

TUMOR BIOLOGY

BREAST CANCER

1. Study of Candidate Genes Associated With Breast Cancer Susceptibility

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Duration : 2001-2007

Aims, Objectives and Background

Among women, breast cancer is the most commonly diagnosed cancer. In 2005, an estimated 212,930 new cases will be diagnosed, and 40,870 deaths from breast cancer will occur.

Germ line variations of the high penetrance types are thought to cause 2-10% of all breast cancers. The two highly penetrant genes involved in increased susceptibility to breast cancer are BRCA1 and BRCA2. The mutation frequency of BRCA1 and BRCA2 in women with breast cancer varies according to family history and age at diagnosis.

Apart from these well defined high penetrance genes, there may be other genes that also increase the susceptibility to breast cancer. These genes are likely to include common low penetrance predisposition alleles that could in principle explain a much larger proportion of total disease. Studies have also been initiated to investigate the role of low penetrance genes, viz.: estrogen receptor, CYP17, androgen receptor and Vit D receptor genes for giving susceptibility to develop both familial and sporadic breast cancer.

BRCA1 and BRCA2 genes

Together, mutations in both the genes account for the great majority of families with hereditary susceptibility to breast and ovarian cancer.

Screening for mutations in coding and intron-exon boundaries of BRCA1/2 has been completed in 204 breast cancer cases and 65 controls. The study group included 122 (59.9%) early-onset (<40yrs) and 82 (40.1%) late-onset cases. Forty-six (22.5%) of those had family history of breast/ovarian or any other cancer. Out of 46, 24 (52.1%) had breast cancer below 40 years while 22 (47.8%) had the disease after 40 years of age.

In total, 19 genetic alterations were found in 23 cases, which were not found in controls. The genetic susceptibility to breast cancer due to BRCA1/2 mutations was noticed in 11.3%(23/204) patients. A sequence alteration was observed in 15 out of 98 early-onset cases without family history, which gives a significant percentage of 15.3%. Among breast cancer cases with a family history of breast, ovarian and other cancers 8.6% (4/46) carried BRCA disease associated mutations. Three deleterious frame shift mutations along with 3 missense, 3 intrinsic and one silent change were found in BRCA1 gene. Similarly, 3 pathogenic protein truncating mutations, one missense, 4 intronic and one silent change were identified in BRCA 2 gene.

Equal distribution of mutations was found in both the genes without any hotspot and founder mutation. This part of study has been completed during the year under report.

E-cadherin Gene

The E-cadherin gene is one of the candidate tumour suppressor genes at 16q22.1, which is one of the smallest deletion regions at 16q. Mutations in E-cadherin gene have been reported so far in gastric carcinomas, cancers of the endometrium, ovary and lobular breast cancers.

A series of 60 human breast cancers were screened for mutations in the entire coding region plus flanking intron sequences of the E-cadherin gene. Patients included 35 early onset, 24 late onset and 16 familial cases. The tumours included 12 infiltrating lobular carcinomas, 28 infiltrative ductal carcinomas, 7 infiltrating ducto-lobular carcinomas (mixed), 4 ductal carcinoma in-situ and 9 miscellaneous breast carcinoma.

Two novel missense mutations (Fig. 1) were identified in two early onset breast cancer without any family history, one presenting as invasive lobular and the other as high-grade invasive ductal case. Both these cases were also harbouring a BRCA2 splice site mutations.

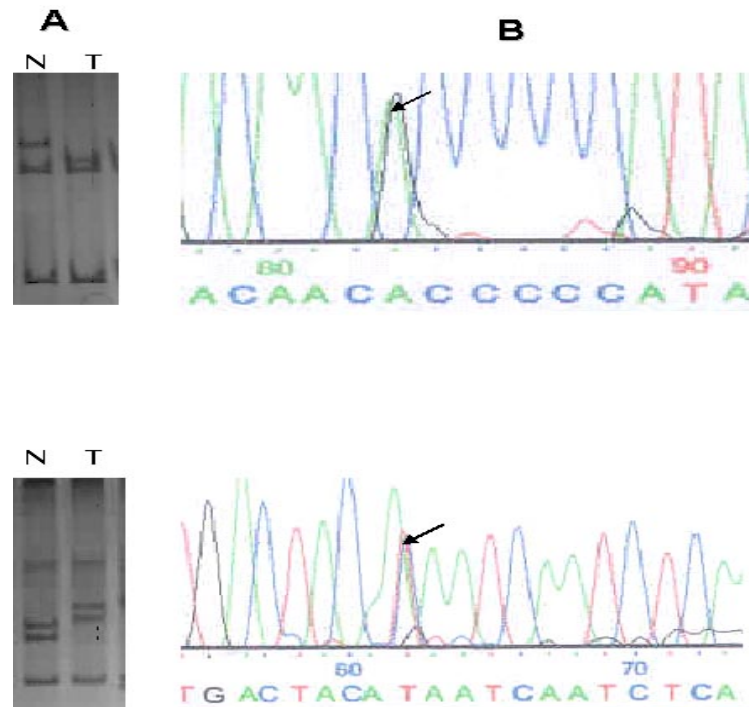


Fig.1: Sequence electrogram of a breast cancer patient showing missense mutation in E-cad gene.

CYP 17 Gene

The CYP17 gene encodes the enzyme cytochrome P450c17 α , which is involved in steroid biosynthesis pathway and a single T \rightarrow C polymorphism in the 5' promoter region of the gene is reported to be associated with increased risk of breast cancer.

Screening for A2 polymorphism in CYP17 gene has already been completed in 242 breast cancer patients as well as 65 controls, which have been also screened for BRCA1/2 mutations. A portion of cases (85.9%) was either heterozygous or homozygous for the A2 variant of CYP17 (Figs. 2-3) compared with 48.9% in control population. A

statistically significant increased risk in carriers of at least one A2 allele was found in young patients [OR, 5.51; 95% CI, 1.53-20.06; p value, <0.001] compared with patients having late onset cancer condition. No statistical significance was found between CYP17 genotype with familial breast cancer cases and BRCA1/2 mutation carriers.

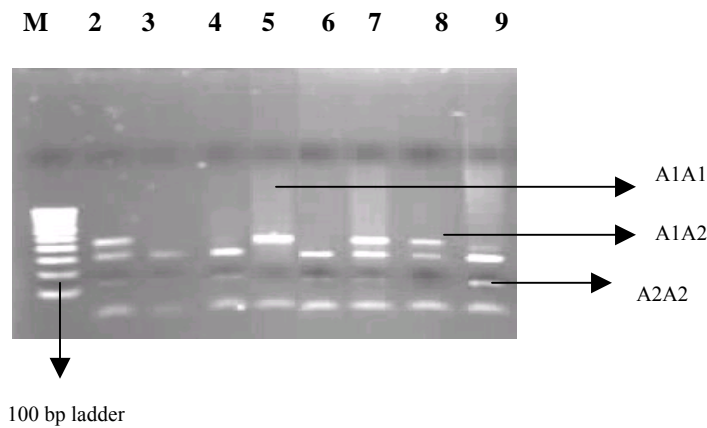


Fig.2: MSPA1 restriction fragments of CYP 17 alleles separated by agarose gel electrophoresis. Lane 1 contains 100bp ladder; homozygous A1A1 genotype is shown in lane 5; the heterozygous A1A2 pattern in lanes 2,7,8; and the homozygous A2A2 genotype in lanes 3,4,6,9.

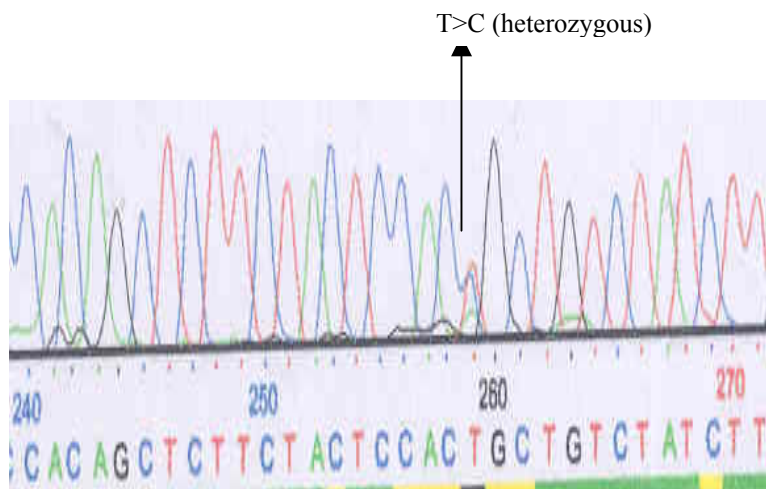


Fig.3: Sequence electrogram of a breast cancer patient showing T>C polymorphism heterozygous for CYP17 A2 allele.

2. Study of Molecular Characteristics of Familial and Early Onset Breast Cancer

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Dr. Amar Bhatnagar, SJ Hospital, New Delhi
Technical Staff : Mrs. Valsamma Mathews, Mr. Jagdish Pant
Duration : 2003-2005

Aims, Objectives and Background

This study was undertaken to characterize molecular changes in oncogenes, tumor suppressor genes, apoptotic genes and DNA mismatch repair genes of early onset breast cancer with or without family history and mutations in BRCA1/2 genes. DNA repair genes encode for enzymes that restore the integrity of the DNA, if radiation or carcinogens damage it, or if mismatches occur during the replication process. Mutations in DNA repair genes which lead to a loss in function, may accelerate the carcinogenic process by allowing mutations to accumulate. The loss of expression of DNA mismatch repair proteins has been studied in breast cancer patients with an aim to study their role in early onset cases showing accumulation of mutations in high and low penetrance genes. The expression of tumor suppressor oncoproteins, p53 and mdm2, and the apoptosis markers, bcl2 and bax was presented in the last year's report.

