

TUMOR BIOLOGY

BREAST CANCER

1. Study of Candidate Genes Associated with Breast Cancer Susceptibility

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<i>In Collaboration with</i>	:	<i>Dr. D. Bhatnagar, Dr. Chintamani, SJH</i>
<i>Technical Staff</i>	:	<i>Mrs. Valsamma Mathews</i>
<i>Duration</i>	:	<i>2006-2007</i>

Aims, Objectives and Background

The high penetrance of the autosomal dominant inherited condition results in multiple cases of cancers among first and second degree relatives, generally at a younger age, e.g. BRCA1 and BRCA2 gene mutation. Such mutations have been well studied genotypically and phenotypically. Besides, analysis of genetic risk of breast cancer has shown that most non-hereditary, sporadic cancers develop in genetically predisposed individuals; this predisposition is the result of several low penetrance genes. These genes grouped as low penetrance have a major role to play in carcinogenesis and serve as markers for predicting cancer risk.

The present study is aimed to identify low penetrance genes associated breast cancer risk in North Indian population. We have already studied the role of polymorphisms in CYP17 gene in breast cancer. During the year under report, polymorphisms/mutations in low penetrance genes, VDR, GST and AR were analyzed in breast cancer patients and in controls.

Vitamin D Receptor Gene

A series of 140 breast cancer patients along with 100 age-matched controls were screened for Apa I (intron 8) and TaqI (exon 9) polymorphisms in VDR gene. Patients included 63 (45%) early onset, 43 (31 %) late onset and 34 (24.2%) familial cases. No statistical significance was found between polymorphic VDR genotype with breast cancer risk. Further finding the genetic linkage of ApaI/TaqI cluster with Poly A repeat in the

3'UTR region of VDR gene will be important to understand the regulation of mRNA stability of the same.

Glutathione S Transferase family (GSTM1, GSTT1, GSTP1)

Study was undertaken to examine if glutathione S-transferase (*GSTM1* and *GSTT1*, P1) genotypes affected breast cancer risk. Total 80 breast cancer cases and 17 controls were analyzed for *GSTM1* and *GSTT1* (Fig 1). Genotyping analysis was performed by multiplex PCR. 27.5% (22/80) of the cases and 29.4% (5/17) of the population controls lacked the *GSTM1* gene. Among 27% of cases, 68.1% (15/22) were early onset and 27.2% (6/22) were late onset. Similarly 37% (30/80) of patients and 29% (5/17) of the population controls lacked the *GSTT1* gene. Among 37%, 63.3% (19/30) and 33.3% (10/30) were found in early and late onset cases respectively.

The *GSTP1* ¹⁰⁵Ile/ Val ¹⁰⁵ was detected in 38% (26/69) of cases and ¹⁰⁵Val/Val¹⁰⁵ was found in 4% (3/69) of the patients. More age matched controls will be included in the study for comparative genotypic analysis of GST family and to calculate significance.

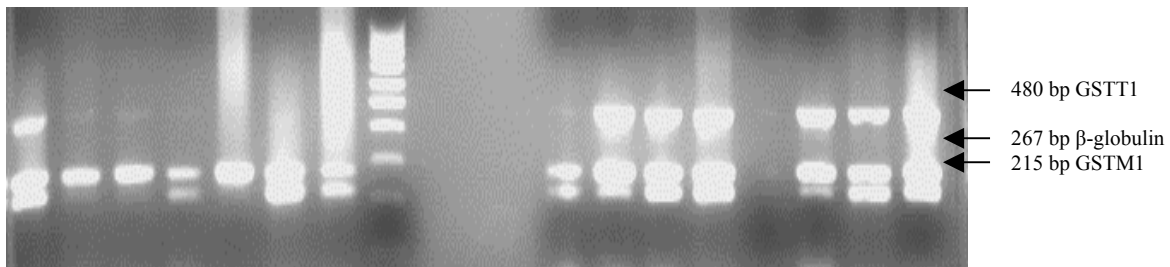


Fig. 1: Genetic alterations in *GSTM1* and *GSTT1* in breast cancer patients. Presence of 480 bp PCR product shows the presence of wild type *GSTT1* gene, 215 bp PCR product shows presence of wild type *GSTM1* gene. β -globulin gene was used as internal control detected by the presence of 267 bp PCR product.

Androgen Receptor Gene

Study has been initiated to identify CAG repeat length in androgen receptor gene in breast cancer. This study is to determine whether AR exon 1 polymorphic CAG repeat length (CAG_n) is a risk factor for early onset breast cancer in North Indian population.

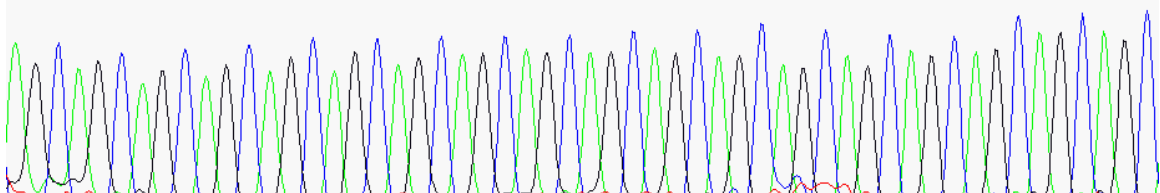


Fig. 2: Sequence electrogram of a breast cancer patient showing CAG repeats (33 repeats) for AR gene

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

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<i>In collaboration with</i>	:	<i>Dr. D Bhatnagar, Dr. R Mohil, Dr. Chintamani, SJH</i>
<i>Technical Staff</i>	:	<i>Mrs. Valsamma Matthews, Mr. Jagdish Pant</i>
<i>Duration</i>	:	<i>2003-2006</i>

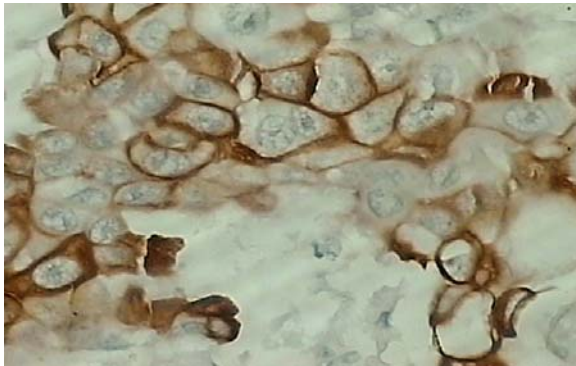
Aims, Objectives and Background

Studies on molecular characterization of early onset and familial cancer have previously shown decreased expression of ER in early onset and familial cases and increased expression of cyclin D in familial cases. 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 BRCA 1 /2 genes. During the year under report, we have studied cytokine profile of early onset breast cancer to understand histogenesis.

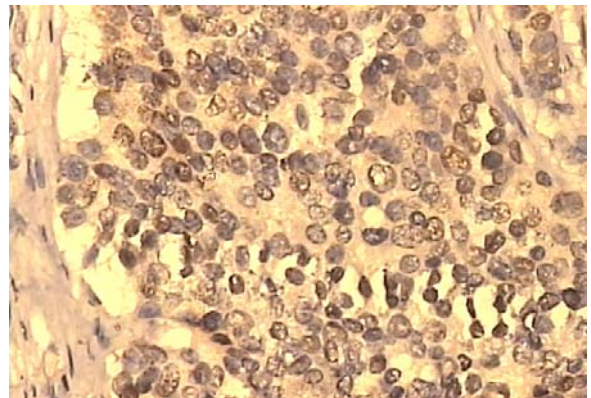
Work done during the year

The cytokeratin profile of breast cancer cases was studied to evaluate the cellular origin, viz. : basal or luminal cells and functional classification of breast cancer. The study included 110 cases of breast cancer and immunohistochemical profile of CK5/6 and CK7 was studied in paraffin embedded sections. The basal cytokeratin CK5/6 was expressed in 26.3% cases and the luminal cytokeratin CK7 in 8.69% cases. CK14, studied in 30 cases was seen to be expressed in 6/30 (20%) cases. Correlation of these cytokeratins with molecular profile of breast cancer cells in different groups is being studied.

Androgen receptor positivity was observed in 40 cases (40%) of carcinoma breast. Estrogen receptor immunostaining was seen in 35% of cases while 44% of cases were positive for progesterone receptor expression. Immunostaining for EGFR was positive in 40% of cases while TGF-beta receptor showed positivity in 32% of cases. Statistically significant higher positivity for ER, PR and TGF-beta receptor was observed in cases positive for AR. EGFR positivity also showed a higher trend in AR positive cases but was not statistically significant. Of the total 30 familial cases of breast cancer, ER and PR expression was seen in 10 cases (33.3%) and 17 cases (56.6%) respectively. AR positivity was found in 15 cases (50%) while EGFR was expressed in 14 cases (46.6%) of familial breast cancer. TGF-beta receptor positivity was seen in 10 of these cases (33.3%).



