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Editorial

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Assessment of Minimal Residual Disease in Management of Cancer Is it just a concept or a true reality?

In this era of personalized medicine and targeted therapy, 'One size does not fit all' is very appropriate when it comes to management of cancer. Clinicians shall agree that in cancer; natural history, biological behavior and outcome of different treatments are variable among patients. Gene expression profiling has supported the heterogeneity of cancer at the molecular level. 'Divide and conquer' is the new model for cancer classification[1]. Molecular classification of tumor is getting popular and so it is imperative to tailor therapy based on individual patient's disease profile. Conventional prognostic criteria are based on pretreatment profile of the patient and tumor. It is based on clinical (age, performance status), pathological (tumor size, nodal status, grade, etc), immunophenotypic and cytogenetic parameters; and it is commonly used but it has its own limitations[2]. Induction failure and early response to treatment has been incorporated into the decision making of acute lymphoblastic leukemia (ALL)[2,3]. Efficacy of treatment assessed at molecular level when disease in still under morphological remission is an evolving concept. Minimal residual disease (MRD) is the detection of residual leukemic/cancer cells not detectable by light microscopy[4]. Assessment of MRD gauges the treatment response at molecular level and it is a stepping stone for personalizing therapy for many cancers.

Concept of MRD: Cancer chemotherapy destroys cancer cells by unique mechanism. It destroys fixed proportion of cells and not the fixed number of cells. There is 99.9% cell kill (i.e.3log) with each dose of chemotherapy and there is re growth of tumor in between the cycle (i.e.1 log) which results into net 2 log reduction[5]. So despite achieving morphological remission, there is significant number of cells present in the body which is responsible for subsequent relapse. Induction of morphologic remission in ALL may result into reduction from 10^{12} to 10^{10} leukemic cells, but still there is significant number of leukemic cells present in the body. There are sensitive techniques available to detect very low levels of leukemic cells in either peripheral blood (PB) or bone marrow[6]. These assays can detect as high as 1 abnormal cell in 10,000 normal cells which corresponds to MRD level of less than 0.1%(10^{-3}) or as low as 1 abnormal cell in 1 million cells which corresponds to MRD of 0 .0001% (10^{-6})[7,8].

Targets and methods used for detecting MRD

Various tumor specific targets are used in detecting residual cells in the body. Various leukemia associated immunophenotypic markers, gene rearrangements, gene fusion transcripts and circulating tumor cells are used as targets for detecting MRD. Table: 1 shows the list of targets useful for detecting the MRD in specific types of cancer[2,7,8,11-33].

Detection of MRD requires very sensitive molecular tests based on DNA, RNA and/ or proteins. There are number of laboratory techniques available for detecting MRD. Broadly speaking, two techniques are widely used and extensively validated and standardized till date. Polymerase chain reaction[7] (PCR) based method: Tests for MRD using the PCR can identify malignant cells based on their characteristic chromosomal rearrangements. PCR is a very powerful tool for detecting MRD at very low level however; it requires expertise, it is laborious to perform the test, it is time consuming and expensive. *Multiparameter flow cytometer[8] (MFC) based method:* It is an immunologic method which is based on detection of leukemia associated phenotypes. Aberrant immunophenotypes are detected in majority

Table 1: List of targets used in detection of MRD

Acute lymphoblastic leukemia	t(9;22)BCR-ABL,t(12;21) TEL-AML1,Patient specific assays for immunoglobulin(Ig) and T cell receptor (TCR)genes
Acute myeloid leukemia	t(15;17) PML-RARA, t(8;21) AML1-RUNX1T1 (AML-ETO), inv(16)
Chronic myeloid leukemia	t(9;22) BCR-ABL
Chronic lymphoid leukemia	Cell surface proteins, patient-specific assays for Ig and TCR genes
Follicular lymphoma	t(14;18) IgH/BCL2, patient-specific assays for Ig and TCR genes
Mantle cell lymphoma	t(11;14) IgH/CCND1, patient-specific assays for Ig and TCR genes
Multiple myeloma	M-protein levels in blood, patient-specific assays for Ig and TCR genes
Hodgkin's lymphoma	?circulating tumor cells in peripheral blood or bone marrow
Solid tumors	Circulating tumor cells in peripheral blood or bone marrow

of leukemias and that allows detection of MRD via flow cytometry. It detects protein with the help of fluorescent tag antibodies. This technology can detect 1 leukemic cell among 10,000 or more normal cells. It is easily available, faster and less expensive method for detecting MRD. Most international studies have used PCR based methods. PCR based methods has longer and larger experience. Various studies have tried to compare the results of flow cytometry and PCR based method and there is high concordance rate between the two methods [9,10].

MRD as a prognostic factor in lymphoid malignancies

Assessment of MRD is a guide to prognosis or relapse risk in many haematological malignancies. Several clinical studies evaluated MRD and its ability to predict outcome. In childhood ALL, multiple studies[11,12] have shown that patients with detectable MRD are at high risk for relapse in the future. Large prospective studies of MRD in pediatric ALL have conclusively demonstrated that the probability of long-term relapse-free survival is directly related to the level of residual disease, both early in the course of treatment, and at later time points[11]. In another example, study by van Dongen JJ et al[12], in 240 children with ALL MRD was evaluated by semi-quantitative PCR on bone marrow samples from several time points during treatment MRD result was used to identify three risk groups with significantly different rates of relapse at three years. Low-risk patients (those with no MRD detectable at either time point) had a relapse rate of 2 percent. Intermediate-risk patients (those that were neither low nor high risk by MRD assays) had a relapse rate of 23 percent while high-risk (with .01% ($\geq 10^{-3}$) blasts detected at both time points) had a relapse rate of 75 percent[12].

Marco Ladetto et al[13] studied predictive value of the persistence of MRD in bone marrow in follicular lymphomas (FL) treated with a rituximab-intensive program. MRD for bcr/IgH translocation was assessed on bone marrow using PCR. In his study, PCR positivity at the end of induction was an independent adverse predictor. Conversion to PCR negativity predicted better progression-free survival (PFS) at all post-treatment times (e.g., end of therapy: 3-year PFS, 72% vs 39%; P < .007). MRD was predictive in both maintenance (83% vs 60%; P < .007) and observation (71% vs 50%; P < .001) groups. MRD is a powerful independent outcome predictor in FL patients who receive rituximab-intensive programs. In intergroup trials of the European mantle cell lymphoma (MCL) network, Christiane Pott et al [14] studied MRD eradication as the therapeutic goal to tailor treatment approaches. MRD was studied on patients receiving high intensive therapy including autologous stem cell transplantation by MFC on PB. This study showed positive MRD status in PB was highly associated with a shorter progression free survival in MCL. In classical Hodgkin's lymphoma (HL), Yasuhiro Oki et al[15]were able to detect circulating clonal tumor cells with the help of high-throughput sequencing-based method. He analyzed additional cases to determine the sensitivity and specificity in early stage versus advanced stage HL and to determine whether the detection of MRD post therapy would correlate with clinical relapse[15].

MRD as a prognostic factor in myeloid malignancies

In acute myeloid leukemia (AML) there is increasing evidence that level of MRD after induction and consolidation therapy is independently associated with risk of relapse and survival[16,17,18,19]. In one report[20] MRD assessed by MFC reveal a level of MRD greater than or less than 3.5 x 10^{-4} following consolidation therapy was associated with relapse rates of 77 %and 17% respectively ($p<0.001$). Buccisano et al[21] have shown that patients with good or intermediate karyotypes who have MRD detected by MFC at the end of post remission therapy have outcomes resembling those in patients with FLT3 ITD + or unfavorable karyotypes AML. In turn patients who are FLT3 ITD + but without MRD fare better than those with FLT3 ITD + with MRD. In acute promelocytic leukemia (APL), study by Jurcic JG et al[22] reported that 40 of 47 patients (85%) who were induced by ATRA alone had residual disease detected by RT-PCR. However, after three cycles of consolidation therapy with idarubicin and cytarabine, residual disease was found in only 10% of patients. APL relapsed in only 3 of 41 patients (7 %) who had two or more negative RT-PCR assays for MRD compared with all four patients with two or more positive results. In chronic myeloid leukemia (CML), quantitative RT –PCR is regularly monitored during treatment. Eight years follow up of International Randomized Study[23] of Interferon Vs STI571 (IRIS) results have documented that achievement of major molecular response (MMR) at 12 months is predictor of low relapse rate in CML-chronic phase. None of the patients with documented MMR progressed to accelerated or blastic phase at the end 8 years.

MRD as a prognostic factor in plasma cell disorder

In multiple myeloma, Barlogie et al[24] have shown that the vast majority of complete remission (CR) patients (94%) achieving long-term survival (10 years relapse free), were MRD negative. By contrast, at least one-third of MM patients achieving CR after initial therapy will not experience a survival benefit because of persistent MRD.

MRD and circulating tumor cells (CTCs) in solid tumors

In solid tumors, development of metastasis is the major obstacle for cure. Dissemination of tumor cells in other organs or bone marrow is thought to be

responsible for metastatic process. CTCs have been identified in blood and marrow in many solid tumors with the help of highly sensitive PCR assays. Role of CTCc and its prognostic and therapeutic implications has been extensively studied in many solid tumors like breast cancer, prostate cancer, lung cancer, gastrointestinal carcinoma, malignant melanoma, neuroblastoma, hepatoblastoma and many other solid tumors[25]. Metastatic process is poorly understood in human beings. Whether presence of CTCs always leads to relapse or metastasis is still not clear. Majority of tumor cells that enter into the circulation is killed by mechanical, immunological or unknown mechanisms[26]. In a very excellent review article by Michail Ignatiadis et al[27], concluded that in early breast cancer, the presence of MRD after completion of standard adjuvant treatment may contribute to a better selection of patients to evaluate secondary adjuvant treatment strategies.

Incorporation of MRD in day to day clinical practice

1. MRD based risk stratification is widely used in most of the protocol for childhood ALL[28]. Assessment of MRD is now becoming an integral component in the risk stratification of ALL by many cooperative group protocols like BFM[29], St Jude[30] etc.
2. MRD based response criteria has been proposed to define uniform clinical response and clinical end points. European study[31] groups on childhood and adult ALL have proposed the definitions for assessing response based on MRD status.
3. MRD monitoring of disease is well established in CML and APL and it is regularly used for determining molecular response and early detection of molecular relapse.
4. Tailoring therapy on the basis of MRD status is becoming standard of care for e.g. in CML, those who do not achieve molecular remission are qualified for change of therapy. Rising transcript level in CML also calls for testing for mutational analysis and change of therapy[32].
5. Escalating therapy on basis of MRD status has been proposed in Ph negative adolescent and young adult ALL, national comprehensive cancer network (NCCN)-2015[33] recommends use of allogeneic transplantation in MRD + disease following induction remission.
6. In UK ALL 2003 study, by Ajay Vora et al[34] suggest that escalating care in patients with MRD at the end of induction therapy improves clinical outcome. It is a large randomized study which has evaluated the role of augmentation of post induction therapy based on MRD-defined high-risk subgroup of children and young people with clinical standard-risk and intermediate-risk ALL.
7. Tailoring therapy on the basis of MRD in AML is found to be beneficial in AML02 trial[35] with significant improvement in 3 year event free survival (63%) and overall survival (71%) compared to previous trial.
8. Therapeutic significance of MRD in lymphoma and multiple myeloma is under active investigation.
9. Detection of circulating tumor cell and MRD in solid tumor is still in infancy and its role is under exploration.

Conclusions

Assessment of MRD is no longer a research tool; it is an integral part of comprehensive management of many cancers. Its role has been proven beyond doubt in CML, ALL and APML. Its role in other haematological malignancies is under extensive evaluation. Role of MRD is still in infancy in solid tumors however extensive research is ongoing in breast cancer and neuroblastoma. Detection of MRD will remain as a hallmark for the diagnosis, prognosis, monitoring, and treatment of malignant conditions in future clinical trials.

Unanswered questions

There are still many unanswered questions related to MRD. It remains to be established which sample? peripheral blood or bone marrow, whether MRD should be assessed at multiple times during various phases of treatment, what should be the cut-off value, at what level of MRD should inspire a change in therapy and whether these levels are reproducible at different centers, whether reduction in MRD will lead to better relapse free or overall survival or it will remain as a surrogate marker of drug resistance/refractory disease?

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Medicine is not only a science; it is also an art. It does not consist of compounding pills and plasters; it deals with the very processes of life, which must be understood before they may be guided.

Paracelsus

Shri Madanmohan Ramanlal GCRI Luminary Award

Dr. Anila S Kapadia
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Journey of Four Decades at the Gujarat Cancer Research Institute

I am very grateful for giving me an opportunity to talk about my experience at Gujarat cancer and research institute.

My journey to cancer hospital was started from august 1973 till 2005, now at present I am emeritus professor at GCRI.

When I joined the cancer hospital newer technology like USG, CT Scan, etc were not available still we were treating patients and we were getting comparable results. New advances in chemotherapy have changed the prognosis of patients. Over the time newer radio therapeutic machines have reduce the complication of radiotherapy.

GCRI gave me a chance to attend UICC conference in Germany and also in Delhi. I also went as observer at Sloan-Kettering hospital and Clevaland clinic in USA. I attended technology transfer programmer at UK with British council.

I was lucky to become president of Ahmedabad obstetrics and gynecological society in 1994-1995 and received trophy for best society in India. I also become president of association of gyn-oncology of India.

In Gynec department over and above routine oncology work, we have to do so many camps. We do camp not only in city of Ahmedabad but also all over Gujarat. At times very few persons take part in camp, and those were very unhappy moments for me. I feel we should do the camps at places were cervical cancer is more common.

My self and my staff of Gynec department were lucky to organize workshop with European school of gyn-oncology and conference of association of gyn-oncology of India. It was very informative and we learned a lot and the delegates were also happy.

At the end, so many things to say but I am lucky that all the staff of GCRI was always helpful to me. The Gynec department was like family to me and very helpful to me.

Thanks to all!

Formerly, when religion was strong and science weak, men mistook magic for medicine; now, when science is strong and religion weak, men mistake medicine for magic.

Thomas Szasz

Significance of Glycosylation Changes in Oral Cancer

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Summary

Oral cancer is the major health burden in India. Glycosylation changes are associated with malignant transformation. Therefore, we investigated alterations in several glycoconjugates in oral cancer. Total 50 healthy controls and 50 oral cancer patients were enrolled for the study. Serum total sialic acid (TSA) and fucose were analyzed using spectrophotometric method, serum glycoprotein profile was performed by native PAGE followed by glycoprotein staining and for ST3GALI and FUT6 mRNA expression, reverse transcriptase PCR (RT-PCR) was performed. The result depicted that TSA/TP ratio and fucose were found to be increased in oral cancer patients as compared to the controls. Receiver Operating Curve (ROC) analysis indicated that fucose/TP ratio could significantly distinguish between controls and oral cancer patients. Fucose/TP ratio was found to be higher in tobacco habituates oral cancer patients as compared to non-habituates controls and habituates controls. ST3GAL1 mRNA expression was found to be higher in malignant tissues as compared to normal tissues. The mRNA expression of FUT6 was comparable between malignant and adjacent normal tissues. The glycoprotein profile showed an increase in 44 kDa in oral cancer patients as compared to the controls. Moreover, levels of 230 kDa glycoprotein were observed to significantly increased in oral cancer patients as compared to controls. The study illustrated that glycosylation has significant role in development of oral cancer. Further in-depth studies with a larger sample size can give insights into the molecular mechanism underlying glycosylation in oral cancer.

Keywords: Fucose, fucosyltransferease glycoprotein, oral cancer, sialic acid, sialyltransferase

Abbreviations: AJCC: American Joint Committee on Cancer; AUC: Area under curve; FUT: Fucosyltransferase; IDV: Integrated density value; LN: lymph node; NHT: no habit of tobacco; RT-PCR: Reverse transcriptase polymerase chain reaction; SEM: Standard error of mean; TP: Total proteins; TNM: Tumor node metastasis; TSA: Total sialic acid; ST: Sialyltransferase; WHT: With habit of tobacco

Introduction

Cancer is one of the most common causes of mortality and morbidity today, with more than 14.1 million new cases and more than 8.2 million deaths each year worldwide. It has been estimated that around 43% of cancer deaths are due to tobacco use, unhealthy diets, alcohol consumption and infections.[1] The occurrence of oral cancer is particularly high among men, and is the eighth most common cancer worldwide.[2] It is estimated that 390,000 new cases occur worldwide accounting for 2% to 3% of all malignancies.[2] An increasing trend has been observed in a number of oral cancer cases registered in the hospital cancer registry (2010) of Gujarat Cancer and Research Institute. At The Gujarat Cancer and Research Institute, Ahmedabad oral cancer is more common among male with incidence of 31.1% while among females the incidence are comparatively lower having incidence of 11.5%.

Many properties of mammalian cells are expressed at, or mediated through the cell surface. Immense increase in knowledge of altered characteristic of malignant cells has shown that, altered cell surface is the hallmark of malignant cells.[3] Cell surface glycosylation changes are associated with diverse types of neoplastic transformation.[4] Glycoproteins and glycolipids are the major constituents of cell membrane. Glycoproteins are usually defined as protein carbohydrate complexes in which oligosaccharides and/ or polysaccharides are joined by covalent linkage to specific amino acids of proteins. The carbohydrate portion contains amino sugars (glucosamine, galactosamine and sialic acid) and hexoses (galactose, mannose) or fucose. These glycoconjugates especially the terminal epitopes of glycoproteins are thought to play important role in cell-cell interactions, development of cell adhesion, malignant transformation and metastasis.[5] Sialic acid is thought to be important in determining surface properties of cells and has been implicated in cellular invasiveness, adhesiveness and immunogenicity. Elevated serum total sialic acid (TSA) levels are reported to be correlated with the clinical staging, prognosis and recurrence of malignancies.[6] Fucosylation of glycoproteins at terminal ends is one of the most important features that mediate several specific biological functions. It has been documented that tumor cells modulate their surface by increasing fucosylation levels to escape recognition, thus contribute to decreased adhesion and uncontrolled tumor growth.[7] Protein glycosylation is increasingly being recognized as one of the most prominent biochemical alterations associated with malignant transformation and tumorigenesis.[8-10] Changes in the glycosylation pattern also affect the levels of glycoproteins[11]. High serum glycoconjugates levels have been reported in many cancers including head neck and oral cancers indicating their usefulness in diagnosis and monitoring therapy.[12]

The alterations in serum sialyltransferases activity was significantly correlated with disease status during follow-up in oral cancer patients. Various studies have documented elevated levels of serum sialic acid and sialyltransferases along with ST3GAL1 mRNA expression in cancer patients.[11,13,14] Analysis of sialyltransferases (ST) along with sialic acid levels has been suggested to be important in understanding the mechanism of elevation sialic acid values during malignancy. Fucosyltransferase (FUT)

is a group of enzyme that catalyze incorporation of fucose from activated nucleotide donor Guanosine diphosphate fucose to reducing end of complex glycans in the linkage specific manner. FUT genes from human genome are divided in three sub-families, α-1,2 FUT, α-1,3/4 FUT, and α-1,6 FUT. FUT3–FUT8 and FUT9–FUT11 belong to the group of α-1,3/4 FUTs.[12] This enzyme is expressed by many tissues and is found to be increased in serum and tumors of cancer patients.[13,15,16] As there is lack of study correlating all these parameters simultaneously, the present investigation aimed to evaluate sialic acid, fucose, glycoprotein profile together with ST3GAL1 and FUT6 mRNA expression in oral cancer.