Work done during the year

The study comprised of 127 breast cancer patients including 51 (40.15%) early onset, 41(32.2%) late onset and 35 (27.5%) familial cases. Seventeen patients had mutations in either BRCA1 (6) or BRCA 2 (11) gene. Tumor tissue was studied for the expression of ER, c-erbB2, E-Cadherin, bcl-2, bax, p21waf, cyclin D, mdm2, DNA mismatch repair genes (MSH2 and MLH1) by immunohistochemistry alongwith histopathologic examination (Fig.1).

Histopathologic examination showed high incidence of infiltrating lobular carcinoma and mixed morphologic pattern including both ductal and lobular features in early onset and familial cases. ER expression was found to be low while p21 waf was found to be high in early onset cases and familial cases as compared to non-familial cases

($p < 0.008$). Cyclin D Expression more in familial (early + late) than non familial cases ($p < 0.001$). BRCA1 associated tumours showed significant overexpression of c-erbB2 oncoprotein as compared to BRCA2 associated tumors. This study suggests that BRCA1 mutation initiates oncogenesis through c-erbB2 stimulation.

MMR protein expression was studied in 78 cases. A loss of expression of MSH2 was seen in 57 (73%) cases and of these 31 cases were of early onset and 26 of late onset. The MLH1 expression was seen to be lost in 54 (69.2%) cases, 28 of early and 26 of late onset. Both MSH2 and MLH1 expression was seen to be lost in 41 (52.6%) cases. Of the 33 familial cases the loss of expression of MSH2 was 57.6% and that of MLH1 was 42.4%.

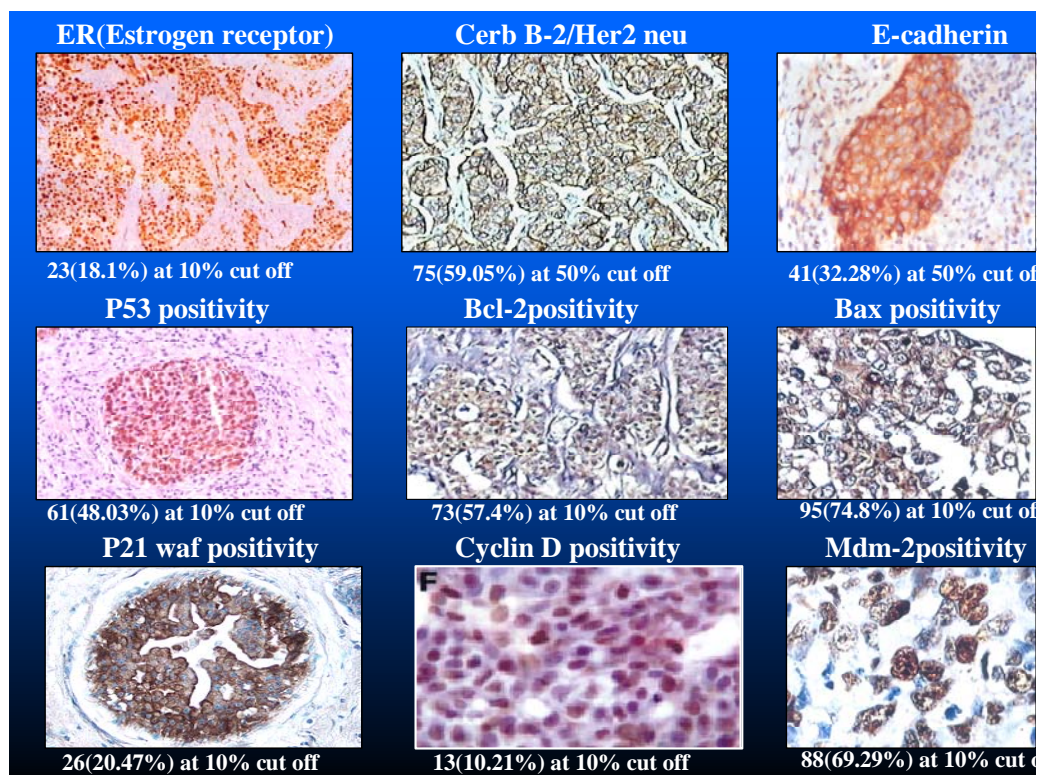


Fig. 1: Microphotograph showing representative sections of Breast Cancer with biomarker expression.

MOLECULAR BIOLOGY OF PROSTATIC NEOPLASIA

1. Study on microsatellite instability and defects in mismatch repair system in prostate cancer

Scientific staff : Dr. Sunita Saxena, Dr. Anju Bansal
In collaboration with : Dr. N K Mohanty, SJ Hospital, New Delhi
Duration : 2005-2008

Aims, Objective & Background

Prostate cancer represents one of the leading causes of cancer related deaths in males. Knowledge regarding molecular mechanisms involved in this tumour initiation and progression is sparse. Androgen receptor (AR) has an important role in the progression of prostate cancer. The CAG repeats encode long glutamine homopolymeric amino acid chains in the amino-terminal domain of the AR gene. AR CAG repeats impose a transactivation activity on the receptor and have a binding affinity for androgens. Another molecular marker of prognostic importance is the p53 protein that constitutes as a significant component of cellular response to DNA damage. Its accumulation leads to cell cycle arrest preventing the replication of damaged DNA. In case of non-repair, it can lead to apoptosis in cells with severe DNA damage. This study is planned to analyze the role of polymorphic CAG repeat sequences in exon 1 of AR gene in conferring susceptibility to develop prostate cancer and progression of prostate cancer. Correlation would be done with p53 mutations, germ line mutations in mismatch repair genes and expression of MMR proteins in prostate gland. Identification of microsatellite instability in androgen receptor gene along with other molecular mechanisms would help in understanding molecular mechanisms underlying occurrence and progression of prostate cancer. The study is approved for financial funding from DST in Jan.,05.

Work done during this year

Cases of prostatic carcinoma and cases of premalignant lesions are being included for present study. Blood and tumor tissue (paraffin embedded) would be collected from these cases. DNA extraction would be done from blood as well as formalin fixed paraffin

embedded tissue by Phenol chloroform method. DNA amplification would be done by polymerase chain reaction using exon specific primers. Further, SSCP analysis and DNA sequencing would be done. Immunohistochemical staining would be done for MLH1, MSH2, PMS2 and PMS1 on prostate biopsy cases and TURP cases. The staining protocols for these antibodies are being standardized.

BLADDER CANCER

1. Role of In-vitro Cytotoxicity Assay and Adjuvant Intravesical Therapy in the Management of Superficial Bladder Cancer

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Technical Staff : Mr. Jagdish Pant
Duration : 2001-2005

Aims, Objectives and Background

Management of superficial bladder cancers includes transurethral resection of tumour followed by adjuvant chemotherapy and immunotherapy used as a prophylactic measure to reduce the frequency of recurrences. However, these agents have been used in a random manner for reducing the recurrence rate giving variable success rates. The objective of this study has been to assess the role of intravesical administration of chemotherapeutic/immunomodulating agents in management of patients with superficial transitional cell carcinoma after *in vitro* autologous tumour cell culture so as to tailor the dosage to individual needs. This study has been financially supported by DST and completed during the year under report.

Work Done during the year

Of the 91 cases enrolled in the study, 41 patients received BCG, 120 mg according to the International Protocol and 50 patients received treatment based on *in vitro* cytotoxicity assay after TURBT. Of these, 3 patients (6%) in the *in vitro* group and

1 patient (2.4%) receiving International Protocol developed muscle invasive disease in the first 6-8 months of follow-up. These patients were excluded from the study group and remaining 47 patients in the *in vitro* group and 40 among control group were followed. The recurrence of the tumor in the *in vitro* group was seen to be lower at 14.8% (7/47) while that in the International protocol group was 37.5% (15/40) ($p=0.02$). *In vitro* cytotoxicity assay showed 16 (34%) patients with maximum cytotoxicity to a single drug while 31(66%) showed maximum cytotoxicity to a combination of immunomodulators and cytotoxic agents. Intravesical therapy was administered as single and double drug regimen as per *in vitro* cytotoxicity results.

Among 16 patients on single drug regimen, 9 (56.25%) were given immunomodulators and 7 (38.9%) cytotoxic agents. Only one patient (6.25%) in single drug regimen group receiving Mitomycin-C showed recurrence. Among 31 patients on double drug regimen, 29 (93.5%) patients were given a combination of immunomodulators and cytotoxic agents, while in the remaining 2 patients, it was a combination of BCG and Interferon $\alpha 2b$ based on results of *in vitro* cytotoxicity assay. In double drug regimen group, 6 (19.3%) patients showed recurrences. It was observed that while patients on BCG in combination with cytotoxic drugs showed no recurrences, those on Interferon $\alpha 2b$ combinations showed a recurrence rate of 28.5% ($p=0.02$). The number of cases receiving cytotoxic therapy was too low to draw any conclusion. The maximum recurrence was found when these drugs were given in combination with Interferon $\alpha 2b$. Between two patients receiving combination of immunomodulators only (BCG+Interferon $\alpha 2b$), one showed recurrence within 5 months. These results suggest that BCG is a more potent immunomodulator and has stronger anti-proliferative activity compared to Interferon $\alpha 2b$.

Fourteen patients showed recurrence in initial 6-10 months follow-up after adjuvant chemo/immunotherapy given on the basis of *in vitro* cytotoxicity assay. *In vitro* cytotoxicity assay was repeated in all of them and 7 (50%) patients are at present recurrence -free till the end of follow-up period of 48 months. In the remaining 7 patients (14.8%), modification of intravesical therapy following repeated cytotoxicity assay has

not helped in preventing recurrence in spite of giving three drugs in combination and maximum doses of each drug.

The comparison of the cumulative probabilities of the survival in the two groups at different points of time showed decrease in recurrence free survival with increasing grade and over time. The Kaplan-Meier survival plots (Fig. 1) shows the cumulative probability of developing recurrences, to be 62.5% in the group receiving therapy based on *in vitro* cytotoxicity results and 83.9% in the patient group treated by the International Protocol ($p=0.08$). The recurrence-free survival was more in the *in vitro* group (75.1%) compared to the International Protocol group (49.2%) at 36 months follow-up.