Cytokeratin 7 expression in a case of infiltrating ductal carcinoma



Androgen Receptor nuclear immunopositivity in infiltrating duct carcinoma, breast

3. Type 1 Growth Factor Receptor Family: Expression and Correlation with Response to Neo-adjuvant Chemotherapy in Locally Advanced Breast Cancer

Scientific staff : ***Dr. Sunita Saxena, Dr. LC Singh,
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In collaboration with : ***Dr. Dinesh Bhatnagar,
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SJH***
Duration : ***2006-2009***

Aims, Objectives and Background

It is planned to study the quantitative analysis of expression of type 1 growth factor receptor genes including (EGFR, c-erbB-2, c-erbB-3) in locally advanced breast cancer and their correlation with MDR1 gene in response to pre and post neo-adjuvant chemotherapy.

Locally advanced breast cancer is the most common breast cancer that has not spread from the breast to distant sites in the body. Stage II-A, II-B and III breast cancer are often consider to be locally advanced. It is most common type of presentation in India. The recommended protocol for management of locally advanced breast cancer is neo-adjuvant chemotherapy with three cycle of CMF (Cyclophosphamide, methotrexate, 5-flourouracil). After 3 cycles of neo-adjuvant chemotherapy, the patients will be taken up for surgery. Then the patients may be grouped into responders, partial responders and non-responders. Several lines of evidence suggest that type 1 growth factor receptor family (EGFR, c-erbB-2, c-erbB-3) is involved in breast cancer development and progression. This receptor family has a critical role to play in normal development, and aberrant expression or activation of the receptor family has been associated with breast cancer. Multidrug resistance gene has been found to be associated with a worse final outcome in locally advanced breast cancer after chemotherapy.

Work done during the year

Fourteen matched samples of pre- and post- NACT have been collected. Of these, 1 was a complete responder, 7 were partial responders and 6 were non-responders. Total RNA has been isolated from the samples by TRIzol method (Figs. 1&2) followed by generation of cDNA using High Capacity cDNA Archive kit (*Applied Biosystems*) according to the manufacturer's protocols.

Standardization of the relative quantitation of expression levels of selective genes (EGFR, c-erbB-2, c-erbB-3, MDR1) in one case of breast tissue (Figs. 1&2) was carried out by real time RT-PCR (*ABI 7000 SDS, Applied Biosystems*) with cDNA as template using TaqMan probe Assay. Primers and Probe for the targets (EGFR, c-erbB-2, c-erbB-3, MDR1) and internal control genes GAPDH were designed by *Applied Biosystems, (Foster city, CA, USA)*. A singleplex reaction mix was prepared according to the manufacturer's protocol of Assays-on-Demand Gene Expression products and included 10 μ L of TaqMan Universal PCR Master Mix, 1 μ L of 20x Assays-on-Demand Gene Expression Assay Mix (all gene expression assays have a carboxyfluorescein reporter dye at the 5'-end of the TaqMan minor groove binder probe and a non-fluorescent quencher at the 3'-end of the probe) and 5 μ L of cDNA (50ng) diluted in Rnase-free water, in a total 20 μ L volume.

The mean expression level of selected genes (EGFR, c-erbB-2, c-erbB-3, MDR1) was calculated for 1 case in breast tissue normalized to a house keeping gene (GAPDH). The data of real time RT-PCR are presented as threshold (C_T) values where C_T is the fractional cycle number at which the fluorescence passes the threshold. The average C_T was calculated for both interest of genes and GAPDH and following equations are used for the calculation ΔC_T and $\Delta\Delta C_T$.

$$\Delta C_T = \text{Gene of interest } C_T - \text{Endogenous } C_T$$

$$\Delta\Delta C_T = \Delta C_T \text{ of sample} - \Delta C_T \text{ of calibrator}$$

Here, ΔC_T of sample is the C_T value for any sample normalized to the endogenous house keeping gene and ΔC_T of calibrator is the C_T value for the calibrator also normalized to the endogenous house keeping gene. The N-fold differential expression of the genes for

a sample compared with pre neo-adjuvant chemotherapy samples (calibrator) was expressed as $2^{-\Delta\Delta CT}$.

The expression level of target genes like EGFR, c-erbB-2, MDR1 were found to be up-regulated in this case, while c-erbB-3 gene was down-regulated by 0.41 in case of BC-7. But, more cases have to be studied for validation of data (Figs. 1 & 2).

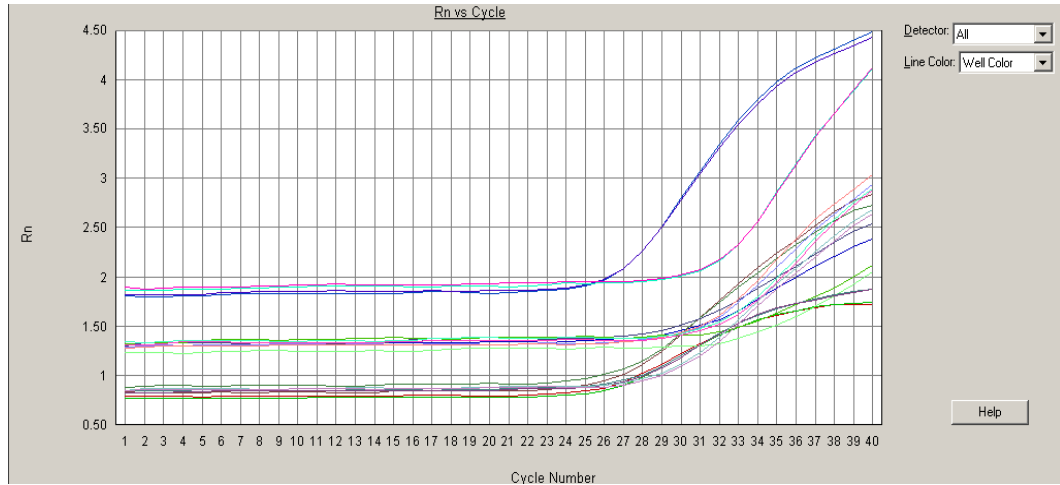


Fig. 1: Real time amplification plot of EGFR, c-erbB-2, c-erbB-3, MDR1 and GAPDH of non-responders patient (BC-7) to NACT

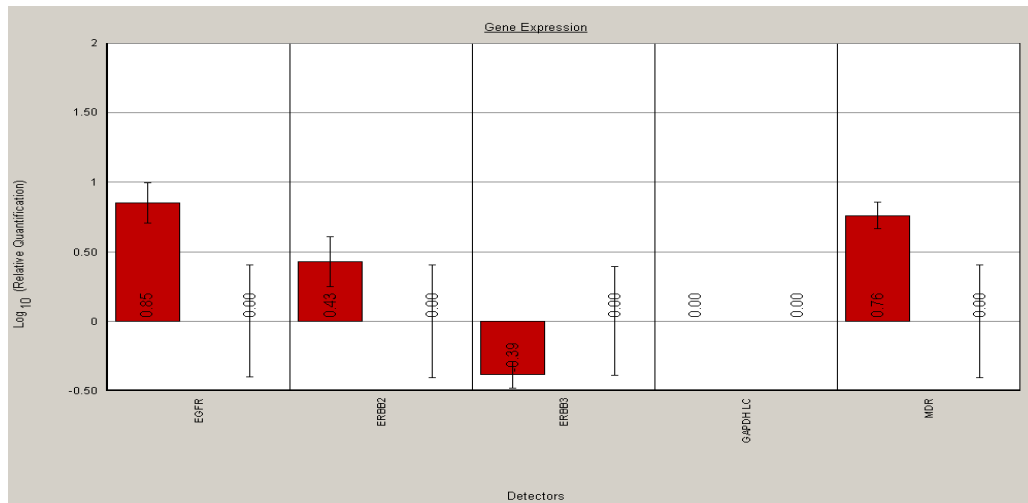


Fig.2: Gene Expression Profile of non-responders patient (BC-7) to NACT

4. Establishment and Characterization of Breast Cancer Cell Lines from Primary Breast Cancers

Scientific staff	:	Dr. Sunita Saxena, Dr. SA Raju Bagadi, Dr. Sujala Kapur
In collaboration with	:	Prof. D. Bhatnagar, Dr. Chintamani, Dr. RS Mohil, SJH
Technical Staff	:	Mr. Jagadish Pant
Duration	:	2006-2009

Aims, Objectives and Background

Cell cultures established directly from human tumours serve as important models for studying and manipulating the potentially relevant molecular and cellular processes underlying malignant breast disease. Cell lines provide an unlimited, self-replicating source of cells that can be widely distributed to facilitate comparative studies. Hence, well-characterised cell lines are powerful research resources for studying cancer cell biology and for the development of new strategies against cancer. The majority of breast carcinoma cell lines have been established from tumour metastases, in particular malignant pleural effusions, while relatively few have been established from primary tumours. In India, the aetiology of breast cancer appears to be different from that in the west, with early onset, short disease-free interval and high mortality. It is aggressive in nature; most of these cases are also not suitable for hormone treatments as these are ER negative. There is no breast cancer cell line available from Indian population in the international or national cell culture repository. Therefore, there is a need to establish cell lines from Indian population, which may be useful for understanding molecular mechanisms involved in breast tumorigenesis in Indian population. Thus, the present study is aimed to develop breast cancer cell line from Indian patients preferably from lower age group primary breast tumours that may serve as a good *in vitro* model for understanding early breast tumorigenesis and mechanisms underlying breast tumorigenesis in Indian population.

