b). Comparison of percent cell death after 5 Gy and 10 Gy radiation exposure at 24 h time point between MPN (n=3) and control (n=2) cell lines (c). Comparison of percent cell death after 48 h time point between MPN (n=2) and control (n=3) cell lines. The difference in the percent cell death after 48 h was significant between MPN and control group with *p value=0.04 at 5 Gy and **p value=0.01 at 10 Gy (d).

Similarly a range of BPDE concentrations, time of treatment duration and various time points post BPDE exposure were evaluated to standardise the optimum BPDE concentration and time of exposure. Few representative graphs showing BPDE concentration standardisation are shown in Fig. 17. A concentration of 5 µM and 8 µM, and a 6 h time of treatment duration were found to be best suited for comparing percent cell death after BPDE exposure between MPN and control cell lines.

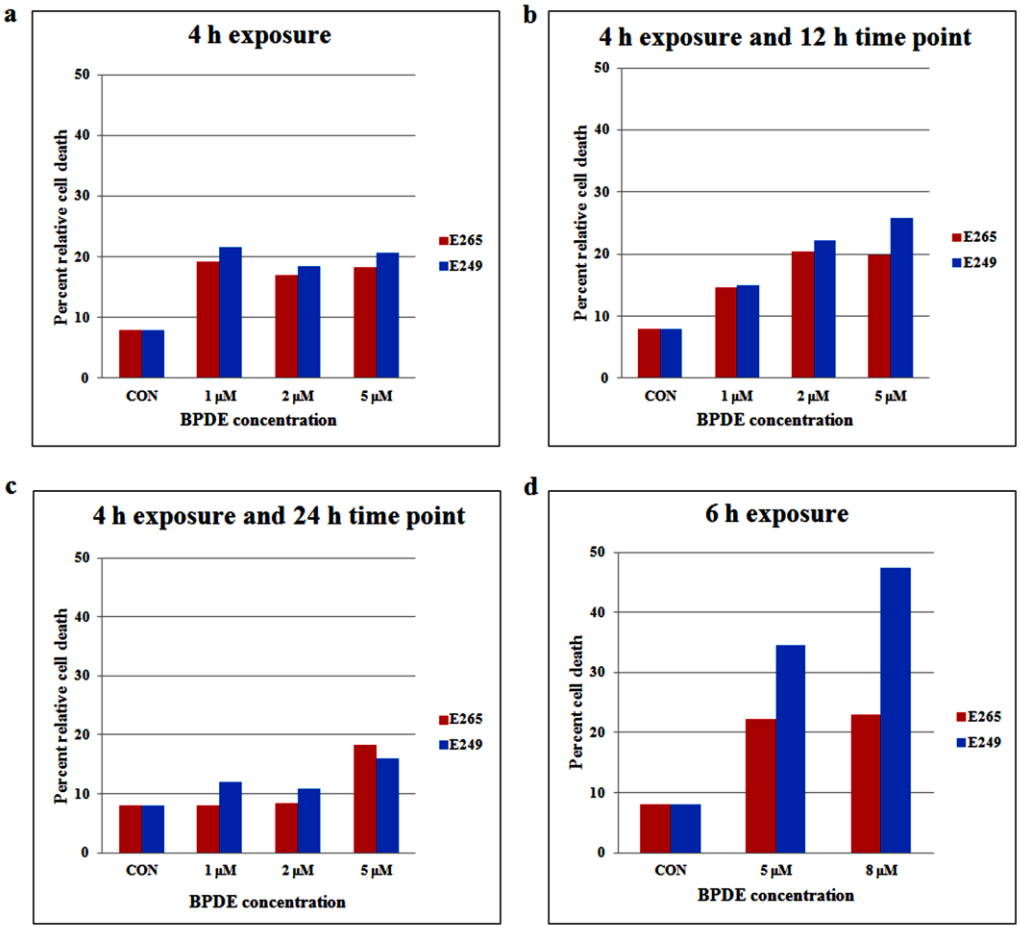


Fig. 17: Standardization of BPDE concentration and time of exposure to measure percent cell death using Annexin-V-FLUOS-PI staining by flow cytometry (representative graphs). Standardization of radiation exposure using E265 (MPN) and E249 (Control) cell lines at indicated BPDE concentrations and 4 h BPDE treatment time, cells were collected at 4 h time point (a). Standardization at indicated BPDE concentration and 4 h BPDE treatment time when cells were further allowed to grow for 12 h (b). Standardization at indicated BPDE concentration and 4 h BPDE treatment time when cells were further allowed to grow for 24 h (c). Standardization at indicated BPDE concentration and 6 h BPDE treatment time, cells were collected at 6 h time point (d).

For cell cycle profiling after γ-radiation exposure, time point of 10 h and radiation doses of 5 Gy and 10 Gy were chosen as optimal conditions to test an effect on G2 delay. The radiation doses were same as used for apoptotic response assay, while the time point was chosen on the basis of a study done by Zhoa et al. [105], where they have assessed percent G2 delay in lung cancer and control LCLs after radiation exposure at 10 h. In the report difference in G2 delay is shown to be most evident between MPN and control LCLs at 10 h time point. Similarly 5 µM and 8 µM concentration of BPDE were selected to assess effect of BPDE exposure on cell cycle profile whereas standardization of exposure time was done by comparing the effect of BPDE exposure at 6 h and 24 h. Although not very remarkable but a slight difference in

percent S and G2/M arrest was observed at both time points, hence were selected for cell cycle profiling (Fig. 18).

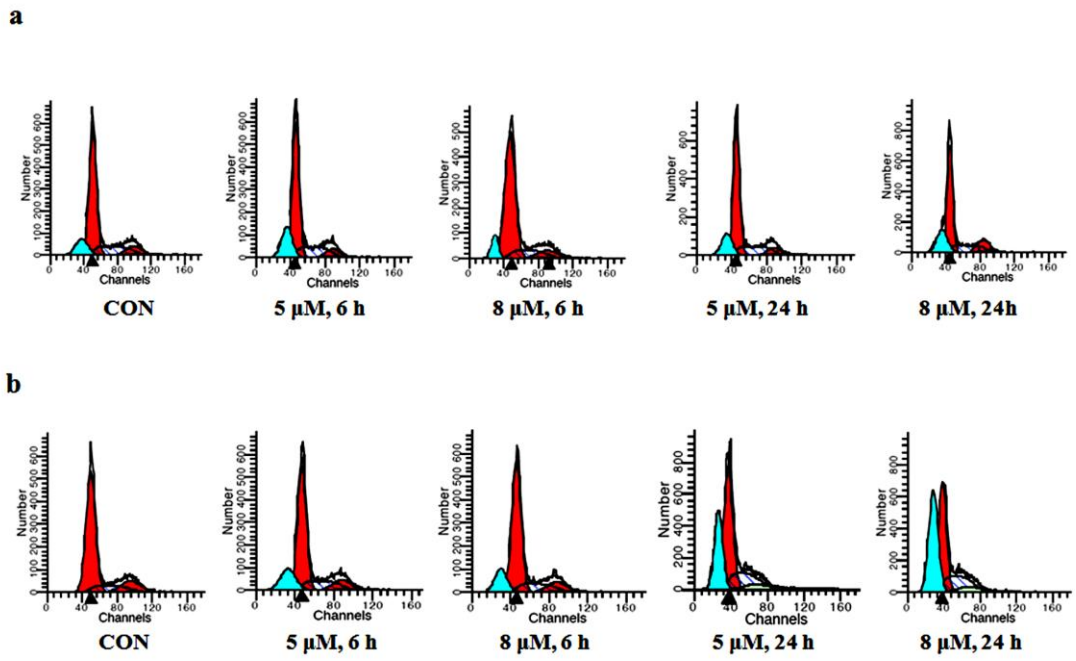


Fig. 18: Effect of BPDE exposure on cell cycle profile. E242 (MPN cell line) (a) and E250 (Control cell line) (b) were treated with BPDE and cells were collected at 6 h and 24 h time points. The cells were subjected to cell cycle analysis by flow cytometry.

Immediately after DNA damage, phosphorylation of H2AX (variant of histone H2A) occurs and rapidly reaches the site of DNA damage resulting in foci formation which can be detected using fluorescently labelled antibodies to γH2AX. On an average, γ-radiation at an exposure dose of 1 Gy results in the formation of 5-15 γ-H2AX foci. Maximum number of γ-H2AX foci can be observed at a time point of 30 min which are directly proportional to the extent of DNA damage. After this, DNA repair begins and γ-H2AX undergoes dephosphorylation. Loss of foci serves as a marker for DNA repair. Considerable dephosphorylation of H2AX molecules leading to reduction in γ-H2AX foci is observed optimally at a time point of 4 h, while at later time points this dephophorylation is slower [97]. Subsequently, 2 Gy and 5 Gy radiation doses were selected to analyze DNA damage/repair in MPN and control cell lines. The number of foci formed at 10 Gy were too many to be quantitated (Fig. 19). A 30 min time point was selected to assess DNA damage, while 4 h time point was found to be ideal to compare the extent of DNA repair.

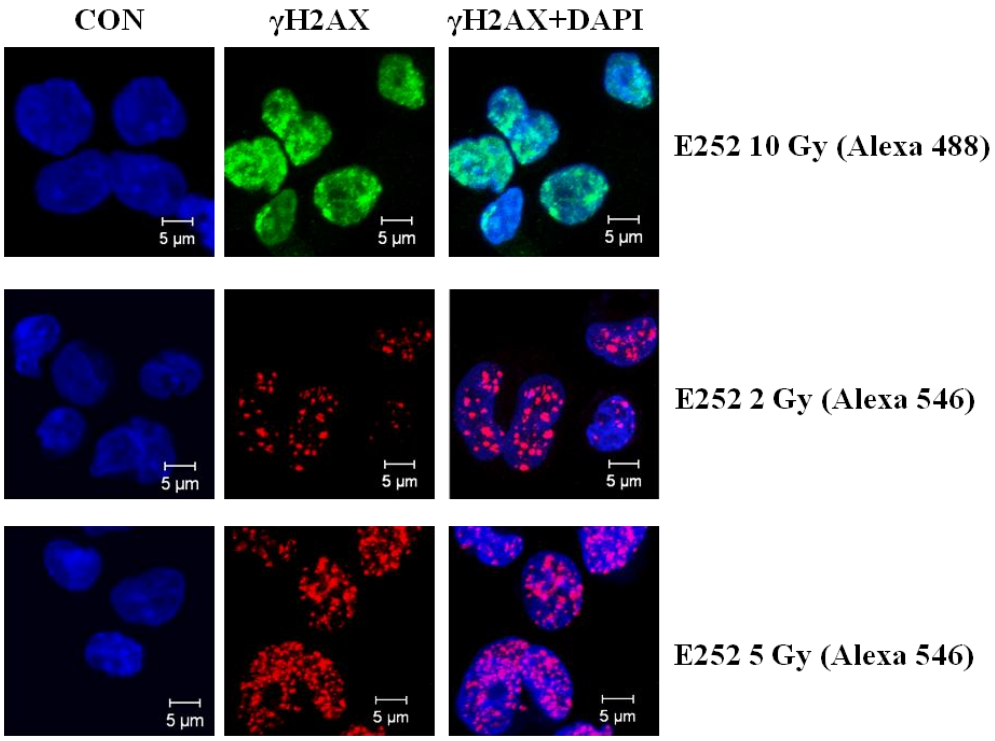


Fig. 19: Standardisation of radiation dose for foci formation assay for γH2AX. The foci formed at 10 Gy radiation exposure were not discrete while foci formed at 2 Gy and 5 Gy radiation exposure were distinct.

4.2.2 DNA damage and repair following γ-radiation exposure

γ-radiation causes DNA double strand breaks. Following DNA damage, appearance and the disappearance of phosphorylated H2AX (γ-H2AX) can be used as a parameter to measure DNA damage and repair respectively.

4.2.2.1 Immunofluorescence foci formation assay for γ-H2AX

To compare the extent of DNA damage between MPN and control cell lines, cells were irradiated with 2 Gy and 5 Gy radiation and γ-H2AX foci were measured by immunofluorescence at 30 min time point. The experiment was conducted on randomly selected 4 MPN and 4 control cell lines. The number of γ-H2AX foci were approximately same between the two groups at 30 min indicating that the amount of DNA damage was almost equal (Fig. 20a, Fig. 20b, Fig. 20c, Fig. 20d). To evaluate the extent of DNA repair between the two groups, LCLs were incubated further for 4 h, providing time for the cells to repair the damage.

At 4 h time point, at both the radiation dose, number of γ-H2AX foci disappeared in LCLs from healthy controls indicating that DNA repair was complete, while in case of the MPN cell lines approximately half of the foci were still present at 4 h indicating that DNA repair was either delayed or impaired (Fig. 20a, Fig. 20b, Fig. 20c, Fig. 20d).

4.2.2.2 Assessment of γ-H2AX by flow cytometry

In order to have a quantitative measurement, DNA damage and repair was measured in cell lines by flow cytometry. This experiment was performed on all 20 MPN and 10 control cell lines by analyzing percent positive cells for γ-H2AX at 30 min and 4 h time points. After radiation exposure, percent γ-H2AX positive cells at 30 min were approximately same in both MPN and control cell lines. As shown in Table 6 average percent γ-H2AX positive cells were 43.40% and 36.43% at 2 Gy; and 77.01% and 76.08% at 5 Gy in patient and control groups respectively. This indicated that the extent of DNA damage was almost equal between patient and control cell lines (Fig. 21, Fig. 22).

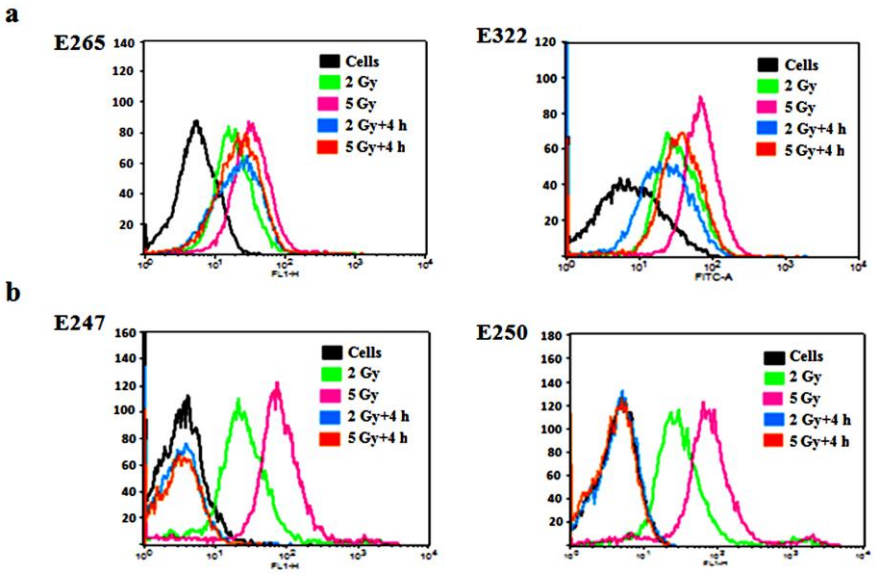


Fig. 21: Percent relative γ-H2AX positive cells. Percent positive cells in MPN cell lines (a) percent positive cells in control cell lines (b). The black peak corresponds to unstained cell, green peak corresponds to percent positive cells after 2 Gy radiation exposure at 30 min, pink peak corresponds to percent positive cells after 5 Gy radiation exposure at 30 min, blue peak corresponds to percent positive cells after 2 Gy radiation exposure at 4 h and orange peak corresponds to percent positive cells after 5 Gy radiation exposure at 4 h.

The cells were incubated further for 4 h so as to allow the cells to repair the damaged DNA. At 4 h time point percent of γ-H2AX positive cells were more in MPN cell lines as compared to control cell lines. The results show that average percent positive cells were 30.72% and 12.17% at 2 Gy; and 44.59% and 20.56% at 5 Gy, in patient and control groups respectively (Fig. 22, Fig. 23). A comparison between the two groups in terms of difference in percent positive cells at both radiation doses and at both time points is shown in Table 9.

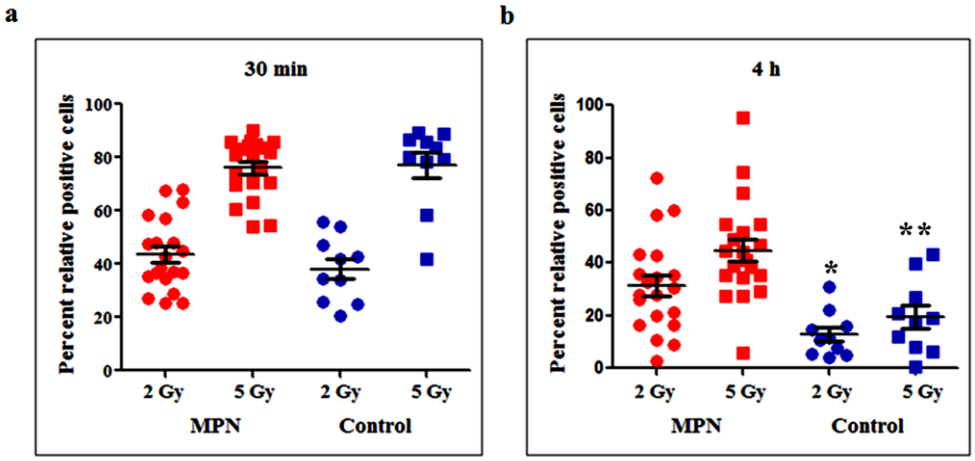


Fig. 22: Percent relative γ-H2AX positive cells in MPN (n=20) and control cell lines (n=10). Percent relative positive cells at 30 min (a) percent relative positive cells at 4 h (b). Mean difference between percent positive cells at 4 h time point between MPN and control cell lines was statistically significant (*p=0.0047 at 2 Gy and **p=0.0009 at 5 Gy). The values shown are the mean values from three separate experiments.

The mean difference in percent positive cells at 30 min between MPN and control cell lines was 6.97% at 2 Gy and 0.93% at 5 Gy. This difference was not statistically significant. While the mean difference between percent positive cells at 4 h time point between MPN and control cell lines was 18.55% at 2 Gy and 24.03% at 5 Gy which was statistically significant with a p value=0.0047 at 2 Gy and p value=0.0009 at 5 Gy, indicating that DNA repair is either delayed or impaired in MPN cell lines which was in concert with immunofluorescence data (Fig. 23).

Table 9: Average percent γ-H2AX positive cells in MPN and control cell lines at 30 min and 4 h time point

	Radiation dose	MPN cell line (n=20)	Control cell line (n=10)
Average percent positive cells-30 min	2 Gy	**43.42%** (Range 25.08 - 67.76%)	**38.08%** (Range 20.59% - 5.61%)
Average percent positive cells-30 min	5 Gy	**75.97%** (Range 53.08% - 0.02%)	**77.13%** (Range 41.77% - 89.34%)
Average percent positive cells-4 h	2 Gy	**31.20%** (Range 2.80% - 72.53%)	**12.80%** (Range 4.11% - 31.09%
Average percent positive cells-4 h	5 Gy	**44.71%** (Range 5.82% - 95.01%	**19.30%** (Range 0.37% - 43.09%)

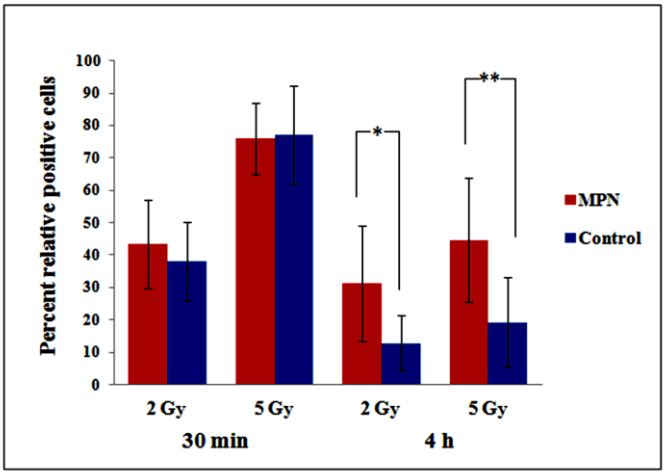


Fig. 23: Comparison of average percent γ-H2AX positive cells between MPN and control groups at 30 min and 4 h time points. The reduction in γ-H2AX positive cells at 4 h is more pronounced in control group as compared to MPN group. The difference in percent γ-H2AX positive cells was statistically significant with a *p=0.0047 at 2 Gy and **p=0.0009 at 5 Gy. Vertical line on each bar shows standard deviation.

4.2.3 Effect of γ-radiation and BPDE exposure on cell cycle profile

After genotoxic exposure cell cycle arrest provides time for the cell to repair damaged DNA before entering into the next phase of the cycle. Radiation induced G2 phase accumulation and BPDE induced S and G2/M phase arrest was performed 20 MPN and 10 healthy control cell lines using flow cytometry.

4.2.3.1 Percent G2 delay after γ-radiation exposure

A considerable G2 phase delay was observed in control cell lines while the extent of G2 phase arrest was lower in MPN LCLs (Fig. 24, Fig. 25). The mean γ-radiation -induced G2-phase accumulation in control cell lines was 19.18% at 5 Gy and 15.83% at 10 Gy which was significantly higher than the values for MPN cell lines where mean G2 phase delay was 5.83% at 5 Gy and 3.86% at 10 Gy.

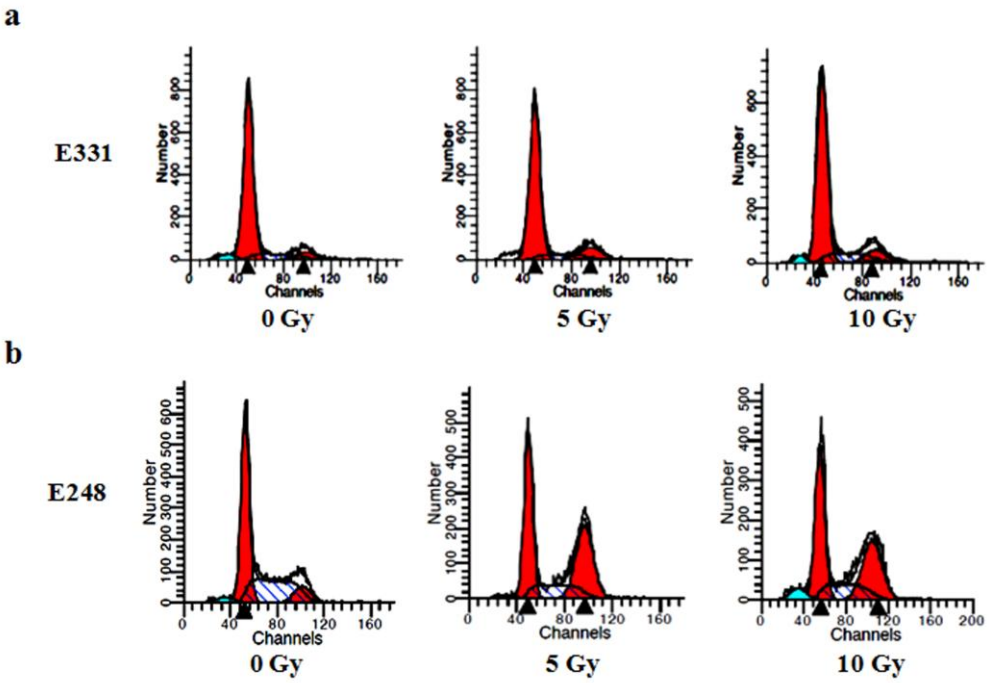


Fig. 24: Effect of γ-radiation exposure on MPN and control cell lines. Effect of radiation exposure on MPN cell line (a) Effect of radiation exposure on control cell line (b).