Materials and methods

The study was approved by institutional review board. Due consent was obtained from all the subjects to participate in the study. The subjects included in the study were 50 controls (healthy individuals who had no major illness in the recent past) and 50 oral cancer patients (with histopathologically proven untreated cancer of the oral cavity). The demographic details of the subjects are summarized in Table 1.Clinical TNM (tumor node metastasis) stage of malignant disease was determined according the American joint committee on cancer (AJCC) criteria[17]. The distribution of oral cancer patients with regard to various clinicopathological characteristics including disease site, tumor differentiation, lymph node status, NHT and WHT, nuclear grade, infiltrating and non-infiltrating is mentioned in Table 2.

Sample collection and processing

Blood samples were collected by venipuncture from all participants; sera were separated and store at -80˚C until analyzed. Tumor and adjacent normal tissues were collected at the time of surgery washed with phosphate buffer saline (PBS) and RNA stabilizing agent was added, the vials were then stored at -80˚C until analysed.

Estimation of serum total ptotein

Total protein from was estimated spectrophotometrically using Biuret method.[18]

Estimation of total sialic acid

TSA was estimated by method as described by Skoza et al.[19] Briefly, 0.1 ml of serum was incubated with 0.8 ml normal saline and 0.1 ml 1N sulphuric acid at 80˚C for 1 hour. Then, proteins was be precipitated by 1.0ml of 10%w/v trichloroacetic acid. The tubes were centrifuged at 454 g for 10 min, 0.1ml supernatant was then mixed with 0.25ml periodic acid. Reaction mixture was incubated at 37˚C for 30 min. To this, 0.25 ml 2% w/v sodium arsenite was added immediately. Further after addition of 0.5 ml thiobarbituric acid the reaction mixture was kept in boiling water bath for 7.5 min and was cooled. 1.5 ml dimethyl sulphoxide was added to intensify the pink choromophores. Absorbance was read at 549 nm spectrophotometrically. The levels were normalized to total protein and TSA/TP ratio was calculated.

Estimation of fucose

Fucose was estimated using the method described by Winzler.[20] Briefly, serum proteins were precipitated out by 95% ethanol. The pellet obtained by centrifugation was dissolved in 2 ml of 0.2 N NaOH. From this, 0.5 ml of aliquots was used for fucose estimation. After addition of concentrated H_2SO_4 and boiling for 3 minutes, Cysteine-hydrochloride was added, which forms yellow chromophores, and absorbance was read at 396 nm. A reaction mixture was also read at 430 nm simultaneously to correct the interference caused by chromophores from other sugars. Fucose levels were normalized to total protein and ratio was expressed as mg/mg protein.

Estimation of mRNA expression of ST3GAL1 and FUT6

RNA was isolated from all the tissue samples (paired adjacent normal and malignant) collected from oral cancer patients using RNA isolation kit (Qiagen, Valencia, CA, USA) and stored at -80˚C. Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for transcripts ST3GAL1 and FUT6 was carried out using specific primer sequences and conditions as mentioned in Vajaria et al.[13] β-ACTIN was used as internal controls in all the reactions, and RT- PCR was carried out using one-step RT-PCR kit (Qiagen, Valencia, CA, USA). The amplifications was performed using thermal cycler (Eppendorf Mastercycler gradient Eppendrof, Hamburg Germany). Reactions contained 500ng of RNA, 0.6µl of primers for the target genes, and 0.3µM of primers for the house- keeping genes (β-ACTIN) in 50µL RT-PCR reaction volume. The reaction products was electrophoresed on 1.5% Agarose gel containing ethidium bromide, and gels were analyzed densitometrically using gel documentation system (Alpha Innotech, USA). For semi- quantitative analysis of ST3GAL1 and FUT6 transcripts, the integrated density value (IDV) of each sample was compared with the IDV of β-ACTIN coamplified in the same tube, and relative expression (IDV) of the glycosyltransferase transcripts /IDV of β-ACTIN) was been measured.

Native PAGE

1000 µg of serum protein was standardized and separated on 10% native polyacrylamide gel by standard protocols as described by Hames et al.[21] in non- denaturing and non- reducing conditions at constant voltage (100V) until the dye front reached the bottom of the gel. The gels were separated from the glass plates and kept in fixative consisting of methanol and acetic acid (20:10) for fixation of

Table 1: Details of the subjects

Variable	Controls (n = 50)	Cancer patients (n = 50)
1. Age	25-50	30-75
Median (Age range)		
2. Sex		
Men/ Women	44/6	36/14
3. Tobacco habits		
Yes/No	27/23	41/9
4. Type of Habits		
Chewing	16	26
Smoking	7	3
Chewing and smoking	—	8
Alcohol	4	4

Table 2: Clinico-pathological details of oral cancer patients

Disease characteristics	No. of patients
1. Disease site	
Buccal mucosa	16
Oral tongue	13
Alveolus	12
Other (lip, Gingival buccal sulcus, Hard plate)	9
2. Histopathology SCC	50
3. Stage	
Stage I	9
Stage II	11
Stage III	10
Stage IV	20
4. Early / Advanced	20/30
5. Tumor Differentiation	
Well	27
Moderate	20
Poor	3
6. Metastasis/Non-metastasis	15/31
7. Undefined	4
8. Infiltrative/Non-infiltrative	17/27
9. Undefined	6

Table 3: Comparison of TSA/TP Ratio with various clinico pathological parameters

Subjects	Mean ± SEM
1. Early stage	0.0088 ± 0.000476
2. Advanced stage	0.0087 ± 0.000339
	p=0.903 (1 vs. 2)
3. Non- Metastatic	0.00881 ± 0.000294
4. Metastatic	0.0085 ± 0.0005374
	p=0.598 (3 vs. 4)
5. Non- Infiltrative	0.00899 ± 0.0002726
6. Infiltrative	0.0078 ± 0.000589
	p=0.069 (5 vs. 6)
7. Well	0.0088 ± 0.00027
8. Moderate	0.0086 ± 0.000501
	p=0.808 (7 vs. 8)

Table 4: Comparison of Fucose/TP Ratio with various clinico pathological parameters

Subjects	Mean ± SEM
1. Early stage	0.00388 ± 0.000014
2. Advanced stage	0.0036 ± 0.00012
	p=0.188 (1vs. 2)
3. Non- Metastatic	0.0037 ± 0.00094
4. Metastatic	0.0085 ± 0.00054
	p=0.219 (3 vs. 4)
5. Non- Infiltrative	0.0090 ± 0.00027
6. Infiltrative	0.0078 ± 0.00059
	p=0.0577 (5 vs. 6)
7. Well	0.0088 ± 0.00027
8. Moderate	0.0087 ± 0.00050
	p=0.145 (7 vs. 8)

Table 5: Comparison of ST3GAL1 mRNA expression various clinico-pathological parameters

Subjects	Mean ± SEM (ST3GAL1/β-ACTIN)
1. Early stage	0.3093 ± 0.03357
2. Advanced stage	0.3799 ± 0.04991
	p=0.0797 (1 vs. 2)
3. Non- Metastatic	0.3148 ± 0.0026
4. Metastatic	0.4327 ± 0.0919
	p=0.319 (3 vs. 4)
5. Non- Infiltrative	0.3532 ± 0.04038
6. Infiltrative	0.376613 ± 0.0669
	p=0.727 (5 vs. 6)
7. Well	0.2726 ± 0.02952
8. Moderate	0.4130 ± 0.0545
	p=0.016 (7 vs. 8)

proteins. Glycoprotein staining was performed as described by Anderson et al.[22]. Destaining was done with 0.1% (w/v) sodium metabisulfite in 0.01 M HCl. The gels were scanned using gel documentation system and IDV values were noted.

Statistical Analysis

Data were analyzed statistically using the SPSS statistical software (version 17). All values were expressed as Mean ± S.E.M. Student's independent 't' test was performed to compare the marker levels between controls and oral cancer patients. Students paired 't' test was used to compare the levels of markers between malignant and adjacent normal tissues. ROC curve analysis was performed to assess the discriminatory efficacy of the markers in distinguishing controls vs. oral cancer patients. The area under curve (AUC) of above 0.5 suggest valid discriminatory efficacy of the markers. 'p' values ≤0.05 were considered as statistically significant.

Results

For standard curve of protein, Bovine serum albumin (BSA-Sigma) was used as a standard for

Table 6: Comparison of FUT6 mRNA expression with various clinico-pathological parameters

Subjects	Mean ± SEM (FUT6 /β-ACTIN)
1. Early stage	0.1787 ± 0.03926
2. Advanced stage	0.3151 ± 0.0685
	p=0.358 (1 vs. 2)
3. Non- Metastatic	0.2278 ± 0.0.0555
4. Metastatic	0.3479 ± 0.0958
	p=0.335 (3 vs. 4)

Biuret method. The standard curve was linear from 10 mg to 80 mg. For total sialic acid, N-acetyl neuraminic acid (NANA-sigma) was used as a standard. The standard curve was linear from 1 µg to 9 µg BSA.

Total Sialic Acid levels in controls and oral cancer patients

TSA (mg/dl) were measured by thiobarbituric acid method spectrophotometrically from 50 controls and 50 oral cancer patients. Then the concentration of serum TSA was normalized with total protein value (estimated by biuret method) and the ratio TSA/TP was calculated. Figure 1 depicts that TSA (mg/dl) levels were significantly higher in oral cancer patients as compared to the controls ($p<0.0001$). As illustrated in Figure 2 the mean levels of TSA/TP ratio was found to be higher in oral cancer patients as compared to controls ($p=0.0041$).

ROC curve were constructed for serum TSA/TP (Figure 3) to evaluate their discriminatory efficiency between controls and oral cancer patients. TSA/TP could moderately discriminate between controls and oral cancer patients with AUC of 0.614 ($p=0.106$).

Correlation of TSA/TP ratio with clinico-pathological parameters

It was observed that TSA/TP ratio was higher in early and advanced stage as compared to controls ($p=0.047$ and $p=0.903$, respectively, Table 3). The levels of TSA/TP ratio were found to higher in patients with lymph node (LN) metastasis and patients without LN non-metastasis as compared to the controls. However the levels were comparable when compared between metastatic and non-metastatic oral cancer patients.

Fucose levels in controls and oral cancer patients

For standard curve, fucose (sigma) was used as a standard. The standard curve for fucose was found to be linear from 5 to 50µg. Serum fucose levels (mg/dl) were measured by cysteine HCl method from 50 controls and 50 oral cancer patients. Normalization of serum fucose was performed with total protein value and the ratio fucose/TP was calculated for all the subjects.

Figure 4 depicts the comparison of serum fucose levels (mg/dl) in controls and oral cancer patients. The fucose levels (mg/dl) were found to be significantly higher in oral cancer patients as compared to controls ($p<0.0001$). The mean level of fucose/TP ratio was found to be elevated in oral cancer patients as compared to controls ($p=0.764$) (Figure 5).

Table 7: Levels of 230 kDa glycoprotein in controls and oral cancer patients

Subjects	230 kDa Glycoprotein evels (IDV)
1. Controls	708078 ± 465606
2. Oral Cancer Patients	5291157 ± 1142242
	p=0.018 (1 vs. 2)

Figure 6 shows the ROC curve which indicated that fucose/TP could significantly discriminate between controls and oral cancer patients which was supported by area under curve (AUC: 0.714, $p=0.006$).

Comparison of Fucose/TP ratio in NHT and WHT subjects

Oral cancer patients and controls were grouped into subjects WHT and subjects with NHT. Fucose/TP ratio was found to be significantly higher in WHT oral cancer patients as compared to WHT controls and was also significantly increased in WHT oral cancer patients when compared to NHT oral cancer patients.

Correlation of Fucose/TP ratio with clinico-pathological parameters

Table 4 depicts that the fucose/TP ratio was higher in early stage and advanced stage disease as compared to controls ($p=0.188$ and 0.182). While the levels were comparable between early and advanced stage of disease as well as between patients with and without LN metastasis.

Comparison of ST3GAL1 expression in controls and oral cancer patients

ST3GAL1 mRNA expression was studied from 25 paired malignant and adjacent normal tissues. Figure 7(a) is the representative pattern of ST3GAL1 mRNA expression and Figure 7(b) depicts that ST3GAL1 mRNA expression was higher in malignant tissues as compared to normal tissues. ROC curve analysis showed that ST3GAL1 could significantly discriminate between controls and oral cancer patients which was supported by area under curve (AUC: 0.572, $p=0.2893$).

Table 5 depicts that the ST3GAL1 mRNA expression ratio was observed to be higher in advanced stage as compared to controls ($p=0.797$). While the levels were comparable between early and advanced stage of disease. The levels were higher in tumors with and without LN metastasis as compared to controls.

Comparison of FUT6 mRNA expression in malignant and adjacent normal tissues

FUT6 mRNA expression was estimated from 25 paired malignant and adjacent normal tissues.

Figure 8(a) is the representative pattern of FUT6 mRNA expression. The mRNA expression of FUT6 was comparable between malignant and adjacent normal tissues as depicted in Figure 8(b). Table 6 depicted that the levels of FUT6 were higher in poorly differentiated tumors as compared to well and moderately differentiated tumors. It was observed that FUT6 transcript levels were higher in advanced disease as compared to the early stage of the disease (p=0.797).

Comparison of glycoprotein profile between controls and oral cancer patients

Figure 9 (a) is the representative pattern of glycoprotein profiling from serum. Glycoprotein profiling was analyzed from 25 controls and 25 oral cancer patients. The glycoprotein profile [Figure 9 (b)] showed that the glycoprotein levels of 192 kDa, 170 kDa and 116 kDa were comparable between controls and oral cancer patients. An increase in 44 kDa was observed in oral cancer patients as compared to the controls (p=0.430). A 230 kDa glycoprotein was observed in 7 oral cancer patients and 4 controls out of 25 subjects. As depicted in Table 7, the levels of 230 kDa glycoprotein was observed to be significantly higher in oral cancer patients as compared to controls (p=0.018).

Glycoprotein expression in NHT and WHT groups

The glycoprotein levels of 190 kDa, 170 kDa, 116 kDa and 44 kDa were higher in WHT controls as compared to NHT controls, respectively. However, as all the oral cancer patients were tobacco habituates, the levels could not be compared with NHT oral cancer patients.

Correlation of Glycoproteins with clinico-pathological parameters

The levels of 192 kDa, 170 kDa and 116kDa in well and moderately differentiated tumors were almost comparable. An increase in 44 kDa was observed in well differentiated tumors as compared to controls.

The levels of all the glycoprotein were comparable between patients with LN metastasis and without metastasis. The levels of all other glycoproteins in various stages were almost comparable with controls. An increase in 230 kDa was observed in early stage as compare to controls.

Discussion

Glycosylation being the most important post-translational modification of proteins that plays a key role in malignant transformation, the present study evaluated glycosylation changes in oral cancer patients. Many studies have shown significant clinical usefulness of total sialic acid in oral precancerous conditions and oral cancer.[10,14,23] In the present study, the TSA/TP ratio was found to be higher in oral cancer patients as compared to controls. This was again consistent with our earlier findings.[10,14] Our recent studies showed that the salivary levels of total bound sialic acid along with other sialylation markers were higher in oral cancer patients than normal subjects.[14,24] In the present study, the levels of TSA/TP ratio were higher in well and moderately differentiated tumors as compared to controls. The TSA/TP ratio was found to be higher in early and advanced stage of disease as compared to controls. Earlier reports have suggested that lipid-bound sialic acid levels correlated with stage of the disease, degree of metastatic involvement and recurrence of disease.[10] Serum and salivary sialic acid elevated levels have been also reported in in oral potentially malignant disorders and oral cancer.[25]

We have observed that fucose/TP ratio was found to be higher in oral cancer patients as compared to the controls. Also ROC curve analysis indicated that serum fucose/TP ratio could significantly discriminate between controls and oral cancer patients. Earlier studies have suggested increased fucose levels in various cancers including oral cancer.[16] It has been documented that tumor cells modulate their surface by increasing fucosylation levels to escape recognition, which contributes to several abnormal characteristics of tumor cells, such as decreased adhesion and uncontrolled tumor growth. Several studies have suggested that monitoring serum/tissue fucose levels could be a promising approach for the early detection, diagnosis and prognosis of various cancer types, including oral carcinoma.[16,26] The increased levels of fucose have been attributed to tissue destruction and tissue proliferation.[26] Earlier investigations have shown that serum fucose levels were comparable between early and advanced stage, as well as between patients with and without LN metastasis.[27]

In the present study, elevated ST3GAL1 mRNA expression was found in malignant tissues as compared to adjacent normal tissues. Earlier studies from our laboratory have observed similar results with increased expression in oral cancer tissues[13] Our results indicated that ST3GAL1 mRNA levels were higher in metastatic tumors as compared to the non-metastatic tumors of oral cancer patients. Schneider[28] et al., have shown that ST3GAL1 mRNA expression was found to be significantly increased in cases showing invasion of lymph vessels in colorectal cancer. Earlier study by Wang[29] et al., have reported no correlation of ST3GAL1 and ST6GAL1 transcripts with stage, differentiation, amount of ascites and serum levels of CA125 in ovarian cancer. On the other side, Wang et al. found down regulation of ST3GAL1 expression in squamous cell carcinoma of the cervix.[30]

In the present study, the levels of FUT6 transcripts were comparable between oral cancer tissues and adjacent normal tissues. Moreover, FUT6

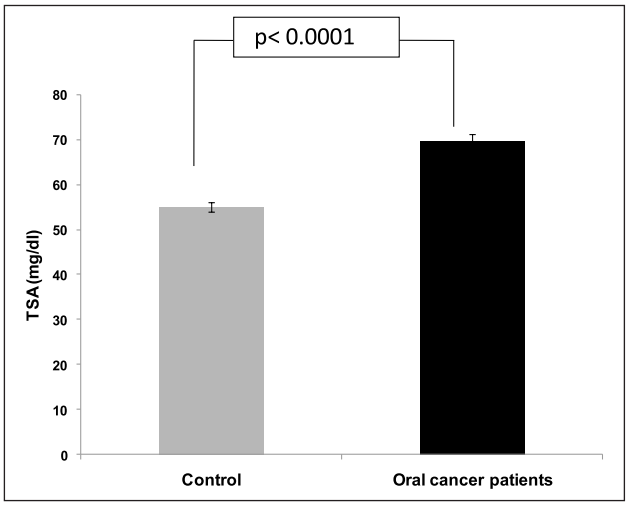

Figure 1: Comparison of serum TSA (mg/dl) between controls and oral cancer Patients