Work done during the year

This project has been designed and presented to Indian Council of Medical Research for funding as Task Force Project in the NCD division of ICMR in the month of January 2006. The project has been accepted and recommended for the funding in the month of May 2006 and approved. The routine tissue culture laboratory work has been streamlined in order to achieve and maintain a sterile environment in the tissue culture facility. MCF7 cell line (Fig. 1) has been procured from the National Center for Cell Science in order to use as a positive control for the future project and for checking the tissue culture procedures.

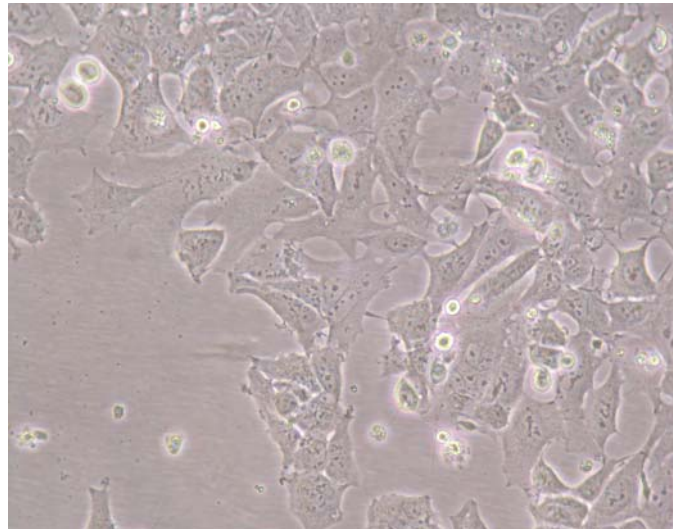


Fig. 1: MCF7 cells at 20X

MOLECULAR BIOLOGY OF PROSTATIC NEOPLASIA

1. Study on Microsatellite Instability in Androgen Receptor Gene, p53 Gene Polymorphisms and Expression Profile of Mismatch Repair Genes in Prostate Carcinoma

Scientific staff : ***Dr. Sunita Saxena, Dr. Anju Bansal, Ms. Abha Soni***
In collaboration with : ***Dr. NK Mohanty, SJH***
Duration : ***2005-2008***

Aims, Objectives and Background

Prostate cancer is the most common malignancy in men and the second leading cause of cancer death in the western world. Prostate cancer is a heterogeneous disease with multiple loci contributing to susceptibility. But the molecular pathogenesis of prostate cancer is poorly understood. The genetic predisposition to prostate cancer is well established, as genomic instability is a common feature of many human cancers. The DNA-damage–signaling pathway plays a critical role in maintaining genomic stability in response to a variety of DNA-damaging events. Disruption of this pathway has been shown to be pivotal in cancer development, since several proteins involved in this pathway (such as TP53 or p53) are frequently mutated in human cancers. The androgen receptor gene variants have been examined in relation to both prostate cancer risk and disease progression. Altered activity of the androgen receptor due to inherited variants of the androgen receptor gene may influence risk of prostate cancer. Knowing the genetic markers involved in prostate cancer could help determine which men are at highest risk. The present study aims at studying the genetic polymorphisms in androgen receptor gene, p53 gene in prostate cancer and correlation of the microsatellite instability in androgen receptor gene and mutations/polymorphisms in p53 gene with progression of prostate carcinoma. The

study will also aim at profiling the expression of mismatch repair genes using real time PCR.

Work done during the year

Blood samples from 70 patients of Cancer Prostate and 87 Benign Prostatic Hyperplasia have been collected. DNA has been extracted from all these blood samples using standard Phenol-chloroform extraction procedure. DNA extraction has also been done from paraffin embedded tissue blocks of biopsy samples. PCR for the CAG Repeat in Androgen Receptor gene has been successfully performed on patients' samples. Standardization of SSCP for CAG repeat polymorphisms (Fig. 1) has been done using 5% acrylamide gel containing glycerol. For mutational analysis of p53 gene, exons 5, 6, 7 and 8 were selected and exon specific primers were designed. Standardization for all the exons was also performed at different $MgCl_2$ concentration and at different annealing temperatures. Sequencing for CAG repeat in AR gene (Fig. 2) and exons 5 and 7 of p53 gene has been standardized using Big-dye terminator v 3.1 reaction mixture (*Applied Biosystems*) and the initial screening for mutations has been done on few patients and control samples.

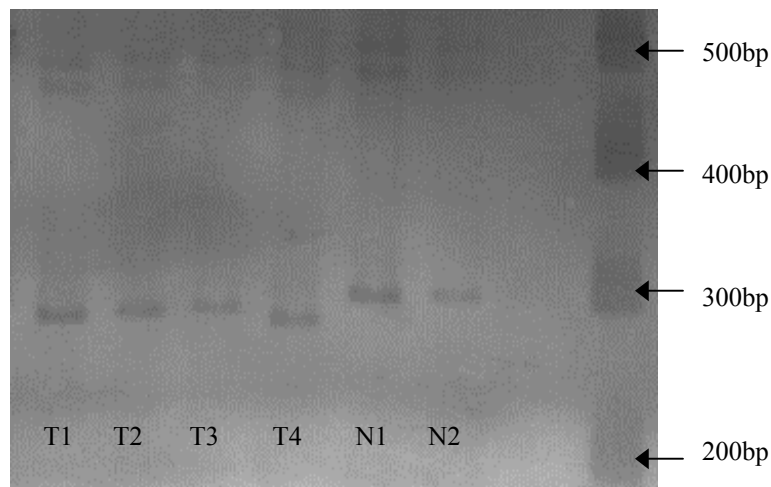


Fig. 1: Picture showing SSCP for CAG repeat analysis of AR gene on patients' samples (T1-T4) control individuals (N1 and N2)

BLADDER CANCER

1. Study of the host immune response in patients with superficial transitional cell carcinoma

Scientific staff : *Dr. Sunita Saxena, Dr. Usha Agrawal, Dr. Saurabh Varma, Dr. Abhilasha Agarwal*

In collaboration with : *Dr. NK Mohanty, SJH*

Technical Staff : *Mr. Jagdish Pant, Mr. PD Sharma*

Duration : *2001-2006*

Aims, Objectives and Background

The study evaluates the Th1 and Th2 balance in PBMCs of Transitional Cell Carcinoma patients in order to assess the immunological factors influencing the anti-neoplastic activity of intravesical combination BCG/IFN α 2b in patients. While it has been previously reported that an imbalance exists between the Th1 and Th2 immune responses which attributes to the immune dysregulation in superficial TCC patients, the role played by Th1/Th2 cytokines in tumor recurrence and progression after TURBT and intravesical immunotherapy in these patients has still not been well elucidated. Therefore, the present study was designed to measure the dynamics of systemic Th1 and Th2 cytokine responses in patients who had not received any therapy prior to TURBT (pre-therapy) and compare the immune responses in the same group of patients following TURBT and intravesical combination therapy (post-therapy) using flowcytometry.

Work done during the year

Forty-one patients with histologically confirmed superficial transitional cell carcinoma treated by transurethral resection followed by intravesical combination immunotherapy formed the study group. Patients were followed up every third month for duration of 6 months to 3 years (median follow-up of 22.5 months).

The expression of circulating levels of Th1 and Th2 cytokines were determined in the sera of normal healthy subjects (20) and superficial TCC patients prior to therapy (41, pre-therapy) and following combination BCG/IFN α 2b intravesical therapy (37, post-therapy). The mean levels of Th1 cytokines, IL-2 and TNF α were significantly reduced whereas significantly enhanced levels of Th2 cytokines like IL-4, IL-6 and IL-10 were observed in pre-therapy patients as compared to healthy volunteers.

The mean values of IFN- γ and IL-2 were reduced following combination therapy (BCG+IFN- α 2b) in post-therapy patients as compared to normal healthy subjects, however the mean difference between these groups was not statistically significant whereas, post-therapy patients showed significantly higher mean value of TNF- α as compared to normal controls ($p < 0.004$). No significant difference was observed in the means of IL-4, IL-6 and IL-10 values between normals and post-therapy patients (Fig. 1).