The mean difference of percent G2 delay between the two groups was 13.35% at 5 Gy and 11.97% at 10 Gy which was statistically significant with a p value=0.0001 at 5 Gy and p value=0.0017 at 10 Gy. A comparison between the two groups in terms of difference in percent G2 delay at 5 Gy and 10 Gy radiation dose is shown in Table 10 (Fig. 26).

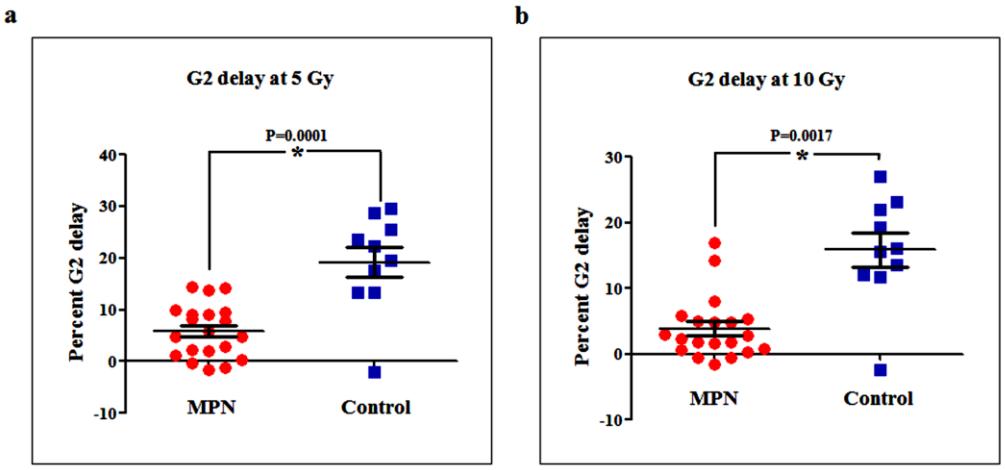


Fig. 25: Relative G2 delay after radiation exposure in MPN (n=20) and control cell lines (n=10). Percent relative G2 delay after 5 Gy radiation exposure (a) percent relative G2 delay after 10 Gy radiation exposure (b). Mean difference between percent relative G2 delay between MPN and control cell lines was statistically significant (p=0.0001 at 5 Gy and p=0.0017 at 10 Gy). The values shown are the mean values from three separate experiments.

Table 10: Average G2 phase delay in MPN and control cell lines after radiation exposure

Radiation dose	MPN cell line (n=20)	Control cell line (n=10)
5 Gy	**5.83%** (Range -1.53% - 14.41%)	**19.18%** (Range -1.95% - 29.65%)
10 Gy	**3.86%** (Range -1.59% - 16.98%)	**15.83%** (Range -2.38% - 23.10%)

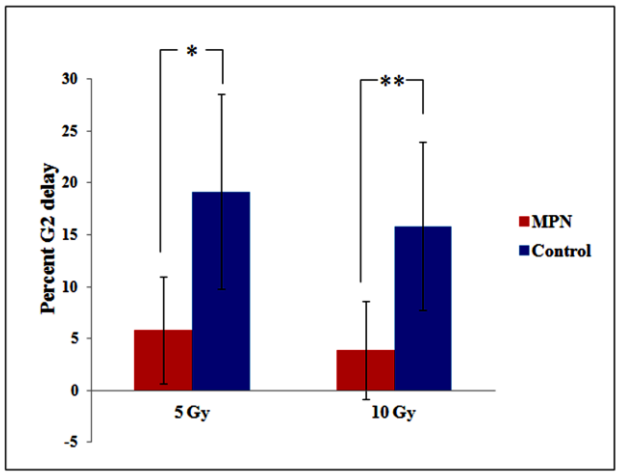


Fig. 26: Comparison of average G2 delay between MPN and control groups at 30 min and 4 h time points. Percent G2 delay is more pronounced in control group as compared to MPN group. The difference in percent G2 delay is statistically significant between the two groups with *p=0.0001 at 5 Gy and **p=0.0017 at 10 Gy. Vertical line on each bar shows standard deviation.

4.2.3.2 Effect of BPDE exposure on Cell cycle profile

Flow cytometry analysis of cell cycle phase distribution after BPDE exposure revealed that there was no considerable difference in the mean percent cell arrested in S and G2/M phase at both 6 h and 24 h time point between MPN and control cell lines.

Mean percent S phase arrest in MPN cell lines at 6 h was 18.54% at 5 µM and 19.14% at 8 µM BPDE concentrations. In control cell lines mean S phase arrest was 13.85% at 5 µM and 13.58% at 8 µM BPDE concentration. Mean percent S phase arrest at 24 h was 19.53% at 5 µM and 20.09% at 8 µM in MPN cell lines. In control cell lines mean S phase arrest was 17.68% at 5 µM and 16.67% at 8 µM (Fig. 27). A comparison between the two groups in terms of difference in cells arrested in S phase after BPDE exposure at both time points is shown in Table 11.

Mean percent G2/M arrest in MPN cell lines at 6 h was 4.86% at 5 µM and 4.87% at 8 µM BPDE concentration. In control cell lines G2/M arrest was 9.57% at 5 µM and 9.55% at 8 µM. Mean percent G2/M arrest in MPN cell lines at 24 h was 3.65% at 5 µM and 3.39% at 8 µM and control cell lines it was 5.19% at 5 µM and 4.83% at 8 µM (Fig. 28). A comparison between the two groups in terms of difference in cells arrested in S phase after BPDE exposure at both time points is shown in Table 11 (Fig. 29).

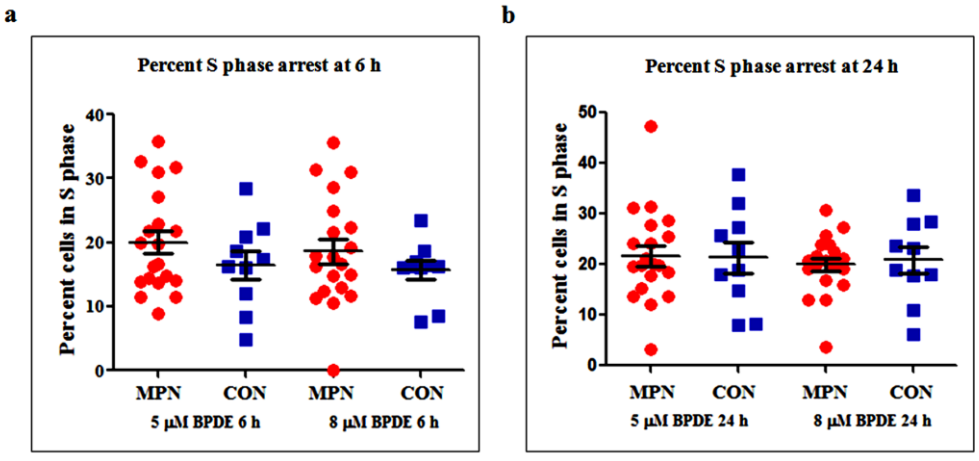


Fig. 27: Relative S phase arrest after BPDE exposure in MPN (n=20) and control cell lines (n=10). Percent relative S phase arrest at 6 h time point (a) percent relative S phase arrest at 24 h time point (b). Mean difference between percent relative S phase arrest between MPN and control cell lines was not statistically significant.

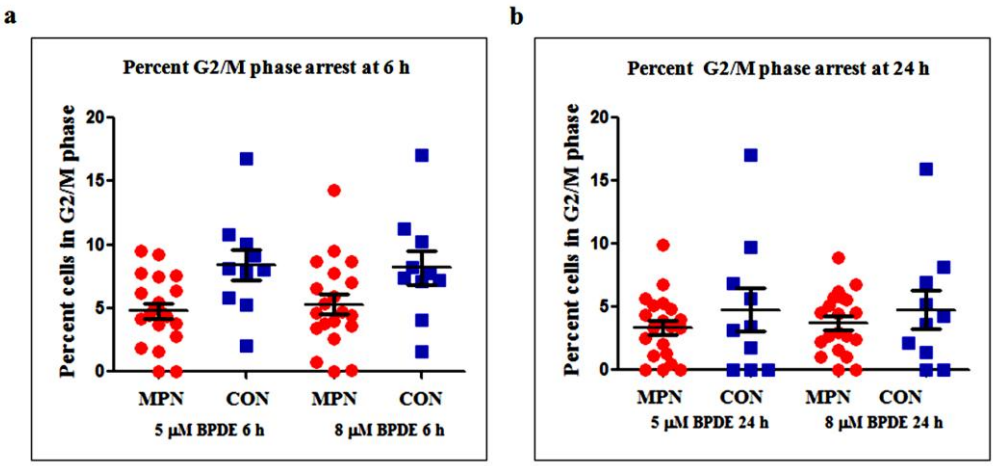


Fig. 28: Percent relative G2/M phase arrest after BPDE exposure in MPN (n=20) and control cell lines (n=10). Percent relative G2/M phase arrest at 6 h time point (a) percent relative G2/M phase arrest at 24 h time point (b).

Table 11: Average S and G2/M phase arrest in MPN and control cell lines after BPDE exposure

	BPDE concentration	Time point	MPN cell line (n=20)	Control cell line (n=10)
Average S phase arrest	5 µM	6 h	**19.99%** (8.84% - 35.08%)	**16.49%** (4.77% - 28.47%)
	8 µM	6 h	**18.57%** (0 - 35.52%)	**15.62%** (7.5% - 17.01%)
	5 µM	24 h	**21.74%** (3.26% - 47.31%)	**21.33%** (8.10% - 37.68%)
	8 µM	24 h	**19.96%** (3.73% - 30.72%)	**20.90%** (6.28% - 33.62%)
Average G2/M phase arrest	5 µM	6 h	**4.79%** (0 - 9.54%)	**8.39%** (2.05% - 16.82%)
	8 µM	6 h	**5.29%** (0 - 14.27%)	**8.20%** (1.55% - 17.05%)
	5 µM	24 h	**3.36%** (0 - 9.9%)	**4.77%** (0.01% - 17.01%)
	8 µM	24 h	**3.71%** (0 - 8.8%)	**4.76%** (0 - 15.9%)

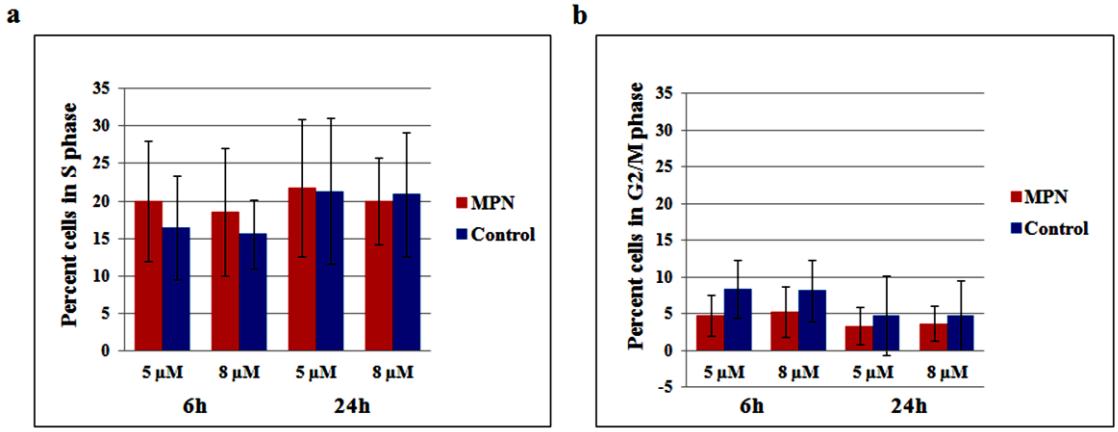


Fig. 29: Comparison of percent S and G2/M phase arrest between MPN and control groups after BPDE exposure. There difference between mean percent cell arrested in S (a) and G2/M phase (b) at both 6 h and 24 h time points is no statistically significant between the two groups. The difference was not statistically significant between the two groups at both BPDE concentrations and both time points. Vertical line on each bar shows standard deviation.

4.2.4 Apoptotic response in MPN and control samples in vitro

A method of Annexin-V-PI double labelling was used to measure percent cell death after γ-radiation and BPDE exposure. The apoptotic response was performed on LCLs from 20 MPN and 10 controls.

4.2.4.1 Percent cell death after γ-radiation exposure

Percent cell death after radiation exposure was higher in control cell lines with average percent cell death of 19.22% at 5 Gy and 22.09% at 10 Gy, as compared to MPN cell lines where the average percent cell death was 9.84% at 5 Gy and 10.84% at 10 Gy. The mean difference between the two groups was 9.38% at 5 Gy and 11.25% at 10 Gy which was statistically significant with a p value=0.0003 at 5 Gy and p=0.0002 at 10 Gy (Fig. 30). A comparison between the two groups in terms of percent cell death after γ-radiation exposure at both radiation doses is shown in Table 12 (Fig. 32).

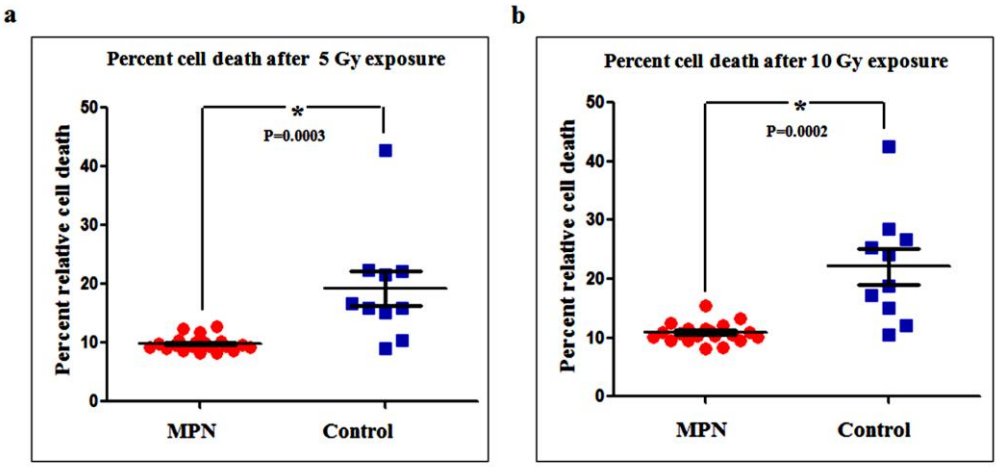


Fig. 30: Percent cell death after radiation exposure in MPN (n=20) and control cell lines (n=10). Percent cell death after 5 Gy radiation exposure (a) percent cell death after 10 Gy radiation exposure (b). Mean difference between percent cell death between MPN and control cell lines was statistically significant (p=0.0003 at 5 Gy and p=0.0002 at 10 Gy). The values shown are the mean values from three separate experiments.

4.2.4.2 Percent cell death after BPDE exposure

A statistically significant difference was observed in percent cell death between MPN and control cell lines on exposure to BPDE. Percent cell death was higher in control cell lines when compared to MPN cell lines (Fig. 31). The results demonstrate that the mean BPDE induced percent cell death was 23.25% at 5 µM and 24.98% 8 µM in control cell lines which was higher as compared to MPN cell lines where mean percent cell death was 13.57% at 5 µM and 14.02% at 8 µM BPDE concentrations. Untreated cells and cells treated with THF were taken as control. The mean difference between the two groups was 9.68% at 5 µM and 10.98% at 8 µM which was statistically significant with p value=0.0009 at 5 µM and 8 µM BPDE concentration. A comparison between the two groups in terms of percent cell death after γ-radiation exposure and after BPDE exposure is shown in Table 12 (Fig.27).

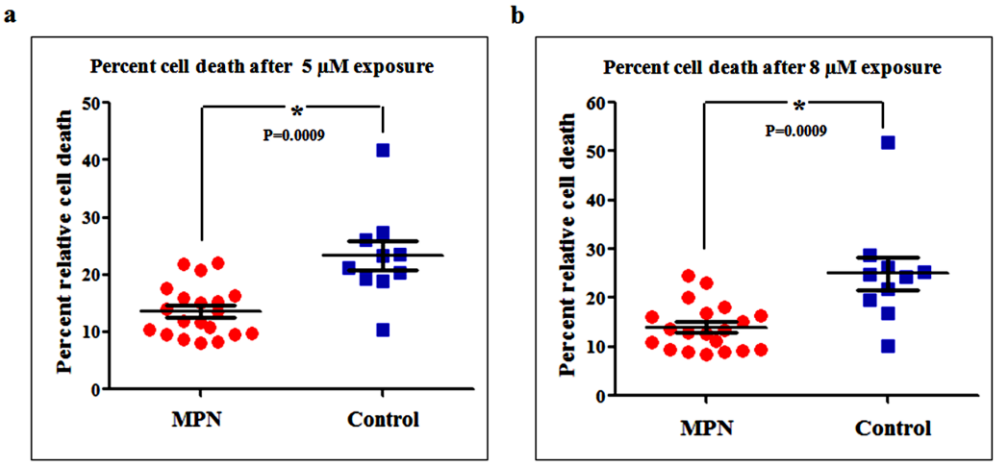


Fig. 31: Percent cell death after BPDE exposure in MPN (n=20) and control cell lines (n=10). Percent cell death after 5µM radiation exposure (a) percent cell death after 8µM radiation exposure (b). Mean difference between percent relative cell death between MPN and control cell lines was statistically significant (p=0.0009 at 5µM and p=0.0008 at 8µM). The values shown are the mean values from three separate experiments.

Table 12: Average cell death after γ-radiation and BPDE exposure in MPN and control cell lines

Genotoxic exposure	Dose/concentration	MPN cell line (n=20)	Control cell line (n=10)
Radiation exposure	5 Gy	**9.84%** (Range 8.3% - 12.7%)	**19.22%** (Range 9.05% - 42.82%)
	10 Gy	**10.84%** (Range 8.3% - 12.7%)	**22.09%** (Range 12.04% - 42.4%)
BPDE treatment	5 µM	**13.57%** (Range 8.02% - 21.77%)	**23.25%** (Range 10.49% - 41.79%)
	8 µM	**14.02%** (Range 8.46% - 24.69%)	**24.98%** (Range 10.23% - 51.76%)

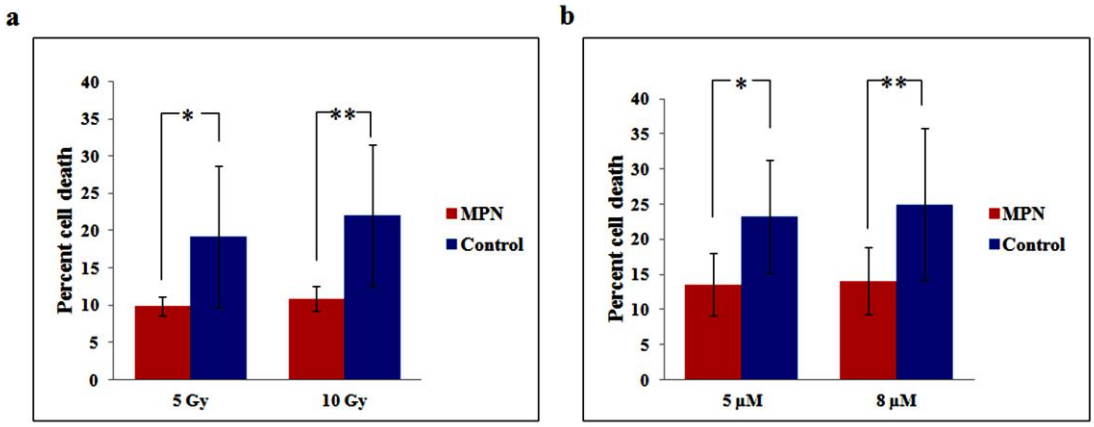


Fig. 32: Comparison of percent cell death between MPN and control groups. Percent cell death after genotoxic exposure is more pronounced in control group as compared to MPN group after γ-radiation. The difference was statistically significant between the two groups with *p=0.0003 at 5 Gy and **p=0.0002 at 10 Gy (a) and BPDE exposure with *p=0.0009 at 5µM and **p=0.0009 at 8µM BPDE (b). Vertical line on each bar shows standard deviation.

4.2.5 Global gene expression profiling after γ-radiation exposure

It is well established that the way in which an individual deals with genotoxic exposure is related to the probability to develop cancer. Sensitivity to genotoxic agents in terms of phenotypic responses can reflect and individual's susceptibility to neoplastic evolution. At present, apart from performing standard tests to assess genotoxic effect the focus has started to shift to search for pathways involved in differential genotoxic sensitivity and phenotypic responses. These differences in cellular responses can be traced back to differential gene expression hence whole genome expression profiling was done in order to find any association between varied phenotypic response after genotoxic exposure and gene expression in patient and control cell lines.

4.2.5.1 Gene expression data analysis

To assess the effect of γ-radiation exposure between MPN and control cell lines whole genome expression profiling was done using randomly selected 5 MPN and 5 control cell lines, at 5 Gy radiation exposure and 24 h time point. After processing the slides as described in Materials and Methods (Toronto microarray centre slides 19K and 8K were used) they were analyzed on AXON Scanner 4200A and raw data was obtained as ratio of two fluorescent dyes for each spot, corresponding to different genes along with other parameters. This data was subjected to analysis using GeneSpring GX 10.0 software. The raw data was first normalised by Lowess

normalization with baseline transformation to median of all the samples. Lowess normalization is a method used to normalize a two colour array to compensate non linear dye bias. After normalization it was observed that for all the normalized intensity values the median was lying on zero which implies that the hybridizations were good and inter-sample hybridization variation was negligible (Fig. 33).