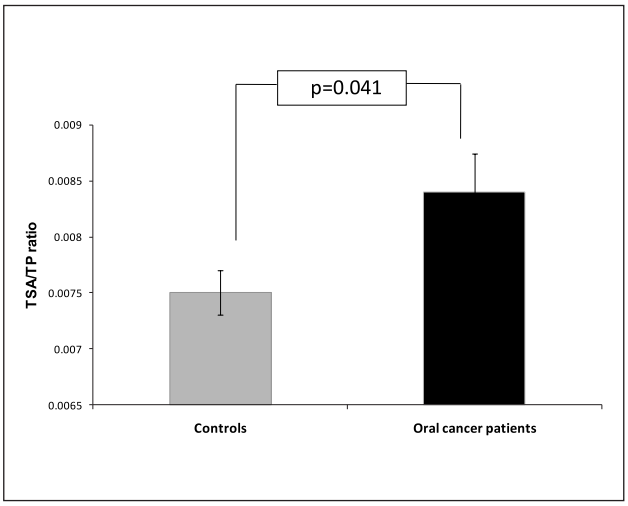

Figure 2: Comparison of serum TSA/TP ratio between controls and oral cancer patients

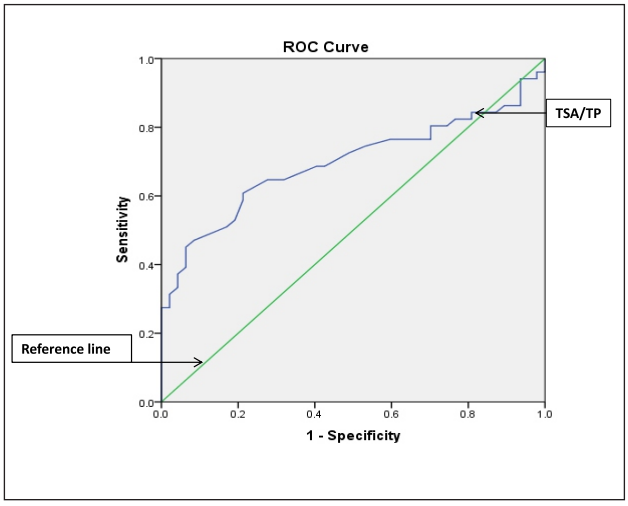

Figure 3: ROC curve for TSA/TP ratio to distinguish controls and oral cancer patients

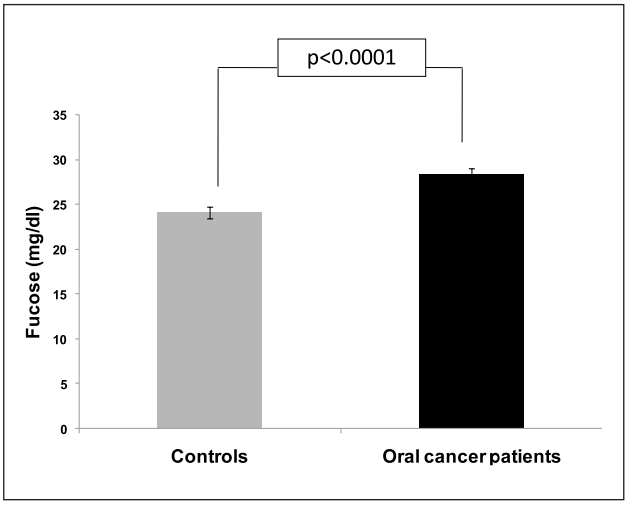

Figure 4: Comparison of serum fucose levels (mg/dl) in controls and oral cancer patients

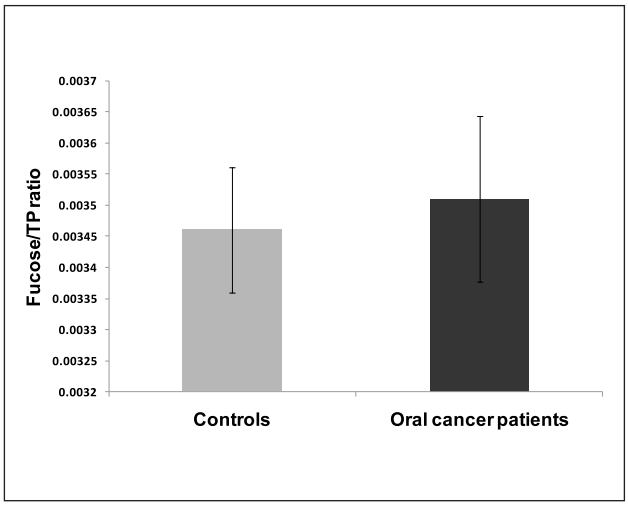

Figure 5: Comparison of serum fucose/TP ratio between controls and oral cancer patients

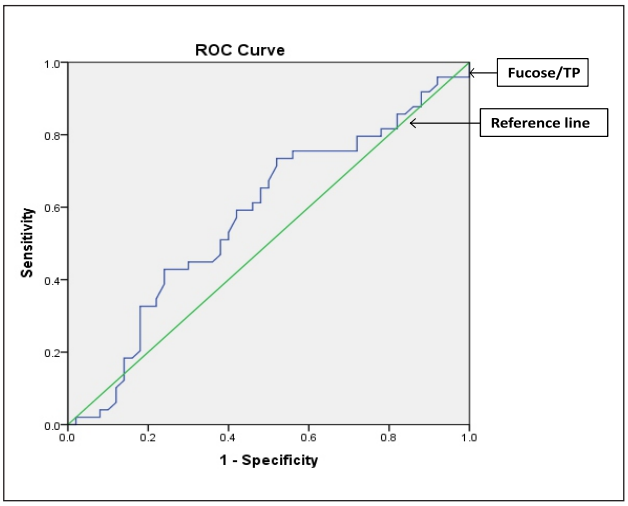

Figure 6: ROC curve for Fucose/TP ratio to distinguish controls and oral cancer patients

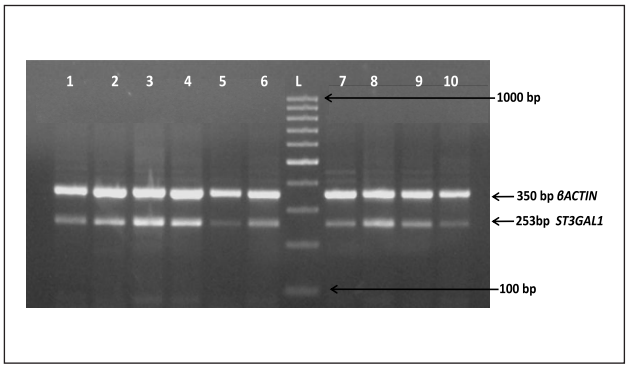

Figure 7 (a): Representative pattern of ST3GAL1 mRNA expression in paired malignant and adjacent normal tissue. Lanes 1,3,5,7 and 9 represent the amplicon pairs of ST3GAL1 (253 bp) and β-ACTIN (350 bp) from adjacent normal tissues, whereas lanes 2,4,6,8,10 represent the amplicon pairs of ST3GAL1 (253bp) and β-ACTIN (350 bp) from malignant tissues. Lane L represents DNA ladder (100-1000 bp).

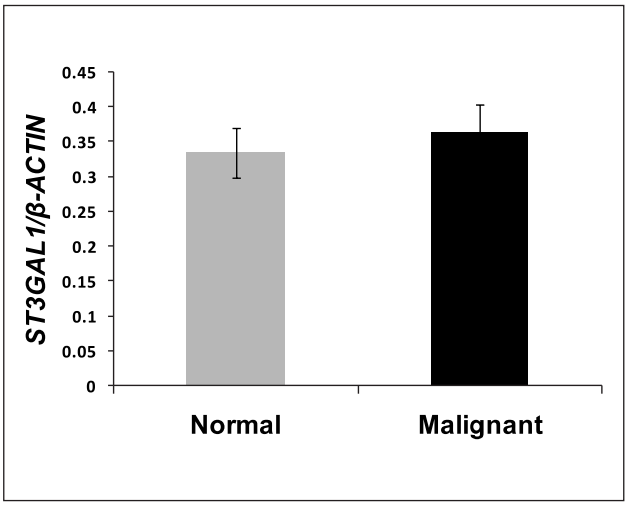

Figure 7 (b): Comparison of ST3GAL1 mRNA expression in malignant and adjacent normal tissues

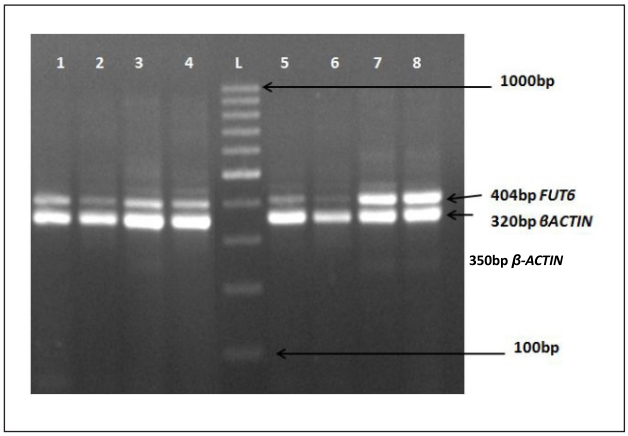

Figure 8 (a): Representative pattern of FUT6 mRNA expression in paired malignant and adjacent normal tissues. Lanes 1,3,5 and 7 shows the amplicon pairs of FUT6 (404 bp) and β-ACTIN (350 bp) from adjacent normal tissue, Lanes 2,4,6 and 8 shows the amplicon pairs of FUT6 (404 bp) and β-ACTIN (350 bp) from malignant tissues, and Lane L represent the DNA ladder (100-1000bp).

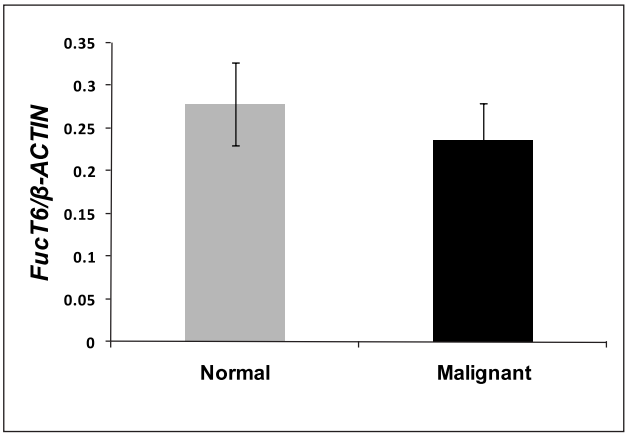

Figure 8 (b): Comparison of FUT6 mRNA expression in malignant and adjacent normal tissues

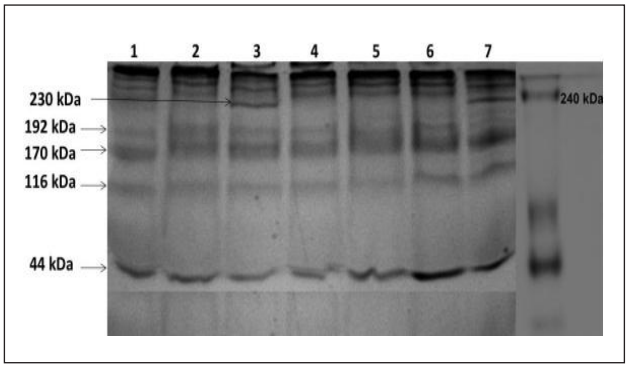

Figure 9 (a): Representative pattern of glycoprotein profiling from controls and oral cancer patients. Lanes 1, 2 and 3 represent the amplicon from normal controls, lanes 4, 5, 6, and 7 represent the amplicon from the oral cancer patients.

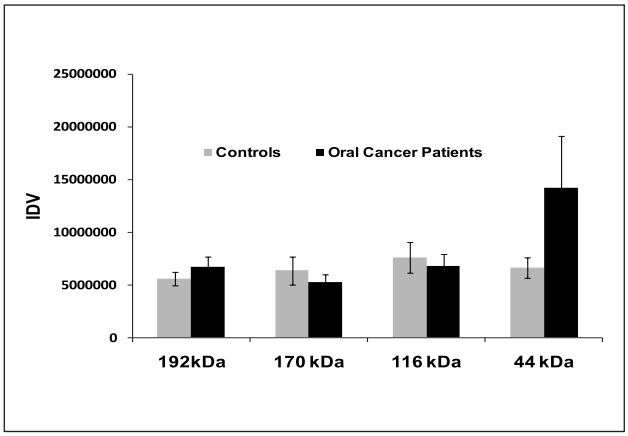

Figure 9 (b): The levels of glycoprotein in controls and oral cancer patients

expression depicted moderate increase in metastatic tumors as compared to non-metastatic tumors and was also higher in advanced stage of disease as compared to early stage of disease. The results were consistent with our previous studies in malignant oral cancer tissues[13] Hiraiwa[31] et al, have shown increase in FUT3, FUT6 and FUT7 in colon cancer tissues along with increase in SLea antigen. Increased expression of FUT5 and FUT6, and decreased expression of FUT4 expression has been observed earlier in gastro intestinal carcinoma cells.[12] FUT6 expression has been shown to be involved in SLeX expression on breast cancer cells.[32] The present study depicted higher expression of FUT6 in metastatic and advanced tumors, which is known to be involved in up regulation of SLeX in metastatic disease. Our earlier studies have indicated increased SLeX in oral cancer and a significant positive correlation with advanced and metastatic disease[33] Earlier reports have indicated increase in FUT6 in colon cancer and have observed that its knockdown caused decrease in FUT6 mRNA and inhibition of SLeX expression.[11]

The altered glycoforms are released into the bloodstream due to increased shedding from the malignant cells. In the present study, the levels of 192 kDa, 170 kDa, and 116 kDa glycoproteins were comparable in oral cancer patients as compared with controls. Our results from our laboratory have indicated elevated expression of these glycoprotein in oral cancer patients. Moreover, in the present study, a 44 kDa glycoprotein was observed to be increased in oral cancer patients as compared to controls. The altered 44 kDa glycoprotein in the current investigation was characterized as being the α1-acid glycoprotein in previous studies.[34] Earlier studies from our laboratory have indicated a greater number of glycoprotein bands in the gamma region in patients with OPC and patients with oral cancer.[35] In the present study, a 230 kDa glycoprotein was present in 7 oral cancer patients and 4 controls. The levels of this glycoprotein were observed to be significantly increased in oral cancer patients as compared to the controls. The results were consistent to our previous studies that depicted increased expression of 230 kDa glycoprotein in oral cancer patients.[36] The presence of 230 kDa glycoprotein in tobacco users and it absence among non-users signifies changes occurring in glycoproteins among consumers of tobacco. Further characterization of the 230 kDa band can give insight into the role of this glycoprotein in oral cancer development.

Further characterization of 192 kDa, 170 kDa and 116 kDa glycoproteins by mass spectrometric analysis is required for identification of the structure and function of these proteins. Further analysis with a larger sample size could give a better idea of utility of glycoprotein profiling for disease monitoring of oral cancer patients.

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FLT3 Protein Expression and Mutation in B Cell Acute Lymphoblastic Leukemia (B-ALL)

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Summary

FLT3 is a receptor tyrosine kinase, which play important role in normal hematopoiesis activating mutations of FLT3 are commonly found in acute leukemia patients and reported to be associated with poor clinical outcome. This study aims to evaluate the incidence of FLT3 mutation and CD135 protein expression and their role in prognosis of B cell acute lymphoblastic leukemia (B-ALL). Detection of FLT3 internal tandem duplication (ITD) was carried out in total 32 patients diagnosed as B-ALL using reverse transcriptase - polymerase chain reaction (RT-PCR) method. The quantification of (CD135) FLT3 protein on leukemic blasts was done by flowcytometery in total 51 B-ALL patients. The incidence of FLT3 ITD mutation and CD 135 expression was to be found 19% and 51% respectively in 51 de novo B ALL patients. High incidence of FLT3 ITD mutation was noted in patients with low blast count, normal WBC count, low platelet count, low RBC count and low Haemoglobin level but showed no correlation with disease status. High incidence of CD135 over expression was noted in patients with high WBC count, low platelet count, low RBC count and low haemoglobin level and significant association was found with aberrant expression of CD13 and CD33. In relation to disease status, one patient showed disease relapse which had wild type FLT3 gene but CD135 over expression at diagnosis. CD135 over expression was found to be associated with poor prognostic markers and its presence at diagnosis in patient showing disease relapse confirms its role in disease aggressiveness of B-ALL. It might be suggested that CD135 protein is of more clinical relevance as compared to the FLT3 mutations.

Keywords: B-ALL, FLT3 protein, Flowcytometry.

Introduction

The FMS-like tyrosine kinase -3 (FLT3), which belongs to the class III receptor tyrosine kinase family, plays important role in normal hematopoiesis and FLT3 ligand (FL) is expressed on most cell lineages.[1-3] FLT3 is important for lymphocytes (B cell and T cell) development as compared to the development of other blood cells, including myeloid development.[4] FLT3 is also involved in the development of most leukemia including acute myeloid leukemia (AML) and B-lineage acute lymphoblastic leukemia (B-ALL).[4] Commonly observed activating mutations of FLT3 gene are internal tandem duplication (ITD) and FLT3 point mutation (D835). These mutations are commonly found in acute leukemia and associated with poor clinical outcome.

FLT3 has proven role in acute myeloid leukemia and recently, it has been implicated in pathogenesis of infant and childhood acute lymphoblastic leukemia. However, little is known about its role in acute lymphoblastic leukemia.[5] this prompted us to determine the FLT3 protein in patients with ALL. Thus, the present study evaluated the FLT3 ITD mutations along with FLT3 protein at diagnosis in B-ALL. The FLT3 mutations and protein expression were then correlated with clinical and haematological parameters as well as disease status.

Material and methods

Patients in this study, 51 patients with de novo B-cell acute lymphoblastic leukemia (B-ALL) who were diagnosed and treated at GCRI were enrolled. The patients enrolled included 34 (67%) pediatric and 17 (33%) adult B-ALL patients. Bone marrow (BM) or peripheral blood (PB) samples were collected at diagnosis from B-ALL patients. The diagnosis and classification of the acute leukemia samples were based on morphology and cytochemistry according to the French-American-British classification and on the presence of maturation and differentiation antigens as determined by immunophenotyping criteria. The detailed clinical history (age, gender), hematological findings, treatment given as well as disease status after induction therapy was noted from the case files maintained at the medical record department of the institute. This study was approved by the institutional scientific review board and ethics committee. Patients provided informed consent to use their sample for study.