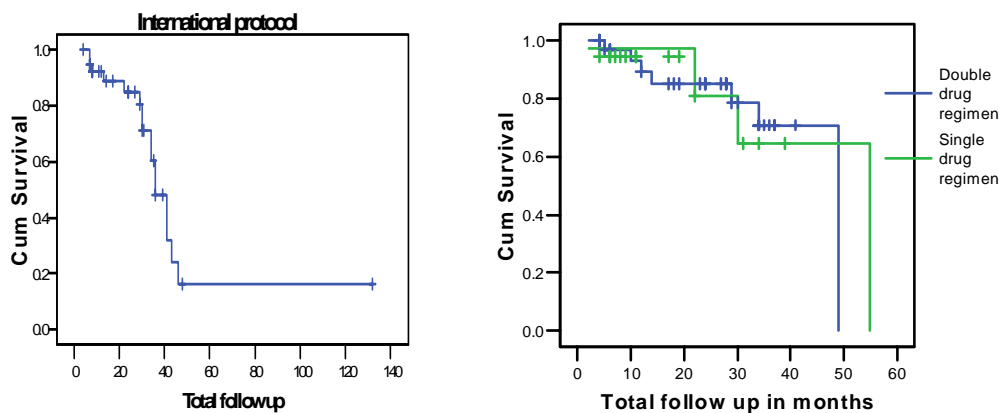


Fig. 1: Kaplan-Meier survival plots showing cumulative probability of developing recurrences.

2. Study of the Host Immune Response in Patients with Superficial Transitional Cell Carcinoma

Scientific staff : Dr. Sunita Saxena, Dr. Usha Agarwal, Dr. Saurabh Verma, Dr. Abhilasha Agarwal

In collaboration with : Dr. N K Mohanty, SJ Hospital, New Delhi

Technical Staff: : Mr. P D Sharma

Duration :2002-2005

Aims, Objectives and Background

An imbalance between the Th1 and Th2 immune responses has been attributed to immune dysregulation in various malignancies, particularly in bladder carcinoma. The method of dual immuno-fluorescent labeling can be useful for enumerating cells from human peripheral blood that bear the helper T-cells. The helper and suppressor T-lymphocytes can be quantified simultaneously by flow cytometry, which enables to correlate the phenotypic activities of antibodies against cell surface receptors and permits the measurement of a large number of samples in minimal time. The present study was initiated to evaluate the Th1 and Th2 balance in Peripheral Blood Mononuclear Cells (PBMCs) of Transitional Cell Carcinoma patients (TCC) and normal healthy subjects in order to assess the immunological and cellular factors influencing the anti-neoplastic activity in terms of their cytokine expression and various cell surface markers using flow cytometry.

Work done during the year

Immunological responses in bladder cancer patients were evaluated by studying cell surface antigen expression in PBMCs for CD3/CD4/CD8/CD56 and cytokine expression for IFN- γ , IL-2, IL-4 IL-6 IL-10 and TNF- α . The present study was carried out in 21 normal healthy subjects (control) and 41 patients (tested) of superficial TCC of bladder using flow cytometry and presented in the previous year (2003-2004). The percentage positive cells were recorded by carefully excluding all cellular debris and identifying the number of antibody-positive cells. These data have been expressed as the values of the actual percentage of cytokine expressing cells, i.e. double positive for CD4+ and cytokine positive.

All the quantitative parameters were expressed as percent mean \pm standard deviations. The mean values between the recurrent and the non-recurrent groups were statistically tested for significance by employing Mann-Whitney U test or the Kruskal Wallis test as appropriate. The relationship between the various Th1 and Th2 cytokines in both groups was determined using Pearson's correlation test.

The cell surface markers CD3, CD4, CD8 and CD56 along with NK cells were significantly reduced in bladder cancer patients as compared to the healthy controls. CD4+T-cell subset was significantly reduced in recurrent patients as compared to non-recurrent patients showing greater immunodeficiency in them. Patients with recurrence showed a significant decrease in the CD8+T-cells and NK cells as compared to normal healthy subjects. Deficiency of Th1 cytokines like IFN- γ and particularly IL-2 along with TNF- α was significantly reduced in patients as compared to normal healthy controls. Patients showed enhanced production of Th2 cytokines like IL-4, IL-6 and IL-10 as compared to normal healthy controls that demonstrates the possible existence of Th2 dominant status in bladder cancer patients. Synergistic effects of IFN- γ and TNF- α along with local presence of IL-2 *in vivo*, and down-regulated Th2 response seems to be linked primarily to inhibition of tumor cell proliferation and an up-regulated protective cell-mediated immunity in non-recurrent patients.

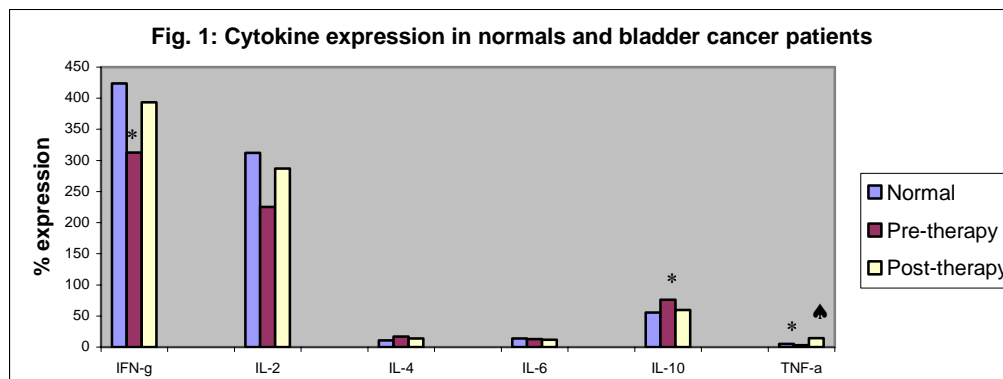
This study demonstrates an apparent dysregulation in the function of Th1 and Th2 subsets of CD4+T-cells in bladder cancer patients, with an expansion of Th2 cells and a deficiency of Th1 cells. Assessing Th1/Th2 homeostasis in peripheral blood lymphocytes may prove to be useful to screen for cancer and to monitor cancer therapy including immunotherapy, particularly in recurrent patients.

Cytokine Bead Array

The expression of circulating levels of Th1 and Th2 cytokines were estimated in serum of TCC patients prior to therapy (pre-therapy) and following intravesical immunotherapy (post-therapy) using a cytokine bead array system (*BD Biosciences, San Diego CA, USA*). The flow cytometric analysis was done using special software that allowed the calculation of concentrations in the unknown serum samples. The present study included 41 (pre-therapy patients) and 37 (post-therapy patients) with histologically confirmed superficial TCC treated by transurethral resection. 20 healthy subjects, whose age ranged from 25 to 55 years, were used as controls.

The expression of the levels of various Th1/Th2 cytokines was determined in the sera of normal healthy subjects along with both pre-therapy and post-therapy bladder cancer patients. The mean levels of Th1 cytokines, viz.: IFN- γ , IL-2 and TNF- α were significantly reduced in pre-therapy patients as compared to normal healthy controls ($p < 0.04$, $p < 0.01$ and $p < 0.02$ respectively) as shown in Fig. 1. On the contrary, significantly enhanced levels of Th2 cytokines, viz.: IL-4 and IL-10 were observed in pre-therapy patients as compared to healthy volunteer ($p < 0.0001$ and $p < 0.002$ respectively). The present study revealed statistically significant enhanced level of TNF- α in post-therapy TCC patients as compared to normal healthy controls ($p < 0.05$), while none of the other Th1/Th2 cytokines revealed any statistical significance between these two groups. Post-therapy patients who received intravesical therapy expressed statistically significantly higher levels of Th1 cytokines, viz.: IFN- γ and TNF- α as compared to pre-therapy patients ($p < 0.03$ and $p < 0.0007$ respectively). Also, significantly reduced level of Th2 cytokine IL-10 was observed in post-therapy patients as compared to pre-therapy patients ($p < 0.002$).

* $p < 0.05$ as compared to pre-therapy, \blacktriangle $p < 0.05$ as compared to post-therap



Enumeration of CD markers and Cytokines in Locally Infiltrating Lymphocytes (TILs)

The immunophenotypic characterization of Tumor Infiltrating Lymphocytes (TILs) was studied in 40 patients of superficial TCC of bladder on paraffin sections by immunohistochemistry. On immunohistochemical examination, an infiltration of T-

lymphocytes (CD3+), helper T-cells (CD4+), suppressor T-cells (CD8+) and natural killer cells (CD56+) was observed in the lamina propria of the bladder. The positive expression of CD3+T-cells was (75.3%), of CD4+T-cells was (48.6%), of CD8+T-cells was (21.4%) and of NK cells was (14.2%) of the total number of mononuclear cells

3. To Study the Expression and Relevance of Tissue Transglutaminase(tTG) in Invasive and Non Invasive Human Bladder Transitional Cell Carcinoma (TCC)

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In collaboration with : Dr. NK Mohanty, SJ Hospital, New Delhi
Technical Staff : Mr. PD Sharma
Duration : 2003-2006

Aim, Objectives and background

Despite significant advances in the treatment of bladder cancer, predicting and preventing metastasis remains a clinical challenge. Metastasis is a multistep process that involves invasion, adhesion to a blood vessel wall, extravasation, infiltration, and the proliferation of cancer cells in the target tissue. Many of these steps require interaction between tumor cells and the Extracellular Matrix (ECM). Tissue Transglutaminase (tTG) plays an important role in stabilizing the ECM by cross-linking its component proteins and rendering it resistant to mechanical and proteolytic degradation. tTG, a member of the Ca^{2+} -dependent family of mammalian enzymes, catalyzes irreversible cross-linking of proteins by inserting highly stable $\epsilon(\gamma\text{-glutamyl})\text{lysine}$ bonds between them. The ability of tTG to affect the physicochemical properties of the ECM may influence the invasive properties of tumor cells by modulating cell-matrix interactions or by facilitating the assembly of the matrix and tissue remodeling. This study attempts to study the expression and relevance of tTG in invasive and non-invasive Transitional Cell Carcinoma (TCC) of bladder and to evaluate its further role in metastases.