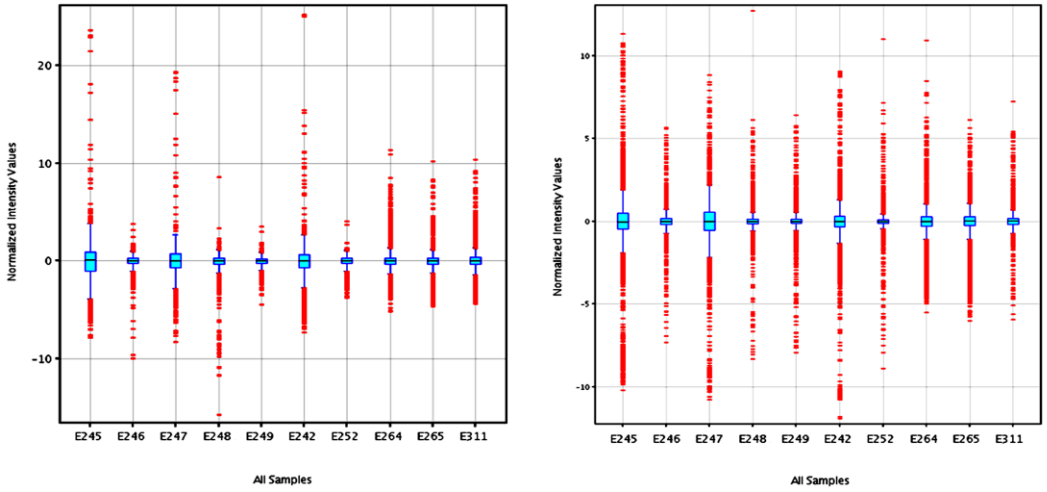


Fig. 33: Normalized intensities of all samples used for microarray. A box-and-whisker plot of the microarray data for 8K (left side) and 19K (right side) slides revealed a similar distribution of normalized intensity values across all 5 MPN and 5 Control cell lines used in the microarray hybridization analysis.

In this experiment we were interested in looking at the differential response after radiation exposure. It is well known that exposure to genotoxic γ- radiations will mainly affect the expression of genes falling in pathways like signal transduction, DNA damage and repair, cell cycle regulation and apoptosis. Hence from the total 27K genes arrayed, approximately 500 genes from above mentioned pathways were selected using KEGG pathway database. The normalized data was subjected to supervised clustering and unpaired students‘t’ test with unequal variance to obtain statistically significant, differentially expressed genes, between the patient and control groups. Since the number of genes subjected for initial analysis was very low, only 20 genes were obtained that were differentially regulated between the two groups, with p value<0.05 and fold change >1.2. These genes were mainly signal transduction genes, genes involved in DNA repair, cell cycle regulation and apoptosis. Since the cut off for the difference in expression was only 1.2 fold the genes could not be validated by real time PCR.

We could not observe a difference in expression level of genes obtained by first analysis therefore the next set of analysis was done by taking all 27K genes together. After normalization the data was again subjected to supervised clustering and paired students't' test with unequal variance to obtain statistically significant, differentially expressed genes between the patient and control groups (Fig. 34). After analysis, 226 genes were found to be differentially expressed between the two groups with p value<0.05, fold change >1.5. Although there was no marked difference in expression of genes falling in important pathways related to cancer we observed a variation in expression of genes like MAFB, ZNF429 and HMGA2 some of these are cancer genes that are involved in transcription regulation, along with MAPK10, ARHGAP25, and GNG12 signal transduction genes which may in turn have an effect on downstream molecules. Few other differentially expressed genes like TMEFF1, AREG and SMAD6 which are known to be associated with cancers were also present in the list. All these genes had a difference in expression level >2 fold between the two groups therefore were subjected to real time validation.

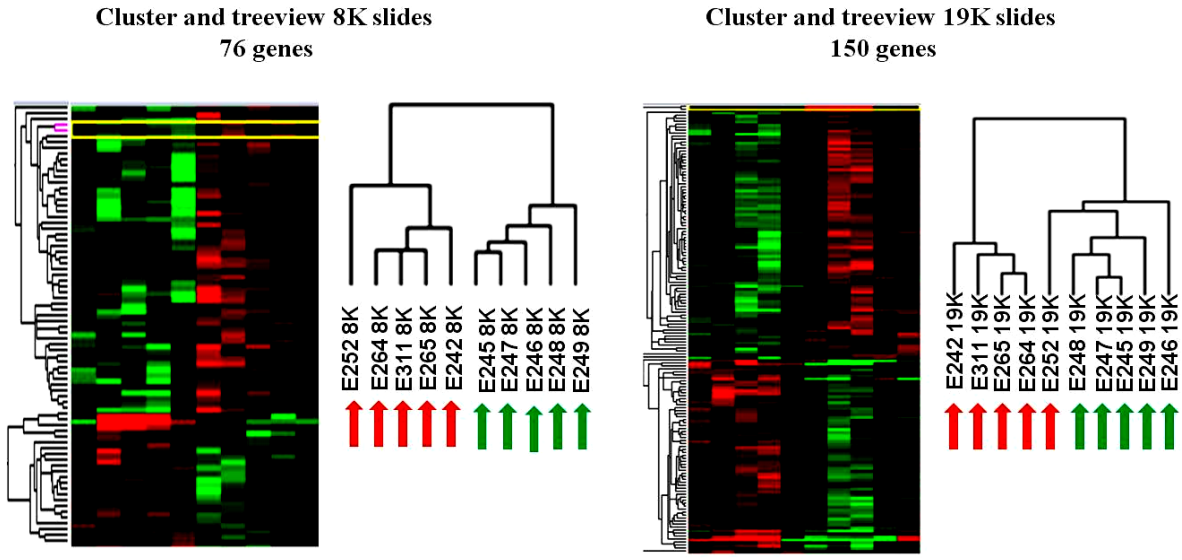


Fig. 34: Supervised hierarchical clustering for differential gene expression. MPN and control cell lines after radiation exposure were subjected to expression profiling using 8 K and 19 K microarray slides. The variable portion of the data set was visualized by the Eisen Cluster and TreeView program. Red, green and black pixels indicate relatively high, low and neutral expression, respectively. Clustering of various cell lines is shown in the right. Red arrows indicate MPN cell lines and green arrows indicate control cell lines. Except one MPN cell line (E252) all cell lines clustered in expected clusters.

4.2.5.2 Real time PCR validation of differentially expressed genes

The genes that were found to be differentially expressed in microarray data were validated by real time PCR along with GAPDH as an internal control. The genes included AREG, ARHGAP25, MAFB, MAPK10, TMEFF and ZNF429. Total RNA isolated from MPN and control cell lines after 5 Gy radiation exposure and 24 h time point was subjected to cDNA was synthesis which was used for real time validation of genes. The expression of all the genes was calculated with respect to GAPDH of the individual samples and a ΔCt value was calculated. This ΔCt value was plotted as relative fold change by calculating:

$$\text{Fold change} = 2^{-\Delta Ct} \times 1000$$

Out of these deregulated genes, ARHGAP25, MAFB, MAPK10, and ZNF429 were up regulated in MPN cell lines while TMEFF was down regulated. Real time validation was conducted on 13 MPN and 7 control cell lines including the cell lines taken for microarray experiment. The difference in the expression of genes that was observed in microarray was also observed in the real time validation to some extent, although it was not statistically significant. Cluster plots showing differential expression of these genes is shown in Fig. 35.

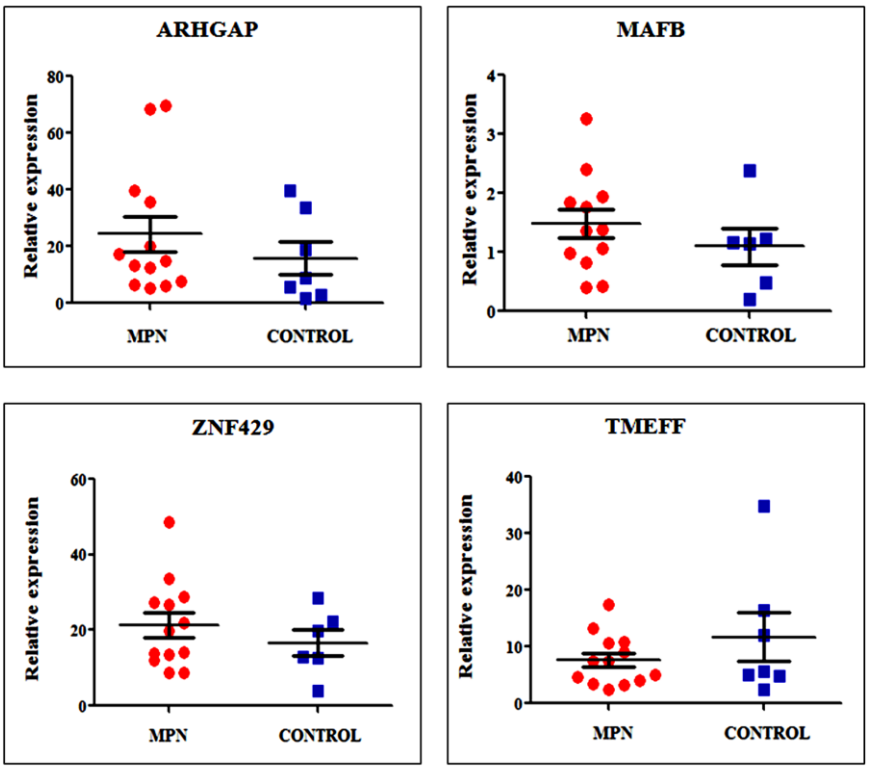


Fig. 35: Representative cluster plots showing real time validation of differentially expressed genes. ARHGAP25, MAFB, ZNF429 were up regulated in MPN cell lines while TMEFF was down regulated.

Objective 3

To genotype selected candidate genes involved in DNA repair, carcinogen metabolism, apoptosis and cell cycle regulation.

The association of various gene polymorphisms with cancer has been frequently reported. The candidate genes influencing susceptibility to cancer includes genes falling mainly in important carcinogenesis pathways of carcinogen metabolism, DNA repair, cell cycle regulation and cell death control. Variation in any of these mechanisms could result in accumulation of cell with genetic alteration in critical genes leading to tumourigenesis. In the present study 22 single nucleotide polymorphisms (SNPs) in candidate genes involved in DNA repair, apoptosis, cell cycle regulation and carcinogen metabolism were genotyped and a G Score was calculated from the number of variant alleles.

4.3.1 Genomic DNA extraction

High molecular weight genomic DNA was isolated from whole blood or the pellet obtained after isolating PBLs from the whole blood for cell line preparation. The quality of DNA was assessed on 0.8% agarose gels stained with ethidium bromide. Approximately 5-20µg of good quality DNA was obtained from each sample. Extreme care was taken to avoid cross contamination of samples. DNA was diluted to a concentration of 50ng/µl for performing PCR amplification while the stock DNA was stored at 0 °C till further use.

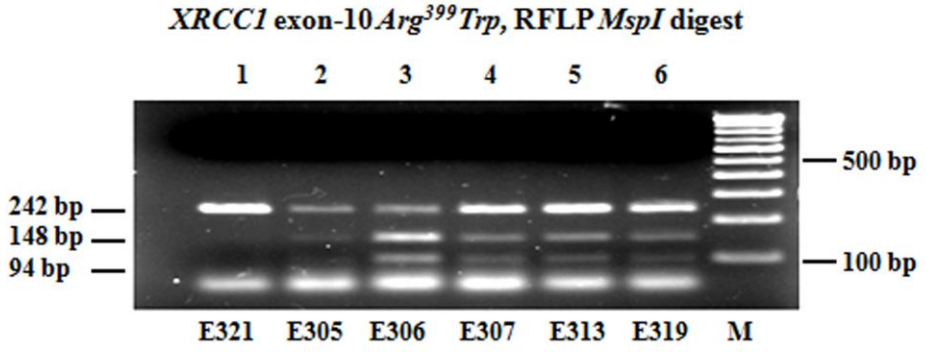
4.3.2 Genotyping of genes by PCR-RFLP

Genotyping of 15 SNPs was performed by PCR and PCR-RFLP in 30 samples. Each sample was subjected to PCR for amplification of the genes of interest and to confirm the successful amplification PCR products were run on agarose gel. Multiplex PCR was done to assess the null genotype of GSTT1 and GSTM1 genes. In the PCR, to investigate null genotype, IFN was used as internal control to rule out amplification failure. For RFLP, PCR products were digested with respective restriction enzymes and the digested products were analyzed on 2% agarose gel (Fig. 36a, Fig. 36b, Fig. 36c). Depending upon the obtained digested product the genotype was recorded as

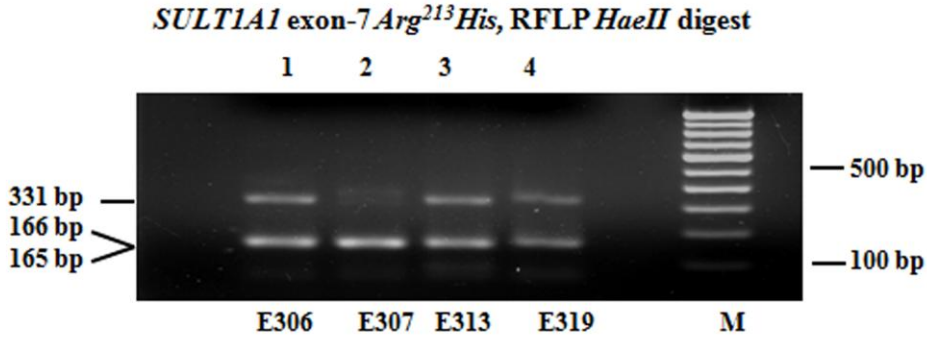
homozygous wild-type, heterozygous or homozygous variant type. Consolidated genotyping data is shown in Table 13.

4.3.3 Genotyping of genes by SNaPshot

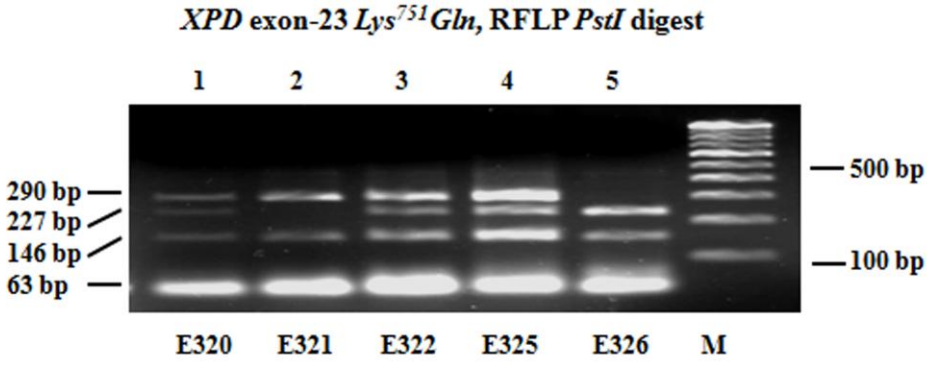
Genotyping of 7 SNPs was performed by SNaPshot reaction. Each sample was subjected to PCR for amplification of genes of interest. To confirm successful amplification of the samples PCR products were run on agarose gel. Positive samples were utilized for genotyping by SNaPshot assay. When the processed sample was run on sequencer the extended primer was read by the fluorescent signal of labelled ddNTP and the SNP was interpreted (Fig. 37a, Fig. 37b). The genotype thus obtained was recorded as homozygous wild type, heterozygous or homozygous variant type. Consolidated genotyping data is shown in Table 13.



a. Genotype of *MspI* digested *XRCC1* PCR products. Lane 1, *Trp/Trp*; lane 2-6, *Arg/Trp*

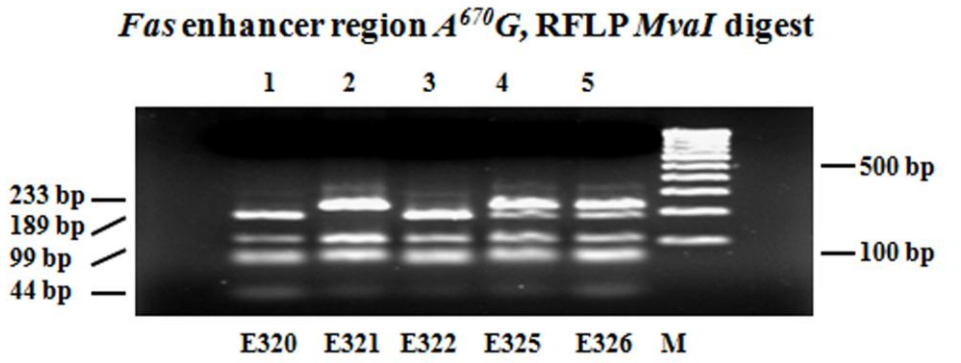


b. Genotype of *HaeII* dig ested *SULT1A1* PCR products. Lane 1,3,4, *Arg/His*; lane 2, *Arg/Arg*

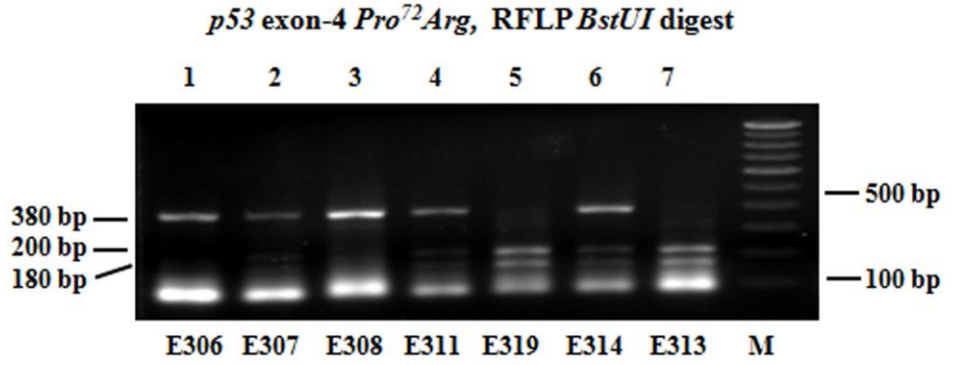


c. Genotype of *PstI* digested *XPD* PCR products. Lane 1,3,4, *Lys/Gln*; lane 2, *Lys/lys*, lane 5 *Gln/Gln*

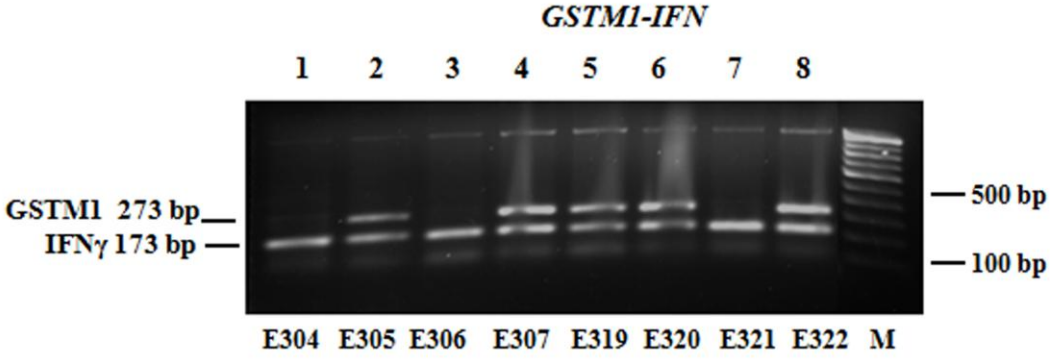
Fig. 36a: Electrophoresis of PCR products after restriction enzyme digestion on 2% agarose gel. RFLP image showing digestion of *XRCC1*-exon10 with *MspI* (a); RFLP image showing digestion of *SULT1A1* exon-7 with *HaeII* (b); RFLP image showing digestion of *XPD*- exon23 with *PstI* (c). Interpretation of respective genotype of all samples is documented below each gel.



d. Genotype of *MvaI* digested *Fas* PCR products. Lane 1,3, *GG*; lane 2, *AA*; lane4,5, *AG*

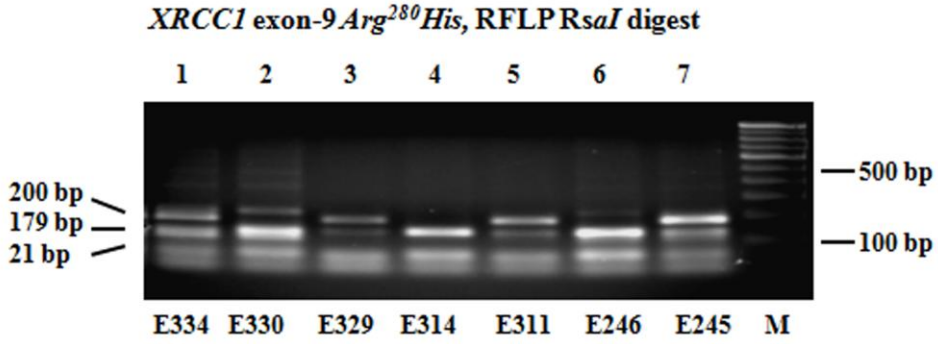


e. Genotype of *BstUI* digested *p53* PCR products. Lane 1,3, *Pro/Pro*; lane 2, 4, 6, *Pro/Arg*; lane 5,7 *Arg/Arg*

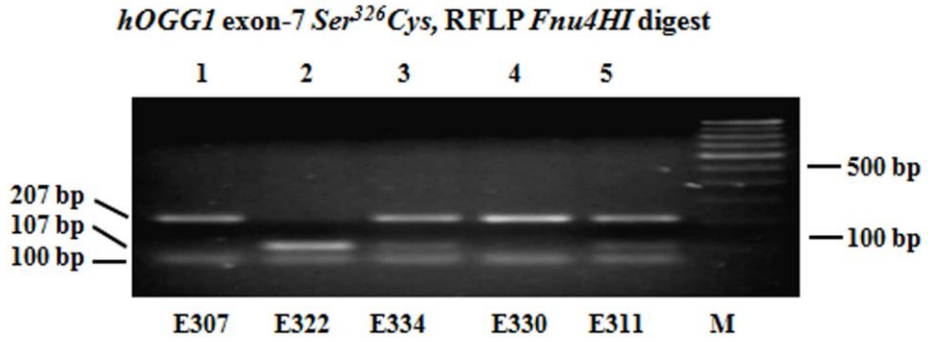


f. Multiplex PCR products of *GSTM1* and *IFN*. Lane 1,3, 7 *Null genotype*; lane 2, 4, 5, 6, 8, *Wildtype genotype*

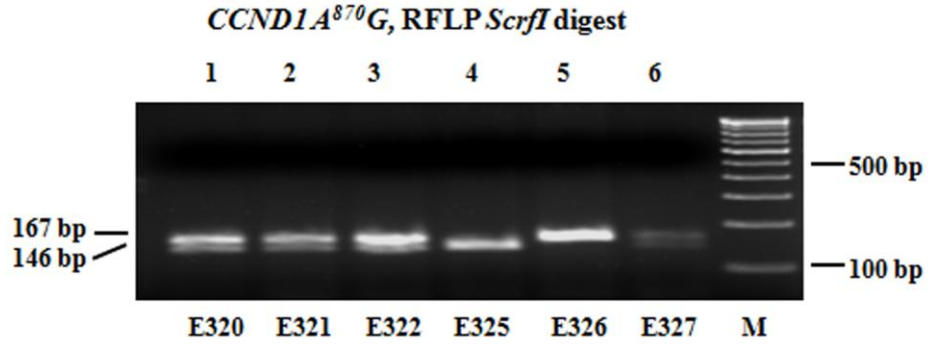
Fig. 36b: Electrophoresis of PCR products after restriction enzyme digestion on 2% agarose gel. RFLP image showing digestion of *Fas* with *MvaI* (a); RFLP image showing digestion of *p53* exon-7 with *BstUI* (b); agarose gel image showing *GSTM1 null* genotype (c). Interpretation of respective genotype is documented below each gel.



g. Genotype of *RsaI* digested *XRCC* PCR products. Lane 1, 2, 3, 5, 7 *Arg/His*; lane 4, 7 *Arg/Arg*



h. Genotype of *Fnu4HI* digested *hOGG1* PCR products. Lane 1, 4 *Ser/Ser*; lane 3, 5 *Ser/Cys*, lane 2, *Cys/Cys*



i. Genotype of *ScrfI* digested *CCND1* PCR products. Lane 1, 2, 3, 6 *AG*; lane 4 *GG*, lane 5, *AA*

Fig. 36c: Electrophoresis of PCR products after restriction enzyme digestion on 2% agarose gel. RFLP image showing digestion of *XRCC1*-exon9 with *RsaI* (a); RFLP image showing digestion of *hOGG1* exon-7 with *Fnu4HI* (b); RFLP image showing digestion of *CCND1* with *Scrf1* (c). Interpretation of respective genotype is documented below each gel.