Flow Cytomery analysis

The bone marrow or peripheral blood samples were used for flow cytometry study. The number of cells were quantified and adjusted 1x106 in each tube. CD135 (FLT3 protein) expression was analyzed using phycoerythrin (PE) conjugated anti-CD 135 monoclonal antibody (4G8) and peridinin-chlorophyll protein(PerCp)-conjugated CD45 monoclonal antibody was added to each tube for blast gate. The surface and cytoplasmic antigens related to leukemia associated phenotype were analyzed using reagents procured from BD Bioscience (San Jose,CA, USA) and followed the manufacture's protocol. For lineage assignment the following combination of monoclonal antibodies were used as the primary panel: Cd22/ Cd34/ Cd45/ Cd5/ Cd10/ Cd19,Cd7/ Cd13/ CD45/ CD33/ CD117/ HLADR, and MPO/ CD79a/ CD45/ CD3/ TdT. The negative controls for surface and cytoplasmic antigens were simultaneously stained with omission of antibody. To study the surface antigen expression, 10 µl / 5 µl of antibody (manufacturer's protocol followed) was added to 1 x 106 mononuclear cells (100 µl) and incubated for 15 minutes. After addition of 2mL lysing solution for RBC lysis (1:10 dilution, BD Biosciences), the samples were incubated for another 15 minutes. The samples were centrifuged at 400 g for 5 minutes, supernatant was

discarded and the remaining pellet was washed twice with phosphate buffered saline (PBS) and then resuspended in 500 µl PBS.

Data acquisition and analysis: A FACSCanto II flow cytometer with FACSDiva software (BD Biosciences) was used for the acquisition and analysis of the samples. At least 10,000 events/tube were acquired. For analysis, lymphoblasts were gated on dot plot of side scatter vs. CD45 PerCp. If the percentage of positive events was more than 20% the leukemic samples was considered to be positive for that surface or cytoplasmic marker.

Mutation detection

FLT3 ITD mutation analysis was carried out by RNA extraction followed by cDNA synthesis and polymerase chain reaction (PCR).

Ribonucleic acid (RNA) was extracted from fresh BM or PB samples using QIAamp RNA mini kit (Qiagen, Germany) according to manufacturer's protocol. cDNA synthesis and polymerase chain reaction (PCR) was carried out. For the study of FLT3 ITD mutation, cDNA synthesis was done using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). Approximately, 5 µl RNA was added to the master mix that included 2 µl (10X) RT buffer, 0.8 µl dNTP (100mM each), 1.0 µl MultiScribe reverse transcriptase, 2 µl (10X) random primers and final volume in each tube was made upto 20 µl. cDNA synthesis was carried out in Mastercyclergradient (Eppendorf, Germany) with conditions consisting of first step at 25°C for 10 minutes followed by 37°C for 2 hours, 85°C for 5 minutes and holding at 4°C for 5 minutes. FLT3 ITD mutation was studied using mutation specific primers specific for Exon 14 Forward (F)5'- tgtaaaacgacggccagtc aatttaggtatgaaagcc-3' and Exon 15 Reverse ® 5'- gaggaaacagctatgaccc tttcagcattttgacggcaacc-3' (Bangalore Genei, India). Approximately 100ng of genomic DNA was added to PCR mix containing 5 µl (10X) PCR buffer, 10 µl (5X) Q solution, 2 µl 25 mM MgCl2, 1.5 µl dNTP (10mM each) and 0.5 µl Taq DNA polymerase of PCR core kit (Qiagen, Germany) and 5 µl (0.8 µM final concentration) each of forward as well as reverse primers, to make a final volume of 50 µl. PCR was carried out in Mastercyclergradient (Eppendorf, Germany) with conditions consisting of initial denaturation step at 95°C for 2 minutes followed by 40 cycles each of denaturation at 95°C for 30 seconds, annealing at 70°C for 1 minute and extension at 72°C for 1 minute and final extension step at 72°C for 10 minutes. Amplified products were resolved on 2% agarose gels stained with ethidium bromide and visualized on Gel Documentation System (Alpha Innotech Corp., USA). An amplified product of wild type FLT3 was obtained at 366 bp was observed on agarose gel electrophoresis. Additional band longer than the wild type was considered as FLT3 ITD positive.

Statistical analysis

Statistical analysis was carried out using SPSS statistical software version 19 (SPSS Inc., USA). Pearson's Chi-square test with Pearson's correlation coefficient (r) was used to assess correlation and significance between two parameters. P values ≤0.05 were considered significant.

Results

FLT3 ITD mutation in B-ALL

Incidence

FLT3 mRNA amplification for mutation specific (FLT3 ITD) analysis, was carried out in total 40 B-ALL patients, where, FLT3 mRNA amplification was observed in 80% (32/40) patients. Out of 32 B-ALL patients, FLT3 ITD mutation was noted in 19% (06/32) of patients (Table 1, Figure 1).

Correlation with clinical parameters

With reference to age and gender, a trend of high incidence of FLT3 ITD mutation was noted in young adults (15 to 25 years; 25%, 01/04) as compared to pediatric (<15 years; 17%, 04/23), and older (> 26 years; 20% 01/05) age group patients while no significant correlation was noted with gender (Table 1).

Correlation with hematological parameters

With hematological parameters, patients with FLT3 ITD mutation (19%, 06/32) had lymphoblasts <60%. Regarding WBC counts, a trend of higher incidence of FLT3 ITD mutation was noted in 19% (06/32) patients with normal WBC count (4 – 11 x 103/µl; 29%, 02/07) than patients with low WBC count (<4.0 x 103/µl; 14%, 1/7) and high WBC count (≥11 x 103/µl ; 14%, 2/14). A trend of high incidence of FLT3 ITD was noted in high platelet count (33%, 1/3) as compared to low platelet count (16%, 4/25). All FLT3 ITD patients had low RBC count. With respect to hemoglobin level, a trend of higher FLT3 ITD mutation of was found in patients with hemoglobin level <7.9 gm/dL (27%, 04/15); than patients with ≥7.9 gm/dL; 08%, 01/13) (Table 1).

Correlation with leukemia associated immunophenotype (LAP)

In relation to B cell markers with B lineage markers, patients exhibiting FLT3 ITD mutation were CD19 positive and cCD79a positive. However, 3 patients with wild type FLT3 were CD19 negative. Further, a trend of high incidence of FLT3 ITD was noted with CD22 negative B-ALL (25%, 04/16) as compared to CD22 positive B-ALL (12%, 02/16) (Table 2).

In relation to CD34 expression

With CD34 (a marker of immature cells), incidence of FLT3 ITD was high in CD34 negative (29%, 02/07) as compared to CD34 positive (16%, 04/21) B-ALL (Table 2).

In relation to non lineage markers

With non lineage markers, all patients exhibiting FLT3 ITD mutation were TdT positive, HLADR positive and CD10 positive. However, 9 patients and 3 patients with wild type FLT3 were TdT negative and CD10 negative, respectively (Table 2).

In relation to aberrant myeloid and T lineage markers

With myeloid lineage markers (CD13, CD33, CD117 and MPO), aberrant expression of CD13 was noted on lymphoblasts (06%; 02/32), where one patients was FLT3 ITD positive and the other had wild type FLT3 gene. A trend of high incidence of FLT3 ITD was found in CD13 positive B-ALL (50%, 01/02) as compared to CD13 negative B-ALL (17%, 05/30). Aberrant expression of CD33, CD117 and cMPO was not seen in these patients (Table 2).

With T cell markers, aberrant expression of CD5 was noted in only one patient and had wild type FLT3 gene. All patients exhibiting FLT3 ITD mutation were cCD3 negative, CD5 negative and CD7 negative (Table 2).

Correlation with Philadelphia chromosome (t(9;22)) status

Out of 32 B-ALL patients studied for FLT3 mutation, Ph+ status was available for 18 patients. A trend of high incidence of mutation was noted in Ph+ patients (50%; 1/2) as compared to ph-patients (25%; 4/16) (Table 1).

Correlation with disease status

Out of 32 B-ALL patients studied for FLT3 mutation, 25 patients were lost to follow up and disease status of 7 patients treated with BFM-90 protocol was available, of which, 86% (06/07) patients showed disease remission and 14% (01/07) patients showed disease relapse.

Out of 6 patients with disease remission, 1 (17%, 01/06) had FLT3 ITD at diagnosis while 1 patient with disease relapse had wild type FLT3 at diagnosis (Table 1).

Correlation with BFM risk stratification

Disease status of 7 B-ALL patients was available of which, BFM risk stratification was carried out in 5 patients. Only one patient from medium risk group of BFM90 showed FLT3 mutation (50%, 1/2) while no mutation was observed in patients from standard risk and high risk group (Table 1).

CD135 expression in B-ALL

Incidence CD135 expression was estimated by flow cytometry in 51 B-ALL patients. CD135 over expression (≥0.30%) was obtained using median as cut off and noted in 51% (26/51) of B-ALL patients (Table 3, Figure 2).

Correlation with clinical parameters with reference to age and gender, high incidence of CD 135 over expression was noted in young adult (15 to 25 years; 67%, 04/06) as compared to pediatric (<15 years; 47%, 16/34) and older (26 to 59 years; 54%, 06/11) age group patients while no significant correlation was noted with gender (Table 3).

Correlation with hematological parameters with hematological parameters, a trend of high incidence of CD135 over expression was found in patients with high blast count (≥60; 52%, 24/46), high WBC count (>11 x 103/µl; 61%, 11/18), low platelet count (<1.5 x 105/µl; 55%,21/38), low hemoglobin level (<7.9 gm/dL; 60%, 12/20) and low (<3.8x 106/ µl; 59%, 20/34) and high (>4.8x 106/ µl; 50%, 01/02) RBC counts as compared to their respective counterparts (Table 3).

Correlation with leukemia associated immunophenotype (LAP)

In relation to B cell markers: With B cell marker, 98% patients (50/51) were cCD79a positive. However, in patients showing CD135 over expression, 25% patients (01/04) and 46% patients (11/24) did not express CD19 and CD22 B cell markers (Table 4).

In relation to CD34 expression: With CD34 (a marker of immature cells), incidence of CD135 over expression was high in CD34 negative (67%, 02/03) as compared to CD34 positive (50%, 24/48) B-ALL (Table 4).

In relation to non lineage markers: With non lineage markers, a trend of high incidence of CD135 over expression was noted in TdT negative B-ALL (57%, 8/14) as compared to TdT positive B-ALL (47%, 18/37), and in CD10 negative B-ALL (67%, 2/3) as compared to CD10 positive B-ALL (50%, 24/48). All 51 patients were HLADR positive and showed CD135 over expression in 51% (26/51) of patients (Table 4).

In relation to aberrant myeloid and T lineage markers

With myeloid lineage markers (CD13, CD33, CD117 and MPO), aberrant expression of CD13 and CD33 was noted in 14% (07/51) and 08% (04/51) patients, respectively. A significantly higher incidence of CD135 over expression was noted in CD13 positive (100%, 07/07, X2=7.802, r=+0.391, p=0.005) and CD33 positive (100%, 04/04, X2=4.173, r=+0.286, p=0.042) patients than CD13 negative (43%, 19/44) and CD33 negative (47%, 22/47) patients, respectively. All 51 patients were CD117 negative and cMPO negative with 51% (26/51) of CD135 positivity in both CD117 negative and cMPO negative patients (Table 4).

With T cell markers, aberrant expression of CD5 and CD7 was noted in 02% (01/51) and 06% (03/51) patients, respectively. A trend of higher incidence of CD135 over expression was noted in CD5 positive (100%, 01/01) and CD7 positive (67%, 02/03) patients than CD5 negative (50%, 25/50) and CD7 negative (50%, 24/48) B-ALL patients. Out of 51 patients having CD3 negativity, CD135 over expression was seen in 51% (26/51) of patients (Table 4).

Correlation with Philadelphia chromosome (t(9;22)) status

Out of 51 B-ALL patients studied for CD135 expression, Ph+ status was available in 24 patients. A trend of high incidence of mutation was noted in Ph+ patients (75%; 3/4) as compared to ph-patients (50%; 10/20) (Table 3).

Correlation with disease status

Out of 51 B-ALL patients studied for CD135 expression, 35 patients were lost to follow up and disease status of 16 patients treated with BFM-90 protocol was available, of which, 06% (01/16) patient

Table 1: Correlation of FLT3 ITD with clinical and hematological parameters

	N (%)	FLT3 ITD Negative N (%)	FLT3 ITD Positive N (%)	$\chi 2$	r	P value
Total Patients	**32(100)**	**26(81)**	**06(19)**			
		Clinical parameters N=32				
Age	32(100)	26(81)	06(19)			
0-14	23(71)	19(83)	04(17)	0.136	+0.040	0.827
15-25	04(13)	03(75)	01(25)			
26-59	05(16)	04(80)	01(20)			
Gender	32(100)	26(81)	06(19)			
Male	14(56)	12(86)	02(14)	0.326	-0.101	0.583
Female	18(44)	14(78)	04(22)			
		Hematological parameters N=32				
Blasts	32(100)	26(81)	06(19)			
< 60	32(100)	26(81)	06(19)			
≥ 60	-	-	-			
WBC count (x 103/µl)	28(82)	23(82)	05(18)			
< 4	07(25)	06(86)	01(14)	0.730	-0.028	0.887
4-11	07(25)	05(71)	02(29)			
>11	14(50)	12(86)	02(14)			
Platelet count (x 105/µl)	28(100)	23(82)	05(18)			
< 1.5	25(89)	21(84)	4(16)	0.549	0.140	0.459
≥4.5	03(11)	02(67)	01(33)			
RBC count (x 106/µl)	26(100)	22(85)	4(15)			
< 3.8	23(88)	19(83)	04(17)	0.617	-0.144	0.482
3.8-4.8	02(08)	02(100)	00(00)			
> 4.8	01(04)	01(100)	00(00)			
Hemoglobin level (gm/dL)	28(100)	23(82)	05(18)			
< 7.9	15(54)	11(73)	04(27)	1.709	-0.247	0.205
≥ 7.9	13(46)	12(92)	01(08)			
		Philadelphia chromosome status N=18				
	18 (100)	13 (72)	05 (28)			
Ph-	16 (89)	12 (75)	04 (25)	0.554	0.175	0.457
Ph+	02 (11)	01 (50)	01 (50)			
		Disease status N=7				
	07(100)	06 (86)	01 (14)			
Remission	06(86)	05 (83)	01(17)	0.194	+0.167	0.721
Relapse	01(14)	01(100)	00 (00)			
		BFM risk stratification N=5				
		04 (80)	01 (20)			
Standard risk	02 (40)	02 (100)	00 (00)	1.875	0.134	0.392
Medium risk	02 (40)	01 (50)	01 (50)			
High risk	01 (20)	01 (100)	00 (00)			

P value ≤ 0.05 is significant

*N is the number of patients whose data was available for statistical analysis

showed disease relapse and 94% (15/16) patients showed disease remission.

One patient with disease relapse showed CD135 over expression at diagnosis while patients with disease remission showed CD135 over expression in 47% (07/15) of patients (Table 3).

Correlation with BFM risk stratification

Disease status of 16 B-ALL patients was available of which, BFM risk stratification was carried out in 7 patients. CD135 over expression was noted in high risk group (100%, 1/1), medium risk group (25%, 1/4) and standard risk group (50%, 1/2) of BFM90 protocol though, no significant correlation was observed (Table 3).

Intercorrelation of CD135 expression with FLT3 ITD mutation

When intercorrelated in a group of 32 patients analysed for FLT3 ITD, no significant correlation was observed except a trend of high incidence of CD135 over expression in patients with wild type FLT3 (42%, 11/26) than patients with FLT3 ITD (33%, 02/06) (Table 3).

Discussion

This study evaluated the clinical relevance of FLT3 ITD mutation (N=32) and CD135 protein expression (N=51) in B-ALL patients. The FLT3 ITD was detected in 19% and CD135 over expression (median cutoff; ≥0.30%) in 51% of B-ALL patients.

FLT3 ITD mutations were first detected by Nakao et al (1996) reporting approximately 23% incidence in AML patients with similar incidence reported so far in AML patients.[6-10] On the contrary, B-ALL patients particularly childhood Leukemia, show low incidence of FLT3 ITD mutation (5% - 16.5%). Later it was reported that FLT3 mutations were commonly present in two subtypes of acute lymphoblastic leukemia - B-ALL and T-ALL.11 These include infant ALL with translocations involving the mixed lineage leukemia (MLL) gene and in hyperdiploid ALL, where incidence of FLT3 mutations was 18.2% and in 21.5% - 25%, respectively.[11,12] Various study groups have reported the incidence of FLT3 ITD in the range of 1% to 10% in B-ALL.[6,11-15]

In the present study, FLT3 ITD was analyzed in 23 pediatric and 09 adult out of 32 B-ALL patients. Although no significant correlation of FLT3 ITD was noted with clinical parameters, the incidence of FLT3 ITD in younger adults (15-25 years, 25%) was higher compared to pediatric patients (17%) and older adult (26-59 years, 20%) patients. Similar findings have been noted by Ishfaq et al (2012) showing higher incidence of FLT3 ITD mutation in age group 21 to 30 years, and no such correlation with gender was observed.[16]

In relation to hematological parameters, no statistically significant correlation was found with FLT3 ITD mutation. All FLT3 ITD patients had <60% blast counts and normal WBC counts, but low platelet counts, low RBC counts, and low hemoglobin counts (<7.9%) which suggest the ITD mutation associated with abnormal haemogram correlated with disease aggressiveness. A study by Ishfaq et al (2012) found no statistically significant relationship between FLT3 ITD mutation and WBC count in ALL.[16] However FLT3 ITD mutation has been demonstrated to be associated with high WBC count and high blast count in AML.[8] Another study conducted on adult leukemia patient's revealed association of FLT3 ITD mutation with high WBC count.[9]

When correlated with Philadelphia chromosome status, the incidence of FLT3 ITD mutation tended to be high in Ph+ patients as compared to Ph- patients. The pediatric patients were treated with BFM90 protocol and therefore FLT3 ITD mutation was further correlated with risk groups associated with BFM90 protocol[17], but no significant correlation was noted.