Work done during the year

Paraffin sections were studied for tTG expression using immunohistochemistry in 32 cases of TCC of human bladder. Out of the total , 60% of the cases were positive to tTG expression. Most of the tTG positive samples were grade II and few were grade I. Experiments were done to study the expression of alpha6 and beta4 with CD44 to correlate it with tTG expression and its further role in invasion. Fig.1 shows expression of tTG in endothelial cells and vessels. However, tumor cells did not show tTG expression. Fig.2 shows the tTG expression in tumor cells.

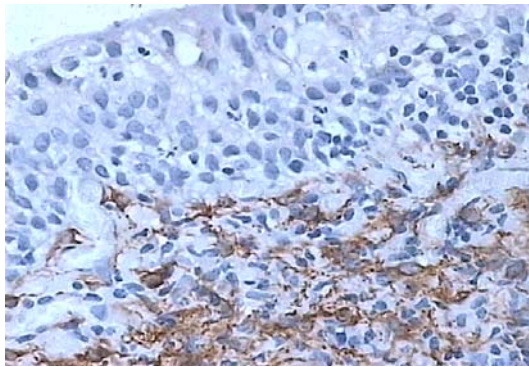


Fig. 1: Showing expression of tTG in endothelial cells and in vessels of TCC in bladder. Tumor cells are negative here.

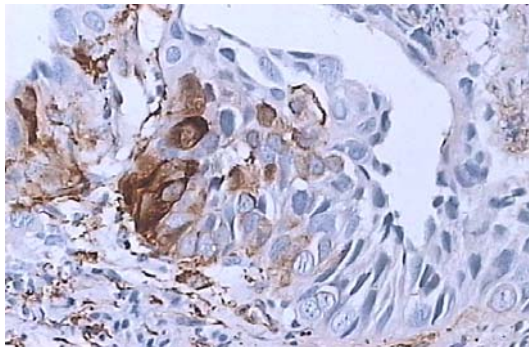


Fig.2: Showing expression of tTG in tumour cells of TCC in bladder.

Future plan of the work

Samples will be processed to study the tTG gene in different phases of cell cycle as we are standardizing the sorting technique in case of biological tissues and to study the expression adhesive proteins like alpha6 beta4 , CD44 to correlate with tTG expression and relevance of these adhesive proteins in TCC of human bladder.

CANCERS IN NORTH-EAST REGION OF INDIA

North-Eastern states of our country have reported a very high incidence of several cancers including those known to be associated with use of tobacco and with pesticide exposures. However, the available data on tobacco usage and pesticide exposure alone is not sufficient to explain the high incidence.

North-East region of India has diverse ethnic groups with different customs, food habits and life-style. The type and pattern of tobacco use in this region is different from that of the rest of the country. The carcinogenic potentiality of tobacco depends on its carcinogenic contents, viz.: nitrosamines, nicotine and other constituents which vary from place to place and also depend on the pattern of use. Moreover there is extensive use of pesticides in tea gardens in North-East which can lead to widespread occupational and environmental exposures.

ICMR has set up Cancer Registries in the North-East under the NCRP (National Cancer Registry Programme). These centres have a good working relationship with the populations harbouring the cases of cancers and have earlier provided data showing the high prevalence of certain types of cancers in the North-East. Multicentric studies have been initiated by ICMR to find out if genetic factors, in addition to common environmental exposure and dietary habits such as tobacco smoking, alcohol consumption and pesticide exposure could possibly explain the high prevalence of certain cancers in North-East India. This forms the basis of the current studies.

Studies on oesophageal cancer started in 2004. Interesting leads have been obtained from the ongoing multicentric project on oesophageal cancer at IOP, ICPO, RMRC Dibrugarh and BBCI Guwahati. In 2005, this study was extended to include other tobacco and pesticide related cancers such as oral cancers, stomach cancer, lung cancer, breast cancer and malignant lymphoma. These multicentric studies will investigate the link between carcinogenic contents of tobacco and pesticides used in North-East and genetic variation including polymorphism/mutations associated with ethnic variation. The study will be carried out in the six population based Cancer Registry area of NE with a

case control epidemiologic design. NIOH will be doing pesticide and chemical residue analysis of tobacco that are present in micro-quantities in environmental and biological samples while the genetic studies on blood samples and gene expression analysis in tissue samples will be done at IOP and ICPO.

Ongoing Work

- I Comprehensive Study of Carcinoma Oesophagus at North-East India – Multidisciplinary Approach. *Collaborative, Multicentric, ICMR Task Force Project*

New Projects

- II Effect of Pesticide Exposure in Causation of Cancer in North-East India. *Collaborative, Multicentric, ICMR Task Force Project*
- III Role of Tobacco Use in Causation of Cancer in North-East India. *Collaborative, Multicentric, ICMR Task Force Project*

1. Comprehensive Study of Carcinoma Oesophagus at North-East India - Multidisciplinary Approach

Scientific Staff : Dr. Sunita Saxena, Dr. Sujala Kapur, Mr. Indranil Chatterjee, Ms. Afsan Noor

In collaboration with : Dr. J Mahanta, Dr. R K Phukan, RMRC Dibrugarh
Dr. J Purkayastha, Dr. AC Kataki, BBICI, Guwahati,
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Dr. BC Das, Dr. J Sharma, ICPO, New Delhi

Duration : 2004-2007

Aims, Objectives and Background

Epidemiological studies have identified several countries, including India, that have reported a high incidence of oesophageal cancer. Cancer data generated from six hospital based Cancer Registries under National Cancer Registry Programme (NCRP, Annual Report, 1984-93) has revealed that in India, Assam has the highest incidence of oesophageal cancer. In addition, aggregation of oesophageal cancer in families is a long-observed and well-documented phenomenon in Assam. Epidemiological studies indicate that tobacco smoking and alcohol consumption are the major factors for oesophageal

cancer. However, the role of genetic factors for familial aggregation, have not yet been elucidated.

Recent studies have focused on genetic factors in the etiology of oesophageal cancer by attempting to identify genes associated with its development and progression. Chromosomal regions with frequent allelic loss may point to major susceptibility genes that will assist in understanding the molecular events involved in oesophageal carcinogenesis and may serve as the basis for the development of markers for genetic susceptibility and screening for early detection of this cancer. Identifying the genes upregulated and downregulated in oesophageal cancer and their association with familial aggregation and tobacco use may help in identifying targets for therapy and understanding the carcinogenic mechanisms. With this background, the current study has been planned to investigate the role of genetic factors and tobacco smoking in the etiology of oesophageal cancer.

Work done during the year

Selection of Patients

Blood samples from 22 patients with oesophageal cancer were obtained from Dr. Bhubaneshwar Borooah Cancer Institute (BBCI), Guwahati, Assam, during the 1st year of study. In addition, oesophageal biopsies were obtained from 11 of these 22 patients. Tissues were obtained from the tumour areas as well as from the surrounding normal area. The age range of these patients was 32 to 75 years; 20 of the 22 patients were male; five patients had family history of oesophageal cancer and one had family history of liver cancer; 16 patients were tobacco users.

Loss of Heterozygosity (LOH) or Microsatellite Instability (MSI)

For this study, microsatellite markers that have earlier been analyzed for association with genetic susceptibility in other high-incidence regions of oesophageal cancer (*Clinical Cancer Research*, (5), 3476-82, 1999) were selected. PCR was done on genomic DNA extracted from whole blood samples using suitable primers to amplify these loci. Amplified PCR products were screened for LOH or MSI by SSCP (Single Stranded Conformation Polymorphism) using non-radioactive method.

Results of LOH / MSI

Using DNA from blood samples and various microsatellite markers, specific loci were amplified using PCR. PCR conditions of 5 microsatellite markers have so far been standardized (Figs. 1-3).

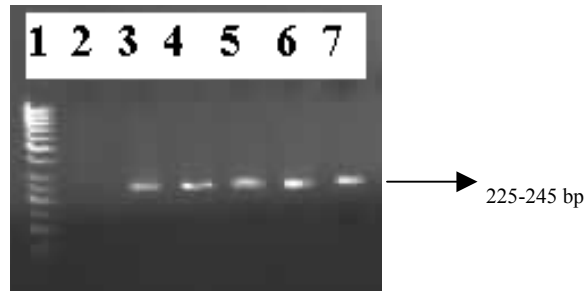


Fig.1: Agarose gel electrophoresis shows 225-245 bp PCR products in DNA extracted from blood samples (Lanes 3 to 7) using D17S1303 microsatellite marker

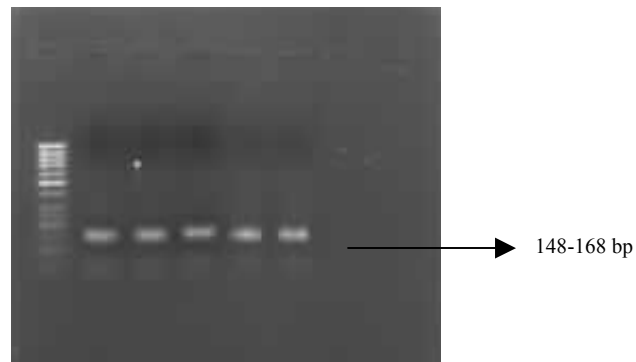


Fig. 2: PCR products of 148-168 bp seen with D13S796

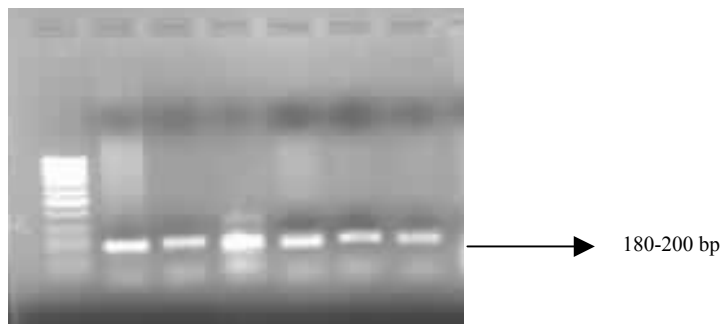


Fig. 3: PCR products of 180-200 bp, D13S1894

Altered band patterns in SSCP were found in 46% samples using marker D11S1984; in 44% samples using marker D9S910; in 38% samples using marker D17S1303 and in 34% samples using marker D13S796. However they were found only in 10% samples using marker D13S894 (Table I, Fig. 4)