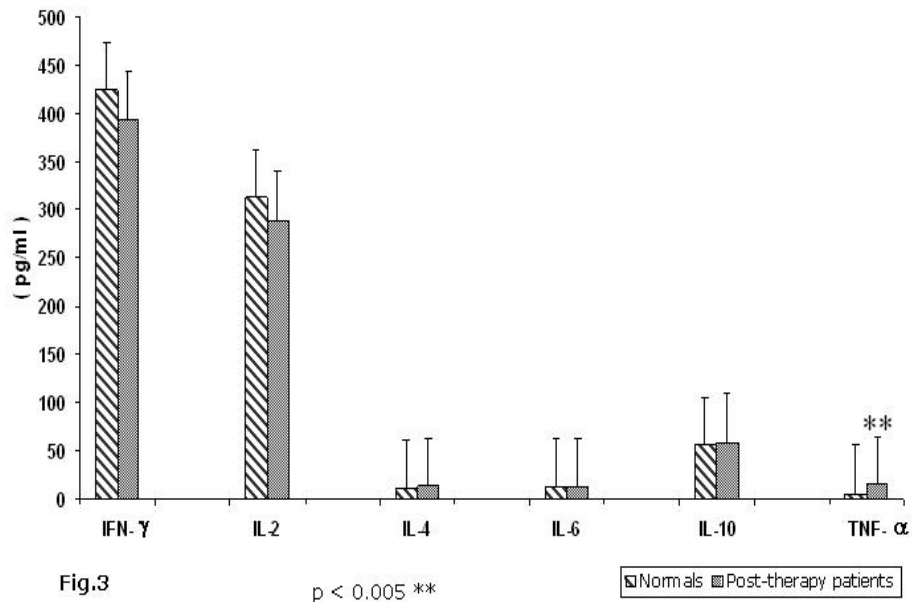


Fig. 1: Graph showing significant expression of TNF- α in post therapy patients as compared to normals

2. Study of Tumor Markers in Patients of Superficial TCC of Bladder

Scientific staff : **Dr. Sunita Saxena, Dr. Usha Agrawal, Dr. Saurabh Varma, Dr. Abhilasha Agarwal, Dr. Payal Salgia**

In collaboration with : **Dr. N K Mohanty, SJH**

Technical Staff : **Mr. Jagdish Pant, Mr. P D Sharma**

Duration : **2001-2006**

The expression of p53, p21^{waf1} and growth factor receptors such as VEGFR and TGFβR was studied in 32 cases of superficial transitional cell carcinoma to evaluate the relevance of transforming proteins in the progression of superficial bladder cancer. Expression of p53 was seen in 68.75% cases, and that of VEGFR in 46.87%. Loss of p21^{waf1} was observed in 32.75% cases and TGFβR in 56.25% cases. The study showed that while p53 and VEGFR expression was increasing with increasing grade of tumor (p<0.01), TGFβR decreased with increasing grade of tumor (p<0.01). The study also showed that the biomarker profile of p53+/VEGFR+/p21+/TGFβR – was a predictor of recurrence, progression and decreased survival. TGFβR expression is shown on confocal microscope (Figs. 1-2).

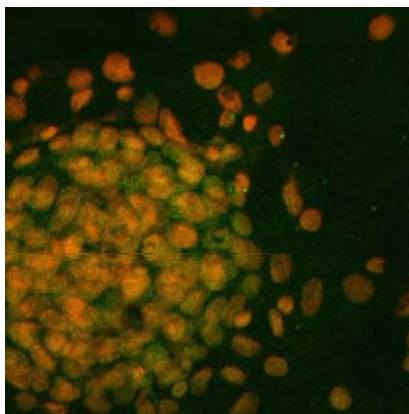


Fig. 1: VEGFR Expression in a case of TCC bladder on cell spots

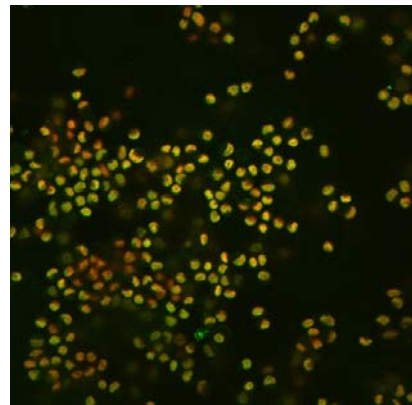


Fig.2: TGFβR expression in TCC bladder

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

Scientific staff : ***Dr. Saurabh Varma, Dr. Usha Agrawal, Dr. Sunita Saxena***
In collaboration with : ***Dr. NK Mohanty, SJH***
Technical Staff : ***Mr. PD Sharma***
Duration : ***2003-2006***

This study was done to evaluate the role, pattern in expression of tissue transglutaminase in invasive and non-invasive TCC of human bladder in correlation with tumor grades and other adhesive proteins like $\alpha 6\beta 4$ and CD44.

The bladder biopsy samples were collected from patients from Urology Department of Safdarjung hospital with some normal tissue around the tumor portion. The samples were performed for DNA ploidy to get the DNA contents. On the basis of DNA contents, the cells of different phases of cell cycle like G0G1 and G2M were separated in different tubes by putting gates on these sub-populations using flow cytometer (Fig. 1). The separated cells of different phases of tumor cell cycle were again acquired to observe that they should fall in same gate to confirm their purity. The separated cells were then taken to study the expression of tTG using immunohistochemistry to evaluate its expression and its pattern in tumor cells (Fig. 3). The expression of tTG was more in higher-grade tumors and invasive one as well in comparison to internal control.

Immunohistochemistry of the paraffin embedded tissue of the samples was done to study the expression of adhesive proteins like $\alpha 6\beta 4$. The alpha 6 beta4 showed loss of expression from low-grade tumors to high-grade tumors.

The biopsy samples were also studied for the expression CD44 on flowcytometer (Fig. 2), which plays important role in adhesion. The samples were performed to disintegrate the cells to make single cell suspension and then it was centrifuged to get pellet and incubated with primary antibody for CD44 and thereafter with anti-mouse secondary antibody conjugated with FITC. The higher expression of CD44 was inclined towards high-grade tumors.

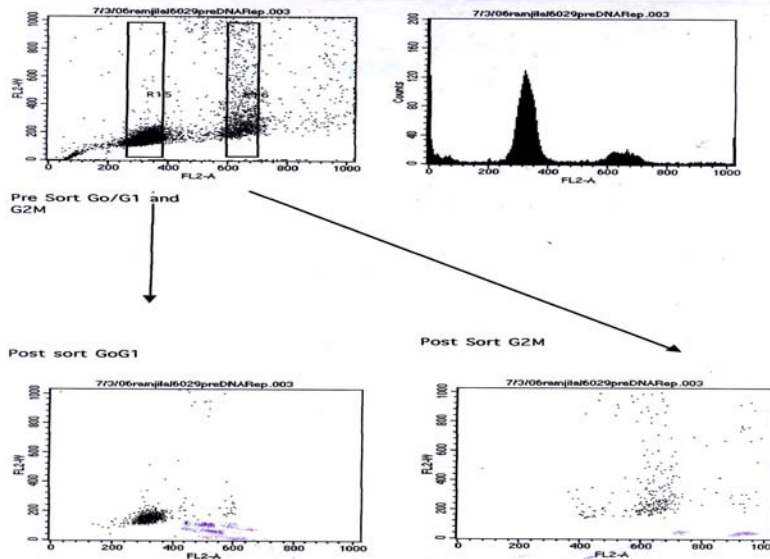


Fig.1: Dot plots showing post -sorted cells of Go/G1 and G2/M phases of cell cycle using Flowcytometer.

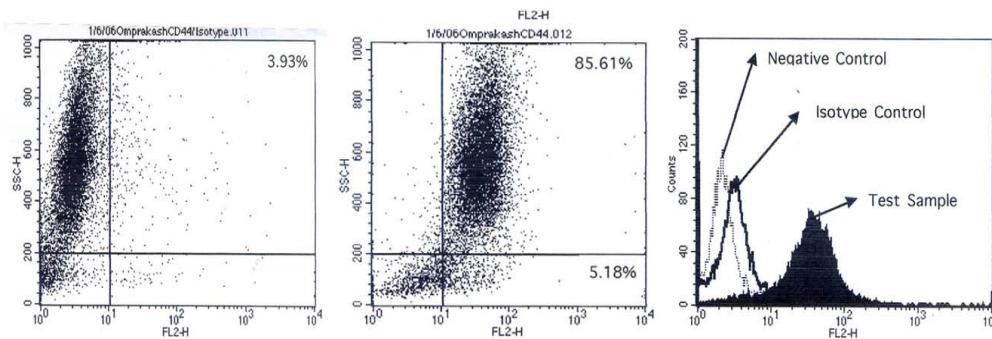


Fig.2: Showing the histogram of control or unstained PBMCs, isotype control of CD44 and expression of CD44 in TCC of human bladder

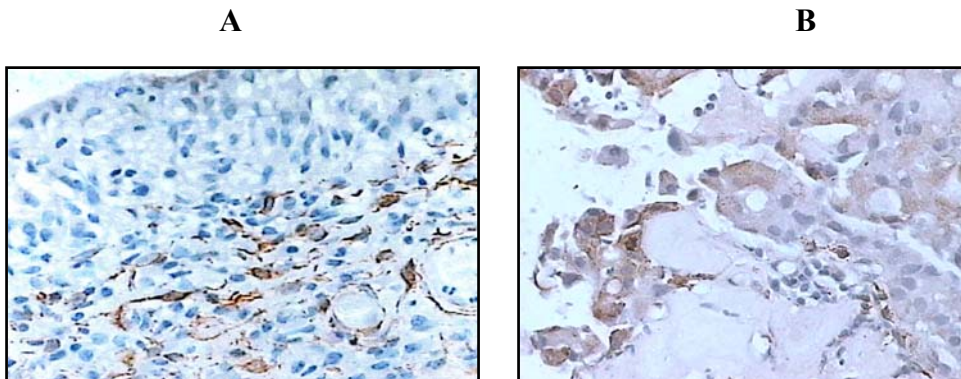


Fig. 3: A Showing the expression of tTG as internal control.
B Tumor cells showing the expression of tTG in TCC sample using immunohistochemistry.

Cancers in Northeast Region of India

A high incidence of several cancers, particularly those associated with use of tobacco / pesticides (NCRP, ICMR), have been reported from NE India. Further, aggregation of oesophageal Ca in families is a long-observed and well-documented phenomenon in Assam. Multi-centric studies have been initiated to find out if genetic factors, in addition to common environmental exposure and dietary habits could possibly explain the high prevalence.