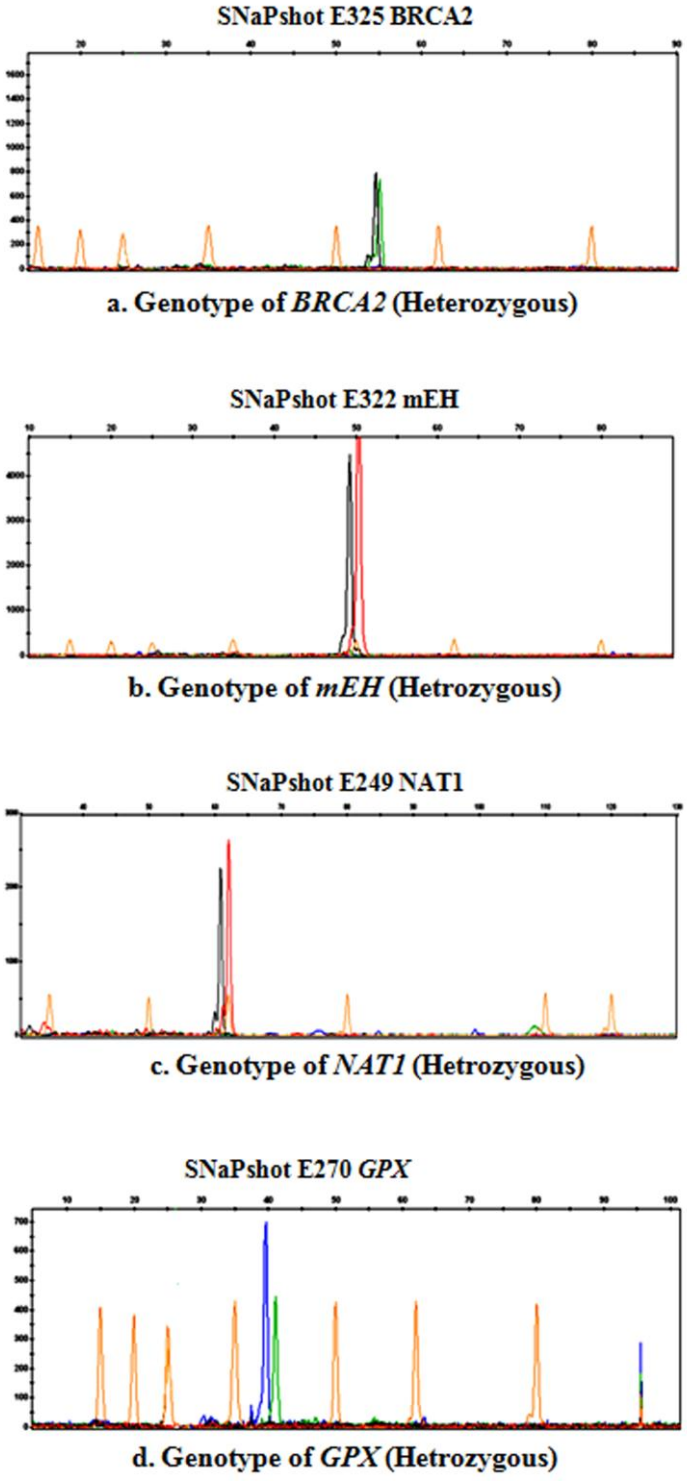


Fig. 37a: Representative electropherogram showing genotyping done by SNaPshot reaction. Identification of single SNP in different samples by fluorescent signal. Two peaks at the same point indicate heterozygous genotype. The location of the peak run along the size standard marker (orange) displays the identity of the SNP. Results are interpreted on the basis of colour of the peak.

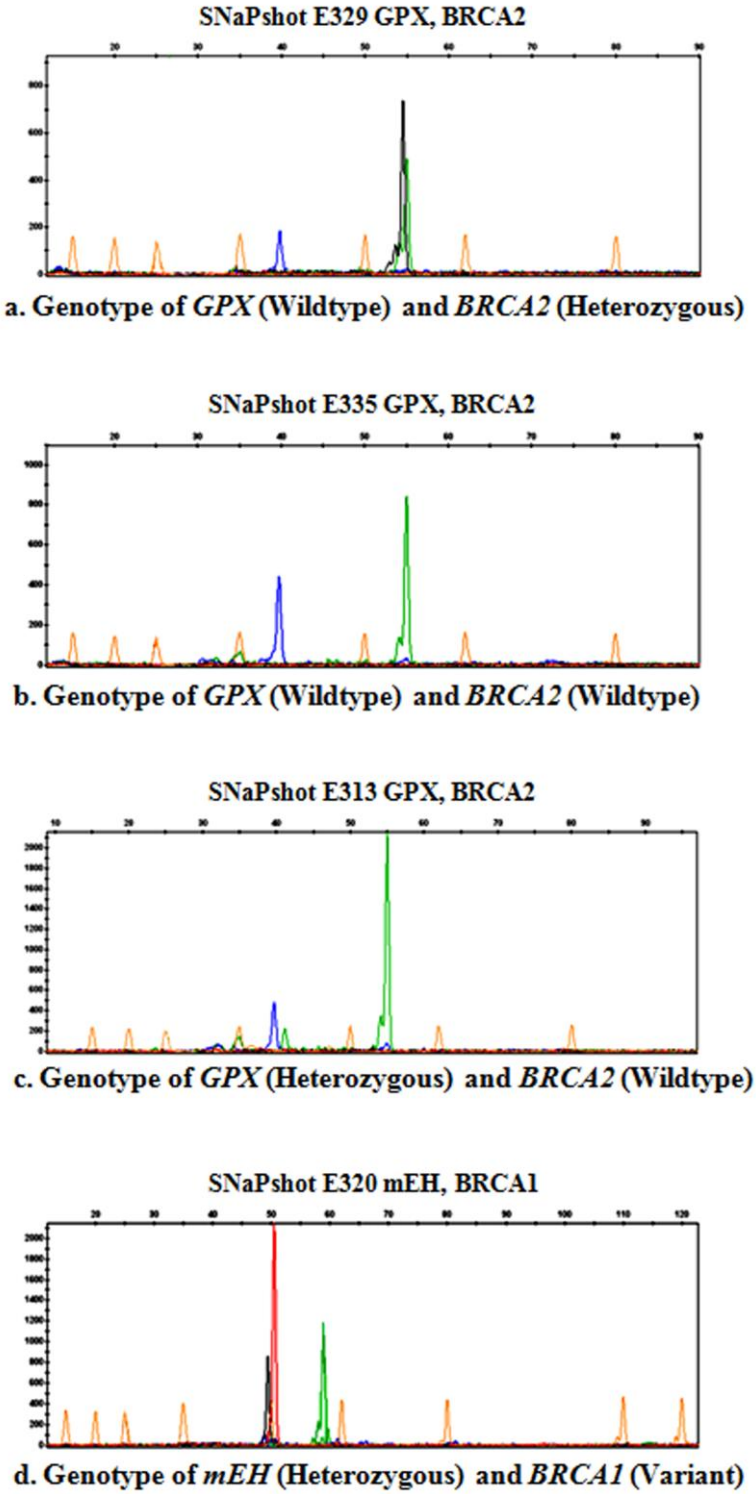


Fig. 37b: Representative electropherogram showing genotyping done by SNaPshot reaction. Identification of two SNPs in two different genes in different samples by fluorescent signal. Single peak indicates either wildtype or variant genotype while two peaks at the same point indicate heterozygous genotype. The location of the peak run along the size standard marker (orange) displays the identity of the SNP. Results are interpreted on the basis of colour of the peak.

Table 13: Consolidated genotyping data for 22 SNPs in 17 genes for all 30 samples

LCL	Genes							
	GSTT1	**GSTM1**	**GSTP1**	**SULT1A1**	**MPO**	**hOGG1**	**p21**	**p53**
E242	Wt	Wt	Wt	Wt	Ht	Wt	Hm	Ht
E252	Wt	Wt	Ht	Ht	Hm	Wt	Ht	Ht
E253	Null	Null	Wt	Wt	Wt	Ht	Hm	Ht
E264	Wt	Wt	Wt	Ht	Wt	Wt	Hm	Wt
E265	Wt	Wt	Wt	Ht	Wt	Wt	Ht	Wt
E270	Wt	Null	Hm	Ht	Wt	Wt	Wt	Hm
E305	Wt	Wt	Hm	Ht	Wt	Ht	Wt	Ht
E306	Wt	Null	Ht	Ht	Wt	Ht	Wt	Wt
E307	Wt	Wt	Ht	Wt	Wt	Wt	Wt	Ht
E308	Wt	Null	Hm	Ht	Wt	Ht	Wt	Wt
E311	Wt	Wt	Hm	Ht	Hm	Ht	Wt	Ht
E319	Null	Wt	Hm	Ht	Wt	Ht	Ht	Hm
E320	Wt	Wt	Ht	Ht	Wt	Ht	Wt	Ht
E321	Wt	Null	Wt	Ht	Wt	Ht	Wt	Ht
E322	Wt	Wt	Wt	Ht	Ht	Hm	Wt	Ht
E325	Wt	Null	Ht	Ht	Wt	Wt	Ht	Ht
E326	Wt	Wt	Ht	Ht	Wt	Ht	Ht	Hm
E329	Null	Null	Hm	Ht	Wt	Ht	Ht	Ht
E330	Wt	Wt	Hm	Ht	Wt	Ht	Wt	Ht
E331	Wt	Wt	Ht	Ht	Wt	Ht	Ht	Wt
E245	Wt	Wt	Wt	Ht	Ht	Ht	Ht	Hm
E246	Wt	Wt	Ht	Wt	Wt	Wt	Wt	Wt
E247	Null	Wt	Ht	Wt	Wt	Wt	Wt	Hm
E248	Wt	Wt	Wt	Wt	Wt	Wt	Hm	Hm
E249	Wt	Null	Ht	Wt	Wt	Wt	Hm	Wt
E250	Null	Wt	Wt	Ht	Wt	Wt	Ht	Wt
E313	Wt	Null	Ht	Wt	Wt	Wt	Wt	Hm
E314	Wt	Null	Hm	Ht	Ht	Wt	Wt	Ht
E334	Wt	Null	Hm	Ht	Wt	Ht	Wt	Ht
E335	Wt	Wt	Hm	Ht	Wt	Wt	Ht	Ht

	MPN
	Control

LCL	Genes						
	XRCC1-6	**XRCC1-9**	**XRCC1-10**	**XPD**	**Fas**	**Cyclin D1**	**XRCC3-7**
E242	Ht	Ht	Wt	Hm	Wt	Hm	Ht
E252	Wt	Wt	Ht	Hm	Hm	Ht	Hm
E253	Wt	Wt	Wt	Ht	Ht	Wt	Ht
E264	Ht	Wt	Ht	Ht	Ht	Ht	Hm
E265	Wt	Wt	Hm	Ht	Ht	Wt	Hm
E270	Wt	Ht	Hm	Hm	Ht	Hm	Ht
E305	Wt	Ht	Ht	Ht	Ht	Ht	Ht
E306	Wt	Wt	Ht	Hm	Ht	Wt	Ht
E307	Wt	Wt	Ht	Ht	Hm	Wt	Hm
E308	Ht	Ht	Wt	Ht	Hm	Ht	Hm
E311	Wt	Ht	Wt	Wt	Hm	Wt	Wt
E319	Wt	Ht	Ht	Wt	Hm	Wt	Ht
E320	Ht	Wt	Wt	Ht	Hm	Ht	Hm
E321	Wt	Wt	Hm	Wt	Wt	Ht	Ht
E322	Wt	Wt	Wt	Ht	Hm	Ht	Ht
E325	Wt	Wt	Hm	Ht	Ht	Hm	Ht
E326	Wt	Wt	Wt	Hm	Ht	Wt	Hm
E329	Ht	Ht	Ht	Wt	Ht	Wt	Wt
E330	Wt	Wt	Hm	Ht	Hm	Wt	Ht
E331	Wt	Wt	Hm	Ht	Ht	Wt	Hm
E245	Wt	Ht	Ht	Wt	Ht	Wt	Ht
E246	Wt	Wt	Wt	Wt	Wt	Hm	Hm
E247	Wt	Wt	Ht	Wt	Ht	Wt	Hm
E248	Wt	Wt	Hm	Wt	Ht	Wt	Ht
E249	Wt	Wt	Wt	Ht	Wt	Hm	Hm
E250	Wt	Wt	Wt	Wt	Ht	Wt	Hm
E313	Wt	Wt	Ht	Wt	Ht	Ht	Hm
E314	Ht	Wt	Ht	Ht	Hm	Wt	Ht
E334	Wt	Ht	Hm	Ht	Ht	Wt	Ht
E335	Wt	Ht	Ht	Ht	Ht	Hm	Hm

	MPN
	Control

LCL	Genes						
	NAT2(1)	**NAT2(2)**	**NAT2(3)**	**BRCA2(1)**	**BRCA2(2)**	**GPX**	**mEH**
E242	Wt	Hm	Wt	Ht	Wt	Hm	Hm
E252	Ht	Wt	Wt	Wt	Ht	Wt	Hm
E253	Wt	Ht	Wt	Wt	Hm	Hm	Ht
E264	Wt	Wt	Wt	Hm	Hm	Ht	Wt
E265	Hm	Wt	Wt	Wt	Hm	Ht	Wt
E270	Ht	Ht	Wt	Wt	Hm	Ht	Wt
E305	Wt	Wt	Wt	Ht	Hm	Hm	Ht
E306	Ht	Wt	Wt	Hm	Hm	Ht	Ht
E307	Wt	Wt	Ht	Ht	Wt	Wt	Wt
E308	Wt	Wt	Wt	Ht	Ht	Ht	Ht
E311	Wt	Wt	Wt	Wt	Hm	Hm	Wt
E319	Wt	Hm	Ht	Wt	Wt	Ht	Wt
E320	Ht	Ht	Hm	Hm	Hm	Hm	Ht
E321	Wt	Ht	Ht	Wt	Wt	Wt	Wt
E322	Wt	Ht	Wt	Wt	Wt	Wt	Ht
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E246	Wt	Hm	Wt	Wt	Ht	Ht	Ht
E247	Wt	Ht	Wt	Wt	Ht	Ht	Ht
E248	Wt	Ht	Wt	Ht	Wt	Ht	Ht
E249	Ht	Ht	Wt	Wt	Ht	Wt	Hm
E250	Wt	Hm	Wt	Wt	Ht	Wt	Hm
E313	Ht	Ht	Wt	Ht	Wt	Ht	Ht
E314	Ht	Ht	Ht	Ht	Wt	Ht	Wt
E334	Wt	Ht	Wt	Ht	Ht	Ht	Ht
E335	Wt	Ht	Wt	Hm	Wt	Wt	Wt

	MPN
	Control

4.3.4 Genotype Score (G Score)

For quantitatively analysing the genotype, a value of 2, 1 and 0 was allotted to variant, heterozygous and wildtype forms of each gene polymorphism respectively and a consolidated Genotype Score or G Score was calculated. For all samples (n=30) value of total G Score ranged from 10 - 22. A high G Score designated more number of risk alleles in an individual. Our assumption was that the individuals with a high G Score might have a higher probability of developing MPN as compared to those with the low G Score. It is evident from the genotyping data that most of the MPN patients displayed a high G Score value (≥15) while controls showed a low G Score. Of the total samples, 13/20 MPN patients had a G Score ≥15 (median total G Score) while 7/10 controls had a G Score <15.

Similarly G Score was calculated for various subgroups of genes including only DNA repair genes (XRCC1-Arg^{194}Trp, XRCC1-Arg^{280}His, XRCC1-Arg^{399}Trp, XRCC3-Thr^{241}Met, XPD-Lys^{751}Gln, hOGG1-Ser^{326}cys, BRCA2-26G>A (5'UTR), BRCA2-Asn^{372}His); MPN risk association signature (SULT1A1-Arg^{213}His, hOGG1-Ser^{326}cys, BRCA2-Asn^{372}His, mEH-Try^{113}His, XRCC1-Arg^{280}His); carcinogen metabolising enzymes genes (GSTT1-Null, GSTM1-Null, GSTP1-Ile^{105}Val, SULT1A1-Arg^{213}His, MPO--463G>A (promoter region), NAT2-Ile^{114}Thr, NAT2-Arg^{197}Gln, NAT2-Gly^{286}Glu, mEH-Tyr^{113}His, GPX-Pro^{198}Leu); and cell cycle regulation and apoptosis genes (Fas- -670A>G (enhancer region), p53-Pro^{72}Arg, p21-Ser^{31}Arg, CCND1-870A>G).

There was no significant difference between the G Scores of MPN and controls for carcinogen metabolising enzymes genes and cell cycle regulation and apoptosis genes subgroups (Fig. 38). However, G Score of DNA repair genes ranged from 3 - 9 and MPN risk association signature ranged from 0 - 6 and was found to be significantly higher in MPN group as compared to control group. In case of DNA repair genes 14/20 MPN patients has a G Score ≥6 (median G Score DNA repair genes) while 8/10 controls had a G Score <6. In case of MPN risk association signature 16/20 MPN patients had a g Score ≥3 (median G Score MPN risk association signature) while 7/10 controls had a G Score <3. Although total G Score was not statistically significant between the two groups at the 5% level (p=0.12), G Score of DNA repair genes and MPN risk association signature was statistically significant (Fig. 38). G Score for DNA

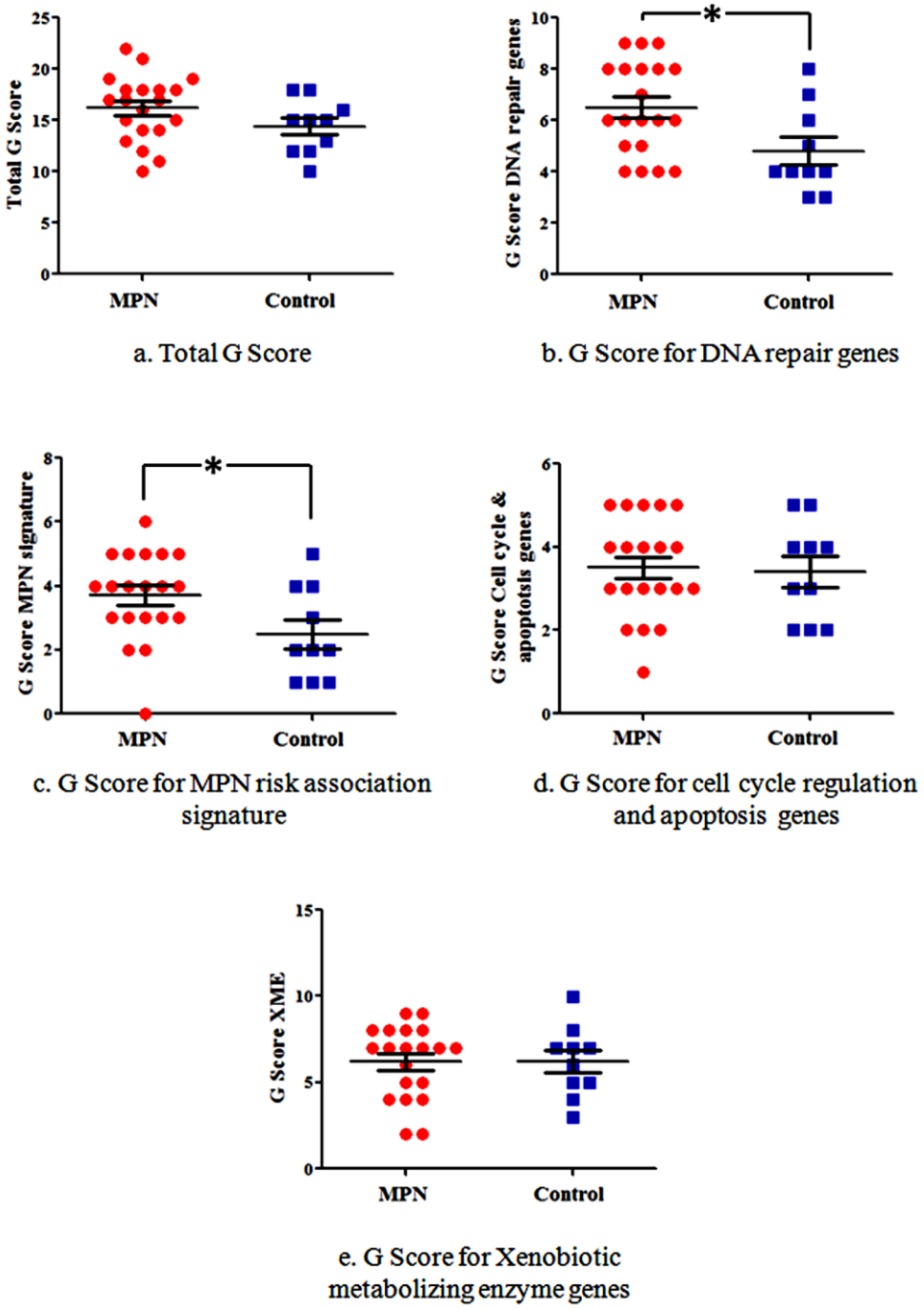

Fig. 38: Distribution of Genotype Scores for all 22SNPs and various subgroups of genes between MPN and control samples. A statistically significant difference was observed between MPN and control samples for G Score DNA repair genes (p=0.012) and MPN risk association signature (p=0.039). A borderline significance was observed for total G Score (p=0.12).

repair genes and MPN risk association signature could significantly differentiate between MPN and control group with a p value=0.012 and p value=0.039 respectively.