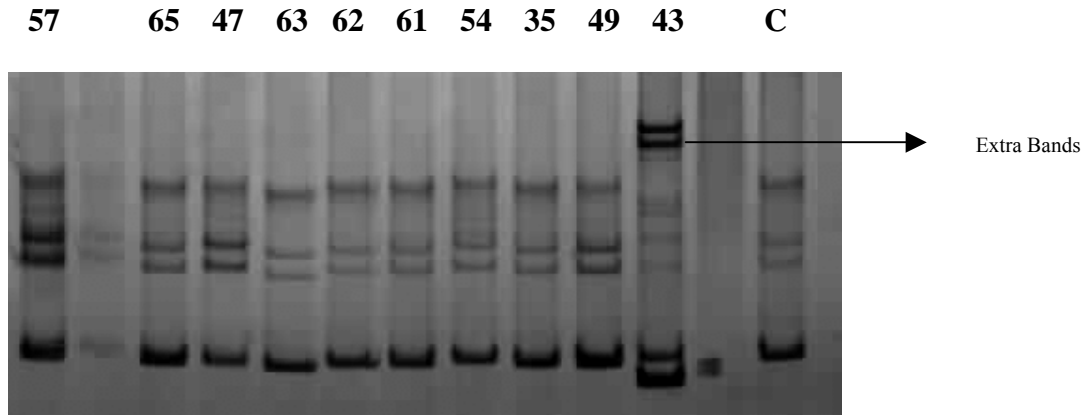


Fig. 4: SSCP analysis of D13S894 microsatellite locus to screen for LOH or MSI. Silver stained non-denaturing PAGE showing band patterns of amplified PCR products. Lane C is DNA sample from healthy individual. All others lanes denote DNA samples of oesophageal cancer patients. Patient No. 43 shows two extra bands due to abnormality in D13S894 locus.

Markers	Designation	Tm	% samples with band shift
D17S1303	M1	56	38
D13S796	M2	57	34
D11S19844	M3	60	46
D13S894	M7	60	10
D9S910	M6	55	44

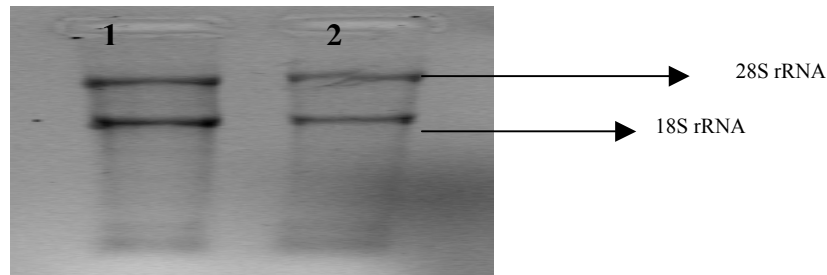
Table I: Number of samples with altered band pattern in SSCP

Studies on Gene Expression

For this, isolation of RNA from tumor tissue and normal surrounding area was done using Trizol reagent (*Invitrogen*) and chloroform - isopropyl alcohol method. Determination of quantity and quality of RNA was done by UV spectrophotometry (Table II) and agarose-formaldehyde gel electrophoresis (Fig. 5), respectively.

Sample ID	Ng/il	A2 60	A280	260/280	260/230	Constant
71+72 N	1238.44	30.962	13.884	2.23	1.83	40
71 T	1805.21	45.13	20.028	2.25	1.66	40

Table II: Determination of quantity of RNA by UV spectrophotometer



**Fig. 5: Determination of quality of RNA by agarose-formaldehyde gel electrophoresis Lane 1: RNA from tumor tissue
Lane 2: RNA from normal tissue**

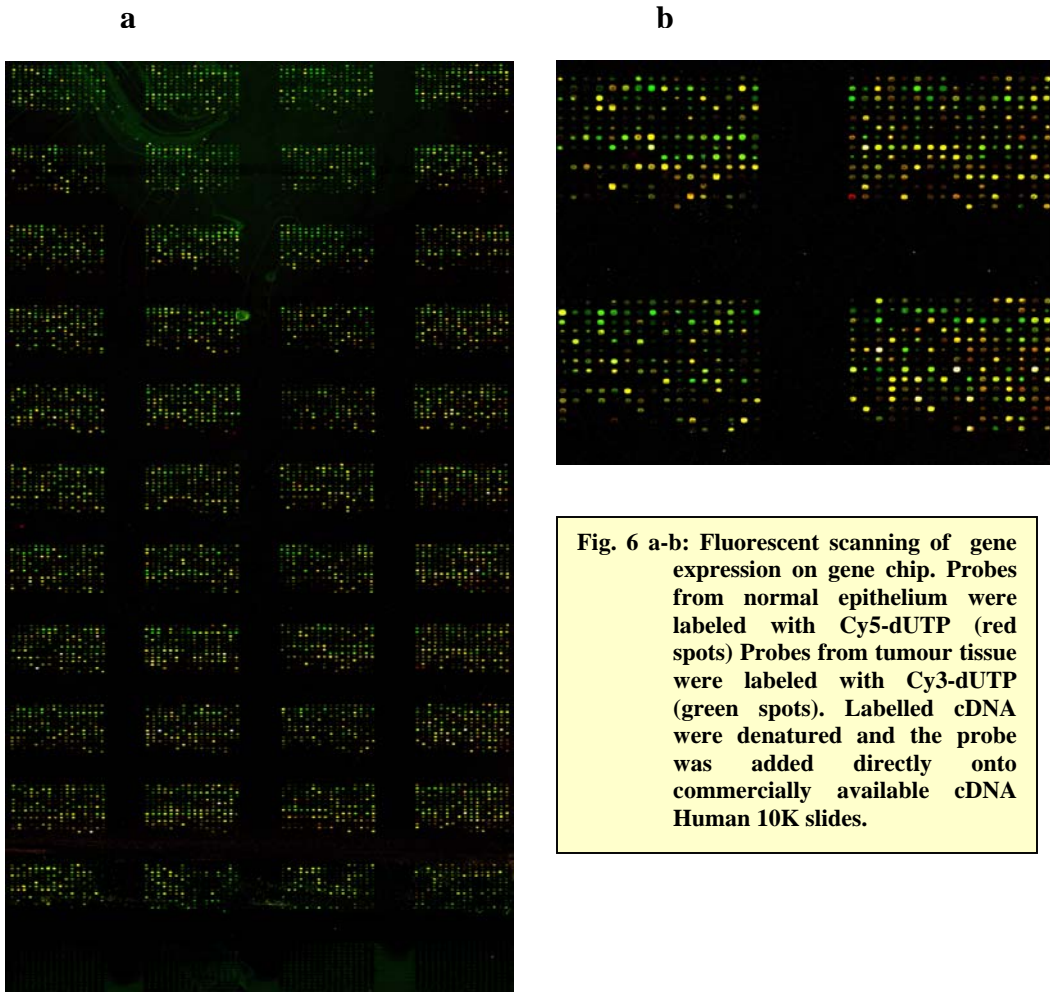
Gene expression analysis

Preparation and purification of Cy-3 and Cy-5 labelled cDNA was done from total RNA. For this RNA samples were reverse transcribed and labelled with Cy-3 and Cy-5. Labelled cDNA pellet was washed with cold 70% ethanol, dried and dissolved in TE buffer. Hybridization of Cy-3 and Cy-5 labelled cDNA was done with human cDNA microarray. Labelled cDNA were denatured and the probe was added directly onto commercially available cDNA microarray slides. Hybridization was done at 65°C. Washing was done using both medium and high stringency buffers. Slides were scanned on Axon Scanner (Figs. 6 a-b)

Results on cDNA microarray

Probes from normal epithelium were labeled with Cy5-dUTP (red spots) and probes from tumor tissue were labeled with Cy3-dUTP (green spots). Both the digitised images were overlaid to form a pseudo-colored image (yellow spots). Cy3 and Cy5 pixel

values and overall intensities were determined and normalised to obtain the Cy3/Cy5 ratio (Figs. 7a -b).



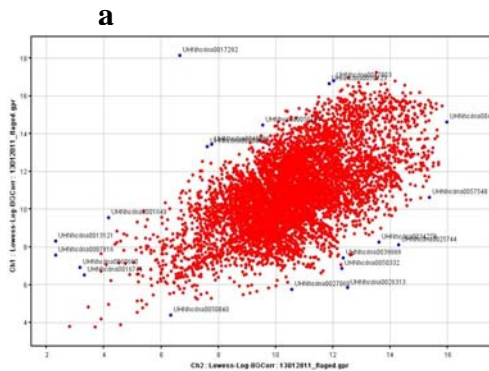


Fig. 7a: Scatter plot showing upregulated and downregulated genes in cDNA microarray

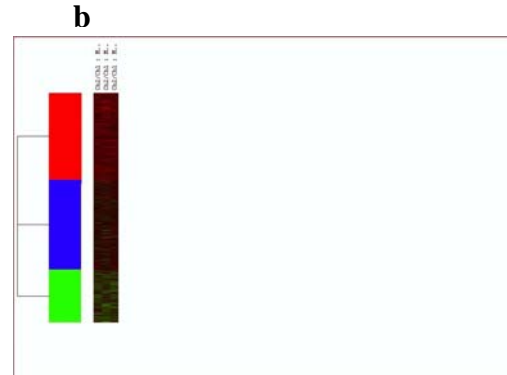


Fig. 7b. Clustering of microarray gene expression

Quantitative real time RT-PCR analysis

Validation of the microarray data will be done using Real Time PCR. For standardization of this technique, a gene, known to be over-expressed in tobacco smokers, (DDH) was used. This part of the work was done at IISc, Bangalore. Quantitation is done relative to the control gene by subtracting the cycle threshold, or C_t of the control gene from the C_t of the gene of interest. Resulting difference in cycle number (ΔC_t) is the exponent of the base2 (due to doubling function of PCR), representing the fold difference of template for these two genes. These C_t values are directly proportional to the amount of starting template and are the basis for calculating mRNA expression. Relative gene expression of DDH is determined based on the threshold cycles of DDH in relation to the threshold cycle of the corresponding internal standard GAPDH (Figs. 8- 9).