Studies on oesophageal cancer started in 2004 (IOP, ICPO, RMRC Dibrugarh and BBCI Guwahati). In 2005, this study was extended to include other tobacco and pesticide related cancers (oral, stomach, lung, breast cancer and NHL). The aim is to establish link between carcinogenic contents of tobacco and pesticides and genetic variation including polymorphism/mutations and gene expression pattern in this region. Identification of risk of developing certain cancers with relation to low/high penetrance genes and their effects on environmental carcinogen exposure including exposure to tobacco, pesticides and their by products will be analyzed. A study of tobacco and pesticide-exposed populations will provide opportunities to characterize the somatic or germline mutations and polymorphisms that give rise to specific cancers and to identify the inherited genetic traits that confer susceptibility or resistance.

Collaborating Institutes

Institute of Pathology, New Delhi

Institute of Cytology and Preventive Oncology, Noida

Regional Medical Research Center, Dibrugarh, Assam

Dr. B Barooah Cancer Institute, Guwahati, Assam.

Six PBCRs of NE Region (Aizawl, Gangtok, Guwahati, Silchar, Dibrugarh, Imphal)

Ongoing Work

- I Comprehensive study of carcinoma oesophagus at North East India - Multidiscipline approach.
Collaborative, Multicentric, ICMR Task Force Project (2004-07)
- II Effect of Pesticide Exposure in Causation of Cancer in North East India.
Collaborative, Multicentric, ICMR Task Force Project (2005-08)
- III Role of Tobacco Use in Causation of Cancer in North East India.
Collaborative, Multicentric, ICMR Task Force Project (2005-08)

1. Comprehensive Study of Carcinoma Oesophagus at Northeast India-Multidiscipline Approach

Scientific staff : ***Dr. Sunita Saxena, Dr. Sujala Kapur, Mr. Indranil Chatterjee***
In collaboration with : ***Dr. J Mahanta, Dr. RK Phukan, RMRC. Dibrugarh, Dr. J Purkayastha, Dr. AC Katak, BBCI, Guwahati, Assam Dr BC Das, Dr JK Sharma, ICPO, NOIDA***
Duration : ***2004-2007***

Aims, Objectives and Background

The objective is to study the frequency of allelic alteration at specific chromosome loci in the blood samples of familial and non-familial oesophageal cancer patients. In addition, differential gene expression pattern is also being evaluated in these patients. The study aims to find out frequency of allelic alteration and to identify alteration in gene expression pattern that may be responsible for the increased susceptibility to development of oesophageal cancer in these patients

Work done during the year

Blood samples of 42 oesophageal cancer patients were obtained from Dr. Bhubaneshwar Borooah Cancer Institute (BBCI), Guwahati, Assam. Age range was 32-85 years, 33 patients were males and 9 were females. 11 patients had family history of cancer and 36 patients were tobacco users.

Genotyping of Microsatellite Loci

Microsatellite markers that have earlier been analyzed for association with genetic susceptibility in other high-incidence regions of oesophageal cancer (*Clinical Cancer Research*, 5:3476-3482, 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 genotyping of microsatellite loci by SSCP (single stranded conformation polymorphism) using non-radioactive method (Fig. 1).

Preliminary results show high frequency of allelic alterations in D17S1303, D11S1984 and D3S4545 microsatellite loci. Of 42 patients, two patients showed alterations in four-microsatellite loci (D13S796, D3S4545, D3S1766 and D17S1303) and 8 patients showed alterations in three-microsatellite loci (D17S1303, D13S796 and D11S1984). There was no significant association of allelic alterations between tobacco users and non-users and between familial and non-familial patients with oesophageal cancer.

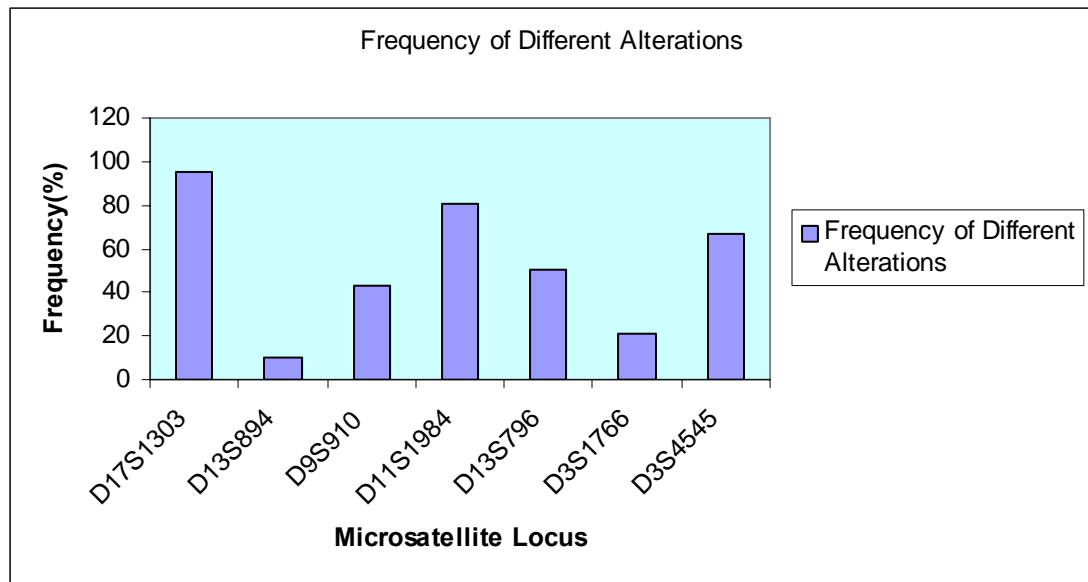


Fig. 1: Frequency of allelic alterations at different microsatellite loci in esophageal cancer patients

Studies on Gene Expression

Total RNA was isolated from tumor and matched normal tissue of 16 patients with oesophageal squamous cell carcinoma. Pooled tumor tissues RNA was labeled with Cy3-dUTP and pooled normal tissue RNA was labeled with Cy5-dUTP by Direct labeling method. The labeled probes were hybridized with Human 10K cDNA chip and expression profiles were analyzed (Fig. 2).

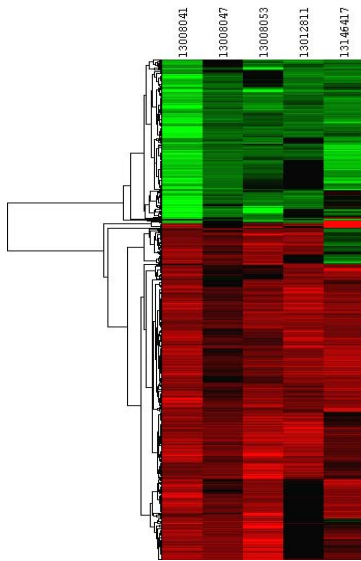
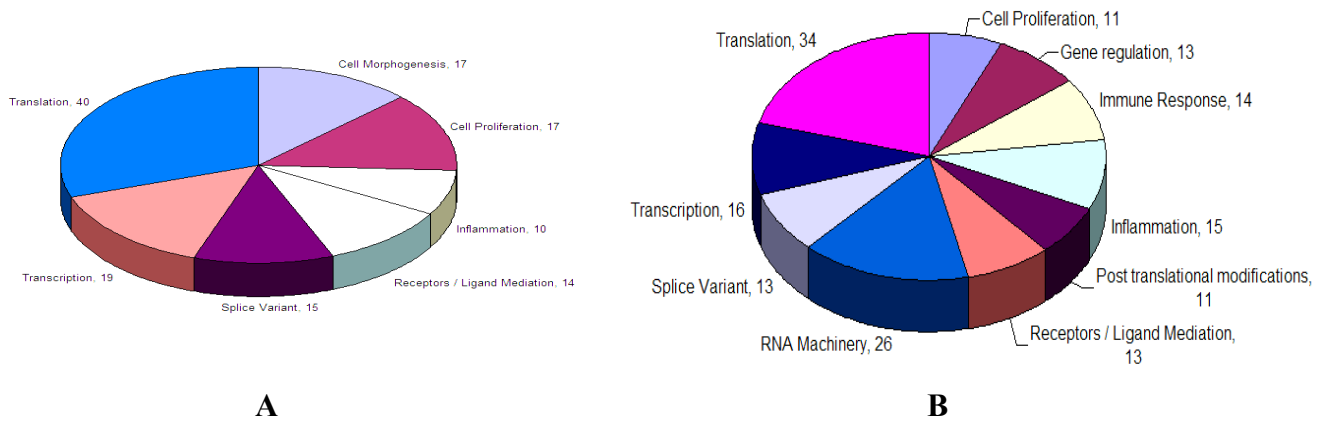


Fig. 2: Hierarchical Clustering (Average Linkage Clustering) of the genes that were over or under expressed in tumor versus normal tissue in 16 patients with esophageal cancer

Preliminary experiments have shown several genes with differential expression in tumor tissue of esophageal cancer when compared with normal esophageal tissue. Most of these genes involve cellular physiological process, metabolism, cell communication, morphogenesis and cell death (Figs. 3A-3B). Genes involved in G-protein coupled receptor activity, anti-apoptosis activity, MAPK signaling pathway, cellular proliferation and calcium-activated potassium channel activity were significantly up-regulated. Genes involved in keratinization, structural constituent of cytoskeleton, endopeptidase inhibitor activity, humoral immune response, base-excision repair and protein biosynthesis were significantly down-regulated.