Objective 4

To establish correlation between the phenotype (e.g., poor DNA repair capacity, apoptosis) with the genotype (e.g., polymorphisms in the genes)

4.4.1 Genotype-phenotype correlation

The probable effect of genotype combinations including total G Score, G Score of DNA repair genes or MPN risk association signature was evaluated by summing the number of adverse alleles across subgroups and comparing with phenotypic responses after radiation and BPDE exposure (Fig. 39). Apart from total G Score, G Score for only DNA repair genes, and MPN risk association signature, were also correlated with the phenotype as the G Score of these subgroups had shown a statistically significant difference between MPN patient and control subjects.

As the difference in the cell cycle profile after BPDE exposure was not statistically significant between MPN and control groups, it was not correlated with the genotype. To measure the strength of dependence between genotype and phenotype method of Pearson correlation was used, when data followed a normal distribution or Spearman correlation was used, when data did not follow normal distribution.

A comparison between total G Score and G Score of other subgroups with the phenotypic response is shown in Table 14. The negative and positive value of correlation coefficient indicated a negative or positive correlation between the genotype and phenotype. A comparison of total G Score of all 22 SNPs, G Score of DNA repair genes and MPN risk association signature showed a negative correlation with percent cell death and percent G2 delay. This correlation was statistically significant for most of the groups (Table 14, Fig. 40). While a positive correlation was observed between G Score (all three groups) and percent γ-H2AX positive cells at 4 h time point, although it was not significant.

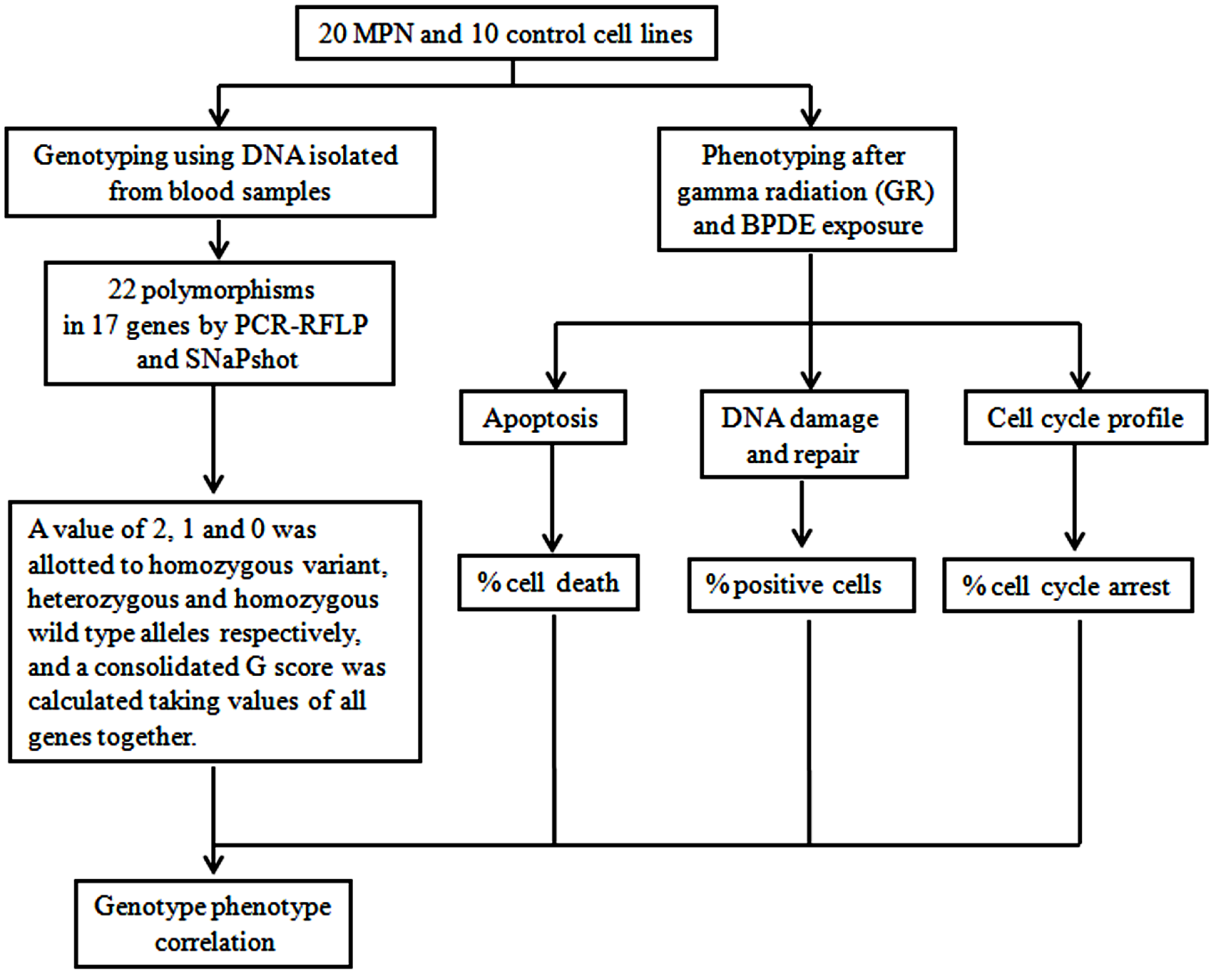

Fig. 39: Various combinations of genotype-molecular phenotype correlation undertaken in present study

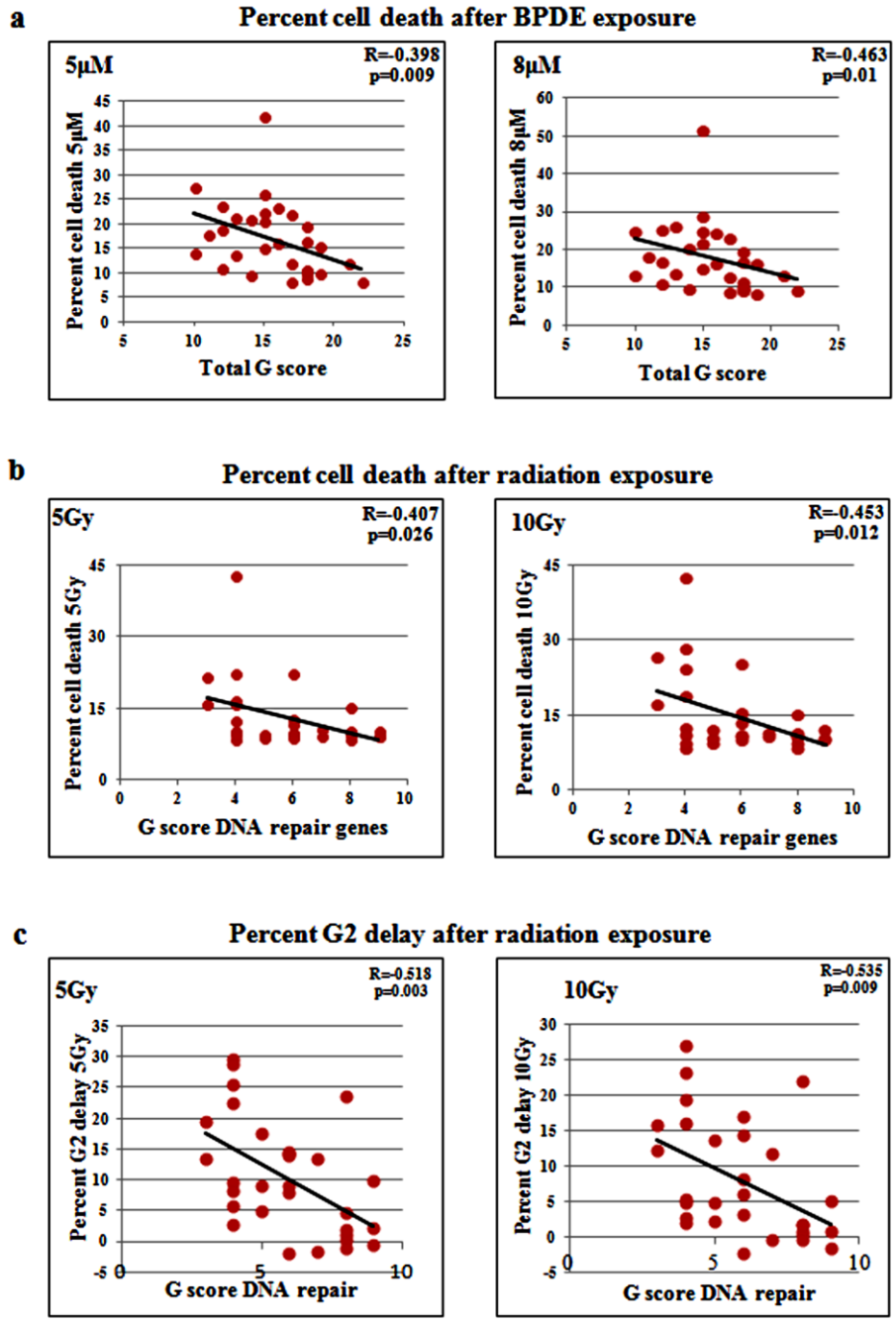

Fig. 40: Representative genotype-phenotype correlation graphs. Correlation between Total G Score and percent cell death after BPDE exposure (a), correlation between G Score DNA repair genes and percent cell death after radiation exposure (b), correlation between G Score DNA repair genes and percent G2 delay after radiation exposure (c). Respective Correlation coefficients (r) are shown in each graph. The slope of the line indicated a negative correlation which was statistically significant with $p \leq 0.05$ in the above groups.

Table 14: Genotype-phenotype correlation between G Score (number of variant alleles) and phenotypic responses after γ-radiation and BPDE exposure

Phenotype	Genotype Score	r value	p value
Percent cell death at 5 Gy	Total G Score	-0.210	0.264
	G Score DNA repair	-0.361	0.049*
	G Score MPN sign	-0.235	0.210
Percent cell death at 10 Gy	Total G Score	-0.291	0.118
	G Score DNA repair	-0.388	0.034*
	G Score MPN sign	-0.353	0.055#
Percent γ-H2AX positive cells (DNA repair at 2 Gy)	Total G Score	0.061	0.751
	G Score DNA repair	0.104	0.588
	G Score MPN sign	-0.067	0.728
Percent γ-H2AX positive cells (DNA repair at 5 Gy)	Total G Score	0.245	0.198
	G Score DNA repair	0.181	0.346
	G Score MPN sign	-0.014	0.939
Percent cell death 5µM BPDE	Total G Score	-0.471	0.008*
	G Score DNA repair	-0.403	0.026*
	G Score MPN sign	-0.348	0.058#
Percent cell death 8µM BPDE	Total G Score	-0.462	0.010*
	G Score DNA repair	-0.412	0.023*
	G Score MPN sign	-0.334	0.070
G2 delay at 5 Gy	Total G Score	-0.214	0.255
	G Score DNA repair	-0.519	0.003*
	G Score MPN sign	-0.451	0.012*
G2 delay at 10 Gy	Total G Score	-0.189	0.315
	G Score DNA repair	-0.466	0.009*
	G Score MPN sign	-0.379	0.038*

Key: * p value significant (≤0.05), # Borderline significance

Chapter 5

Discussion

Introduction

Squamous cell carcinomas of UADT are the most common cancers found in India and occurrence of MPN in early stage survivors is presently one of the major concerns. Predominant risk determinant for these cancers include exposure to carcinogens like tobacco and alcohol, however only a fraction of exposed individuals develop the disease suggesting the importance of interplay of intrinsic genetic susceptibility to environmental genotoxic exposures. Since the time occurrence of MPN in UADT cancers was discovered, numerous studies have been conducted in order to investigate the association of genetic polymorphisms with risk of such multiple primary cancers [10, 19, 126, 179, 190-192].

Results from few such studies reveal association of genetic variants of important genes like p53 and p73 with increased risk of second primary malignancies in patients with index squamous cell carcinoma of the head and neck [190, 191, 193]. Whereas, few other studies report involvement of polymorphisms in DNA repair genes with approximately 2.4 fold increased risk of second primary neoplasms in UADT cancer patients [19, 126]. Apart from this, genetic variation in carcinogen metabolising genes as well as genes involved in cell death regulation are found to be associated with increased risk of MPN [10, 44, 172, 179, 192]. However, all these studies have been conducted on populations other than Indians. Previous reports from our group are the only studies done on Indian patients with tobacco-related MPN where association of genetic polymorphisms falling in major tobacco carcinogenesis pathways was studied with occurrence of MPN in UADT [7, 11, 45].

Similarly, there are studies where only phenotypic response with either presence or absence of genotoxic exposure has been conducted in order to understand susceptibility to MPN [194-199]. Such studies done specifically on UADT MPN cancers reveal mutagen hypersensitivity as an indicator of genetic susceptibility to multiple primary malignancies [198, 199]. Also, similar reports demonstrating association of altered phenotype with occurrence of MPN in other cancers are available [194-197]. However, the need for completely understanding the pathogenesis of UADT MPN warrants concerted efforts focusing on developing a correlation between genotype and intermediate/ molecular phenotype, since such studies might lead to improved current understanding of the disease perspective.

In the present study we have investigated the combined effect of multiple genetic variations and phenotypic effects, after genotoxic exposure *in vitro*, in order to

assess UADT MPN risk, by developing a genotype-phenotype correlation. It is believed that patients with MPN provide a genetically enriched resource of risk alleles and hence are ideal to study predisposition to cancer [200]. The correlation observed in the present study supports the notion that inherent deficiencies in response to carcinogen-induced DNA damage/repair, apoptosis and cell cycle control may contribute to the risk of developing UADT MPN and the risk is modulated by genetic polymorphisms in genes regulating these cellular events. Similar hypothesis has been tested by other groups in order to investigate the functional significance of genetic polymorphisms in conferring cancer risk by modulating intermediate phenotypes [49, 51, 52, 54, 55, 201]. These studies were conducted on patients with mostly lung cancer and also breast cancer and head and neck cancers and correlated polymorphism in either single gene or genes falling in single pathway, with occurrence of cancer.

Generation of Lymphoblastoid cell lines

One of the prime requisites for such long term genotype-phenotype correlation studies is the continuous supply of staring material. Therefore, we successfully generated continuously growing LCLs following infection of isolated PBLs with EBV-containing supernatants from UADT MPN patients and cancer free, healthy, control individuals. Large scale population based studies requiring patient derived material have also been conducted using isolated lymphocytes, short term lymphocyte cultures or blood cultures [202-204]. However the major limitation of using isolated lymphocytes and short term lymphocytes cultures is that multiple experiments cannot be conducted on single sample due to limited sample availability. Also in such cases, repeated collection of the starting material from individuals becomes unethical as well as impractical, especially from patients who are lost to follow up or due to death of the subject during an ongoing study. Therefore the generation of EBV cell lines is a practical approach to tackle the limitation.

LCL have been regularly used as source of basic biomolecules like DNA, including mitochondrial DNA, RNA and protein as well as for studies assessing DNA damage/repair and apoptosis [57, 61, 62, 70, 73, 74, 205, 206]. However, there are a couple of reports where the potential use of LCL as a surrogate of isolated lymphocytes has been questioned and re-evaluated by comparing the results with freshly isolated or cryopreserved lymphocytes [78-80, 207]. One of the reasons for occurrence of such variation observed between LCL and isolated or cryopreserved lymphocytes could be the use of

stimulated lymphocytes which consists mainly of T cell population. A variation in carcinogen sensitivity mainly to γ-radiation between B-lymphocytes compared to T-lymphocytes has been reported [208, 209]. Moreover, differences in the mutagenic response can occur as LCL are exposed in the G1/S/G2 phase of the cell cycle as they are continuously proliferating and undergo continuous cell division; whereas stimulated T lymphocytes remain quiescent. Difference in the efficiency of pathways, owing to differential expression of genes, activated on genotoxic exposure in quiescent and proliferating lymphocytes are known to contribute to the dissimilar sensitivity [210, 211]. Furthermore the type of assay used to compare the sensitivity of LCL with lymphocytes can also give rise to variation due to varied sensitivity of the assays [78]. Therefore care must be taken to ascertain the suitability of using LCL in certain experiments and selecting the type of assay.

Despite the studies where the utility of LCL in place of isolated lymphocytes has been shown to be compromised, there are reports which provide evidence for the relevance of their use in biological research. Talebizadeh et al. [212] have assessed the feasibility of using LCL to study the role of miRNAs in the etiology of autism. To test for genotypic errors potentially caused by EBV transformation, Herbeck et al. [213] compared SNP using Affymetrix GeneChip Human Mapping 500k array set, in peripheral blood mononuclear cells (PBMCs) and LCL from the same individuals. They reported that genotypic changes found in PBMCs and LCLs were not significantly different and hence LCL constitute a reliable DNA source for host genotype analysis. Whole-exome sequencing of DNA from PBMCs and EBV transformed lymphocytes from the same donor demonstrated that there occur very minor changes in EBV LCL and that too at a higher population doubling, thus providing evidence that LCL are an appropriate representation of the donor [214].

LCLs as an in vitro model

Comparison and characterisation of representative EBV LCLs generated in our study with the respective parent lymphocytes revealed comparable genotypic and phenotypic characteristics in terms of DNA ploidy, population doubling time, expression and activity of ATM gene and cell surface markers. Apart from this, using LCLs generated as a part of previous study in the lab, a comparison was done for the genotype of genes including GSTT1 and GSTM1 between DNA isolated from LCLs and respective PBLs. No difference in the genotype of parent lymphocyte and

respective cell line genotype was observed. All this emphasizes the utility of LCLs as a surrogate for isolated lymphocytes. The cell lines developed in the present study thus provide a valuable, cost effective, *in vitro* model system for genotypic and phenotypic assays ensuring adequate starting material for current and future analysis.

Genotype – phenotype correlation using MPN derived LCLs

In order to elucidate a combination of genetic alterations that drive tobacco carcinogenesis, in a previous study from our laboratory, we have explored a unique model system and analytical method for an unbiased qualitative and quantitative assessment of gene-gene and gene-environment interactions [11]. This was the first Indian study associating multiple SNPs in tobacco carcinogenesis in patients with MPN [11]. Current study was undertaken to investigate implications of the same gene polymorphisms on intermediate phenotypes, after γ-radiation and BPDE exposure *in vitro* and to develop a genotype-phenotype correlation. Both, γ-radiation and BPDE are well known genotoxic and carcinogenic agents [215, 216]. In our study after genotoxic exposure we analyzed phenotypes that are frequently reported to be altered in cancer including DNA damage and repair, cell cycle regulation, percent cell death and gene expression profile. It is evident from our results using LCLs that there is a marked difference in γ-radiation and BPDE induced phenotypic responses between MPN patient and healthy control cell lines.

DNA damage and repair after genotoxic exposure

Inter-individual differences in DNA repair capacity have been suggested to be an important source of variability in cancer risk [82]. Deficiency in DNA repair capacity contributes to the accumulation of DNA damage and accelerates genetic variations involved in human carcinogenesis. A number of epidemiologic studies, primarily in lung, breast and head and neck cancer suggest association of variation in extent of DNA damage and repair with cancer risk [60, 83-86, 197, 217, 218]. We therefore hypothesized that deficiency in DNA repair capacity following exposure to γ-radiation would be more apparent in patients with UADT than in control subjects.

Exposure to ionising radiation results in DNA double strand breaks (DSB) and one of the first cellular responses is the rapid phosphorylation of histone H2AX at ser139 (γ-H2AX), which is a sensitive marker for measuring DNA damage [96, 219]. Measurements of γ-H2AX phosphorylation using fluorescently labelled antibodies to γ-

H2AX is currently the most sensitive way to detect IR-induced DNA damage and to measure the extent of DSB repair. Foci of γ-H2AX can be quantified by immunofluorescence microscopy or flow cytometry [220, 221]. Measurement of association of cancer risk with DNA damage and repair is often done by measuring γ-H2AX following genotoxic exposure [98-100, 222].

Mutagen sensitivity and DNA repair

We observed that after radiation exposure the level of DSB between the two groups was almost similar as the number of γ-H2AX foci and percent γ-H2AX positive cells at 30 min time point was approximately same. However the extent of DNA repair at 4 h time point was significantly different between the two groups as revealed by residual γ-H2AX foci in MPN cell lines as compared to control cell lines implying that DNA repair is either delayed or impaired in these cells. The result was replicated when we quantitatively measured γ-H2AX positive cells by flow cytometry. Our observations are in concert with studies done on breast cancer by Kennedy *et al.* [60] and Machella *et al.* [77], where they observed that deficient DNA repair capacity may represent a risk factor which predisposes women to breast cancer. Similarly findings by Cheng *et al.* [83], suggest that individuals with reduced DNA repair capacity may be at increased risk of developing head and neck cancer [83]. In numerous reports by Spitz *et al.*, higher mutagen sensitivity, which is an indirect assessment of DNA repair capacity, has been found to be associated with head and neck cancers including UADT cancers as well as increased risk of developing second malignant tumours in such patients [223, 224].