Correlating FLT3 ITD mutation with the disease status, one out of seven patients showed disease relapse and had wild type FLT3 at diagnosis. A study by Nakao et al (2000), reported that ALL with FLT3 ITD may not be associated with a poor prognosis, although the very low incidence of this molecular alteration in ALL currently prevents a definite conclusion.[6]

Further, FLT3 ITD was correlated with the leukemia associated immunophenotype, where no significant correlation was noted with immature marker CD34, B- cell markers, non lineage markers and aberrantly expressed myeloid and T cell markers. FLT3 is associated with immature cells and is lost during maturation to committed lineages.[18] FLT3 ITD has been found to be associated with immature cell CD34 and TdT (Terminal deoxynucleotidyl transferase), immature progenitor cell markers which are common aberrancies with uniform positive expression in lymphoblasts of B-ALL.[19] Although FLT3 ITD was not found to be associated with CD34 but a trend of high incidence of FLT3 ITD mutation was noted in patients showing TdT positivity in the present study.

In this study, CD135 protein was analyzed using flow cytometry and found positive in 51% patients with B ALL. This finding was in accordance with our previous findings.[9] Till now most of the study groups have analyzed CD135 protein in AML by different methods such as western blot, ELIZA and flow cytometry method.[5,15,20,21] Flow cytometry provides advantage of quantitation and less variance over other methodologies. CD135 is expressed in most of the acute leukemias including B- ALL[5] but no study till date have correlated CD135 over expression with clinical or hematological parameters. Also, no prognostic significance of CD135 over expression in B-ALL has been observed so far.

The present study analyzed CD135 in 34 pediatric and 17 adult out of 51 B-ALL patients. No significant correlation was noted between CD135 and clinical parameters except a trend of high incidence of CD135 over expression was noted in younger adult (15-25 years, 67%) as compared to pediatric patients (47%) and older adult (26-59 years) (55%) patients. The result is in line with the results obtained for FLT3 ITD mutations of the present study.

In relation to haematological parameters, no significant correlation was noted with CD135 over expression. Similar incidence of CD135 over expression was seen in both the subgroups of blast count. All CD135 over expressing patients had low platelet count and low RBC count. A trend of high incidence of CD135 over expression was noted in high WBC count and low median hemoglobin level. High WBC count is the major clinical feature of prognostic significance in acute leukemias and therefore, association of CD135 over expression might suggest prognostic significance of the protein.

In relation to Philadelphia chromosome status, the incidence of CD135 over expression tended to be high in Ph+ patients as compared to Ph- patients. Further, CD135 over expression was correlated with risk groups associated with BFM90 protocol, but no significant correlation was noted.

Table 2: Correlation of FLT3 ITD with leukemia associated immunophenotype (LAP)

	N (%)	FLT3 ITD Negative N (%)	FLT3 ITD Positive N (%)	$\chi 2$	r	P value
Progenitor cell marker N=32						
Cd34	**32(100)**	**26(81)**	**06(19)**			
Negative	07(22)	05(71)	02(29)	0.567	-0.133	0.468
Positive	25(78)	21(84)	04(16)			
B cell markers N=32						
cCD79a	**32(100)**	**26(81)**	**06(19)**			
Negative		-	-			
Positive	32(100)	26(81)	06(19)			
CD19	**32(100)**	**26(81)**	**06(19)**			
Negative	03(09)	03(100)	00(00)	0.764	+0.155	0.398
Positive	29(91)	23(80)	06(20)			
CD22	**32(100)**	**26(81)**	**06(19)**			
Negative	16(50)	12(75)	04(25)	0.821	-0.160	0.381
Positive	16(50)	14(88)	02(12)			
Non-lineage markers N=32						
TdT	**32 (100)**	**26(81)**	**6(19)**			
Negative	09(28)	09(100)	00 (00)	2.890	+0.301	0.095
Positive	23(72)	17(74)	06(26)			
HLA-DR	**32(100)**	**26(81)**	**06(19)**			
Negative	-	-	-			
Positive	32(100)	26(81)	06(19)			
CD10	**32(100)**	**26(81)**	**06(19)**			
Negative	03(9)	03(100)	00(00)	0.764	+0.155	0.398
Positive	29(91)	23(80)	06(21)			
Myeloid lineage markers N=32						
Cd13	**32(100)**	**26(81)**	**06(19)**			
Negative	30(94)	25(83)	05(17)	1.368	+0.207	0.256
Positive	02(06)	01(50)	01(50)			
CD33	**32(100)**	**26(81)**	**06(19)**			
Negative	30(94)	24(80)	06(20)	0.492	-0.124	0.499
Positive	02(06)	02(100)	00(00)			
CD117	**32(100)**	**26(81)**	**06(19)**			
Negative	32(100)	26(81)	06(19)			
Positive	-	-	-			
cMPO	**32(100)**	**26(81)**	**06(19)**			
Negative	32(100)	26(81)	06(19)			
Positive						
T cell markers N=32						
cCD3	**32(100)**	**26(81)**	**06(19)**			
Negative	32(100)	26(81)	06(19)	0.238	0.086	0.639
Positive	-	-	-			
CD5	**32(100)**	**26(81)**	**06(19)**			
Negative	31(97)	25(81)	06(19)			
Positive	01(03)	01(100)	00(00)			
CD7	**32(100)**	**26(81)**	**06(19)**			
Negative	30(94)	24(80)	06(20)	0.492	-0.124	0.499
Positive	02(06)	02(100)	00(00)			

P value ≤ 0.05 is significant; TdT, Terminal deoxynucleotidyl transferase; HLA-DR, Human leukocyte antigen – DR MPO, Myeloperoxidase

*N is the number of patients whose data was available for statistical analysis

In correlation with disease status, one out of sixteen patients showed disease relapse and had CD135 over expression at diagnosis. Contrary to the findings related to FLT3 ITD in the present study, CD135 over expression shows more association with poor prognosis. Though, specific deductions cannot be made due to very less number of patients included in the study. Our study on AML suggested CD135 protein over expression a potential prognostic marker.[9,10]

Further, CD135 was correlated with the leukemia associated immunophenotype, where no significant correlation was noted with immature marker CD34, B- cell markers, non lineage markers and aberrantly expressed T cell markers. CD135 over expression was found to be significantly associated with CD13 and CD33 positivity. The frequency of aberrant expression of CD13 and CD33 was high in B-ALL patients of the present study which is in line with the

Table 3: Correlation of CD135 expression with clinical and hematological parameters

	N (%)	Cd135 Negative N (%)	Cd135 Positive N (%)	χ2	r	P value
Total Patients	**51(100)**	**25(49)**	**26(51)**			
Clinical parameters N=51						
Age	**51(100)**	**25**	**26**			
0-14	34(67)	18(53)	16(47)	0.856	+0.082	0.567
15-25	06(12)	02(33)	04(67)			
26-59	11(21)	05(46)	06(55)			
Gender	**51(100)**	**25(49)**	**26(51)**			
Male	26(51)	12(46)	14(54)	0.174	-0.058	0.684
Female	25(49)	13(52)	12(48)			
Hematological parameters N=51						
Blasts	**51(100)**	**25(49)**	**26(51)**			
< 60	05(10)	03(60)	02(40)	0.267	+0.072	0.614
≥ 60	46(90)	22(48)	24(52)			
WBC count (x 103/µl)	**41(100)**	**20(49)**	**21(51)**			
< 4	11(27)	06(55)	05(46)	1.290	+0.143	0.372
4 to 11	12(29)	07(58)	05(42)			
>11	18(44)	07(39)	11(61)			
Platelet count (x 105/µl)	**42(100)**	**21(49)**	**21(51)**			
< 1.5	38(93)	17(45)	21(55)	3.399	-0.227	0.068
1.5-4.5	01(01)	01(100)	00(00)			
> 4.5	03(6)	03(100)	00(00)			
RBC count (x 106/µl)	**39(100)**	**18(46)**	**21(54)**			
3< 3.8	34(87)	14(41)	20(59)	3..851	-0.182	0.267
3.8 to 4.8	03(08)	03(100)	00(00)			
>4.8	02(05)	01(50)	01(50)			
Hemoglobin level (gm/dL)	**41(100)**	**20(49)**	**21(51)**			
< 7.9	20(49)	08(40)	12(60)	1.205	-0.774	0.284
≥ 7.9	21(51)	12(51)	09(43)			
Philadelphia chromosome status N=24						
	24 (100)	**11 (46)**	**13 (54)**			
Ph-	20 (83)	10 (50)	10 (50)	0.839	0.187	0.360
Ph+	04 (17)	01 (25)	03 (75)			
Disease status N=16						
	16(100)	**08 (50)**	**08 (50)**			
Remission	15 (94)	08 (53)	07(47)	1.067	-0.258	0.33
Relapse	01 (06)	00(00)	01 (100)			
BFM risk stratification N=07						
		04 (57)	**03 (43)**			
Standard risk	02 (29)	01 (50)	01 (50)	1.896	0.194	0.388
Medium risk	04 (57)	03 (75)	01 (25)			
High risk	01 (14)	00 (00)	01 (100)			
Intercorrelation of CD135 expression with FLT3 ITD mutation N=32						
ITD	**32(100)**	**19(59)**	**13(41)**			
No mutation	26(82)	15(58)	11(42)	0.163	-0.071	0.698
Mutation	06(19)	04(67)	02(33)			

P value ≤ 0.05 is significant

*N is the number of patients whose data was available for statistical analysis

findings of Seegmiller et al (2009).[19] Furthermore, these markers indicate poor prognosis compared to ALL cases without myeloid antigens and a poor response to drug therapies targeting conventional ALL.[22] Intercorrelation of FLT3 ITD mutation with CD135 was carried out where, no significant association was noted.[19]

In summary, CD135 over expression and FLT3 ITD were both found in younger adults as compared to pediatric and older adult age group. High incidence of FLT3 ITD mutation was noted in patients with low blast count, normal WBC count and showed no correlation with disease status while CD135 over expression was found to be associated with the conventional risk factor high WBC count, and also with aberrant CD13 and CD33 expression. Further, CD135 over expression was noted in one patient who underwent disease relapse after conventional induction therapy had FLT3 wild type gene. Thus, it might be suggested that CD135 protein

Table 4: Correlation of CD135 expression with leukemia associated immunophenotype (LAP)

	N (%)	Cd135 Negative N (%)	Cd135 Positive N (%)	χ2	r	P value
		Progenitor cell marker N=51				
Cd34	**51(100)**	**25(49)**	**26(51)**			
Negative	03(06)	01(33)	02(67)	0.314	-0.078	0.584
Positive	48(94)	24(50)	24(50)			
		B cell markers N=51				
cCD79a	**51(100)**	**25(49)**	**26(51)**			
Negative	01(02)	01(100)	00(00)	1.061	+0.144	0.313
Positive	50(98)	24(48)	26(52)			
CD19	**51(100)**	**25(49)**	**26(51)**			
Negative	04(08)	03(75)	01(25)	1.172	+0.152	0.288
Positive	47(92)	22(47)	25(53)			
CD22	**51(100)**	**25(49)**	**62(51)**			
Negative	24(47)	13(54)	11(46)	1.172	+0.152	0.288
Positive	27(53)	12(45)	15(55)			
		Non-lineage markers N=51				
TdT	**51(100)**	**25(49)**	**26(51)**			
Negative	14(28)	06(43)	08(57)	0.293	-0.076	0.597
Positive	37(72)	19(53)	18(47)			
HLA-DR						
Negative	-	-	-			
Positive	51(100)	25(49)	26(51)			
CD10	**51(100)**	**25(49)**	**26(51)**			
Negative	03(06)	01(33)	02(67)	0.314	-0.078	0.584
Positive	48(94)	24(50)	24(50)			
		Myeloid lineage markers N=51				
Cd13	**51(100)**	**25(49)**	**26(51)**			
Negative	44(86)	25(57)	19(43)	7.802	+0.391	0.005
Positive	07(14)	00	07(100)			
CD33	**51(100)**	**25(49)**	**26(51)**			
Negative	47(92)	25(53)	22(47)	4.173	+0.286	0.042
Positive	04(8)	00(00)	04(100)			
CD117	**51(100)**	**25(49)**	**26(51)**			
Negative	51(100)	25(49)	26(51)			
Positive	-	-	-			
cMPO						
Negative	51(100)	25(49)	26(51)			
Positive	-	-	-			
		T cell markers N=51				
cCD3	**51(100)**	**25(49)**	**26(51)**			
Negative	51(100)	25(49)	26(51)			
Positive	-	-	-			
CD5	**51(100)**	**25(49)**	**26(51)**			
Negative	50(98)	25(50)	25(50)	0.981	+0.139	0.332
Positive	01(02)	00(00)	01(100)			
CD7	**51(100)**	**25(49)**	**26(51)**			
Negative	48(94)	24(50)	24(50)	0.314	+0.078	0.584
Positive	03(06)	01(33)	02(67)			

P value ≤ 0.05 is significant MPO, Myeloperoxidase
TdT, Terminal deoxynucleotidyl transferase; HLA-DR, Human leukocyte antigen – DR
*N is the number of patients whose data was available for statistical analysis

over expression had more clinical relevance as compared to the FLT3 ITD mutations in B-ALL patients.

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Medicine is a science of uncertainty and an art of probability.
William Osler

Significance of Variable Number Tandem Repeats (VNTR) Polymorphism of Thymidylate Synthase Gene in Patients with Colorectal Cancer: A Preliminary Study

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Summary

5-fluorouracil (5-FU) is the most frequently used chemotherapeutic agent in the treatment of colorectal cancer (CRC) both in adjuvant and in palliative therapy. This drug mainly acts by inhibiting the activity of its target enzyme thymidylate synthase (TS) which leads to DNA damage. TS gene contains variable number of 28 bp tandem repeats (VNTR) in the 5'UTR region. This VNTR polymorphism can alter the TS levels. As the response of 5-FU is dependable on the level of TS in the cells, TS VNTR polymorphism could be an informative tool in clinical handling of CRC patients being treated with 5-FU based therapy. Therefore, the aim of the present study was to determine the significance of TS gene VNTR polymorphism in patients with colorectal cancer. In this retrospective study, 40 untreated CRC patients were included. TS gene VNTR polymorphism was studied using PCR technique. The data was analyzed statistically using SPSS software. There was predominance of 2R/3R heterozygous genotype by 53% (21/40) as compared to 2R/2R and 3R/3R homozygous genotype, 22% (9/40) and 25% (10/40), respectively. Patients with 2R/2R genotype had significantly higher rate of recurrence as compared to other two genotypes. However, a larger cohort and longer follow-up will be required for final conclusion.

Keywords: Thymidylate synthase (TS), 5-fluorouracil (5-FU), VNTR polymorphism, Colorectal cancer

Introduction

5-Flourouracil (5-FU) has remained the mainstay of chemotherapeutic treatment of colorectal cancer (CRC) for more than 50 years. This drug is generally prescribed as a single agent or in combination with other drugs in treatment of colorectal cancer.[1] The metabolic pathway of 5-FU mainly targets thymidylate synthase (TS) enzyme. TS catalyzes the transformation of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), essential pre-cursor for DNA synthesis,[2] which intracellularly gets converted to deoxythymidine triphosphate (dTTP) that is utilized to form nucleotides. 5-FU acts by inhibiting the activity of TS leading to disruption of nucleotide production and eventually DNA damage. The therapeutic response to 5-FU has been shown to be associated with the extent of inhibition of TS activity.[3] Therefore, TS is used as target for 5-FU to treat CRC.

TS gene is polymorphic in nature. The 5'-UTR promoter region of TS contains a variable number of tandem repeat (VNTR) sequences. The most common alleles found are double repeats (2R) or triple repeats (3R) of 28 bp tandem repeat sequences. This polymorphism can influence the efficiency of translation leading to alteration in levels of TS, eventually effecting 5-FU response.[4]

In a number of studies, the presence of TS VNTR polymorphism, especially in a homozygous fashion has been correlated with response to 5-FU-based treatment.[5,6] Contrarily, some other studies showed no relationship between the TS polymorphism and response to different 5-FU based chemotherapeutic regimens.[4,9] Most reports suggested that TS expression can be a prognostic and predictive marker for treatment of CRC, but still no clear results are available.[7,8]

The present study aimed to study the distribution of 28 bp VNTR polymorphism of TS gene in primary CRC patients and further to compare its expression with various clinicopathological parameters and survival.

Materials and Methods

Patients: In this retrospective study, 40 untreated histologically confirmed CRC patients registered at the Gujarat Cancer and Research Institute (GCRI) were enrolled. Informed consent was obtained from all patients enrolled in the study. Detailed clinical history was noted from the case files maintained at the Medical Record Department of the institute. The detailed clinicopathological data of patients are mentioned in Table 1. Disease staging was done according to AJCC TNM classification system and Dukes' staging system. Histopathological grading was done according to WHO system. The treatment of the patients was decided by the clinicians of the institute. The primary treatment offered to all patients was surgery or surgery followed by adjuvant chemotherapy (CT) and /or radiotherapy (RT). The main chemotherapeutic treatment included were 5-FU and leucovorin, oral Capecitabine or in combination with oxaliplatin and/or RT. Disease status was assessed by clinical examination, radiological and biochemical investigations including serum CEA levels.

Sample collection: Specimen was collected from operation theatre (OT) on ice. Tumor tissues (N=40) and adjacent normal tissues (N=11) was selected by

the pathologist. These tissues were stored at -80°C until analysis.

Follow-up: Patients were followed for a period of 15 months or till death within that period.

Survival analysis: For survival analysis, only those patients who were treated with adjuvant chemotherapy and/or radiotherapy were included (N=35). Out of these 35 patients, one patient was lost to follow-up within a short period and one patient with persistent disease died at the end of 11 months. Therefore, these two patients were excluded for the study of relapse-free survival (RFS, N=33). Similarly, for overall survival (OS), out of 35,the patient lost to follow-up within short period was excluded (N=34).

Methods

DNA extraction: DNA was extracted from the frozen tumor tissues obtained immediately after the surgery. Approximately, 30-50 mg of tumor tissue was homogenized properly and washed with PBS (phosphate buffer saline) buffer, centrifuged and pellet was dissolved in TNE (Tris Nacl EDTA) buffer (pH-8.0), subsequently followed by Proteinase K (100 ng/ml) digestion and overnight incubation at 37°C. DNA was then extracted by phenol-chloroform extraction method. The quantification of extracted DNA samples were performed by agarose gel electrophoresis using Lambda Hind III digests. Also, the purity of the DNA samples was checked spectrophotometrically at 260 and 280 nm.