**Fig. 3: A: Significant functional classes of upregulated genes
B: Significant functional classes of downregulated genes**

Future Plan of Action

Differential gene expression pattern will be compared in patients with familial and non-familial oesophageal cancers. More microsatellite loci will be analysed to see if alterations in these loci can be related to familial cancer.

2. Effect of Pesticide Exposure in Causation of Cancer in North East India

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<i>In Collaboration With</i>	:	<i>Dr. J Mahanta, Dr. HK Chaturvedi, Dr. NC Hazarika Dr. HK Das, RMRC Dibrugarh Dr. HN Saiyed, Dr. VN Gokani, Dr. VK Bhatnagar, NIOH, Ahmedabad Dr. BC Das, Dr. Alok C Bharti, Dr. Maushmi Bhardwaj, Dr. JK Sharma ICPO, Noida Dr A Nandakumar, NCRP Bangalore</i>
<i>Duration</i>	:	<i>2005-08</i>

Aims, Objective and Background

North-eastern states of the country have reported a very high incidence of cancer of all anatomical sites together as well as high incidence of sites of cancer related to pesticide exposures. There is extensive use of pesticides in tea gardens in Northeast which can lead to widespread occupational and environmental exposures. This study envisages to investigate the link between exposure to pesticides and genetic variation including polymorphism/mutations associated with ethnic variation. The study is being carried out in the six population based cancer registry areas of the NE (Guwahati, Aizwal, Sikkim, Dibrugarh, Silchar and Imphal) with a case control epidemiologic design. At our Institute,

samples are obtained from three PBCRs (Guwahati, Aizawal, Sikkim) from patients with breast cancer and lymphoma.

The objectives of the study are to investigate the role of pesticide exposure in the occurrence of cancer, to study mutation/polymorphism known to confer susceptibility to develop specific cancers and to study the gene expression profile in cancers associated with pesticides. The specific objectives for studies in Breast Cancer include identification of sequence variants in high penetrance and low penetrance genes. For Lymphoma, study of the association of pesticide exposure with allelic alterations and bcl-2-IgH translocation will be done.

Work done during the year

Breast Cancer

For the samples received from patients with Breast Cancer, genomic DNA was extracted and quantified from 16 whole blood samples and matched control samples. Ten exons of BRCA2 genes were amplified using specific primers. Radioactively labelled Heteroduplex gel analysis was done for identification of sequence variants of BRCA 2 gene. Shifts in Exon 2 of BRCA 2 gene with three visible patterns were seen in samples and controls. Sequencing later confirmed these to be polymorphism (Fig. 1).

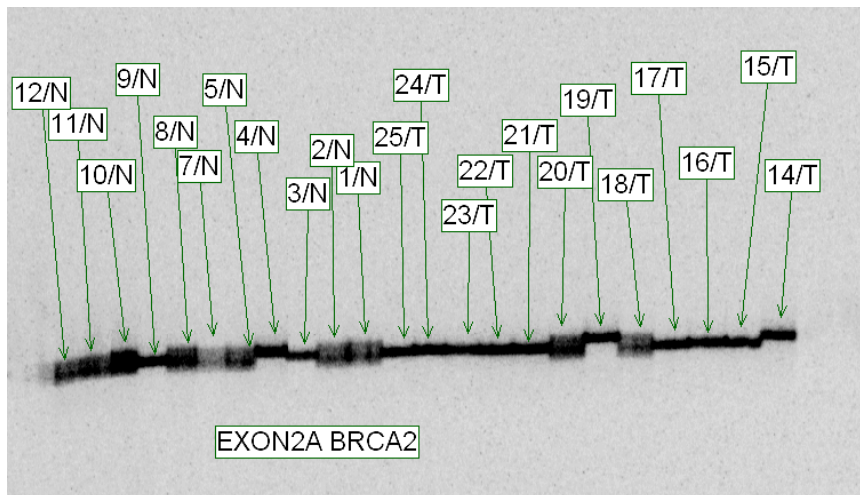
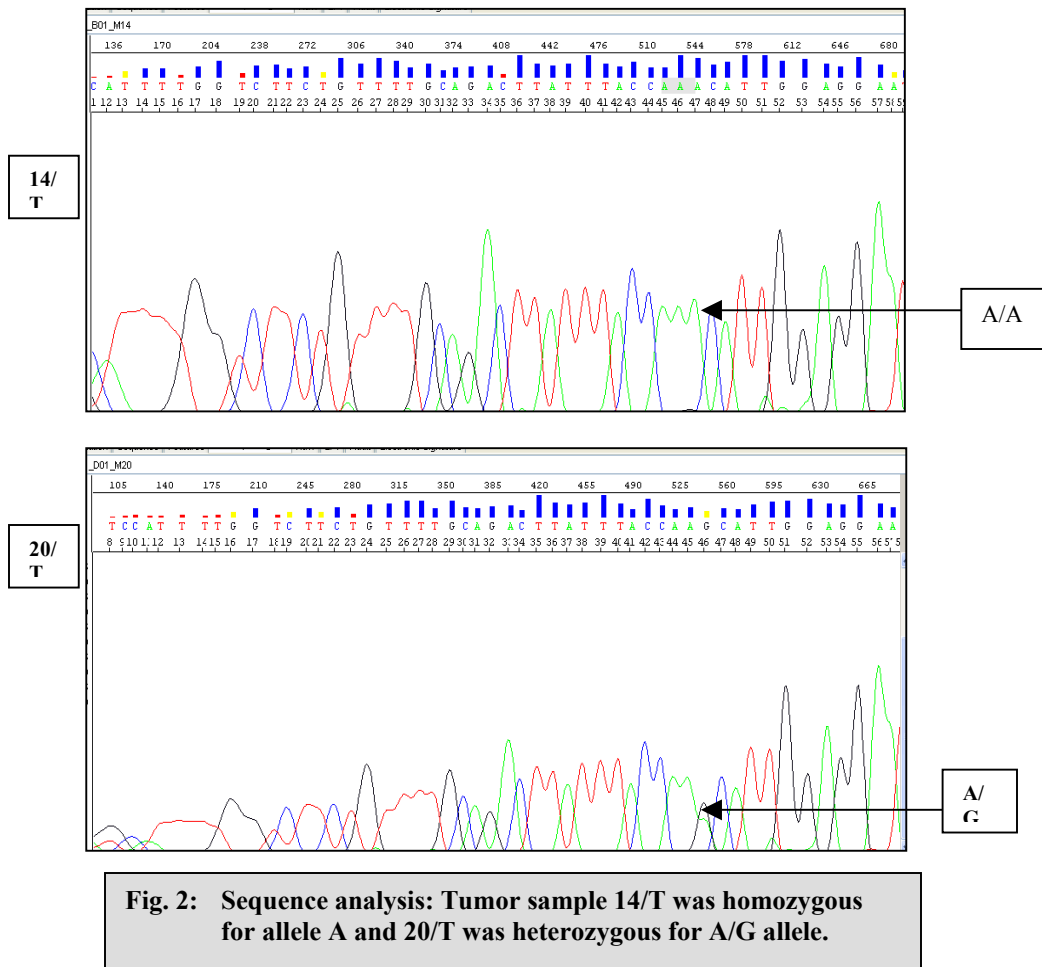


Fig. 1: Heteroduplex analysis of the tumor and control samples in which shifts in exon 2A of BRCA2 gene were seen. Three patterns are visible.

Sequence analysis was done on the two tumor samples in which shifts were seen. A G>A transition was seen in the 276th nucleotide of BRCA2 gene. This change is a same sense mutation coding for Lysine and therefore a polymorphism (Fig. 2). Sample 14/T is homozygous for the A allele and sample 15/T is heterozygous for the A/G allele.



Lymphoma

Alterations at 9p21 have been reported to be associated with the development and progression of T- and B-cell lymphomas. For studies on Lymphomas, microsatellite markers located at 9p21 have been selected for their possible association with pesticide exposure (Table 1).

Marker	Repeat	Size	%HET	Position
D9S169	Dinucleotide	259-275	83.86	9p21
D9S1604	Dinucleotide	163-175	56.00	9p21
D9s1752	Dinucleotide	150-160	50.00	9p21
D9S1748	Dinucleotide	130-150	87.00	9p21
D9S1751	Dinucleotide	150-170	65.00	9p21
D9S171	Dinucleotide	159-177	80.43	9p21
D9S958	Dinucleotide	291-303	58.00	9p21
D9S1747	Dinucleotide	130-150	70.00	9p21
D9S161	Dinucleotide	119-135	78.28	9p21

Table 1: Microsatellite markers selected for studies of Non - Hodgkin's Lymphoma

PCR standardization was carried out for the markers at different conditions of temperature and MgCl₂ concentration using genomic DNA (Fig. 3).