Other than head and neck cancers, altered DNA repair capacity has also been associated with occurrence of multiple primary cancers in other malignancies. In their study, Cianciulli *et al.* [194] have established the usefulness of mutagen sensitivity as an indirect measure of DNA repair and genetic susceptibility to multiple primary cancers. In multiple primary cancers of Hereditary Non Polyposis Colorectal Cancer, genetic instability has been assessed as DNA replication errors and found to play an important role in MPN development [195]. Similarly Orlow *et al.* [197] measured DNA damage by the comet assay and observed its association with the development of multiple primary tumours in individuals with Non Small Cell Lung Cancer [197]. In another study Miller *et al.* [196] demonstrated that if abnormalities in DNA repair and mutagen sensitivity were cancer susceptibility factors, such findings would be seen with regularity in individuals with multiple primary cancers. Their main focus in the study was occurrence of triple

primary cancers [196]. This indicates that compromised DNA repair capacity is an important hallmark of cancer and reinforces our assumption that reduced DNA repair capacity might be a risk factor for UADT MPN development.

Effect of γ-radiation exposure on cell cycle

When a cell is exposed to DNA damaging agent it results in cell cycle arrest and activation of cell cycle check points which play an essential role in DNA repair [225]. A disruption in any of the cell cycle check points may result in accumulation of gene mutations and chromosomal aberrations by reducing the efficiency of DNA repair leading to genetic instability that may drive neoplastic evolution [226]. We hypothesized that individuals with inherited defects in cell cycle control probably are susceptible to UADT MPN development. G2 phase cells are extremely sensitive to ionising radiation induced DNA damage resulting in G2/M arrest and delaying entry into mitosis until the damage has been repaired [226]. In numerous reports defects in cell cycle G2 delay are strongly associated to human carcinogenesis [93, 104-106]. Our results demonstrated that after exposure to γ-radiation, G2 delay was more pronounced and significantly higher in control cell lines as compared to MPN cell lines.

Similar to our results, in earlier studies as well, an abnormal cell cycle control has been associated with cancer risk. In a report by Wu X. *et al.* [93], when a comparison of G2/M check point was carried out with lung cancer risk it was observed that γ-radiation induced S and G2/M phases arrest was significantly lower in cases than in controls. They also compared cell cycle check points with DNA repair capacity measured by comet assay and concluded that defects in cell cycle checkpoints and DNA damage/repair capacity were associated with elevated lung cancer risk [93]. Similar results for lung cancer patients were also observed by Zheng *et al.* [103], Zhoa *et al.* [105] and Xing *et al.* [106]. Their results suggest that a less efficient DNA damage induced G2/M checkpoint is associated with an increased risk of lung cancer.

Effect of BPDE exposure on cell cycle

Tobacco specific carcinogen BPDE forms adducts with genomic DNA resulting in replication errors that can lead to formation of replicative gaps. These replicative gaps can be repaired during S phase by post-replicative repair pathways [226]. If these gaps remain in the genome due to inefficient DNA repair then they may get converted into DSB in G2 phase [226]. Hence BPDE exposure in normal cells results in S

or G2/M phase arrest. As described above, in response to carcinogen exposure, cell cycle checkpoint responses act and suppress initiation of carcinogenesis and therefore aberrant cell cycle control has been associated with neoplastic evolution. It is also been reported that BPDE induces over expression of CDC25B in cancer cells which is known for cell cycle regulation specifically essential for G2/M transition [231]. Therefore we assumed it important to assess the cell cycle profile after BPDE exposure along with gamma radiation in UADT MPN patients and compare it with controls to find any probable difference in cell cycle profile that can be attributed to MPN predisposition.

Contrary to our observations following radiation exposure, there was no marked difference in S or G2/M arrest between patient and control group after BPDE exposure. One of the probable reasons for this observation could be a small sample size. As we have performed this experiment only in 20 MPN and 10 controls cell lines therefore it is possible that effect of BPDE exposure on cell cycle arrest is not so distinctive that it can significantly distinguish between smaller MPN and control groups.

Another very important reason for our observation could be dependent on p53 status. In a study done by Xiao *et al.* [227], it was observed that tumour suppressor p53 plays an important role in regulation of cellular responses to BPDE. p53 tumour suppressor is a well known mutational target of tobacco carcinogen BPDE [228]. They observed that exposure of p53 null H1299 human lung cancer cells to BPDE resulted in S and G2 phase cell cycle arrest. A similar BPDE exposure failed to activate either S or G2 phase checkpoint in H460 human lung cancer cell line which is wild type for p53. On the contrary H460 cell line was relatively more sensitive to BPDE mediated cell death as compared to H1299 cells. [227]. This implies that BPDE induced cell cycle arrest is probably not an appropriate marker for risk assessment in UADT MPN development as the effect is modulated by p53 status. However, as γ-radiation induced G2 delay was a significantly compromised in MPN cell lines, therefore, individuals with inherited defects in the G2 checkpoint may be predisposed to MPN development.

Apoptotic response after genotoxic exposure

Under normal circumstances when cell encounters DNA damage, a cascade of reactions takes place resulting first in cell cycle arrest followed by DNA repair so that the cell with unrepaired DNA is not replicated. However in cases where the damage is severe and cannot be repaired, the cells will undergo apoptosis or programmed cell

death which is a safe way to inhibit risk acquiring neoplastic autonomy [107]. Therefore a failure in proper apoptotic response after extreme genotoxic exposure may also contribute to MPN development. There are reports available where disruption of apoptosis is associated with cancer [51, 53, 104, 105]. We observed that after radiation as well as BPDE exposure there was a significant difference in the apoptotic response between MPN and control cell lines. Our results reveal that UADT MPN patients have a reduced apoptotic capacity following an *in vitro* challenge by γ-radiation and BPDE which can be associated with risk of multiple primary cancers. We believe that low apoptotic response in MPN cases could enable cells with DNA damage to accumulate, possibly conserving mutations in critical genes and inducing carcinogenesis.

There are reports demonstrating a reduced apoptotic capacity in individuals affected with cancer. A study done by Zheng et al [104] showed a lower level of apoptosis, along with defects in cell cycle regulation as a biomarker of genetic susceptibility for salivary and thyroid carcinoma. Similarly, defective apoptotic response after γ-radiation has been associated with lung cancer risk and is suggested to be a susceptibility marker [53, 105]. Alteration in the apoptotic pathway as a risk factor for lung cancer has also been observed by Wang *et al.* where they assessed cell death after BPDE exposure [51]. Our results support the hypothesis that defects in apoptotic response can be attributed as a risk factor for UADT MPN development.

Differential gene expression after γ-radiation exposure

Differences in cellular responses after genotoxic exposure can be attributed to differential gene expression. Differential expression of genes in important carcinogenesis pathways can be considered as driving malignant changes [111-113]. Deregulated expression of DNA repair genes in pathways like nucleotide excision repair is found to be associated with in lung cancer [111]. A more than 2 fold increased of risk of head and neck cancer has been observed in individuals expressing lower levels of nucleotide excision repair genes [112]. Similarly, in a study done on Indian population reduced expression of DNA repair genes was observed in head and neck cancers [113]. However most of these studies were done by classical way of selecting known genes in important pathways already identified to be altered during malignant transformation.

In order to explore the involvement of novel molecules in cancer predisposition there is a need to measure global gene expression alterations for which cDNA microarrays provide an obvious method of choice [109, 110]. The association of

differential expression of important genes upon genotoxic exposure with UADT MPN predisposition has not been investigated. Therefore we compared the altered gene expression after γ-radiation exposure between MPN and control LCLs. Our aim was to determine how cells with certain innate genetic variations would respond to carcinogenic events. It is apparent that exposure to genotoxic γ-radiations will mainly affect the expression of genes falling in pathways like signal transduction, DNA damage and repair, cell cycle regulation and apoptosis. However, using LCL, in our analysis we could not observe noticeable difference in expression of genes falling in above mentioned pathways.

One of the possible reasons for this observation could be the constraint of choosing a single radiation dose and time point i.e. 5 Gy and 24 h respectively. Therefore, if we had assessed the difference in gene expression between MPN and control group at few other time points and a range of radiation doses then probably we would have observed a significant difference in terms of expression of important genes. Moreover, we cannot rule out the possibility that the difference in responses between MPN and control groups to a genotoxic insult may not be due to differential gene expression but due to alterations at the protein level. Therefore it is conceivable to undertake multiple parameters and combination of genomic as well as proteomic approaches in order to obtain a better insight for global variations after genotoxic exposure in UADT MPN patients. Nevertheless, our data revealed certain important transcriptional regulators, few cancer associated genes and signal transduction genes that were differentially expressed between the MPN and control groups.

Genotyping and Genotype Score

It is evident that the phenotypic responses after genotoxic exposure varied between different individuals. As described earlier, this difference in genotoxic response can be attributed to inter-individual difference at genetic level to deal with carcinogens which can arise due to gene polymorphisms between the two groups. Our hypothesis was that genetic variations present in patients may give rise to phenotypes, leading to multiple cancers on genotoxic exposure. Therefore we considered it important to compare phenotypic findings with the genotype and develop a correlation.

We genotyped candidate SNPs in genes falling in important carcinogenesis pathways viz. Carcinogen metabolism, DNA damage/ repair, cell cycle regulation and apoptosis. The SNPs that were selected for present study have been shown to be

associated with tobacco related cancers in our previous study, other case-control studies or their meta-analysis [12, 122, 127, 149]. To understand the genotyping data we created a consolidated Genotype Score (G Score) from the number of risk alleles. Such an analysis of the genotyping data, for a panel of SNPs, has previously been reported for some other diseases [229, 230]. This was done by calculating consolidated G Score for the number of variant alleles.

In one such study, G Score for 9 validated SNPs that are associated with modulating levels of low-density lipoprotein (LDL) or high-density lipoprotein (HDL) cholesterol was calculated and risk of cardiovascular events was determined by Kathiresan et al. [230]. Although the G Score did not improve risk discrimination, it did modestly improve clinical risk reclassification for individual subjects beyond standard clinical factors. Similarly, in another study by Meigs et al. [229] a Genotype Score based on 18 risk alleles predicted new cases of diabetes in the community but provided only a slightly better prediction of risk than knowledge of common risk factors alone. However, they did not study interactions among genes or between genes and the environment that might alter the genetic risk in exposed individuals.

G Score for MPN risk assessment

Our assumption was that individuals with a high G Score (more risk alleles) might have a higher probability of MPN development as compared to those with the low G Score. Hence a higher G Score indicated magnitude of cancer predisposing genotype. We calculated a total G Score with all 22 candidate SNPs, G Score of only DNA repair genes and G Score of MPN risk association signature, which included SNPs that are risk predictor to tobacco related MPN from our previous study [11]. In our analysis we observed that although total G Score was not statistically significant between the two groups at the 5% level (p=0.12), G Score of DNA repair genes and MPN risk association signature was statistically significant. This indicated that there existed inter-individual difference at genetic level between MPN patients and controls at least in a subset of important genes falling in the carcinogenesis pathways.

As described earlier, in our previous study, we did genetic analysis with same set of SNPs except p21-Ser^{31}Arg, on tobacco related MPN, to explore multiple pathway based genetic variations associated with such cancers [11]. In order to enhance the robustness of our Genotype Score assessment we calculated G Score for 151 MPN and 210 controls from the earlier study and compared the total G Score, G Score for only

DNA repair genes and MPN risk signature between the two groups (data not shown). Although the difference between the G Score of MPN and control group was not huge a significant difference between the G Score of patient and control samples in all the three categories was observed with our earlier data set also, signifying the importance of measuring G Score for MPN risk assessment. This emphasizes our present observation and demonstrates that combination of risk alleles probably is a risk factor with modest discriminatory ability between patient and control group.

G Score – phenotype correlation

Gene polymorphisms have long been associated with cancer risk; however the real implication of these gene polymorphisms with increased cancer risk can only be interpreted if they have any functional significance. This approach of understanding effect of gene polymorphisms at functional level has been phenomenal and has been applied to deduce the functional significance of genetic predisposition in cancer risk [49-52, 201].

Many such genotype-phenotype correlation studies have been conducted on lung cancer. Comparable to our study, apoptotic capacity was found to be a risk factor for lung cancer development and the risk was observed to be modulated by the Fas - $A^{670}G$ polymorphism [51]. Similarly apoptosis, DNA repair and decreased G2/M cell cycle check point function are found to be compromised in lung cancer with variations in ATM, CDKN1A, BRCA2, ERCC6, TP53, and TP53BP1 genes [53, 201]. On the contrary in another report XPA gene polymorphism, a DNA binding protein in the nucleotide excision repair pathway, has been observed to modulate repair capacity and associated with decreased lung cancer risk, especially in the presence of exposure to tobacco carcinogens [52].

Other than lung cancer, analogous reports are available for breast cancer where polymorphisms in nucleotide excision repair genes and DNA repair capacity phenotype has been associated with breast cancer risk [54, 55]. In another report by Minard *et al.* evaluation of glutathione S-transferase polymorphisms and mutagen sensitivity as risk factors for the development of second primary tumours in 303 patients previously diagnosed with early-stage head and neck cancer has been done [49]. These studies demonstrate the functional significance of genetic variation in different individuals and then their probable implication on various phenotypes and eventually

cancer risk, hence emphasizing the importance of undertaking genotype-phenotype correlation studies in understanding UADT MPN pathogenesis.

When we correlated G Score with phenotypic responses after radiation / BPDE exposure, a negative correlation was observed between the total G Score in all 22 SNPs, G Score of DNA repair genes and MPN risk association signature, and percent cell death and percent G2 delay. This was statistically significant for most of the groups. As higher G Score corresponds to more number of risk alleles in an individual and improper apoptotic and cell cycle response designate enhanced carcinogenesis phenotypes in a cell, our results emphasize the notion that polymorphisms in certain genes modify cellular intermediate phenotypic responses after genotoxic exposure that may have an important bearing on neoplastic evolution. Thus the genotype-phenotype associations observed in this study support our hypothesis that polymorphisms in genes involved in well regulated pathways like DNA repair, cell cycle check point, carcinogen metabolism and apoptosis can affect important cellular functions like, DNA repair capacity, cell cycle regulation and apoptosis.

Chapter 6

Summary and Conclusions

Together, our data suggest that intermediate phenotypes of cancer susceptibility might be potentially useful in identifying UADT MPN risk subgroups. Identifying distinctive polymorphism based G Score signature can differentiate the study participants into two different subsets and its correlation with various phenotypic effects (indicating a gene-environment interaction) may have an important bearing on predisposing an individual to UADT MPN development**.** However one of the limitations of our study was that the 22 SNPs that were included were probably insufficient to account for the absolute risk prediction. It is quiet likely that several other genes and minor or major carcinogens exist that have not been evaluated in our study. Therefore it is possible that the addition of rare risk alleles with greater effects, or a larger number of common risk alleles with small individual effects, could improve discrimination.

Nevertheless combination of our selected SNPs shows a correlation with phenotypes and make us believe that in near future if more number of such studies are conducted we can arrive on a panel of SNPs that probably can be used as a prediction marker for risk assessment of patients with a high risk of MPN development. Another limitation of the study was that our Genotype Score gave all alleles the same weight; this may not be a true reflection of the biological basis of UADT MPN etiology.

One of the highlights of our study was that we have correlated risk genotype with adverse phenotypic characteristics and undertaken interaction between genes and the environment that might alter the genetic risk in exposed individuals that remains the cornerstone of approaches to predicting cancer risk. To the best of our knowledge our study provides the first molecular epidemiological evidence correlating 22 gene polymorphisms (falling in multiple pathways) with alteration in phenotypic effect under three categories (cell-cycle checkpoints, apoptosis and DNA repair capacity) on same subset of patients and generating a genotype and molecular phenotype correlation for understanding UADT MPN risk.

Our findings emphasize the importance of assessing collective effects from a panel of polymorphisms in modulating phenotypic effects following genotoxic exposure. It is an important contribution to available literature wherein, for the first time SNPs in major metabolic and biological pathways implicated in carcinogenesis have been correlated with intermediate phenotype in order to analyse the functional significance of gene polymorphisms and cancer predisposition in the unique Indian UADT MPN cohort.

Statistically significant differences were observed for all the phenotypic responses between the MPN and control groups implying that parameters such as DNA repair, apoptosis and cell cycle delay might potentially be useful in identifying susceptibility to UADT MPN. Distinctive polymorphism based G score signature was also significantly different in the two groups where MPN patients displayed high G score as compared to controls. A significant correlation was also observed for a group of SNPs with intermediate phenotypes. Thus, the most important finding of this study was the correlation between genotype - phenotype, which supports our hypothesis that variations in important genes may alter phenotypic response and contribute to MPN risk. Identification of distinctive polymorphism based G Score signature that can differentiate the study participants into two separate subsets, and its correlation with various phenotypic effects (indicating a gene-environment interaction) may have an important bearing on predisposing an individual to UADT MPN development.

The significance of this study lies in the fact that it guides us in identifying high risk individuals thus increasing the possibility of identifying cohort of patients of clinical relevance for chemo preventive studies. This study can be considered as an encouraging pilot study for understanding UADT MPN pathogenesis and in future the assays may be validated and applied in larger epidemiological studies.

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Publications

1. **Hussain T**, Kotnis A, Sarin R and Mulherkar R. Establishment and characterization of lymphoblastoid cell lines from patients with Multiple Primary Neoplasms in the upper aero-digestive tract and healthy individuals. *Indian J Med Res.* 2012 Jun; 135(6):820-9.
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3. **Hussain T** and Mulherkar R. Lymphoblastoid cell lines: a continuous *in-vitro* source to study carcinogen sensitivity and DNA repair. (Review article; *International Journal of Cellular and Molecular Medicine,* in press).
4. **Hussain T**, Kotnis A, Sarin R and Mulherkar R. Genetic Susceptibility to Multiple Primary Neoplasms in the Upper Aero-Digestive Tract: Genotype Score and Phenotype Correlation. (communicated to *Cancer letters*)

Poster/oral presentations

1. International Society for Cell & Gene Therapy of Cancer (ISCGT) conference, India, 2007. (Participation)
2. 13th Human Genome Meeting "Genomics and the future of medicine", India, 2008. (Poster presentation)
3. 29th Annual Convention of Indian Association for Cancer Research, India, 2010. (Oral presentation)
4. 6th Graduate Students Meet, ACTREC, Navi Mumbai, 2010. (Poster award)
5. 30th Annual Convention of Indian Association for Cancer Research and International Symposium on "Signalling Network and Cancer", CSIR-IICB, Kolkata, 2011. ('Rajnikant Baxi' poster award)
6. 1st Global Cancer Genomics Consortium-Tata Memorial Centre, 2011. (Poster award).
7. American Association of Cancer Research (AACR) international conference on "New Horizons in Cancer Research: Biology to prevention to Therapy", 2011. (Poster presentation)

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Establishment & characterization of lymphoblastoid cell lines from patients with multiple primary neoplasms in the upper aero-digestive tract & healthy individuals

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***Background & objectives*: A major drawback for genetic studies as well as long-term genotype-phenotype correlation studies in cancer is lack of representative human cell lines providing a continuous source of basic biomolecules and a system to carry out various experimental investigations. This can be overcome to some extent by establishing lymphoblastoid cell lines (LCLs) by infecting peripheral blood lymphocytes with Epstein Barr virus (EBV) which is known to immortalize human resting B cells *in vitro* giving rise to actively proliferating B-lymphoblastoid cell lines. The present study involves preparation and characterization of LCLs generated from patients with multiple primary neoplasms (MPN) of upper aero-digestive tract (UADT).**

***Methods*: Thirty seven LCLs were established from UADT MPN patients and healthy age, sex and habit matched controls using EBV crude stock. Characterization was done with respect to expression of CD-19 (Pan B-cell marker), CD3 (T cell specific marker), CD56 (NK-cell specific marker), cell morphology, ploidy analysis, genotype and gene expression comparison with the parent lymphocytes.**

***Results*: LCLs showed rosette morphology with doubling time of approximately 24 h. Ploidy analysis showed diploid DNA content which was maintained for at least 30 population doublings. When compared with parent lymphocytes there appeared no change at genetic and gene expression level.**

***Interpretation & conclusions*: Our results show that lymphoblastoid cell lines are a good surrogate of isolated lymphocytes bearing their close resemblance at genetic and phenotypic level to parent lymphocytes and are a valuable resource for understanding genotype-phenotype interactions.**

Key words Epstein Barr virus - lymphoblastoid cell lines - multiple primary neoplasia - ploidy analysis - population doubling

Squamous cell carcinoma of the upper aero-digestive tract (UADT), comprising head, neck, oesophagus, trachea and lungs, are common cancers worldwide and one of the most common cancers found in Indian men[1]. Further, a 3-7 per cent annual risk of development of a second primary neoplasm among the survivors of early stage UADT cancer poses an additional threat in terms of morbidity and mortality[2]. Cumulative evidence from the case-control studies analyzing polymorphisms in xenobiotic metabolism,

DNA repair and other gatekeeper mechanisms suggest aberrant gene-environment interactions to be an important aetiological factor in the genesis of multiple primary neoplasm (MPN)[3,4]. Thus it is becoming increasingly important to validate the findings of huge number of genotyping studies in at least a subset of the patients by phenotypic assays. Short term studies to assay chromosomal aberrations, mutagen sensitivity assays and DNA repair kinetics have been carried out using lymphocytes or short term lymphocyte cultures derived from study population[5-7]. However, limited availability of biological material and lack of reproducibility has been the major limitation of such studies.

Immortalization of human B lymphocytes by Epstein Barr virus (EBV) *in vitro* is used routinely to establish lymphoblastoid cell lines (LCLs). Infection by EBV transforms resting B cells from human peripheral blood into actively proliferating LCLs[8]. Unlike SV40 or human papilloma virus (HPV), EBV allows cell immortalization with minimal genetic and phenotypic aberrations, with ease of establishment and maintenance making LCLs ideal material for genotypic and phenotypic studies[8]. These provide an unlimited source of biomolecules like DNA, RNA or proteins and are a promising *in vitro* model system for genetic screening studies, genotype-phenotype correlation studies, a variety of molecular and functional assays along with immunology and cellular biology studies[9-12]. Utility of LCLs is also been very well documented in various population based studies especially investigating *in vitro* carcinogen sensitivity and DNA damage/repair[13-15]. Here we report generation of 37 LCLs from patients with UADT MPN and healthy individuals and characterization of a few randomly selected cell lines.

Material & Methods

This study was approved by the Hospital Ethics Committee, Tata Memorial Centre, Mumbai and conducted during 2005-2009 in the Mulherkar laboratory of Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai, Maharashtra, India. Samples from healthy, age, sex and habit matched, control individuals were obtained from staff and students of ACTREC, Navi Mumbai.

Collection of samples: After obtaining IRB approval and patient informed consent, 3 ml whole blood was collected in an EDTA vacutainer from patients with MPN and cancer free healthy individuals by venipuncture. Patients with MPN were accrued from the Cancer Genetics Clinic in Tata Memorial Hospital and samples from healthy control individuals were obtained from Advanced Center for Treatment Research and Education in Cancer, Mumbai, India. The study was approved by the Hospital Ethics Committee, Tata Memorial Hospital, Mumbai. UADT MPN patients were recruited on the basis of criteria given by Hong *et al*[16] as described earlier[17].