TS genetic analysis: For analysis of TS genotype, polymerase chain reaction (PCR) was performed using PCR Core kit (Qiagen, Germany). PCR of the polymorphic region in the 5'promoter of TS gene, having variable number of 28 bp tandem repeats, was

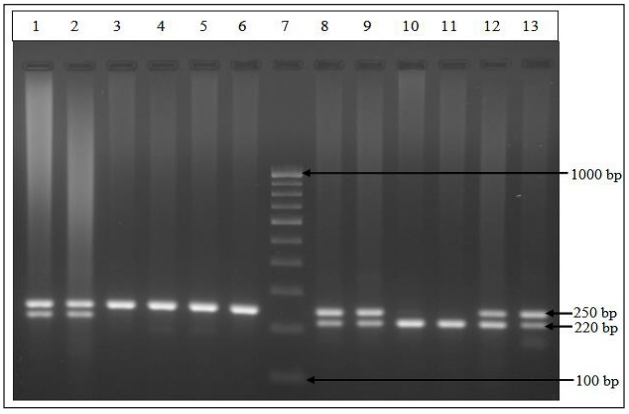

Figure 1: Representative gel image of TS VNTR polymorphism

Lane (1, 2), (8, 9), (12, 13): 2R/3R heterozygous genotype at 220/250 bp for both tumor and its corresponding adjacent normal tissues Lane (3, 4), (5, 6): 3R/3R homozygous genotype at 250 bp for both tumor and its corresponding adjacent normal tissues Lane 10, 11: 2R/2R homozygous genotype at 220 bp for both tumor and its corresponding adjacent normal tissues Lane 7: 100 bp ladder

220/250 bp as shown in Figure 1.

Incidence of TS polymorphism: The incidence of 2R/2R homozygous genotype was observed in 22% (9/40), 3R/3R homozygous genotype in 25% (10/40), and 2R/3R heterozygous genotype in 53% (21/40) of patients (Table 2).

Correlation of 2R/2R homozygous genotype with clinicopathological parameters: The correlation of 2R/2R genotype with clinicopathological parameters is depicted in Table 3.

Table 1: Clinicopathological details of primary CRC patients

Parameters		Number of Patients (N=40)	
		N	%
Age(years) (Range: 32 – 75 years) (Mean: 55.10 ± 1.84 years)	<55	20	50
	≥55	20	50
Gender	Male	24	60
	Female	16	40
Diet	Vegetarian	31	78
	Non vegetarian	4	10
	Mixed	5	12
Habit	Yes	21	53
	No	19	47
Occupation	Sedentary	21	53
	Active	19	47
Site	Colon	22	55
	Rectum	18	45
Tumor size	T2	4	10
	T3	29	72
	T4	7	18
AJCC staging	Early stage (Stage I + Stage II)	23	57
	Advanced stage (Stage III + Stage IV)	7	43
Dukes' staging	B	24	60
	C	15	38
	D	1	2
Astler-Coller staging	B1	2	5
	B2	22	55
	C1	2	5
	C2	10	25
	C3	3	8
	D	1	2
Grade of tumor	Well differentiated	4	10
	Moderately differentiated	30	75
	Poorly differentiated	6	15
Histological type	Adenocarcinoma	30	75
	Mucin secreting adenocarcinoma	10	25
Nodal status	Absent	24	60
	1-3 positive	5	12
	>3 positive	11	28
Lymphatic permeation	Absent	31	78
	Present	9	22
Vascular permeation	Absent	36	90
	Present	4	10
Lymphocytic stromal response	Absent	30	75
	Present	10	25
Pre-operative serum CEA levels (ng/ml) (Mean: 26.37 ng/ml ± 9.15)	< 5.0	16	40
	≥ 5.0	24	60
Treatment	Surgery	5	12
	Surgery + Chemotherapy	23	58
	Surgery + Chemotherapy + Radiotherapy	12	30
Toxicity (N=35)	Absent	19	54
	Present	16	46
Recurrence (N=33)	Absent	26	79
	Local	2	6
	Local + Distant	4	12
	Distant	1	3
Disease outcome (N=34)	Alive	29	85
	Dead	5	15

Table 2: Incidence of TS polymorphism in CRC patients

TS polymorphism	N (%)
2R/2R (homozygous)	9 (22)
3R/3R (homozygous)	10 (25)
2R/3R (heterozygous)	21 (53)

According to Dukes'staging system, an increasing trend was observed from patients with Dukes' B (21%) to patients with Dukes' C (27%). Similarly, according to AJCC TNM staging system, an increasing trend from patients having TNM stage II (23%) to patients having stage III (27%) was observed. However, no correlation was found between 2R/2R genotype and the rest of the parameters.

Correlation of 3R/3R homozygous genotype with clinicopathological parameters: When 3R/3R genotype was correlated with clinicopathological parameters, the following correlation was observed (Table 3).

Similarly as 2R/2R genotype, an increasing trend was observed from Dukes' B stage (21%) to Dukes' C stage (27%) in patients having 3R/3R genotype. According to AJCC TNM staging system also, an increasing trend was observed from patients with TNM stage II (23%) to patients with stage III (27%). However, none of the clinicopathological parameters showed significant correlation with 3R/3R genotype.

Correlation of 2R/3R heterozygous genotype with clinicopathological parameters: The following correlation was observed between 2R/3R genotype and clinicopathological parameters (Table 3).

An increasing trend of 2R/3R genotype was found from patients having T2 tumor size (50%) to T4 tumor size (72%). The 2R/3R genotype dominance increased from patients having lymph node negative (58%) to 1 to 3 lymph nodes positive (80%), but abruptly decreased in patients having more than 3 lymph nodes positive (36%). Contrarily to 2R/2R and 3R/3R homozygous genotype, a decreasing trend of 2R/3R heterozygous genotype was observed from Dukes' B (58%) patients to Dukes' C (46%) patients. Similar results were obtained for TNM staging, a reduced number of patients from stage II (54%) to stage III (46%). Whereas, no correlation was observed between 2R/3R genotype and the rest of the parameters.

Correlation of TS polymorphism with toxicity: Out of total 40 patients, 35 patients had received 5-FU based chemotherapy. Out of these 35 patients who received 5-FU based chemotherapy, 16 patients had developed toxicity. These patients (N=35) when sub grouped according to TS genotype, the frequency trend of toxicity (31%) was observed to be higher amongst patients subgroup having 2R/2R genotype, whereas a reverse trend was noted in the sub group of patients having 3R/3R genotype (19%), In patients having 2R/3R genotype, the incidence was almost similar. (Table 4)

Correlation of TS polymorphism with recurrence: Analysis of association between TS promoter genotype and development of recurrence within 15 month of follow-up period after surgery was evaluated in 33 patients who were treated with 5-FU based chemotherapy. A predominance of 2R/2R genotype (57%) was observed in patients who developed recurrence within 15 months when compared to patients (11%) who developed no recurrence within that period ($\chi 2$= 6.87, df=2, p=0.032; Table 5). On the other hand, significantly higher prevalence of 2R/3R genotype (62%) was observed in patients who did not develop recurrence, as compared to 29% patients who developed recurrence. Similar trend was also noted for 3R/3R genotype in between patients without recurrence (27%) and those patients who developed recurrence (14%) ($\chi 2$= 6.87, df=2, p=0.032; (Table 5).

Correlation of TS polymorphism with disease outcome

At the end of 15 months follow-up period, 34 patients were assessable for the association between TS genotype and disease outcome. Amongst them, 57% patients having 2R/3R genotype were alive till the end of 15 months. In contrast, it was noted that patients who died within 15 months only 25% patients showed the prevalence of 2R/3R genotype. Further, it was observed that the frequency of 3R/3R genotype was 50% in the subgroup of patients who died within 15 months while it was 23% only in this subgroup of patients who were alive till the end of 15 months. The difference between the two groups was statistically non significant. (Table 6)

Univariate survival analysis of TS polymorphism for relapse-free survival: In patients treated with adjuvant therapy, univariate relapse free survival analysis, revealed that patients having 2R/2R (57%) TS genotype polymorphism had early recurrence within 15 months when compared to the subgroup of patients having either 3R/3R (11%) genotype or 2R/3R (13%) genotype. This difference was found to be statistically significant (Log rank $\chi 2$=6.67, df=2, p=0.036). (Figure 2, Table 7)

The Kaplan-Meier survival curve demonstrated that patients having 2R/2R TS genotype was remarkably associated with shorter RFS as compared to subgroup of patients having either 3R/3R or 2R/3R TS genotype (Figure 2). However, larger patient's cohort and longer follow-up period is needed for further conclusion.

Table 3: Correlation of TS polymorphism and clinicopathological parameters

Characteristics		N	TS polymorphism		
			2R/2R	2R/3R	3R/3R
Age	<55	20	5 (25%)	12 (60%)	3 (15%)
	≥55	20	4 (20%)	10 (50%)	6 (30%)
			$\chi2$=1.293, df=2, p=0.524		
Gender	Male	24	6 (25%)	13 (54%)	5 (21%)
	Female	16	3 (19%)	9 (56%)	4 (25%)
			$\chi2$=0.248, df=2, p=0.883		
Habit	Yes	21	5 (24%)	9 (43%)	7 (33%)
	No	19	4 (22%)	13 (68%)	2 (10%)
			$\chi2$=3.52, df=2, p=0.172		
Occupation	Sedentary	21	6 (29%)	11 (52%)	4 (19%)
	Active	19	3 (16%)	11 (58%)	5 (26%)
			$\chi2$=1.104, df=2, p=0.602		
Site	Colon	22	3 (14%)	13 (59%)	6 (27%)
	Rectum	18	6 (33%)	9 (50%)	3 (17%)
			$\chi2$=2.35, df=2, p=0.30		
Tumor size	T2	4	0 (0%)	2 (50%)	2 (50%)
	T3	29	8 (28%)	15 (52%)	6 (20%)
	T4	7	1 (14%)	5 (72%)	1 (14%)
			$\chi2$=3.45 df=4, p=0.48		
Histological type	Adenocarcinoma	30	7 (23%)	16 (54%)	7 (23%)
	Mucin secreting adenocarcinoma	10	2 (20%)	6 (60%)	2 (20%)
			$\chi2$=0.135, df=2, p=0.935		
Histological grade	Well differentiated	4	0 (0%)	2 (50%)	2 (50%)
	Moderately differentiated	30	8 (27%)	16 (53%)	6 (20%)
	Poorly differentiated	6	1 (17%)	4 (66%)	1 (17%)
			$\chi2$=2.92, df=4, p=0.51		
Dukes' stage	B	24	5 (21%)	14 (58%)	5 (21%)
	C	15	4 (27%)	7 (46%)	4 (27%)
	D	1	0 (0%)	1 (100%)	0 (0%)
			$\chi2$=1.34, df=4, p=0.85		
TNM stage	I	2	0 (0%)	2 (100%)	0 (0%)
	II	22	5 (23%)	12 (54%)	5 (23%)
	III	15	4 (27%)	7 (46%)	4 (27%)
	IV	1	0 (0%)	1 (100%)	0 (0%)
			$\chi2$=3.75, df=6, p=0.71		
Nodal status	Absent	24	5 (21%)	14 (58%)	5 (21%)
	1-3 positive	5	0 (0%)	4 (80%)	1 (20%)
	>3 positive	11	4 (36%)	4 (36%)	3 (28%)
			$\chi2$=3.56, df=4, p=0.469		
Mucin	Absent	30	7 (23%)	16 (54%)	7 (23%)
	Present	10	2 (20%)	6 (60%)	2 (20%)
			$\chi2$=0.13, df=2, p=0.93		
Lymphatic permeation	Absent	31	7 (22%)	17 (56%)	7 (22%)
	Present	9	2 (22%)	5 (56%)	2 (22%)
			$\chi2$=0.001, df=2, p=0.999		
Vascular permeation	Absent	36	9 (25%)	20 (56%)	7 (19%)
	Present	4	0 (0%)	2 (50%)	2 (50%)
			$\chi2$=2.514, df=2, p=0.285		
Lymphocytic stromal response)	Absent	30	6 (20%)	18 (60%)	6 (20%)
	Present	10	3 (30%)	4 (40%)	3 (30%)
			$\chi2$=1.212, df=2, p=0.545		
Pre-operative serum CEA levels (ng/ml) (Mean: 26.37 ng/ml ± 9.15)	< 5.0	16	3 (19%)	9 (56%)	4 (25%)
	≥ 5.0	24	6 (25%)	13 (54%)	5 (21%)
			$\chi2$=0.24, df=2, p=0.88		

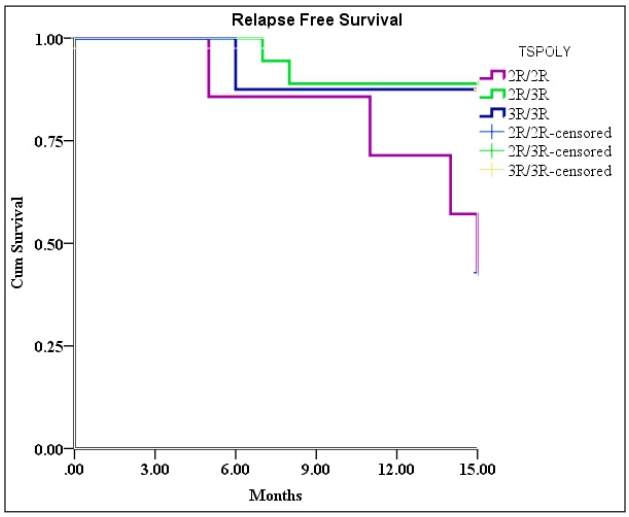

Figure 2: Kaplan-Meier estimates of TS polymorphism for relapse-free survival

Table 4: Correlation of TS polymorphism with toxicity

Parameter		N	TS polymorphism 2R/2R	2R/3R	3R/3R
Toxicity (N= 35)	Absent	19	3 (16%)	10 (53%)	6 (31%)
	Present	16	5 (31%)	8 (50%)	3 (19%)
			$\chi2$=1.47, df=2, p=0.47		

Table 5: Correlation of TS polymorphism with recurrence

Parameter		N	TS polymorphism 2R/2R	2R/3R	3R/3R
Recurrence (N= 33)	Absent	26	3 (11%)	16 (62%)	7 (27%)
	Present	7	4 (57%)	2 (29%)	1 (14%)
			$\chi2$= 6.87, df=2, p=0.032		

Table 6: Correlation of TS polymorphism with disease outcome

Parameter		N	TS polymorphism 2R/2R	2R/3R	3R/3R
Disease outcome	Alive	30	6 (20%)	17(57%)	7 (23%)
(N=34)	Dead	4	1 (25%)	1 (25%)	2 (50%)
			$\chi2$=1.66, df=2, p=0.43		

Table 7: Univariate Log rank relapse-free survival and TS polymorphism in patients treated with adjuvant therapy (N=33)

TS polymorphism	N	Recurrence N (%)	No recurrence N (%)
2R/2R	7	4 (57)	3 (43)
2R/3R	18	2 (11)	16 (89)
3R/3R	8	81(13)	7(87)
	[Log rank $\chi2$=6.67, df=2, p=0.036]		

Cox regression multivariate analysis of TS polymorphism for Relapse-free survival: Cox Regression (Forward Step) multivariate analysis for RFS was performed for TS genotype polymorphism and traditional clinicopathological parameters (age, gender, site, nodal status, Dukes' stage, histologic grade and pre-operative serum CEA levels). It was found that amongst all the variables, TS polymorphism emerged as an independent predictor for RFS in CRC patients (Wald= 4.2, df= 1, p= 0.04). But, a large cohort study is required for proper conclusion.

TS polymorphism for overall survival

TS polymorphism showed no significant correlation amongst the three genotypes for overall survival (data not shown).

Discussion

In this preliminary study, the predominance of 2R/3R genotype was observed in 53% (N=21) of CRC patients as compared to 2R/2R genotype in 22% (N= 9) and 3R/3R genotype in 25% (N= 10) of patients. The results were consistent with the frequencies of study reported by Kristensen et al (2010) in patients with CRC and Hu et al (2012) in patients with NSCLC.[10,11] Contradictorily, Takehara et al (2005) reported 20 cases (6.8%) of 2R/2R, 75 (25.4%) of 2R/3R, 199 of 3R/3R and 1 of 3R/5R (67.8%) in lung cancer.[12] Several studies have suggested that 2R/3R genotype is associated with risk of CRC.[13,14] TS genotypes was found to be identical in tumor and adjacent normal tissues (N=11), probably suggesting that the genetic variants were stable through carcinogenesis.

Further, the present study did not show any significant correlation of TS VNTR polymorphism with clinicopathological parameters. Similar results were reported by Hu et al (2012) and Sulzyc- Bielicka et al (2013).[6,12] One meta-analysis by Popat et al (2004) failed to show any correlation between clinicopathological features with TS genotypes. Toma et al (2010) found no significant association of TS genotype with age, gender, tumor location.[15]

This study reported a decreasing trend of 2R/3R heterozygous genotype from Dukes' B patients to Dukes' C patients while, 2R/2R and 3R/3R genotypes showed increasing trend from Dukes' B stage to Dukes' C stage. Similar results were obtained for TNM staging. Moreover, it was observed that 2R/3R heterozygous TS genotype showed an increasing trend from T2 to T4 tumor size whereas, a decreasing trend was observed from T2 to T4 tumor size in the patients having 2R/2R and 3R/3R homozygous genotypes. In one study by Fariña-Sarasqueta et al (2010), a positive association was reported between tumor T stage and the VNTR genotypes (p = 0.05).16 TS status was also correlated with tumor stage (p =0.02) (Popat et al, 2006). Further, the prevalence of 2R/3R genotype was increased from the subgroup of patients with lymph node negative (58%) to subgroup of patients having 1 to 3 lymph nodes positive (80%), thereafter, it decreased in the subgroup of patients having more than 3 lymph node involvement (36%). However, an reverse trend was noted in patients having 2R/2R genotype.

When TS polymorphism and toxicity was correlated in patients who were treated with adjuvant therapy; a trend of higher frequency of homozygous 2R/2R genotype was noted in patients who developed toxicity. Similarly, Lecomte et al (2004) reported that patients with the 2R/2R genotype were 20 times more likely to have grade 3–4 toxicity compared to those with 3R/3R. [17]

The impact of TS gene polymorphisms on relapse free survival and overall survival in CRC patients treated with adjuvant 5-FU-based chemotherapy alone or in combination with RT was investigated. The study revealed that at the end of 15 months follow-up, TS gene polymorphism failed to emerge as one of the prognostic factor for overall survival in CRC patients treated with adjuvant 5-FU-based therapy. However, in multivariate relapse free survival analysis, TS genotype polymorphism emerged as an independent prognostic factor for relapse-free survival. Out of 35 patients treated with adjuvant 5-FU-based chemotherapy alone or in combination, early recurrence developed in patients harbouring 2R/2R TS genotype polymorphism as compared to patients harboring 2R/3R and 3R/3R TS genotype polymorphism. Fifty-seven percent of patients with 2R/2R TS genotype polymorphism recurred within 15 months whereas only 11% with 3R/3R and 13% patients with 2R/3R TS genotype polymorphism recurred within the same period. Contrarily, Salgado et al (2007) reported lower response rates in colorectal cancer patients with the TS 5'-UTR 3R genotypes (ie, TSER*3), as compared to individuals harboring the homozygous TS 5'-UTR 2R/2R genotype. Jakobsen et al (2005) and Dotor et al (2006) also found longer survival in carriers of TS 5'-UTR 3R genotypes as compared with those carrying the TS 5'-UTR 2R/2R genotypes.[6,18] Similar result was reported by Yim et al (2010) in patient with gastric cancer.[19] While, in studies conducted by Stoehlmacher et al (2004) and Kostopoulos et al (2009) failed to observe any significant difference in the clinical outcome according to the TS genotypes.[20,21] Such conflicting results for predicting outcome from TS genomic status is not surprising.