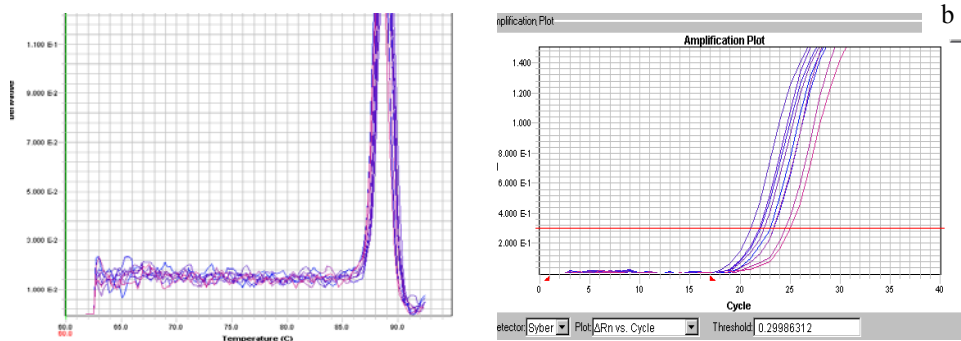


Fig 8. (a -b): Dissociation curve and amplification plot of housekeeping GAPDH gene. No primer-dimer formation or non-specific amplicon are seen.

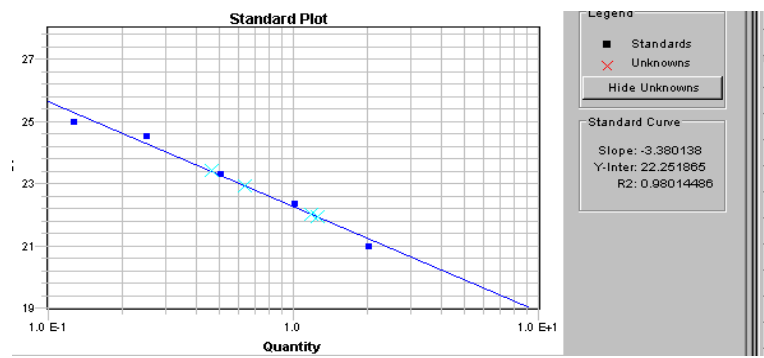


Fig. 9: Standard plot shows linear amplification of GAPDH

2. **Effect of Pesticide Exposure in Causation of Cancer in North-East India.**
Collaborative, Multicentric, ICMR Task Force Project
3. **Role of Tobacco Use in Causation of Cancer in North-East India.**
Collaborative, Multicentric, ICMR Task Force Project

Study will be done in collaboration with the following Centers:

Centre I	Centre II	Center III
Dr. Sunita Saxena, Director, IOP, New Delhi	Dr B. C. Das, Director, ICPO, Noida	Dr. H. N. Saiyed, Director, NIOH, Ahmedabad

The following malignancies are included in these studies:

Tobacco-related malignancies	Pesticide-related malignancies
Oral and oro-pharyngeal cancers	Carcinoma Breast
Oesophageal cancer	Malignant lymphoma
Stomach cancer	
Lung cancer	

Work done during the year

The NCRP has prepared an epidemiological-based questionnaire that has been distributed to all the peripheral collaborating centers. The schedule has been finalized and printed with the approval of all centers. The method of selection of cases, controls and the number of samples to be collected from each center has been planned. It is envisaged that a pre-testing of the schedule within a certain time frame will be necessary. Blood samples and tissue biopsies will be collected for all cases that are included in the study and will be sent for analysis at IOP, ICPO and NIOH.

1. Flow Cytometric Assays to Evaluate Prognosis and Patients Response to Chemotherapy in Patients with Acute Leukemia-Expression of Genes Commonly Involved in Drug Resistance

Scientific staff : Dr. Sujala Kapur, Mr. Bharat Bhushan, Dr. Saurabh Verma, Dr. Aruna Mittal
Technical Staff : Mrs. Rachna
In collaboration with : Dr. Sumita Saluja, SJ Hospital, New Delhi.
Duration : 2002 – 2005

Aims, Objective & Background

Multi-drug resistance is a major obstacle for cancer therapy. It is one of the mechanisms to explain failure of chemotherapy in patients with different hematological malignancies. In a previous study, we have identified chemotherapeutic drugs that could be used in chemosensitivity assay in patients with acute leukemia (AL). In the current study, genes responsible for the development of resistance were evaluated by RT-PCR. The genes commonly involved in chemoresistance include multi drug resistance (MDR) gene, dihydrofolate reductase (DHFR) gene, glutathione-s-transferase (GST) pi and the p53 gene.

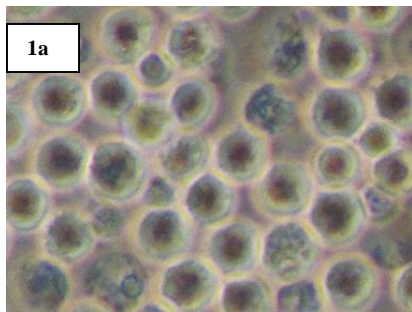
Multidrug resistance (MDR) or P-glycoprotein (P-gp), a pump located in the cell membrane, extrudes several clinically important drugs from the cell, and hence causes multidrug resistance. Overexpression of the multidrug resistance gene, MDR1, is of prognostic relevance in acute myeloid leukemia, while its role in acute lymphoblastic leukemia (ALL) is still under debate. DHFR is an enzyme involved in the metabolism of nucleic acids; it is also an important target for folate antagonists such as methotrexate (Mtx). Methotrexate is an important component in the treatment of childhood leukemia. Mechanisms of Mtx resistance include (1) decreased transport via the reduced folate carrier, (2) altered levels of target enzymes, e.g. DHFR and thymidylate synthase, (3) decreased ratio of folylpolyglutamate synthetase and hydrolase. DHFR gene amplification is a common mechanism of resistance to methotrexate in tumor cell lines, with the exception of a few case reports. Additional studies are necessary to clarify the mechanism for the correlation of low DHFR expression with poor outcome. Drug

resistance in haematopoietic cells may be also be partly related to the expression of the glutathione-s-transferase (GST) pi, a detoxification enzyme that is widely distributed in the human body and is implicated in protection against apoptosis. In normal human tissues, the enzyme protects cells against noxious compounds by catalyzing conjugating reactions with glutathione and protects against reactive oxygen species by reducing organic hydroperoxides via glutathione peroxidase activity. The role of GST-pi in tumor growth and progression is less well known. Increased expression of GST-pi has been reported in several cancers. Down-regulation of GST-pi activity in a T-cell line study appears to favor apoptosis and inhibition of GST-pi function induces apoptosis. Increased expression of GST-pi may account for drug resistance and treatment failure in hematologic malignancies when alkylating agents like cyclophosphamide, chlorambucil, busulfan and melphalan, or doxorubicin are used.

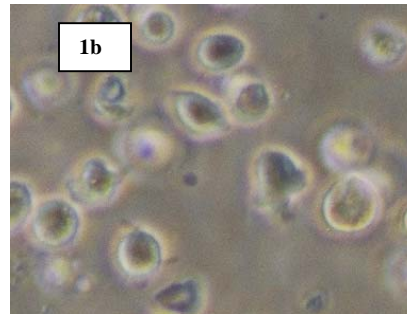
Work done during the year

One hundred and forty-four samples with AL (77 childhood and 67 adult leukemia) have so far been analyzed. 44 samples were of B-ALL, 29 of T-ALL, 56 of AML, 11 of bi-phenotypic and 4 of undifferentiated leukemia. More than 50% patients presented with known high risk factors at the time of diagnosis. These factors included age, sex, total leucocyte and platelet count, hemoglobin, CD 10, MDR, CD34 and FAB type.

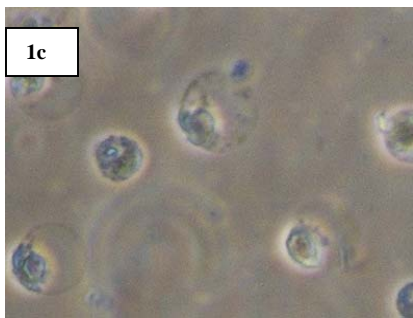
In vitro cytotoxicity assay was done in 79 samples (25 B-ALL, 19 T-ALL, 29 AML and 6 biphenotypic leukemia) of which 56% patients achieved complete remission (CR), and 44% patients failed to achieve CR. Cytotoxicity assays were done using vincristine (Vin), daunorubicin (Dnr), methotrexate (Mtx), cytosine arabinoside (ara-C) and L-asparaginase (L-asp) (Fig. 1).



Control



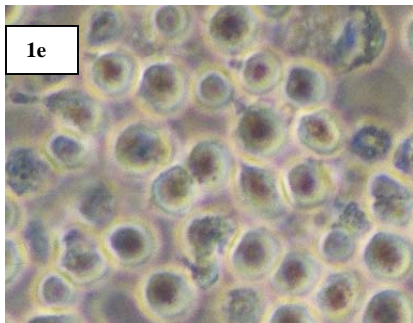
Cytosine arabinoside



Daunorubicin



Vincristine



Methotrexate

Fig. 1: Phase contrast photomicrographs of leukemic cells incubated with cytotoxic drugs in 96-well culture plate. Figure shows a sample with AML that is sensitive to Ara-C, and daunorubicin and resistance to methotrexate and vincristine. (a) is the control well with no drugs; (b) & (c) show cells treated with Ara-C and DNR. Cells are markedly decreased in number and cells remaining in the wells show shrinkage, membrane blebbing, nuclear condensation and pyknosis; (d) and (e) show cells treated with Vin and MTX. Morphologically, no significant difference was seen in these wells in comparison to control wells. This patient achieved complete remission with two cycles of induction therapy.