Generation of viable EBV stock: EBV-transformed B95-8 marmoset cell line was procured from National Centre for Cell Sciences, India and the EBV stock prepared. Briefly, $0.5x10^6$ cells/ml were seeded in RPMI-1640, 15 per cent foetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 200 mM glutamine (Sigma-Aldrich Co., USA) and 1X PenStrep (Sigma-Aldrich Co., USA). After 7 days confluent cultures of B95-8 appearing straw yellow in colour were lysed by freeze thawing at -80°C and 37°C and filtered through 0.22 µM filter (Millipore, Bedford, MA) to obtain EBV crude stock. The filtrate was aliquoted and stored at 4°C for short term or -80°C for long-term storage.

Lymphoblastoid cell line preparation: For separation of peripheral blood lymphocytes (PBLs) approximately 3 ml of blood was separated on a Ficoll-Hypaque gradient (Sigma-Aldrich Co., USA). PBLs were seeded in a sterile 24 well plate at a density of $1.5\text{-}2x10^6$ cells/ml in Dulbecco's minimal essential medium (DMEM) containing 15 per cent FBS 200 m M glutamine and IX Pen Strep. EBV crude stock at 1:1 ratio was added and placed in an incubator maintained at 37°C with 5 per cent CO_2. After 24 h medium containing viral supernatant was aspirated without disturbing the cells and fresh complete DMEM was added**.** After 3-4 wk of incubation rosette morphology of cells ascertained the transformed phenotype of PBLs. Cells were mixed thoroughly to break clumps before splitting to ensure multiclonal population. All the used plasticware (Nunc, Denmark) was treated as biohazard, discarded in 1 per cent sodium hypochlorite after use and autoclaved wherever required.

Characterization of LCL: Characterization of LCLs was done using standard techniques as described below. For all assays freshly grown cells with >90 per cent viability were harvested and washed in 1X PBS at 1500 rpm and used for the genotypic and phenotypic characterization.

Immunophenotyping: $1x10^6$ cells from LCLs were incubated with primary mouse anti-CD3 (T cell

marker), anti-CD19 (pan-B cell marker) and anti-CD56 (NK cell marker) antibodies (BD Pharmingen, BD Biosciences, USA) for 45 min on ice. The cells were washed thrice with FACS buffer (PBS containing 1% FBS and 0.02% Na-azide) and incubated for 45 min on ice with secondary goat anti-mouse FITC antibody (antibodies used in this experiment were kind gift from Dr Shubhada Chiplunkar and Dr Naren Joshi, Immunology department, ACTREC). Cells were passed through BD™ 1ml 26G ½ syringe (BD, Singapore) to break any cell aggregates or clumps and analyzed on Flow Cytometer (FACS Calibur, BD Biosciences, USA) at 488 nm excitation. A minimum of 10,000 events were analyzed for each sample. Cellular debris was removed by gating on Forward vs. Side Scatter. Statistical analysis was done using CELLQUEST software (BD Biosciences, USA).

Ploidy analysis of LCLs: LCLs ($1x10^6$) and control PBLs ($1x10^6$) from healthy volunteer were fixed in 70 per cent ethanol at 4°C for 1 h. The cells were washed twice in PBS followed by incubation with propidium iodide (Sigma-Aldrich Co., USA) and RNaseA (Sigma-Aldrich Co., USA) for 30 min at 37°C. Fluorescence was acquired on Flow Cytometer at 488 nm excitation. Cells were passed through BD™ 1ml 26G ½ syringe before acquisition to break any cell aggregates or clumps and a minimum of 10,000 events were analyzed for each sample. Data were analyzed using ModFit LT V 2.0 software (BD Biosciences, USA). DNA ploidy is defined as diploid DNA represented as single G0/G1 peak on a histogram corresponding to the same DNA content represented as single G0/G1 at the same position in the histogram of the control. Ploidy was measured by calculating DNA index (DI) which is the ratio between the channel number of G0/G1 peak on histogram of the cell line to the channel number of G0/G1 peak of control PBLs.

Expression of ATM gene: RNA was isolated from cell lines and lymphocytes isolated from the same subjects by TRIzol (Invitrogen, Carlsbad, CA) extraction method. β actin PCR[18] was performed on isolated RNA to ensure any DNA contamination and samples were treated with DNase using DNA-free kit (Ambion, Austin, TX) wherever required. cDNA was synthesized using 3 µg of total RNA using Superscript First-Strand synthesis system by RT-PCR (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Expression of *ATM* gene was measured semi-quantitatively by RT-PCR using gene specific primers (Sigma-Aldrich Co., India; Forward 5'-TGTCATTACGTAGCTTCTCC-3'; Reverse 5'-GCTGAGTAATACGC AAATCC-3) and β actin was used as loading control. PCR products were run on 2 per cent agarose gel and stained with ethidium bromide.

Cell population doubling: $5x10^4$ cells from LCLs were seeded in a 24 well plate with 1.5 ml of complete medium. Viable cell count was taken using Trypan blue dye exclusion method[19] at different time points including 0, 12, 24, 36, 48, 72 and 96 h. For each time point four readings were taken.

Results

Establishment of LCLs and morphological analysis: Blood samples from MPN patients (n=24) and cancer free control individuals (n=13) were obtained and subjected to LCL preparation. The demographics of the patients and control individuals are shown in Table I. Considerable cell death of the PBLs was observed after 24 h post EBV infection; however, virus infection promoted B cells to re-populate the culture. The time taken for each LCL preparation varied. On an average culturing the cells 3-4 wk post infection was sufficient to produce >1 million cells.

The average population doubling (PD) time of a few representative LCLs was found to be 24 h, ranging from 12 to 36 h (Fig. 1). The LCLs grew as clusters exhibiting typical rosette morphology in suspension cultures, but single cells were also observed having big nucleus and numerous vacuoles (Fig. 2a, 2b). EBV is known to specifically infect B cells allowing their growth in culture hence the transformed phenotype is expected to have homogeneous cell population, however, flow cytometric analysis revealed the presence of dual population (Fig. 2c). On the basis of morphology and granularity it was revealed that lower (R1) population represents mono cell suspension of interest and the other (R2) population probably represents fraction of cells that have spontaneously differentiated into smaller lymphoid cells with shrunken nucleus that ultimately undergo apoptosis during conventional cell culture thus representing diverse size and granularity[20].

Cell surface marker: Immunophenotyping was done using PBL as positive control for B (CD19), T (CD3) and NK (CD56) cells. Data confirmed that cells from representative randomly selected LCLs showed expression of typical B cell surface marker (CD19) while markers for T cell (CD3) and NK cells (CD56) were absent (Fig. 3), thus ascertaining the purity of growing cultures.

Table I. Demographics of subjects taken for lymphoblastoid cell line (LCL) preparation

No.	LCL	Gender	Age (yr)	Category	1st Cancer	2nd Cancer
1	E245	M	52	Control		-
2	E246	M	62	Control		-
3	E247	M	52	Control		-
4	E248	M	58	Control		-
5	E249	M	46	Control		-
6	E250	M	41	Control		-
7	E301	M	24	Control		-
8	E302	M	26	Control		-
9	E303	M	25	Control		-
10	E313	M	49	Control		-
11	E314	M	61	Control		-
12	E334	F	45	Control		-
13	E335	F	65	Control		-
14	E195	F	48	MPN	Breast	Hard palate
15	E242	M	45	MPN	Tongue	Hard palate
16	E252	M	38	MPN	Buccal mucosa	Buccal mucosa
17	E253	F	65	MPN	Buccal mucosa	Alveolous
18	E254	F	71	MPN	Tongue	Oesophagous
19	E264	M	71	MPN	Lip	Mouth
20	E265	M	48	MPN	Larynx	Tonsil
21	E270	M	60	MPN	Alveolous	Tongue
22	E305	F	64	MPN	Retromolar trigone	Alveolous
23	E306	M	55	MPN	Larynx	Hard palate
24	E307	M	49	MPN	Larynx	Nasal columella
25	E308	M	53	MPN	Tongue	Tongue
26	E311	F	63	MPN	Tongue	Buccal mucosa
27	E319	M	59	MPN	Larynx	Lung+ oesophagus
28	E320	F	45	MPN	Right cheek	Left cheek
29	E321	F	49	MPN	Tongue	Bronchus
30	E322	M	50	MPN	Buccal mucosa	Left lower lip+ right lower lip+ mouth
31	E325	F	43	MPN	Buccal mucosa	Alveolous
32	E326	M	75	MPN	Supraglottis	Oesophagous
33	E329	M	47	MPN	GBS	Alveolous+ buccal mucosa
34	E330	F	63	MPN	Breast	Mouth
35	E331	M	32	MPN	AML	Alveolous
36	E332	M	51	MPN	Lung	Parapharyngeal carcinoma
37	E333	F	55	MPN	GBS	GBS+ RMT+ alveolous

MPN, multiple primary neoplasm; GBS, gingivobuccal sulcus; AML, acute myeloid leukaemia; RMT, retromolar trigone; M, Male; F, female

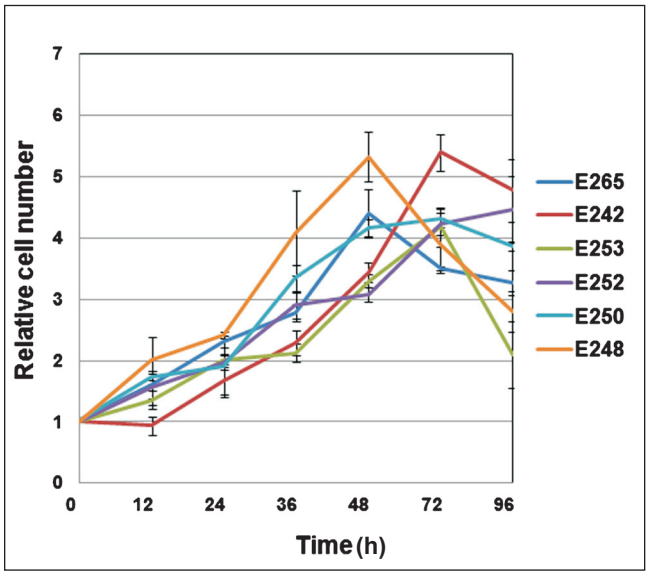

Fig. 1. Graph showing cell population doubling time for 6 representative cell lines at 6 time points till 96 h. The doubling time ranged from 12 to 36 h with an average of approximately 24 h. Values are ± mean SEM (n=4).

DNA ploidy analysis: DNA ploidy status of the LCLs was assessed immediately after cell line preparation at low population doubling (<5 PD) using PBL from healthy individuals as diploid control. A shift in the position of the diploid peak of LCL away from the expected diploid position of the control can be taken as an evidence of DNA aneuploidy. All the cell lines studied had DI values ranging between 0.8-1.3 and were considered diploid (Table II, Fig. 4). Cell lines in continuous culture can show aberrant DNA content hence ploidy was measured even at higher population doublings of 30, 45, 60, 120 and 150 in a few cell lines. The DI values ranged between 0.93 and 1.18; hence were considered to be diploid (Table III).

Expression and activity of ATM gene: Lytic cycle of EBV elicits a cellular DNA damage response resulting in activation of the ataxia telangiectasia-mutated (ATM) signal transduction pathway[21]; hence *ATM* expression in the cell lines and their respective PBL was studied. There was apparently no change in the expression level of *ATM* gene between the LCL and respective PBL from the subject (Fig. 5). The number of pATM Ser1981 foci seen as distinct nuclear foci in response to cellular DNA damage, were similar in both - the cell line as well as lymphocytes (data not shown).

Discussion

We report here generation and characterization of LCLs from MPN patients and healthy individuals. LCLs can be generated efficiently as continuously growing cells from an individual following infection of PBLs with EBV-containing supernatants. Such EBV immortalized cells are derived from B lymphocytes and, unlike cell lines derived from other tissues, remain near diploid in nature. Such LCLs can be used for various *in vitro* studies as well as serve as a source of DNA for genomic studies. Generation of EBV transformed cell lines has proven to be cost-effective, rapid and reliable with minimal deviation from the normal phenotype and genotype.

B95-8 cells derived from Marmoset lymphocytes, infected and immortalized with EBV, are typically used for virus production since these spontaneously

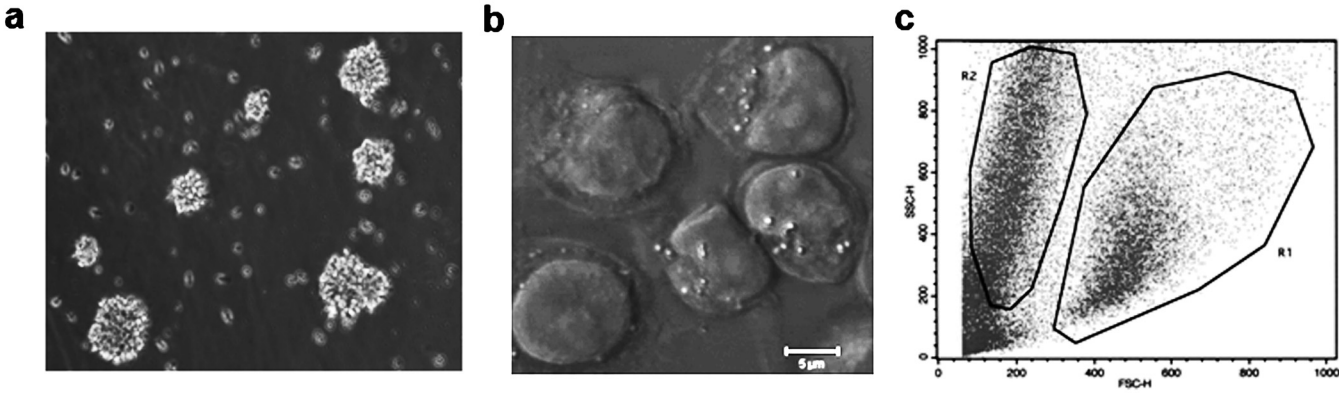

Fig. 2. Morphological analysis of established lymphoblastoid cell lines **(a)** Light microscopy image of LCL showing typical rosette morphology. Cells grow in clumps while single cells are also seen. **(b)** Confocal microscopy image of cells in EBV transformed cell lines showing big nucleus and numerous vacuoles. **(c)** Flow cytometry cluster plot showing two distinct populations in LCL where lower R1 population represents mono cell suspension and R2 population represents cell aggregates.

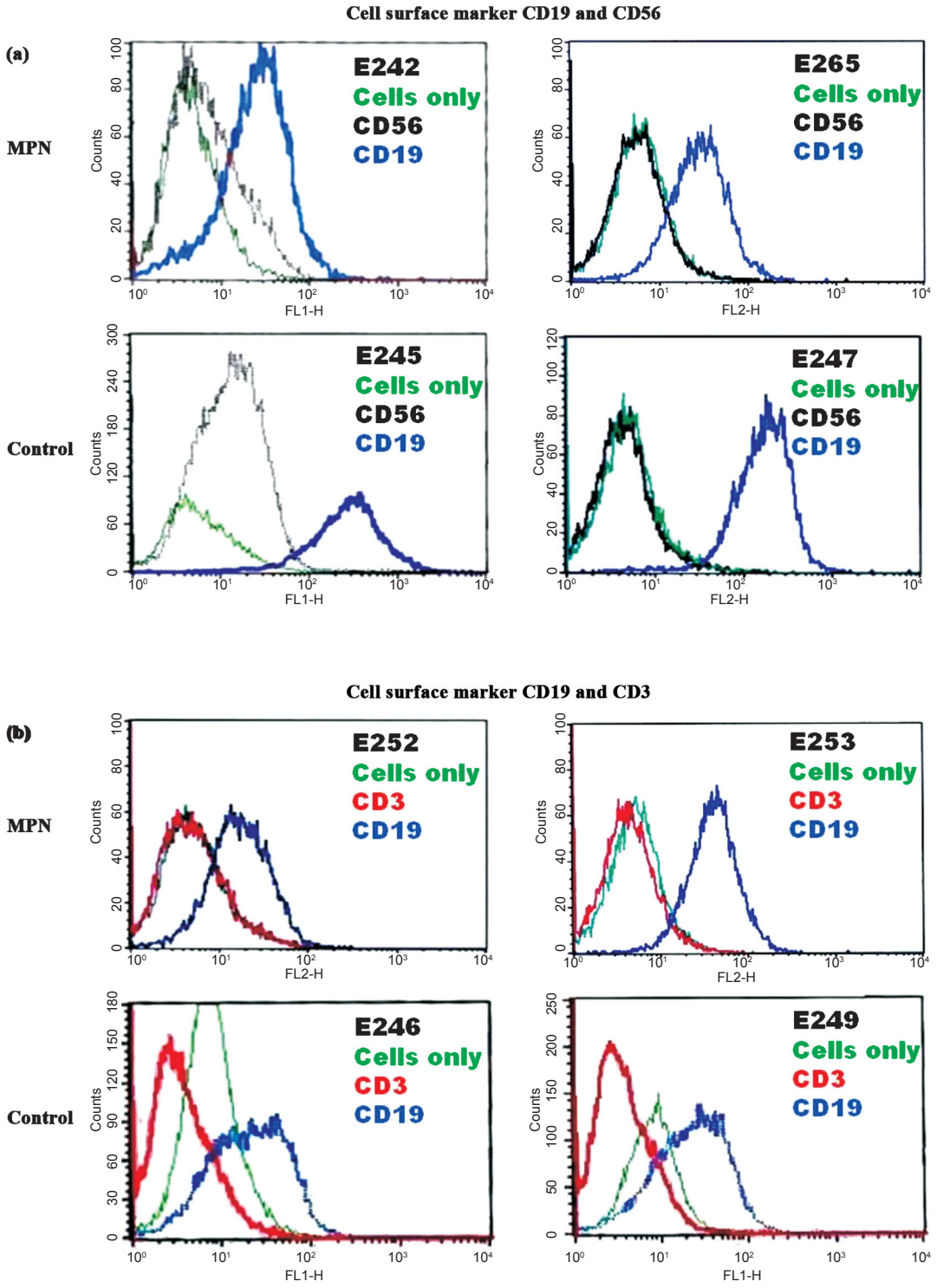

Fig. 3. Flow cytometry analysis for cell surface markers - CD3 (T cell), CD19 (B cell) and CD56 (NK cell) in representative cell lines. Unstained cells were used as internal control. X-axis represents the fluorescence intensity. A forward shift in the peak, caused by binding of fluorophore tagged antibody, as compared to unstained cell is considered positive **(a)** LCLs E242, E265, E245 and E247 were positive for CD19 and negative for CD56 marker while **(b)** LCLs E252, E253, E246 and E249 were positive for CD19 and negative for CD3 marker.

Table II. DNA ploidy status of MPN patient and healthy control cell lines

LCLs	Channel ratio (LCL/PBL)	DNA index
E245	63.75/49.62	1.28
E246	53.83/49.62	1.08
E247	54.62/49.62	1.10
E248	55.16/50.40	1.09
E249	57.64/49.62	1.16
E250	52.55/49.62	1.06
E242	55.31/49.62	1.11
E252	53.98/49.62	1.09
E265	58.95/48.54	1.21
E267	60.93/48.54	1.26
E270	57.52/50.40	1.14
E304	47.23/46.58	1.01
E305	46.40/46.58	0.99
E306	49.37/46.58	1.05
E307	49.61/46.58	1.06

PBLs from healthy individual were used as reference. Values in the range 0.9-1.3 are considered diploid

produce the B95-8 strain of EBV[22]. Average time taken for LCL establishment was 3-4 wk extending up to 5 wk in some cases. Variation in the time taken for cell line establishment and growth rate can be attributed to difference in the transformation efficiency occurring due to possible batch-wise difference in the viral titre. Once the cell lines were established, these behaved similarly showing comparable morphology, doubling time, genotypic and cell surface characters and behaviour in phenotypic assays.

Successful transformation of B cells by EBV resulted in enlargement in size and development of aggregates of proliferative cells. Due to the acquired property of cell aggregation LCLs grew as clumps in suspension cultures, with a mean population doubling time of 24 h and when seeded at a density of 0.5-1X10^6/ml needed to be split twice every week. Early passage cells were cryopreserved immediately after transformation. Established LCLs were cryopreserved at later passages as well, but not later than 30 population doublings.

EBV transformed LCLs are known to exist in two distinguishable forms pre-immortal and post-immortal. In the pre-immortal stage cells proliferate actively and maintain diploid karyotype. These cells are non tumorigenic and die before reaching 160 population doublings. On the other hand, in the post immortalization stage, EBV transformed cells develop a strong telomerase activity and are aneuploid. These also show cellular changes, gene mutations and have the ability to grow indefinitely[9]. Hence, in the present study, for phenotypic assays LCLs were used within 45-60 PD.

Morphological analysis of cell lines by flow cytometry showed two distinct populations although as EBV is known to specifically transform B cell, only one population is expected. The R1 population represents B cells, based on cell morphology and granularity, and R2 population could be proliferating T/NK cells due to immune response elicited by EBV infected B cells. Conventionally the immune suppression of T cells in LCLs is done by supplementing LCL cultures with cyclosporine-A post infection to improve immortalization[8]. However, no cyclosporine A was added in the present study. To study the nature of cells in both clusters immunophenotyping was done using CD19, CD3 and CD56 antibodies in a few representative cell lines. All the cell lines tested were positive for B cell marker in both the clusters (data not shown). The possible reason for occurrence of R2 population could be differential size and granularity of the cells arising due to spontaneous differentiation into smaller lymphoid form with shrunken nucleus. These cells in usual cell culture further undergo apoptosis[20].