In conclusion, the only significant finding in the present study is that patients with more advanced disease had 2R/3R genotype polymorphism. Besides, it is a well established fact that the TS 28 bp VNTR polymorphism is widely studied as a part of

pharmacogenomics to predict response to 5FU based therapy though most studies have found no correlation. The present study concludes that, TS 28 bp VNTR polymorphism may perhaps be useful prognostic and predictive factor for disease-free survival but not for overall survival in patients with primary CRC. However, a larger patient series and longer follow-up study will be essential to attain final conclusion.

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Cross Kingdom CrossTalk: Role of Plant miRNAs in Human Health

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The age old saying "We Are What We Eat" substantiates the scientifically proven fact that our food habit and lifestyle affect our health. Every cell in our body is fed with nutrients assimilated from diet we eat, the water we drink and the air we breathe. In addition to nourishing our body, diet also affects the quality of our life and our overall health and well-being. More attention has been given on phytopharmaceuticals and nutraceuticals due to their medicinal values e.g. curcumin, zingiberin, polyphenols, catechin, emodin, resveratrol etc. At the same time attention has also been given to gene expression and its regulation to understand the disease process and management. Recently focus has been diverted towards noncoding region of the genome responsible for genesis of small RNA called miRNA. These miRNAs are primarily regulatory molecules of gene expression responsible for myriads of cellular functions. They present themselves as double edged sword where it can both downregulate and upregulate a gene target by binding to gene specific transcript or transcript of its transcription factor. Hence, their malfunctioning or presence of a wrong miRNA in a wrong cell at a wrong time contributes to disease pathogenesis including cancer. The miRNA mediated gene regulation was thought to be within the same kingdom either plant or animal. There was a notion that presence of plant miRNA in human body released after digestion of food is merely impossible. However, researchers have now confirmed presence of plant derived miRNA in human blood plama at a relatively high level and revealed that plant miRNAs in food can regulate the expression of target genes in mammals including human. This gives a clue for the basis of Ayurvedic system of medicine where the whole extract, powder or polyherbal formulations are used for rejuvenation and healing various ailments.

Therefore, the best way to stay healthy is to feed ourselves up with nutrient-rich whole foods and fruits which provide all the vitamins, minerals, fiber, antioxidants and phytochemicals needed for chemoprevention and immune-modulation to combat most diseases.

If insurance companies paid for lifestyle-management classes, they would save huge sums of money. We need to see that alternative medicine is now mainstream.

Deepak Chopra

Anaesthetic Management of a One Month Old Child of Dandy Walker Syndrome with Huge hydrocephalus- A Case Report

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Summary

Dandy Walker syndrome characterized by hypoplasia of the cerebellar vermis, cystic dilatation of the fourth ventricle and hydrocephalus. This syndrome was first described by Dandy and Blackfan in 1916. Associated congenital anomalies are said to be present in 48% of cases. Signs and symptoms of this disease become evident in first year of life with slow motor development, bulging anterior fontanel and progressive enlargement in size of skull. This paper presents one month old who reported with huge hydrocephalus which was present since birth for cystoperitoneal shunt. Anesthetic management was challenging due to congenital anomalies associated with syndrome, suspected difficult airway management due to large head circumference and physiological changes of infant.

Keywords: Dandy Walker Syndrome, Hydrocephalus, Congenital anomalies

Introduction

Dandy Walker syndrome classically described as a neuropathological triad consisting of hypoplasia of the cerebellar vermis, cystic dilatation of the fourth ventricle and hydrocephalus.[1] This syndrome was first described by Dandy and Blackfan in 1916. Associated congenital anomalies are said to be present in 48% of cases. These commonly include craniofacial anomalies such as cleft palate, micrognathia, hypertelorism, cardiac, renal and skeletal malformation. Cerebral anomalies include agenesis of corpus callosum with poor intelligence and interference with medullary control of respiration often resulting in respiratory failure. Signs and symptoms of this disease become evident in first year of life with slow motor development, bulging anterior fontanel and progressive enlargement in size of skull. It is at the severe end of the spectrum denoted by the term dandy walker continuum. Clinical manifestation appear in first year of life but can occur during neonatal period.[2] Asai et al[3] (1989) reported as many as 89% of patients diagnosed before first year of age. Genetic factors have a major role in the etiology of this condition. Dandy walker malformation may occur as a part of Mendelian disorders and chromosomal aberrations. Environmental factors including viral infections, alcohol and diabetes have also been suggested to play a role in the genesis of Dandy walker malformation but evidence is uncertain.[4]

Case Report

One month old female child weighing six kg was admitted to neuro intensive care unit because of hydrocephalus. Patient's mother, 28 years old, multigravida was referred for routine antenatal ultraspnography at 22 weeks of gestation. She had no symptoms to offer and obstetrical examination revealed uterine size 22-24 weeks. Her ultrasonography showed single live intra uterine gestation with biparital diameter and foetal femur length corresponding to 22-24 weeks of gestation. It showed 4th ventricle hydrocephalus and corpus callosum agenesis. Parents were informed about scenario of disease but they didn't take any active care of it. This female child, at the age of one month, came to our hospital with large head circumference (approximately 80cm) which always remain in lateral position. She was planned for elective cystoperitoneal shunt operation for hydrocephalus. Along with routine preoperative check up she was also examined for disorders associated with this syndrome like involvement of other areas of central nervous system which include absence of corpus callosum, malformation of face, limbs, fingers and toes.(e.g. polydactyly, syndactyly, cleft palate). CT Brain showed large posterior fossa with high tentorial insertion and torcular inversion, absent cerebellar vermis with hypoplastic bilateral cerebellar hemisphere, 4th ventricle hydrocephalus, corpus callosum agenesis, dilatation of both lateral and 3rd ventricle with mild periventricular interstitial edema.

After obtaining written informed consent of parents, patient was fasted for four hours of breast feeding. As patient had large head always remaining in left (Figure 1) lateral position, we were expecting difficult intubation and were prepared for same. After securing intravenous access monitors applied and inhalational induction done with sevoflurane. Intubation was done in left lateral position after keeping towel below head with zero size Miller's straight blade introduced extreme left side of mouth. Tip of epiglottis visualized by the aid of extreme manipulation of larynx and intubation was performed with number three uncuffed portex endotracheal tube. Endotracheal tube placement was confirmed with capnography and auscultation of lungs and then fixed. (Figure 2) Anaesthesia was maintained with oxygen, sevoflurane and atracurium. Patient was monitored for temperature, heart rate, ECG, SpO_2, $EtCO_2$, NIBP, urine output.

Fluid management was made by intravenous administration of balanced electrolyte solution after the calculation of perioperative fluid deficit and basal requirement of child. At the end of surgery trachea was extubated when the patient was fully awake in operation theatre. Surgery was uneventful. After uneventful recovery period patient was taken to intensive care unit and monitoring of temperature, blood pressure, respiratory rate, SpO_2 done.

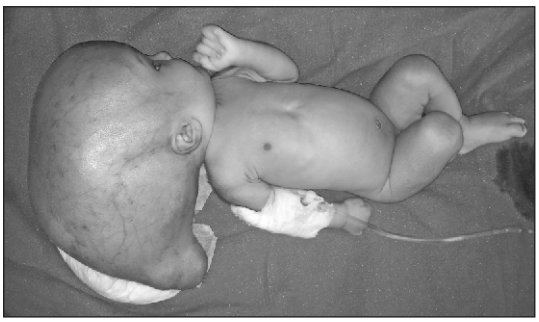

Figure 1: Hydrocephalic head in left lateral position

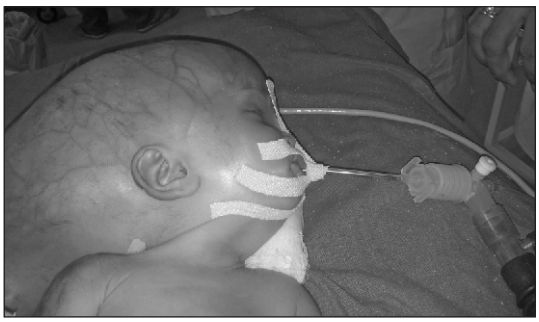

Figure 2: Endotracheal tube insitu

Discussion

Dandy Walker syndrome is not a single entity but several abnormality of brain development coexist.[4] Dandy Walker syndrome requires neurosurgery. Neither marsupialisation nor excision of the membranes of the 4th ventricle has shown definitive improvement. A ventriculo peritoneal shunt operation frequently suffices. Estimated prevalence of about 1:30,000 live births with a slight female prepordence and its responsible for 4-12% of infantile hydrocephalus.[5]

Due to abnormalities associated with this syndrome and common airway changes in children we were prepared for difficult intubation. The tongue is larger in relation to the oral cavity, the epiglottis is large and floppy making it difficult to visualize the airway. The larynx is located higher in infants than in adults hence requiring a straight blade to intubate, the cricoid is the narrowest portion in an infant and hence larger endotracheal tube may lead to airway compromise.

We have reviewed some position for intubation out of them one option is when mild to moderate hydrocepalus is present (most common) tracheal intubation can be performed with the patient in supine position and child's back is supported with the folded towel to avoid direct pressure on the swelling. Another option is after keeping patient's shoulder at the edge of table, head supported by assistant and intubation done. As Rajan S[6] presented a case report of induction and intubation in a Kleeblattschadel syndromic child with post cranial disorder. In his case patient having abnormally shaped large head, hydrocephalus, pancraniosynostoses and posterior distractor so induction and intubation not possible in supine or even lateral position so child was positioned such a way that the body up to shoulder was resting on a table with the shoulder at the edge of table. Head with posterior distractor and neck was supported by an assistant.

In our case swelling was in cystic nature, very huge (head circumference 80cm) and patient couldn't move her head so chances of rupture during manipulation was there, so considering this we put patient in left lateral position and towel below head. As lateral approach provide bypass of tongue and maxillary structures and improve view of glottis.

At induction, we avoided use of succinylcholine in anticipation of difficult intubation. We induced patient with sevoflurane inhalation and put patient on controlled ventilation mode as in these patients inereased intracranial pressure can get exacerbated by any depression of ventilation Hence general anaesthesia with controlled ventilation is preferred and maintain with sevoflurane and regular dose of atracurium so as to get abdominal relaxation for shunt.

Hydrocephalus may cause raised intracranial pressure where anesthetic induction should be performed without increasing cerebral blood flow and cerebral metabolic rate. General measures like maintaining normocapnia, normotension, euglycemia helps in reducing intra cranial pressure.

A period of postoperative ventilation may be required for such patients, as they suffer from recurrent episodes of apnea. Our patient returned back to spontaneous ventilation soon after surgery and did not need ventilator support as respiration was good.

Conclusion

Thorough evaluation of the patient's examination, difficult intubation measures, measures taken to control intracranial pressure and postoperative care is essential to successful management of a patient afflicted with the Dandy Walker syndrome with huge hydrocephalus.

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Methotrexate Induced Hypersensitivity Pneumonitis in a Patient with T cell Lymphoblastic Lymphoma: A Case Report and Review of Literature

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Summary

This case report describes presentation and management of methotrexate induced hypersensitivity pneumonitis. The patient, 17 year old male, was suffering from pre-T cell Lymphoblastic Lymphoma and was on maintenance therapy. He presented with breathlessness on exertion and cough on expectoration. He had tachypnoea with fine crackles in both lung fields. Here we describe importance of early identification and treatment of methotrexate induced hypersensitivity pneumonitis.

Keywords: Methotrexate, Hypersensitivity pneumonitis, Lymphoblastic lymphoma, Steroids

Introduction

Methotrexate is a cell cycle specific antifolate analog which is used in different benign conditions like RA, Psoriasis and in different malignant conditions like leukemia, lymphoma and other solid tumors like GTN, Breast, Osteosarcoma etc.[1,2] Methotrexate induced hypersensitivity pneumonitis is a known complication in patients with rheumatoid arthritis. As it is rare in patients with leukemia it can be easily missed and can lead to fatal outcome. We present a case of one such patient with methotrexate induced hypersensitivity pneumonitis, treated successfully with high dose steroids.

Case Report

Clinical presentation

17 year old male patient resident of Jodhpur and student, diagnosed as case of pre-T cell Lymphoblastic Lymphoma in Nov 2011. He was started on BFM 90 protocol which includes vincristine, daunorubicin, steroids,[1] asparaginase, cytarabine, cyclophosphamide, 6-mercapto purine, methotrexate up to first 6 months followed by maintenance therapy with oral 6-mercapto purine and methotrexate. Patient has completed 18 month of maintenance therapy till March 14.

Patient came to our institute while on 19th month of maintenance with chief complaints of breathlessness on exertion, cough with expectoration without fever for one week. When he presented, he has tachypnoea with normal SPO_2. On systemic examination he had fine crackles heard in both lung fields predominantly in upper lobes. Rest of the systemic examination was normal.

Investigations

All routine investigations including complete hemogram, renal function test and liver function test were normal. Chest x-ray was suggestive of bilateral ground glass opacities. CT Thorax – diffuse ground glass opacities in both lung fields more in upper and middle lobe possibility of hypersensitivity pneumonitis (Figure 1). Sputum sample sent for routine micro, gram stain, acid fast bacilli stain, stain for pnuemocystis carinii and culture was negative. Blood culture and Pneumoslide IgM test were negative. Procalcitonin was normal (0.51).CRP was elevated (90), Galactomannan was normal (0.96). CMV- PCR was neg. CPK MB was 12. 2D echo was normal except tachycardia. BAL culture and AFB was negative. BAL cytology was suggestive of presence macrophages, epithelium, inflammatory cells, and squamous cells in hemorrhagic background. Bronchoscopy guided biopsy was suggestive of inflammatory infiltrate in the wall.

Treatment and follow up

Patient was admitted and started on intravenous broad spectrum antibiotics (piperacilllin-tazobactum, levoflox and vancomycin) and oxygen support at 2-4 L/min. He was given this treatment for three days. He did not improve initially and he developed hypoxia. Then he was shifted to medical intensive care unit and put on ventilator. His antibiotics were upgraded to meropenem and colistin. He was also started on voriconazole and acyclovir. Despite all these, he did not improve so he was started on 100 mg hydrocortisone every 8 hourly. After starting of hydrocortisone, he improved dramatically. He was wean off from ventilator and was shifted on oral prednisolone and later discharged in stable condition. CT scan of chest and chest x-ray done after one month were normal (Figure2). Rest of his maintenance cycles were completed with 6mp only. Steroids were stopped after gradual tapering over one month. At present he is under observation after completion of treatment.

Discussion

Methotrexate is an anti folate drug which is used in various conditions like rheumatoid arthiritis, leukemia, lymphomas and sarcomas.[1] It is used in various doses ranging from 2.5mg weekly to $12g/m^2$ Methotrexate can cause many adverse reactions, most

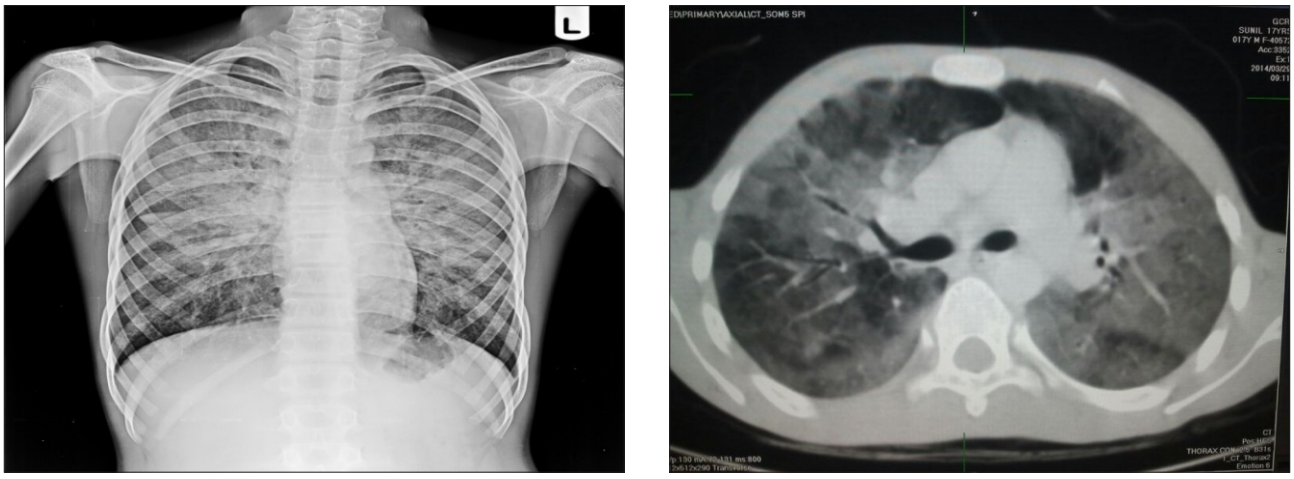

Figure 1: Chest x-ray and CT scan at the time of presentation showing bilateral haziness with pneumonitis

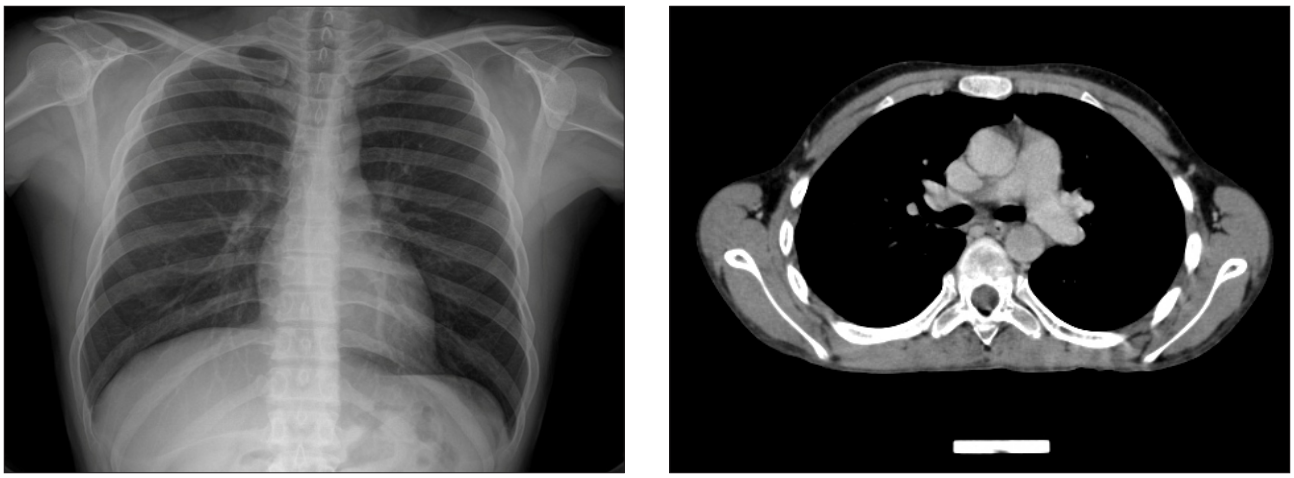

Figure 2: Normal chest x-ray and CT scan on follow up

commonly affecting gastrointestinal tract, hematopoietic, central nervous system, skin, pulmonary and hepatotoxicity.[3,4] Out of all adverse reactions stomatitis, gastrointestinal upset and hematologic abnormalities can be prevented by supplemental folic or folinic acid, without affecting the efficacy of methotrexate. However, this does not reduce the risk for methotrexate induced pulmonary or hepatic toxicity.