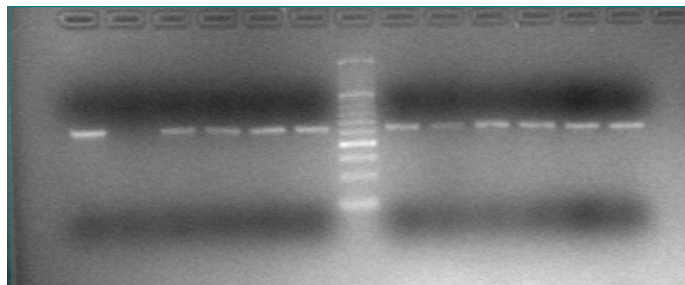
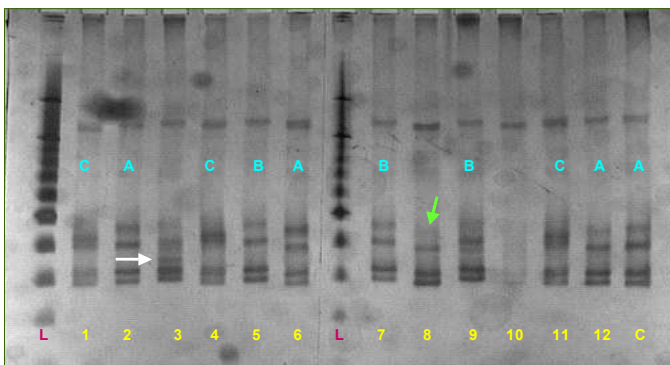


Fig.3: EtBr stained agarose gel showing standardization of marker D9S169

PAGE analysis was performed to determine the variation and alterations in the microsatellite markers (Fig. 4). Three different patterns were obtained (Fig. 4).



**Fig. 4: PCR-PAGE analysis of locus D9S169. Gel shows three patterns (A,B,C)
Lane 2,6,12,13 show pattern A
Lane 5,7,9 show pattern B
Lane 1,4,11 show pattern C
Lane 3 shows a unique extra band
Lane 8 has a missing band
Lane 10 has inadequate DNA loaded**

3. Role of Tobacco Use in Causation of Cancer in North East India

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<i>In Collaboration With</i>	:	<i>Dr. J Mahanta, Dr. HK Chaturvedi, Dr. NC Hazarika Dr. HK Das, RMRC Dibrugarh Dr BC Das, Dr. Alok C Bharti, Dr. Maushmi Bhardwaj, Dr. JK Sharma, ICPO, Noida Dr. HN Saiyed, Dr. VN Gokani, Dr. VK Bhatnagar, Dr. Nandkumar, NCRP, Bangalore</i>
<i>Duration</i>	:	<i>2005-2008</i>

Aims, Objective and Background

Northeastern states have reported a high incidence of cancer associated with use of tobacco. This study envisages to investigate the link between carcinogenic contents of tobacco and genetic variation including polymorphism/mutations associated with ethnic variation. The objectives are to study genetic alterations known to confer susceptibility to develop specific cancers with tobacco exposure. It will also provide information on the role of common dietary and environmental factors that may act as cofactors to bring about genetic changes. The study is being carried out in the six population based Cancer Registry Area of the NE with a case control epidemiologic design. The cancers included in this project include cancer of lung, stomach, oral cavity and oesophagus.

Work done during the year

Lung Cancer

For studies in lung cancer, genetic factors and gene-environment interaction will be analyzed by study of allelic alteration in the chromosomal loci 3p14.2 (FHIT) and 3p21 that are known to have a strong co-relation with lung cancer associated with tobacco use (Table

I). During the year under report, blood samples from nine patients were analyzed for allelic alteration.

Marker	Repeat	Size (bp)	% Max.	Position
D3S1300	Dinucleotide	217-241	83.27	3p14.2
D3S4103	Trinucleotide	165-185	83.00	3p14.2
D3S1312	Dinucleotide	215-225	76.72	3p14.2
D3S1313	Dinucleotide	228-238	68.98	3p14.2
D3S1234	Dinucleotide	99-125	66.00	3p14.2

Table 1: The microsatellite markers selected for the study in lung cancer

PCR standardization of Marker D3S1312 was done with 100ng template DNA at 1.5mM-2.0mM MgCl₂ concentration and with temperature gradient from 61⁰-68⁰C. (Fig. 1) followed by PAGE analysis (Fig. 2).

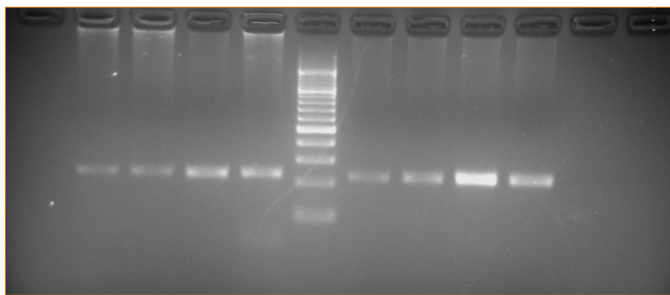


Fig. 1: Agarose gel showing PCR standardization of locus D3S1312

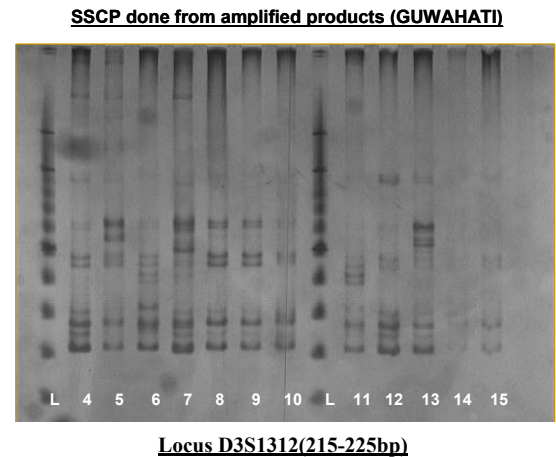


Fig. 2: Polyacrylamide gel electrophoresis shows four patterns (A,B,C,D)
 Lanes 4,8,9,10,12 show pattern A
 Lanes 5,13 show pattern B
 Lanes 6,11 show pattern C
 Lane 7 shows a unique pattern D
 Lanes 5,6,11 have a missing band

Gastric Cancer

For studies in gastric cancer, genetic factors and gene-environment interaction will be analyzed by study of allelic alteration in the chromosomal loci known to have a strong co-relation with stomach cancer and associated with tobacco use (Table 2). The degree of

chromosomal losses and the presence of microsatellite instability in gastric carcinomas have been categorized into low-risk and high risk genotypes. During the year under report, blood samples from 12 and tissue samples from 13 patients were received.

Microsatellite markers	Size	Position
D18S363	177-247bp	18pter-4qter
D4S391	214bp	4p15-4p11
D17S1566	179bp	17p13.3
D4S174	175-190bp	4p15-4p11
D8S552	132bp	8p22-8p22
D9S157	205bp	9p23-9p22
D10S196	99-109bp	Chro.10
D10S220	184bp	Chro.10
D10S225	262-278bp	Chro.10
D5S346	96bp	5q21-5q22
D17S250	151bp	17q11.2-17q12
D2S123	196bp	2p16-2p16
BAT26	121bp	2p16-2p16
BAT25	113bp	4q12-4q12

Table 2: The markers selected for studying microsatellite instability in patients with stomach cancer

PCR amplification of the microsatellite markers were done on genomic DNA of patients with stomach cancer (Fig. 3) and analyzed on polyacrylamide gels (Fig. 4).

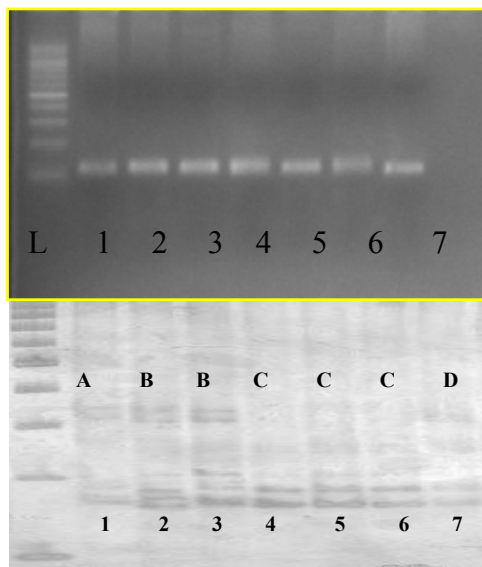


Fig. 3: EtBr stained agarose gel showing PCR amplification of the marker D5S346 (96-121bp)

Fig. 4: PAGE analysis of the PCR product of marker D17S250 in polyacrylamide gel. Pattern A is observed in lane 1
Pattern B in lane 2& 3
Pattern C in 4, 5, 6
Pattern D is observe in lane 7

Oral Cancer

For studies in oral cancer, genetic factors and gene-environment interaction will be analyzed by study of allelic alteration in the chromosomal loci known to have a strong correlation with oral carcinogenesis. Chromosomal regions in 3p and 13q contain regions that are frequently deleted and microsatellite markers have been selected from these regions (Table 3). During the year under report, blood and tissue samples from 8 patients were received.

SL/No.	MS Marker	Size	Location
1.	D3S2447	238-239bp	3p24.2-p22
2.	D3S1286	119-153bp	3p25.1
3.	D3S1260	254-276bp	3p22.2
4.	D3S2450	258-259bp	3pter-p24.2
5.	D3S1560	235-253bp	3p26-p25
6.	D3S3591	148-162bp	3p25.2-p24.2
7.	D3S1300	217-241bp	3p14.2

Table 3: Microsatellite markers selected for study in oral cancer

PCR amplification of the microsatellite markers were done on genomic DNA of patients with oral cancer (Fig. 5) and were analyzed on polyacrylamide gels (Fig. 6).