Results indicate that pattern of *in-vitro* sensitivity of leukemic cells to chemotherapeutic drugs can be correlated with clinical response of the patients (Figs. 2a-b). In B-ALL patients, no significant difference was found in drug sensitivity pattern of Vin in patients who achieved remission versus those who did not achieve CR. Significant difference was found in sensitivity to DNR in responders as compared to non-responders.

In T-ALL patients, no significant difference was found in drug sensitivity pattern of Dnr and Vin. All non-responders were found to be resistant to Ara-C.

In AML patients, significant difference was found in sensitivity to Mtx – only 10% non-responders were sensitive while about half of the responders were sensitive to Mtx. These cells were also sensitive to Ara-C in responders more often than in non-responders.

In biphenotypic leukemia, 50% responders were sensitive to Vin, Mtx and L-asp. All non-responders were resistant to Ara-c, Vin, Mtx and L-asp.

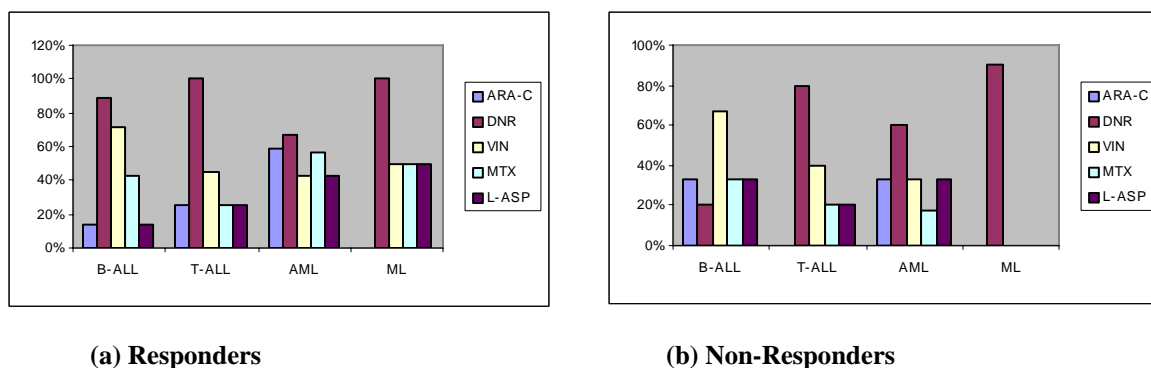


Fig. 2: Drug sensitivity pattern in (a) responders and (b) non responders to chemotherapy. Y-axis denotes the number of patient's samples that were sensitive to the chemotherapeutic agent.

Gene Expression

RT-PCR was done for semiquantitative analysis of the following genes: MDR DHFR, GST-pi and the p53 genes. Leukemic cells were lysed with TRIZOL (*InVitrogen*) and RNA was recovered according to manufacturer's instructions before resuspension in 10-20 μ l of DEPC water. RNA integrity was checked on a 1.2% formaldehyde-agarose gel (Fig. 3a). Human complementary DNA (cDNA) was generated by reverse transcription of total RNA extracted from leukemic cells using high capacity cDNA archive kit (Applied Biosystem). PCR was done using cDNA and suitable primers. Quantification of gene expression was done using densitometry.

Standardization of GAPDH

The cDNA (50 ng) was amplified in a 25 μ l reaction volume containing Taq polymerase, dNTP mix, MgCl₂, 10x PCR buffer and GAPDH primers. cDNA was amplified for different number of cycles to determine log phase of amplification. PCR products were detected by electrophoresis on a 2% agarose gel containing ethidium bromide. Location of products was confirmed using a 100 bp ladder (Fig. 3b).

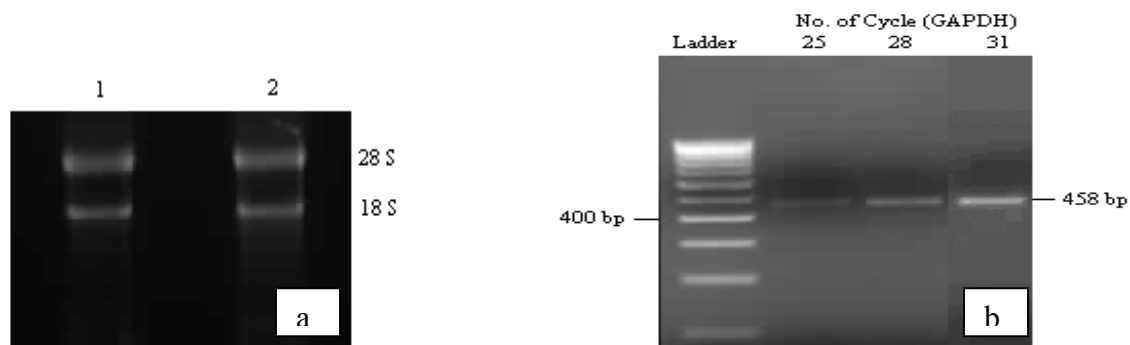


Fig. 3: (a) Formaldehyde-agarose gel showing RNA integrity. (b): 458 bp product of housekeeping GAPDH gene showing amplified PCR products in log phase for quantitative analysis of gene expression.

Expression of GAPDH, GST, DHFR, MDR and p53 genes in normal human volunteers

Quantitative analysis of all the genes that were analyzed was initially done on normal human volunteers. All four genes were found to be expressed on normal peripheral blood mononuclear cells (PBMCs). Quantitation was done using GAPDH as internal control (Fig. 4).

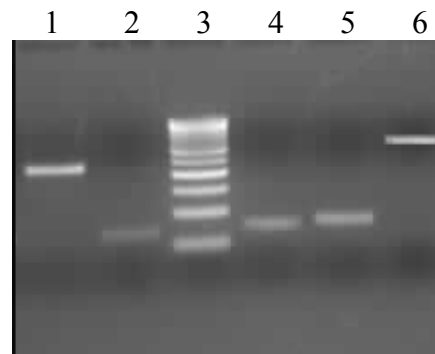


Fig. 4: Expression of genes frequently involved in drug resistance in normal peripheral blood mononuclear cells. All the genes tested were found to be expressed in normal human volunteers. Lane 1: GAPDH, Lane 2: GST-pi, Lane 3:100bp ladder, Lane 4:DHFR, Lane 5: MDR, Lane 6: p53 gene.

Expression of GAPDH, GST, DHFR, MDR and p53 genes in leukemic cells

Expression of MDR, DHFR, GST-pi and p53 was analysed using semi-quantitative RT-PCR on RNA extracted from patient's blood/bone marrow samples. Eight samples have so far been analyzed. Figs. 5 (a-d) shows expression levels of these genes in three samples – samples # 1 and 3 are from patients with AML and sample # 2 is from a known patient with CLL who presented with T-cell blast crisis (T-ALL). The first lane is from a normal human volunteer. AML sample # 1 shows upregulation of the DHFR, MDR and p53 genes as compared to control sample. AML sample # 3 shows downregulation of DHFR, GST-pi and p53 genes with absence of MDR expression. T-

ALL sample # 2 shows downregulation of GST-pi and p53 genes and upregulation of DHFR and MDR genes.

More samples will be analyzed to see if gene expression could be indicator of drug sensitivity pattern in individual patient samples.

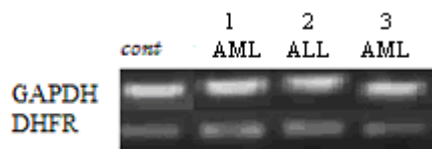


Fig. 5a: 148 bp fragment of DHFR gene. Sample # 1 and 2 show overexpression and sample # 3 shows underexpression of the gene.

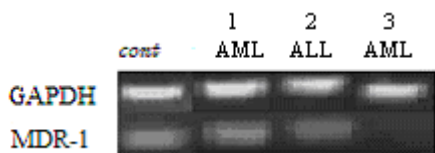


Fig. 5b: 184 bp fragment of MDR-1 gene. Sample # 1 and 2 show overexpression and in sample # 3 MDR is not expressed.

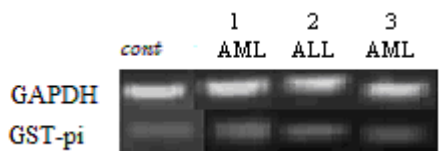


Fig. 5c: 134 bp fragment of GST-pi. Sample # 2 and 3 show downregulation of the gene

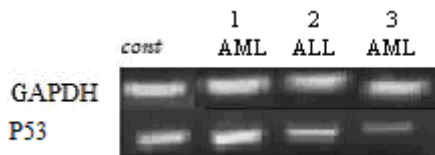


Fig. 5d: 600 bp fragment of p53 gene. Sample # 1 and 2 are upregulated and sample # 3 is downregulated.

Fig. 5: Gene expression in control sample and patients with ALL/AML. First lane is control sample. All other lanes are of patient's samples. Sample # 1: AML, Sample # 2: sample of a patient with CLL in T-cell blast crisis, Sample # 3: patient with AML.