Table III. DNA ploidy status of a few MPN patient and healthy control cell lines at higher population doublings (PD)

LCLs	PD	DNA index
E304	30	0.97
	120	1.00
	150	0.93
E305	30	1.05
	120	1.18
	150	1.17
E311	45	0.99
	60	0.93
E313	30	0.97
	45	1.14

PBLs from healthy individual were used as reference. Values in the range 0.9-1.3 are considered diploid

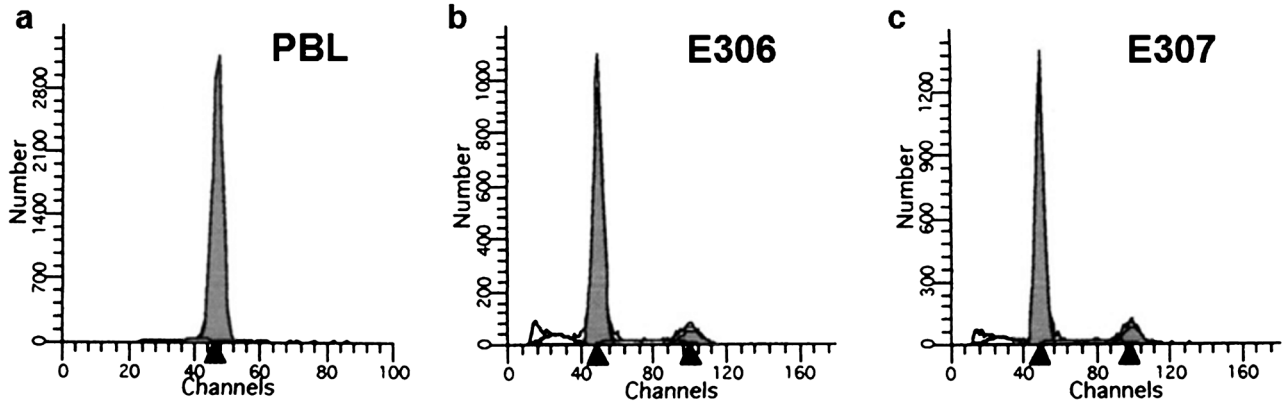

Fig. 4. Flow cytometry analysis showing the diploid status of PBLs isolated from healthy subject and two representative LCLs E306 and E307. The black arrows in the histogram X- axis represent the channel number of the respective cell cycle stage. Arrow at smaller channel number corresponds to G0/G1 stage of cell cycle while the other arrow corresponds to G2/M stage which is located at approximately double position.

Cell immortalization and proliferation in continuous cultures can result in aberrant DNA changes like aneuploidy or tetraploidy. Hence ploidy status of the cell lines was ascertained at both, low and high population doublings ranging from <5 to >100 PD taking normal PBL as control. DNA index (DI) ratio was calculated to establish the ploidy status. DI ratio of 1 was considered as diploid[23] while DI values ranging from 1.9-2.1 with proportion of cells greater than the G2/M fraction of normal control, after correction of the aggregates were considered as tetraploid[24]. All the cell lines studied at lower (<5 PD) as well as higher population doubling (>100 PD) had a DI ratio ranging between 0.9-1.3 and were considered to be diploid.

As described in earlier reports EBV remains episomal in lymphoblastoid cell lines maintaining a latent infection, although there is a small subpopulation of cells that switches spontaneously from a latent stage of infection into the lytic cycle. Induction of EBV lytic replication elicits cellular DNA damage response dependent on *ATM*[19]. DNA damage sensor MRN (Mre II, Rad 50 and Nbsl) complex and phosphorylated *ATM* are recruited to viral replication compartments, presumably recognizing newly synthesized viral DNAs as abnormal DNA structures[21]. The LCLs in the present study were established with an aim to eventually study the contribution of DNA damage repair *in vitro* in UADT MPN patients and to elucidate the mechanism involved. Hence it was necessary to ensure that the process of EBV transformation did not affect expression and activity of DNA repair gene *ATM*. It was observed that EBV transformation did not elicit DNA repair pathway dependent on *ATM* as there were no pATM foci seen in LCLs (data not shown). This was further confirmed by measuring γH2AX foci in cell lines which was also found to be negative (data not shown). Also there was no change in *ATM* gene expression in cell lines and PBLs as revealed by semi-quantitative RT PCR data.

This property of LCLs to be able to grow in continuous culture together with maintaining a close similarity to the parent lymphocytes has been exploited in various studies. There are numerous reports where LCLs have been used as a source of basic biomolecules

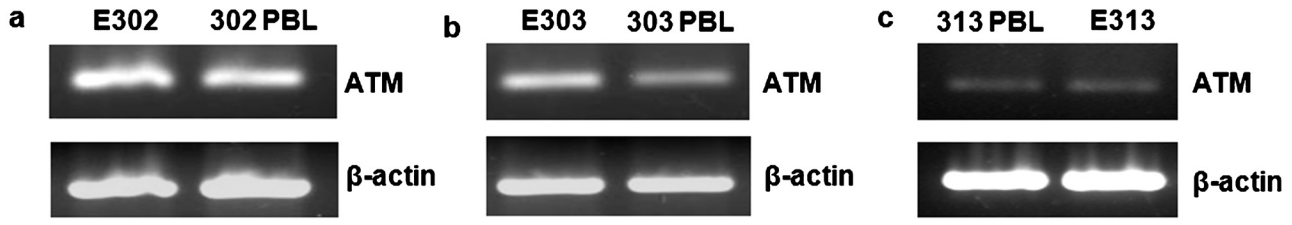

Fig. 5. Expression of *ATM* gene in different cell lines and their respective PBLs **(a)** E302 **(b)** E303 **(c)** E313. RT-PCR products were run in 2 per cent agarose gel stained with ethidium bromide. β-actin gene was taken as loading control.

like, DNA, including mitochondrial DNA, RNA and protein[9]. DNA isolated from LCLs has been widely used for mutation analysis[25,26], while RNA isolated from these cell lines has been commonly used for cDNA library preparation and to assess transcriptional response to genotoxins using high throughput technologies including cDNA microarray[27], together with this LCLs have as well been used for proteomic studies[28].

For large scale population based studies, LCLs provide a constant supply of starting material for a variety of assays, sparing the need of re-sampling. LCLs have been established as an excellent model system not only in basic biomedical studies but also to carry out genomic wide high throughput research thus showing their utility in a broad range of biomedical research[29,30]. All this emphasizes the research utility of LCLs as a surrogate for isolated lymphocytes. The cell lines developed in the present study have been well characterized and provide a valuable, cost effective, *in vitro* model system for genotypic and phenotypic assays ensuring adequate starting material for current and future analysis.

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CASE REPORT

Eben L. Rosenthal, MD, *Section Editor*

SQUAMOUS CELL CARCINOMA OF BASE OF TONGUE IN A PATIENT WITH FANCONI'S ANEMIA TREATED WITH RADIATION THERAPY: CASE REPORT AND REVIEW OF LITERATURE

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Abstract: *Background.* Fanconi's anemia (FA) is a rare autosomal recessive genetic disorder characterized by congenital anomalies, progressive aplastic anemia, and a predisposition for malignancies. Solid tumors in the head and neck region, especially in the tongue, are rarely observed. Management of these patients is a challenge because of hematological complications and increased toxicities.

Methods. We report a case of Fanconi's anemia in a 27-year-old man with carcinoma of the base of tongue (T2N0M0) who was treated with radical radiation therapy to a dose of 70 Gy/35 fractions/51 days. We have also done in vitro radiosensitivity tests.

Results. The patient tolerated the radiation treatment well and completed it without any interruptions. In vitro studies did not show any increased radiosensitivity in this patient.

Conclusion. Head and neck cancer in a patient with FA requires individualized treatment. The decision about opting for different modalities should be based on a balanced approach with respect to locoregional control and toxicities of the treatment. © 2009 Wiley Periodicals, Inc. *Head Neck* **32:** 1422–1427, 2010

Keywords: Fanconi's anemia; base of tongue; radiation therapy; head and neck cancer; radiosensitivity

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Fanconi's anemia (FA) is a rare autosomal recessive genetic disorder characterized by congenital anomalies, progressive aplastic anemia leading to pancytopenia, and a predisposition for malignancies.[1] Congenital anomalies in FA clinically manifest as skeletal, renal, ophthalmological malformations, and chromosomal aberrations. The disease involves multiple organs that include skin, genitourinary, musculoskeletal, renal, and neurological systems. The clinical findings in FA patients are abnormal skin pigmentation like café au lait spots, abnormal male gonads (absent, atrophic, or abnormal

testis, hypospadias, undescended testis), microcephaly, short stature, hypoplastic thumb with or without radial anomalies, renal defects, and developmental delay, mental retardation, and learning disability.[2,3] FA is associated with increased risk of malignancies, and the risk further increases with bone marrow transplantation, which is done for treatment of hemopoietic failure associated with the disease.[4,5]

The cause of development of cancer in FA is thought to be attributable to a defect in maintaining the genome integrity, leading to increased chromosomal instability with defective DNA repair mechanism.[6–8] Although hematological malignancies are 1 of the most common cancers, solid tumors in the head and neck region, especially in the tongue, are also observed.[9–11] Management of cancers in the head and neck region in patients with FA is challenging. Surgery, although considered as an optimal treatment, has its limitations as a result of low counts, risk of infection, and wound-healing issues.[9,12]

Radiation therapy or chemotherapy is associated with increased risk of toxicity in these patients because of defective DNA repair mechanism.[9,13,14] There have been instances of variable clinical radiosensitivity and fulminant radiation toxicities even with low doses of radiation.[13–15] Therefore, in vitro sensitivity studies, although not common, have been performed on fibroblast cells or peripheral blood mononuclear cells to predict the in vivo radiation sensitivity.[15,16] We report a case of a young man with FA who was diagnosed with carcinoma of the base of tongue and was treated with radical radiation therapy. We also report the results of an in vitro test for radiosensitivity in our patient and review the literature for FA patients with head and neck cancer.

CASE REPORT

A 27-year-old man was referred to our hospital for evaluation of a base-of-tongue lesion that had appeared 3 months earlier. Oral examination revealed a well-circumscribed, ulceroproliferative lesion on the left side of the base of tongue measuring 2.5 cm in diameter. Neck examination showed no cervical lymphadenopathy. On general physical examination, he was physically retarded (short stature, microcephaly, and microophthalmia) with atrophic testis and a few café au lait spots on skin, particularly of the lower extremities.

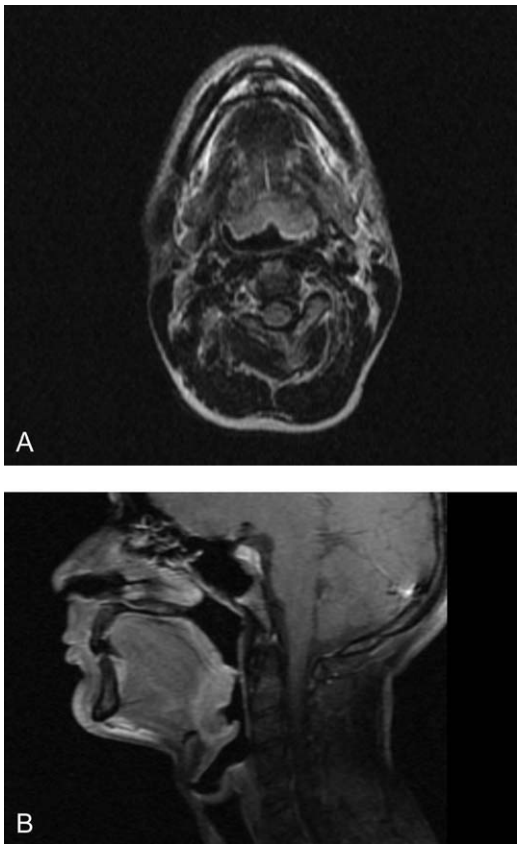

FIGURE 1. (**A** and **B**) Axial and sagittal MR images of the patient with Fanconi's anemia show mass on the base of tongue (left > right).

MRI showed a well-localized soft tissue mass on the base of tongue (left > right), with no significant cervical lymphadenopathy (Figures 1A and 1B). The histopathology review showed squamous cell carcinoma. Therefore, a diagnosis of carcinoma of the base of tongue, T2N0M0 (stage II), was made. The complete blood count, showed features of generalized pancytopenia (total leukocyte count, 1.8×10^9/L; hemoglobin, 7.1 gm/dL; and platelets, 17×10^9). Complete blood count was repeated but pancytopenia persisted. The patient underwent a bone marrow aspiration and biopsy, which revealed hypocellular marrow. In view of his abnormal morphological features and unexplained hypocellular marrow, the patient was advised for chromosomal studies. Subsequently, chromosomal breakage analysis was done in peripheral blood lymphocytes in the presence of DNA crosslinking agents, diepoxybutane (DEB) and mitomycin C (MMC), which revealed a high frequency of chromosomal breakage (6.2

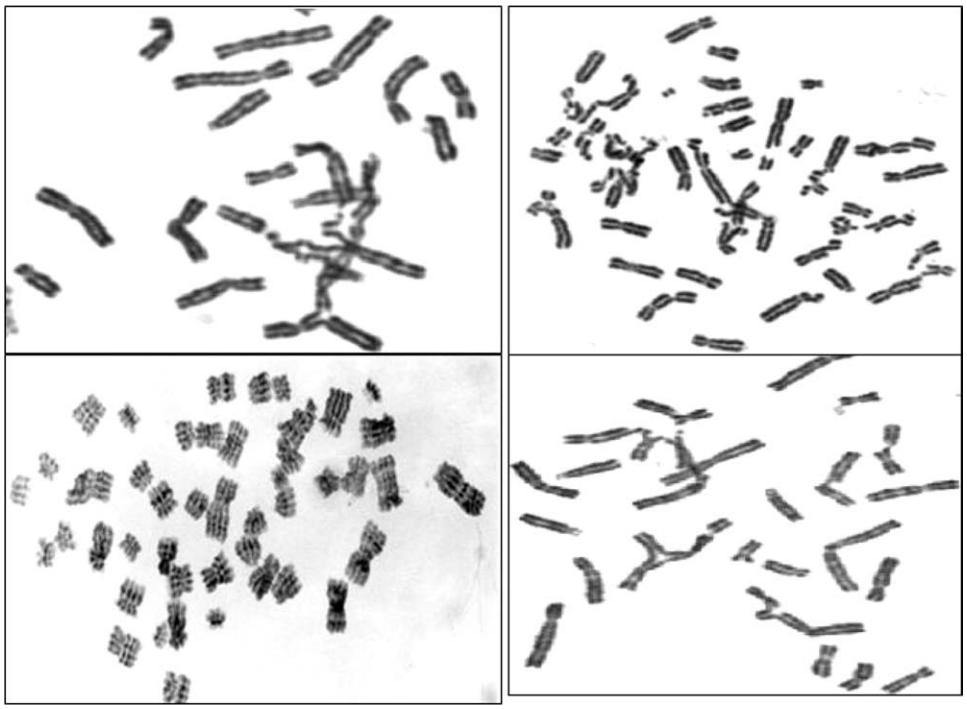

FIGURE 2. Chromosomal breakage analysis in patient with Fanconi's anemia shows high frequency of chromosomal breaks.

chromosome breaks per metaphase) compared with control (1.3 breaks per metaphase). Increased frequency of radial formation was also observed in this patient compared with controls (Figure 2). Bone marrow chromosomal analysis further revealed chromosomal aberrations. Finally, diagnosis of FA was made on the basis of clinical features, hematological picture, and chromosomal breakage analysis.

Hematological parameters precluded surgical interventions and chemotherapy. It was therefore decided to treat him with radical external beam radiation therapy. Radiation to the face and regional lymph nodes was started with conventional fractionation of 200 cGy/day once daily and 5 days/week schedule using bilateral parallel opposed portals. In week 4 of radiation, a nasogastric tube was inserted to improve the nutritional support. The patient was admitted after completion of 54 Gy, given that his total leukocyte count was 2.1×10^9/L and he developed fever. Broad-spectrum injectable antibiotics were started, along with granulocyte colony stimulating factor. He successfully completed the treatment to a total dose of 70 Gy/35 fractions over 51 days with reducing portals respecting the tolerances of critical structures. The treatment was completed without any interruptions. At the conclusion of treatment, he had Radiation Therapy Oncology Group (RTOG) grade 3 toxicity of skin and mucosa. His count was consistently low, for which he was kept on intensive supportive care. Admission was required because of systemic effects, and not because of radiation reaction. He was eventually discharged from the hospital after 4 weeks, when his fever had subsided but pancytopenia persisted.

In vitro study was done to determine the radiosensitivity by radiating peripheral blood mononuclear cells (PBMCs) isolated from blood to increasing doses of radiation. The rate of apoptosis in this patient was compared with a matched control. At each radiation dose as well as in the unirradiated sample, FA had more live cells than that of control. Spontaneous rate of apoptosis (ie, apoptosis at 0 Gy) was found to be less in this case of FA. The percentage of early apoptotic cells increased from 9% to 30% in the case of an FA patient with an increase in radiation dose from 0 Gy to 25 Gy compared with control, in which it increased from 14% to 48%. The percentage of late apoptotic cells was also found to be low in the case of the FA patient, which increased from 2% to only 5%, which in the case of control reached 14%. This experiment suggested that radiation-induced apoptosis was less in our patient. We also studied the

percentage of necrotic cells and observed that the percentage of necrotic cells was greater in the case of the FA patient compared with control. Necrotic cells increased from 1.5% to 12% in the case of the FA patient, compared with control, in which they remained <2% at each radiation dose, suggestive of cell death attributed to the necrotic inflammatory pathway in the FA patient, making them more radiosensitive.

The patient was followed up at 6 weeks postradiation therapy, when he was evaluated for local control and toxicities. The radiation reactions of skin and mucosa had settled and there was no clinical evidence of disease. A follow-up evaluation at 3 months postradiation therapy revealed similar findings. An MRI scan showed evidence of level IV lymph node. This lymph node was not present in the preradiation therapy scan. Fine-needle aspiration cytology from this node showed the presence of squamous carcinoma. Opinion was taken for surgical salvage but was not considered because of fixation to surrounding structures, low counts, and the risk associated with surgery because of his FA. He was therefore considered for palliative reirradiation with electrons, which he completed with good symptom relief. He was alive with disease at his last follow-up, which was 14 months after his initial diagnosis.

DISCUSSION

FA is an autosomal recessive disorder associated with bone marrow suppression, congenital anomalies, and high risk of malignancies.[17] It has been hypothesized that the malignancies occur either because of chromosomal instability or because of immunodeficiencies.[18] Although the association of FA with malignancies was described in 1927, cancer of the head and neck region was reported in 1966 by Esparza and Thompson.[3] Hematological malignancies are the most common malignancies seen in patients with FA, followed by solid tumors, especially head and neck cancers.[9] In a large study of 1300 patients with FA, the incidence of solid tumors was around 5%.[19] In a review of 754 patients from the International Fanconi's Anemia Registry, 3% patients had head and neck cancer.[20] This incidence was significantly higher compared with that of the normal population.[9] Although the data on ethnic origin of these patients are not very clear, there is a scarcity of data from Asian countries. This is thus 1 of the important case reports in an Indian male patient.

The median age of onset of tumors in patients with FA is as early as 16 to 31 years in different series.[12,19] In our patient, the age of onset was at 27 years, which was much earlier compared with the median age of 56 years in our population for head and neck cancer. In our patient, the diagnosis of malignancy was made before that of FA. His routine investigations showed pancytopenia, which led to further investigations, such as bone marrow biopsy and chromosomal studies, which eventually confirmed the diagnosis of FA. Surgery has been considered as the primary modality of treatment in many patients with FA.[12] Of the 19 patients with head and neck cancer in the International FA Registry, 17 have undergone primary surgery. Surgery in these patients was well tolerated.[9] It has been suggested that surgery should be encouraged in these patients, to prevent the issues associated with chemotherapy and irradiation.[9,13,14,20]

Chemotherapy—associated with very high morbidity attributed to DNA damage and impaired repair mechanism—was thus not considered in our patient. In addition, this patient had stage II low-volume disease; therefore single-modality treatment in the form of radiation therapy was considered. Use of alkylating agents that crosslink DNA can have serious adverse effects in these patients.[14] Furthermore, chemotherapy is also known to cause a deleterious effect of myelosupression in patients with baseline bone marrow suppression.[9,12–14]

There have been varying reports of tolerance to radiation therapy in cancer patients with FA and there appears to be increased radiosensitivity in these patients.[13,14] This could possibly be ascribed to an increase in chromosomal breakage and impaired cell repair mechanisms.[9,13,14,16] In 1 of the reported cases, the radiation mucositis was observed as early as 3.2 Gy, whereas in another patient radiation was delivered without much toxicity.[12] Marcou et al[15] reported a patient with tonsillar cancer who developed unusually brisk reactions after 24 Gy. Varying doses of radiation have been documented in the literature, ranging from 3.2 Gy to 80 Gy. Overall, radiation has been associated with increased normal tissue toxicity, delayed healing, and increased supportive care.

FA along with ataxia telangiectasia, Bloom's syndrome, and xeroderma pigmentosum are called cancer breakage syndromes because they are associated with chromosomal instability and defective repair, thereby predisposing to development of cancers.[15] Cells from FA show variable levels of cellular radiosensitivity, and results of an in vitro experimental study done for predicting the hypersensitive response may not coincide with in vivo radiosensitivity results.[21,22] Various experimental techniques including alkaline single-cell gel electrophoresis (Comet assay), colony-forming test, Western blot, and foci immunofluorescence analysis of the expression of DNA repair proteins, and also the cytochalasin-blocked micronuclei (MN) test using FA fibroblasts have been adopted to correlate the in vitro radiation sensitivity with the outcome of therapy.[23,24] However, the limitation of these techniques is the 4-week delay required for colony growth.[15,25–27] Comet assay is widely regarded as a robust and informative method for radiosensitivity measurements immediately after DNA damage,[28,29] but it is only a qualitative assessment of the response. Western blot and foci immunofluorescence analysis give qualitative results, but are labor intensive and also lack correlation between in vitro results and in vivo radiation response.[28] In view of the conflicting data on radiosensitivity of FA cells, and particularly FA fibroblasts, there is a pressing need for the development of new rapid and predictive assays of radiation responses. We have performed our study on PBMCs isolated from blood. Compared with most of the previous studies using fibroblasts, collecting blood has an advantage over invasive procedures of obtaining fibroblasts from patients' skin biopsies, avoids time-consuming methods of generating monolayer cultures, and is also less labor intensive.

Clinical radioresponsiveness is unpredictable in patients of FA with cancer, especially of the head and neck region, and outcome varies.[14] Of the 19 patients in the International FA Registry who had head and neck cancer, 14 died because of disease. The median time to recurrence in their patients was 16 months and the median follow-up of surviving patients was 19 months.[9] Kennedy and Hart[30] noted multiple primary malignancies either synchronous or metachronous in patients with FA to the extent of 36% (5 of 14 patients). Our patient developed a lymph node at level IV at 3 months follow-up and was alive with disease at 14 months.

There are no standard guidelines for management of FA patients with malignancies. This is primarily a result of the scarcity of data and heterogeneous population.[13] It has therefore been recommended that more data in the form of case reports should be encouraged.[13] Until then, the treatment of patients with malignancies with FA should be individualized. Any decision about opting for different modalities should be based on a balanced approach with respect to locoregional control and toxicities of the treatment.

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