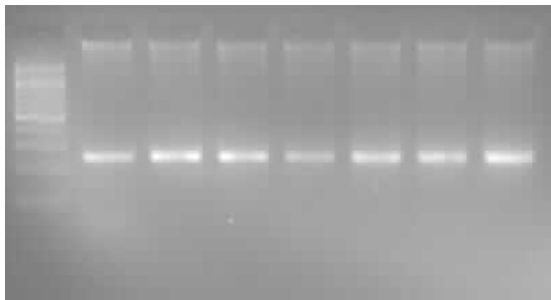


Fig. 5: Etbr stained agarose gel showing PCR amplification of marker D3S2447

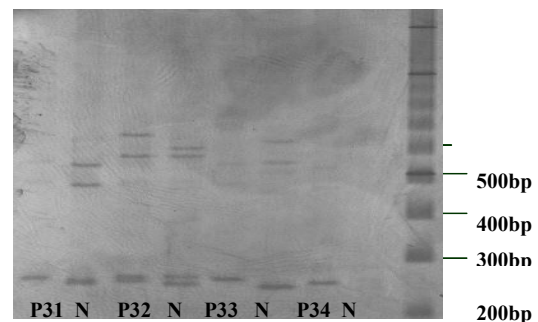


Fig. 6: PCR-PAGE analysis for marker D3S2447 showing several patterns suggestive of it being a highly polymorphic marker

HEMATOPOIETIC LYMPHOID MALIGNANCIES

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*

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<i>Technical Staff</i>	:	<i>Mrs. Rachna</i>
<i>In collaboration with</i>	:	<i>Dr. S Saluja, SJH</i>
<i>Duration</i>	:	<i>2003– 2006</i>

Aims, Objective and Background

Multi drug resistance is a major obstacle and an important mechanism to explain failure of chemotherapy in patients with leukemia. In this study we have identified chemotherapeutic drugs that could be used in chemosensitivity assay in patients with acute leukemia (AL). In addition, genes responsible for the development of resistance such as multi drug resistance (MDR) gene, dihydrofolate reductase (DHFR) gene, glutathione-s-transferase (GST) pi and the p53 gene were evaluated by RT-PCR.

Work done during the year

Flow cytometric immunophenotyping was done in 185 patients (63 samples of B-ALL, 33 of T-ALL, 74 of AML and 11 of Mix lineage leukemia). Four cases were undifferentiated. Flow cytometry based *in vitro* chemosensitivity assay was done in 114 samples using vincristine (Vin), daunorubicin (Dnr), methotrexate (Mtx), cytosine arabinoside (Ara-C) and L-asparaginase (L-Asp).

Evaluation of cell viability and apoptosis

Patients with ALL who achieved complete remission (CR) had significantly lower mean live cell count (70.9%) compared to those patients who did not achieve CR (93.3%) ($p=0.02$). Furthermore, ALL responders had also significantly higher mean apoptotic cell count (19.4%) as compared to non-responders (5%) ($p=0.04$). No significant difference was found in the mean live / apoptotic cell count of responders and non-responders of AML patients. Interestingly, we found that the responders of ALL had significantly lower mean live cell and higher mean apoptotic cell count than the responders of AML. The mean live / apoptotic cell count (%) of ALL responders was 70.9 / 19.4 versus 95.9 / 3.6 of AML responders ($p=0.01 / 0.02$). The probability of obtaining CR in ALL patients was 3.7 and 2.7 times higher in those who had mean live cell count less than 70% and apoptotic cell count more than 10% respectively.

***In vitro* chemosensitivity assay**

In samples of AML, non-responders were significantly more resistant compared to responders to any combinations of three drugs ($p=0.01$). In responder group, the sensitivity of Mtx and Dnr was significantly high ($p=0.004$). In samples of B-ALL, sensitivity to Vin and Dnr was found to be significantly higher in responders when compared to non-responders ($p=0.03$). In samples of T-ALL, responders showed no significant difference in the sensitivity pattern of any of the tested five drugs either alone or in combination. Furthermore, non-responders patients of acute leukemia were more likely to have an *in vitro* drug resistant pattern compared to responders.

Expression of drug resistance related genes

Expression of genes commonly involved in drug resistance (MDR, DHFR, GST-pi, p53) was done by Real-time RT-PCR (*TaqMan probe assay*) to see if gene expression could be indicators of drug sensitivity pattern in individual patient samples. The N-fold differential expression of the genes for a sample compared with the normal was expressed as $2^{-\Delta\Delta C_T}$. Using the $2^{-\Delta\Delta C_T}$ method, the data presented as the fold change in the target gene expression in leukemia normalized to an internal control gene (GAPDH) and relative to the normal sample (Figs. 1-2).

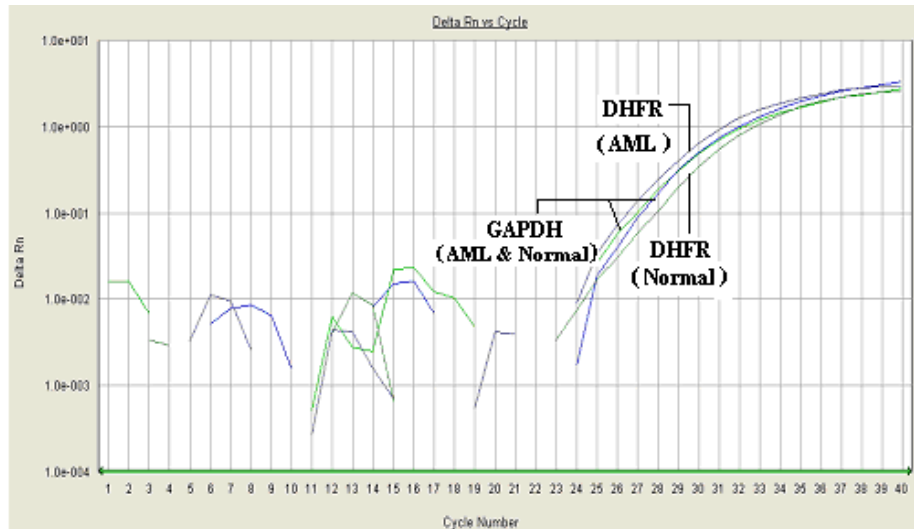


Fig. 1: Representation of Real Time PCR quantification of DHFR mRNA expression in AML patient normalized to GAPDH and relative to normal control

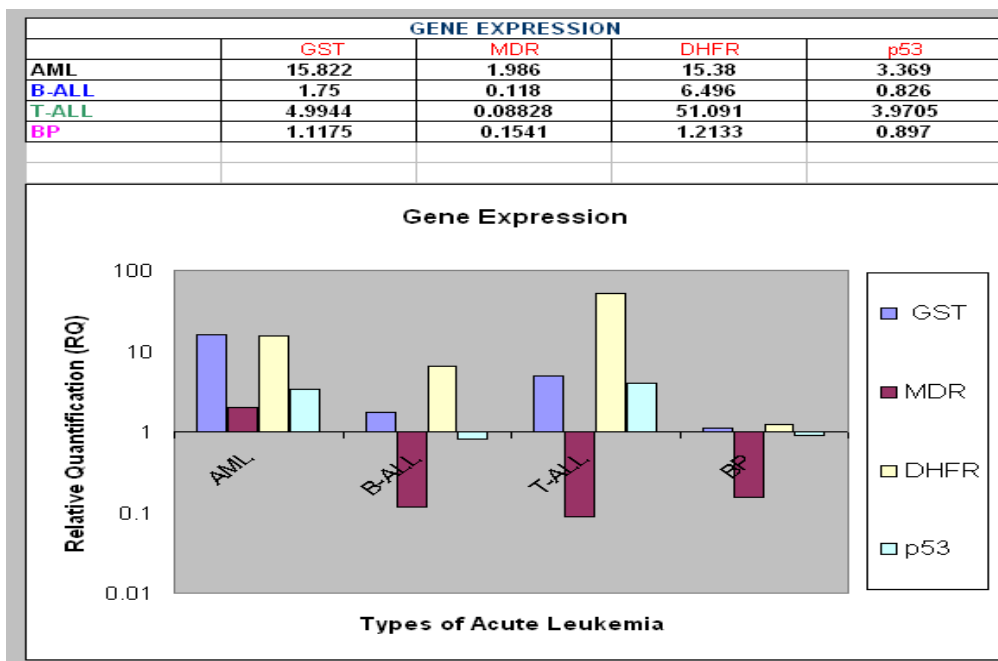


Fig. 2: Gene expression in acute leukemia: Fold change (RQ) normalized to GAPDH and relative to the Normal control.

Results show that GST-pi expression (associated with poor prognosis) was significantly upregulated in AML and T-ALL. MDR expression was significantly upregulated in AML (a poor prognostic indicator) and downregulated in B-ALL, T-ALL and biphenotypic leukemia. DHFR expression was correlated with resistance to methotrexate drug. p53 expression was significantly upregulated in AML and T-ALL. However this could not be correlated with drug sensitivity.