2. Expression of Fusion Oncoproteins and Cell Cycle Regulators in Peripheral Blood and Bone Marrow Specimens of Patients with Acute and Chronic Myeloid Disorders

Scientific staff : Dr. Sujala Kapur, Dr. Aarti Khanna, Ms. Tarannum Siddiqui, Mr. Bharat Bhushan
Technical Staff : Mrs. Rachna
In collaboration with : Dr. Sumita Saluja, SJ Hospital, New Delhi.
Duration : 2004– 2007

Aims, Objective & Background

Chronic myeloproliferative disorders remain stable for years and often transform to a more overtly malignant disease. A common finding in these disorders is a variable course of progression to accelerated (unstable) phase, which merges into blast crisis. However, the cause of this progression is not clearly understood. Recent studies are focusing on the role of cell cycle regulators in the progression of these disorders. Mutations in the signal transduction molecules, which regulate cell proliferation and apoptosis, are thought to be involved in development of acute and chronic myeloid disorders, ineffective hematopoiesis and bone marrow failure. Presence of fusion oncoproteins are also likely to play a role in regulating the cell cycle by p53 dependent or p53 independent pathways in acute and chronic myeloid leukemia as well as in preleukemic conditions such as myeloid dysplastic syndrome.

The current study is being done to understand the significance of presence of fusion oncoproteins in the leukemic cells of patients with acute and chronic myeloid disorders. In addition, expression of cell cycle regulators p53, p21 and mdm2 will be correlated with the proliferative and apoptotic markers, PCNA and CPP32, in bone marrow specimens of patients with acute and chronic myeloid disorders.

Work done during the year

During the year, 48 samples have been collected. These include 38 bone marrow biopsies (10 with AML, 7 with CML, 10 with Myelofibrosis and 1 with hairy cell

leukemia) and 10 bone marrow aspirate specimens (5 with AML and another 5 with CML). Of these, 24 samples have so far been analyzed (Fig. 1).

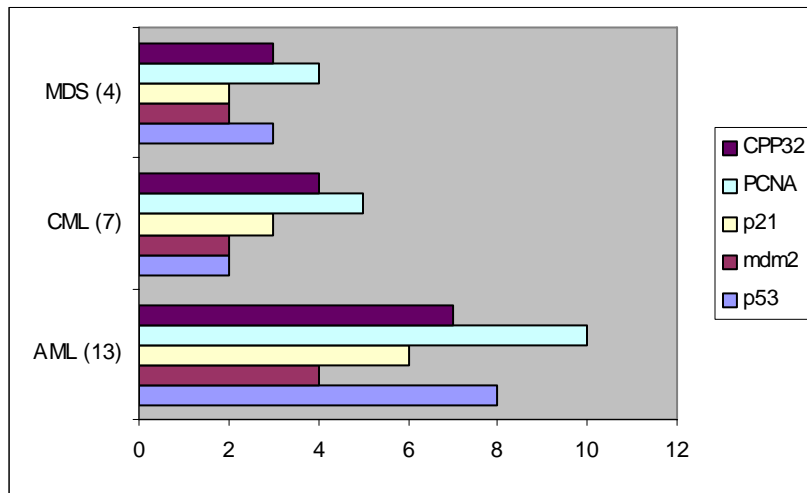


Fig. 1: Immunohistochemical expression of cell cycle regulators in myeloproliferative disorders. Number of samples analyzed are shown in the X-axis.

Immunohistochemistry for p53, p21 and mdm-2 revealed that p53 was positive in 13 of 24 (54%), p21 in 11 of 24 (46%) and mdm-2 in 8 of 24 (33%) samples. 10 of 24 samples were negative for all (p53-/p21-/mdm2-) while 2 of 24 were positive for all 3 markers (p53+/p21+/mdm2+). Other patterns seen were p53-/p21+/mdm2+ (4 samples), p53+/p21+/mdm2- (3 samples), p53-/p21-/mdm2+ (3 samples), p53+/p21-/mdm2- (1 sample) and p53+/p21-/mdm2+ (1 sample) (Fig. 2).

Overexpression of P21 and Mdm-2 in the absence of p53 expression indicates that p53 independent pathway may be involved in cell cycle regulation in these samples.

Mdm-2 expression was high in patients of CML in blast crisis while it was found to be lower in samples with AML and MDS. Our results suggest that Mdm-2 overexpression may be related to a higher grade of myeloid proliferative diseases and its progression (Table I).

	Mean % cells with Mdm-2 expression
AML	9%
MDS	6%
CML	15%
CML in Blast crisis	37%

Table I: Mdm-2 expression in myeloproliferative diseases.

Proliferative and apoptotic index (PCNA and CPP 32) was found to be higher in AML and MDS as compared to chronic myeloid leukemia (Table II).

	PCNA	CPP
AML	41%	22%
MDS	53%	30%
CML	18%	20%

Table II: Proliferative and apoptotic indices (PCNA and CPP) in myeloid diseases.

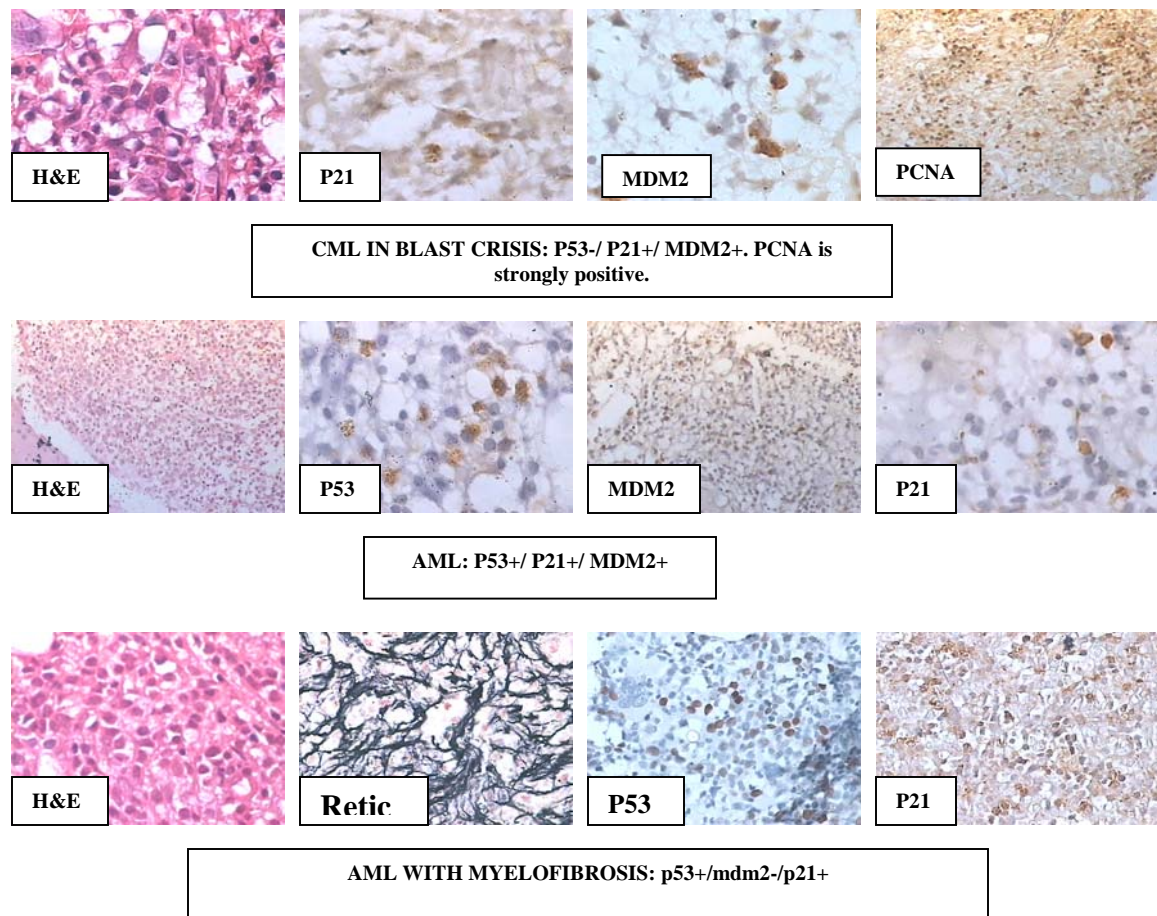


Fig. 2: Expression of cell cycle regulators in myeloproliferative diseases

Identification of fusion oncoproteins

Identification of fusion oncoproteins such as BCR / ABL fusion proteins, RAR α fusion proteins, AML-1 fusion proteins and MLL fusion proteins is being done in AML, MDS and CML using RT-PCR. Mononuclear cells obtained from bone marrow are lysed with Trizol (*InVitrogen*) and RNA is recovered according to manufacturer's instructions. cDNA is generated by reverse transcription of total RNA using high capacity cDNA Archive kit (*Applied Biosystem*). The cDNA is amplified in a 25 μ l reaction volume containing Taq polymerase, dNTP mix, MgCl₂, 10x PCR buffer and suitable primers. PCR products are detected by electrophoresis on a 2% agarose gel containing ethidium bromide. Location of products is confirmed using a 100 bp ladder (Fig. 3).

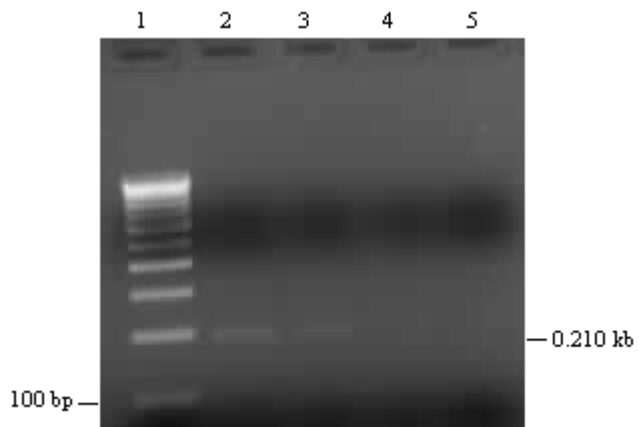


Fig. 3: Agarose gel electrophoresis showing Bcr/Abl fusion product identified using RT-PCR. Presence of a 0.210 kb band confirms the presence of the t (9; 22) translocation (Philadelphia chromosome) in two samples with CML (Lanes 2 & 3). No bands are seen in AML and MDS (Lanes 4 & 5). Lane 1 shows 100 bp size marker.

The presence of the fusion oncoproteins will be correlated with expression of cell cycle regulators. This study is continuing.