Methotrexate can cause various type of lung injuries like hypersensitivity pneumonitis (extrinsic allergic aleveolitis), bronchiolitis obliterans with organizing pneumonia (BOOP), acute lung injury with noncardiogenic pulmonary edema, pulmonary fibrosis and bronchitis with airways hyperreactivity.[6] Among them, hypersensitivity pneumonitis is the most common type of lung injury.[7] Hypersensitivity pneumonitis is more common with low dose, continuous exposure of methotrexate than with high dose. In most literature, this complication is mentioned in treatment of rheumatoid arthritis with low dose methotrexate.[10,13]

Mechanism of action for methotrexate induced lung injury is unknown. But many of the risk factors are identified like old age, previous lung injury, diabetes.8 Proposed mechanism for this injury is damage induced by pneumotoxic agents gives rise to alveolitis and edema. In response to injury to the lung parenchyma, there is an immediate requirement to initiate tissue repair and restore barrier function. Acute injury may progress to chronic inflammation and eventually lead to fibrotic change that ultimately interferes with gas exchange. Chemotherapeutic drugs can additionally cause a direct toxic reaction, and direct toxicity usually occurs over time before manifesting clinically-induced pulmonary fibrosis.[2]

Patients treated with methotrexate can present acutely as early as 12 days after treatment initiation or in a chronic form after 18 years. Most of the patients present with sub acute toxicity of methotrexate. In these patients most common symptoms of presentation are fever, chills, malaise, cough, dyspnea, and chest pain. In severe cases, it may rapidly progress to respiratory failure.

At the time of presentation with such symptoms, routine chest X ray shows widespread interstitial opacities in early stage which later may progress rapidly to patchy acinar consolidation. High resolution CT scan may show patchy areas of ground glass attenuation with small areas of consolidation.[10] They are usually bilateral, symmetric, and predominantly in lower lung zones. Bronchoalveolar

lavage studies may reveal lymphocytic predominance with an increase in the number of CD4+ lymphocytes and the CD4/CD8 ratio.[9] Lung biopsy if done may show alveolitis with epithelial cell hyperplasia and cytologic dysplasia.[11]

There is no specific diagnostic test available for methotrexate induced hypersensitivity pneumonitis. A combination of clinical signs and symptoms, radiology, bronchoalveolar lavage, and pulmonary cytology are used today for diagnosis.[9,10,11] Improvement of patient's signs and symptoms with discontinuation of drug and response to steroid therapy (1mg/kg/day) are used as diagnosis. It also acts as treatment. When diagnosis is suspected, methotrexate should be stopped and prednisolone upto 100 mg/day should be started. With clinical response prednisolone should be continued for 15 days followed by gradual tapering over 4-6 weeks.[13,14]

For diagnosis of methotrexate induced hypersensitivity pneumonitis certain criteria is proposed. They are known as Searles and McKendry diagnostic criteria, which are as below.[11,12,13]

Searles and McKendry diagnostic criteria-

•Major

1. Interstitial Pneumonitis by histopathology without evidence of pathogenic organism
2. Radiologic evidence of pulmonary interstitial or alveolar infiltrates
3. Negative blood and initial sputum cultures

•Minor

1. Shortness of breath for less than eight weeks
2. Nonproductive cough
3. Oxygen saturation ≤ 90 % on room air on initial evaluation
4. DLCO ≤ 70 % of predicted for age
5. Leukocyte count ≤ 15,000 cells/mm^3

Definite diagnosis can be made by either Major criterion 1 with 3 minor criteria or Major 2 and 3 with 3 minor criteria.[11,12] Probable diagnosis can be made by Major 2 and 3 criteria with 2 minor criteria.

Once the diagnosis is made and patients are treated with steroids and then gradually tapered.[13,14] After complete recovery of patient, rechallenging with methotrexate should be done or not is still unclear. Some literature suggests rechallenging can be done without risk of pneumonitis in acute and subacute forms. But most of the literature is not in favoring of rechallenge with methotrexate.

Conclusion

Methotrexate is an essential component of leukemia treatment with low dose is given for 2 years as maintenance therapy. Methotrexate induced hypersensitivity pneumonitis is a rare but treatable complication. The condition should be suspected after ruling out infection and relapse and is diagnosed on base of clinical and imaging picture and diagnostic criteria (Searles and McKendry). It responds very well to steroids. So suspicion for this condition should be high and immediate treatment with steroid should be initiated.

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Bleomycin Induced Pneumonitis: A Case Report and Review of Literature

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Department of Medical and Pediatric Oncology

Summary:
This case report describes presentation and management of Bleomycin induced Pneumonitis. A 60 year old male patient, who was taking chemotherapy including bleomycin for treatment of Hodgkin's Lymphoma, presented with complaint of exertional dyspnoea and dry cough. Patient had tachypnoea, tachycardia and fine crackles in both lung fields. Here we describe importance of early identification and treatment of Bleomycin induced Pneumonitis.
Keywords: Bleomycin, Hodgkin's Lymphoma, Lung injury, Toxicity, Safe use, Treatment

Introduction:

Bleomycin is a potent antineoplastic agent with activity mainly against germ cell tumor and Hodgkin's lymphoma. But its usefulness is limited by life threatening toxicities like hypersensitivity reaction and pulmonary side effects like Bleomycin induced pneumonitis (BIP) which occurs in 20% to 40% of treated patients with high mortality (10% to 20%). 1,2,3 So awareness about such rare but potentially fatal toxicity is helpful in early identification and treatment if it occurs. We report a case of bleomycin induced pneumonitis in a patient of Hodgkin's lymphoma treated with ABVD protocol who was successfully treated with high dose of corticosteroids.

Case Report

Clinical Presentation: A 60 year old male, non-smoker, presented with complaints of neck swelling, anorexia and weight loss since 2 month. He was taking 25 mg of atenolol daily since 5 years for hypertension. Biopsy of cervical node was suggestive of Hodgkin's Lymphoma. PET-CT scan was suggestive of cervical, axillary, mediastinal and abdominal lymphadenopathy with involvement of spleen and bones. Bone marrow was also involved. His blood investigations were within normal range except for high ESR. His blood pressure was controlled with 25 mg of atenolol per day for last 5 years. He was treated with ABVD protocol (each cycle include 2 courses of Adriamycin 25 mg/m^2, Bleomycin 10 U/m^2, Vinblastine 6 mg/m^2 and Dacarbazine 375 mg/m^2 14 days apart).

On the day of his 6th cycle of chemotherapy, he presented with complaint of exertional dyspnea and dry cough. He denied history of fever, chest pain or hemoptysis. On examination he has tachypnoea and tachycardia with normal blood pressure and SPO_2. On auscultation, fine crackles were present in both lung fields predominantly in lower lobes. Other systemic examination was unremarkable.

Investigations:

Blood investigations including hemogram, blood sugar, serum electrolytes and renal and liver function tests were within the normal range. Fluffy soft tissue opacities were seen in both lung fields with internal air bronchogram on chest x-ray(fig.1a). Electrocardiogram was normal. Echocardiogram revealed severe pulmonay arterial hypertension with normal left ventricular function. (Ejection fraction 55% right ventricular systolic pressure 70 mm Hg). HRCT scan of thorax showed ground-glass opacities in both lungs which was suggestive of non-specific pneumonitis and mediastinal lymphadenopathy with pulmonay arterial hypertension(fig.1b). Pulmonary angiography didn't show evidence of pulmonary embolism. Pulmonary function test and DLCO (diffusion capacity of lung for carbon monoxide) revealed restrictive lung disease with decrease in diffusion capacity. All microbiological cultures were negative for organism.

Treatment and follow up:

Patient was hospitalized and given treatment in form of Intravenous broad spectrum antibiotics (cefoperazone+sulbactam 2gm/day and linezolid 1200 mg/day) and oral azithromycin (500mg/day), Oxygen inhalation at 2-4 L/min, and supportive therapy including diuretics. As his symptoms were progressive with no response to antibiotics for 3 days, he was given intravenous dexamethasone (24 mg/day) with suspicion of pneumonitis. His dyspnoea improved in 48 hours and he was able to walk to the washroom without assistance and without oxygen. He was discharged on oral antibiotics (linezolid and cefixime) for 7 days and oral steroids (prednisolone 60 mg/day) which were tapered over next 1 month. He had marked symptomatic improvement over next 15 days. He received last cycle of chemotherapy without bleomycin. Thirty days after completion of chemotherapy, PET/CT scan was done. PET/CT scan report was suggestive of complete metabolic response(fig.2a,b). Afterwards he was kept under observation.

Discussion

Bleomycin-induced pneumonitis is a major pulmonary toxicity that occurs in approximately 20% to 40% of patients who are affected by Hodgkin's lymphoma (HL) and are treated with a bleomycin containing regimen.[1,2] The mortality of this complication is high, ranging from 10% to 20%, and significantly impacts quality of life and 5-year overall

Before Treatment

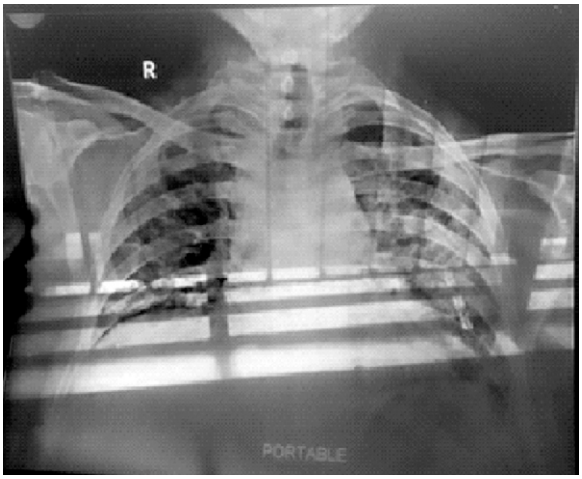

Figure 1 a: Chest X Ray showing bilateral haziness

Figure 1 b: CT scan showing ground glass opacities

After treatment

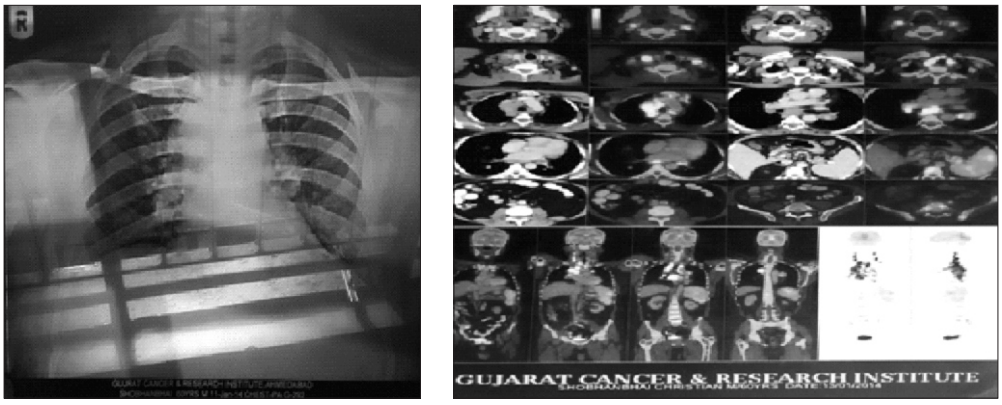

Figure 2: a Normal Chest X-Ray **b** Normal PET scan after 1 month

survival.[3] The risk factors suggested include advanced age, high cumulative dose (>400 - 450 IU/m^2), renal impairment, bolus administration, smoking and giving concurrent high flow oxygen, radiotherapy and granulocyte colony stimulating factor (G-CSF) support.[4,5]

BIP pathogenesis is unknown, but there is consensus that deregulated mechanisms of tissue repair driven by profibrotic cytokines initiate the lung-damaging effects.[6,7] Specifically, tumor growth factor β, platelet-derived growth factor receptor α (PDGFR-α), and tumor necrosis factor α are believed to cause transformation, proliferation, and accumulation of fibroblasts with the deposition of an extracellular matrix. The progressive accumulation of this collagen matrix determines distortion and destruction of the alveolar structures and, eventually, loss of lung function.

Clinical picture of BIP is usually characterized by dyspnea after exertion, persistent nonproductive cough and mild fever. At physical examination, findings may be non-specific and range from tachypnea only to dry crackles. Generally, the possibility of BIP is suggested by chest imaging studies. The presence of respiratory symptoms and bilateral interstitial infiltrates on CT scans along with the exclusion of active lung

infections (defined as negative cultures and lack of response to antimicrobials) are the most important clues to clinical diagnosis. Whenever feasible, the diagnosis relies on a lung biopsy that demonstrates a heterogeneous appearance with alternating areas of normal lung, interstitial inflammation, foci of proliferating fibroblast, dense collagen fibrosis, and honeycomb changes.[9]

Pulmonary fibrosis from bleomycin can develop insidiously during treatment.[10] Patients should be questioned carefully for new dry unproductive cough or new respiratory limitation of exercise tolerance at each visit. Persistence of either of these symptoms for longer than one week without other explanation, such as infection, should prompt consideration of discontinuation of the bleomycin. Some authors have recommended serial measurement of carbon monoxide diffusing capacity by pulmonary function testing, however, this has not proved to be a reliable predictor of bleomycin toxicity and its use must be individualized. A normal chest x-ray is unreliable to exclude BIP.[10] Identification of patients with bleomycin induced pulmonary toxicity can be very difficult due to the non-specific signs and symptoms.[10] Treatment includes corticosteroids for pneumonitis to prevent pulmonary fibrosis and antibiotics for infectious pneumonitis.

Bleomycin therapy should be withheld if the pulmonary diffusion capacity for carbon monoxide (DLCO) falls to 30-35% of initial value, if the forced vital capacity (FVC) falls significantly, or if there are any clinical or radiographic features indicating pulmonary toxicity. Pneumonitis due to bleomycin should be treated with corticosteroids in an effort to prevent progression to fibrosis. Corticosteroids have been of questionable value once interstitial fibrosis has occurred. Patients should be warned that uncontrolled oxygen should be avoided except briefly in an emergency and to avoid increased oxygen pressure as in scuba diving.

The patient was an older male who received 100 IU/m^2 of bleomycin as total dose, was a nonsmoker and did not receive radiotherapy. Clinically the patient had presented 15 days after the last cycle of chemotherapy, with an acute presentation and typical physical exam findings and gas exchange abnormalities. The patient also received G-CSF during previous cycles. Though, Data has been presented to refute the role of G-CSF in augmenting Bleomycin induced Pulmonary Toxicity in both advanced germ cell tumors and Non Hodgkin's Lymphoma, the relationship between G-CSF and bleomycin in promoting pulmonary toxicity in Hodgkin's Lymphoma patients is less clear in the literature. In a retrospective study, Martin et al reported higher rate of Bleomycin induced Pulmonary Toxicity in patients receiving G-CSF (26%) compared with patients who didn't received G-CSF (9%) (p= .014) without any difference in overall survival (OS) or progression free survival (PFS).[11]

Conclusion

Bleomycin induced pneumonitis is uncommon but serious complication of bleomycin containing chemotherapy regimen. It should be considered as differential diagnosis if the patient presents with compatible features especially in presence of risk factors. Prompt diagnosis and aggressive treatment with high dose corticosteroids can reduce morbidity associated with this complication.

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Summaries of Presentations at Clinical Meetings

01. Imaging Predictors of severity assessment in Patients with Pulmonary Embolism

Shah Dwiti
Radiodiagnosis Dept

Summary

Acute pulmonary embolism being the 3rd most common cause of acute cardiovascular death after mi and stroke and its nonspecific clinical presentation; needs a strategic diagnostic approach. Several clinical prediction scores; most commonly used two level well's score are used before proceeding to the imaging procedures. Several imaging criteria for acute and chronic pulmonary embolism on different modalities have been described. Computed tomographic (ct) pulmonary angiography is becoming investigation of choice for the evaluation of patients with suspected pulmonary embolism. This pathologic condition, whether acute or chronic, causes both partial and complete intraluminal filling defects, which should have a sharp interface with intravascular contrast material. Severity imaging predictors and the clinical significance of each of them has to be evaluated. Role of other imaging modalities less often practiced with its shortcomings also needs to be understood.

02.Safety and Efficacy of Eltrombopag in Post-Hematopoietic Stem Cell Transplantation (HSCT) Thrombocytopenia

Choudhary Mukesh
Medical Oncology

Summary

Aim: This study was done to determine the safety and efficacy of Eltrombopag in Post-hematopoietic Stem Cell Transplantation (HSCT) Thrombocytopenia.

Method: This retrospective study was conducted in the Bone marrow transplantation unit of GCRI at Ahmedabad from January 2012 to July 2014.

Results:Twelve adult patients (median age 29.5 years)were started on Eltrombopag 25–50 mg/day for post-hematopoietic stem cell transplantation thrombocytopenia. All patients were having primary thrombocytopenia after HSCT. No patient had other secondary cause for thrombocytopenia. Two patients were allogenic subsets (1 acute myeloid leukemia i.e., AML and 1 aplastic anemia), and 10 were autologous transplants (3 multiple myeloma, 6 lymphoma and 1 AML). Nine patients were males, three were females. The median time of starting Eltrombopag was 21 days post-stem cell infusion (range day 17 to 60) at a median platelet count of 9,000/cmm(range 3,000–11,000/cmm). The median duration for treatment was 29 days. Median total dose of 812.5 mg was received by patients and they had a median platelet increment of 36,000/cmm. We observed that there were no adverse effects in these patients and there was a gradual increase in platelet count so that none of the patients had any complication due to thrombocytopenia. The cost of treatment was less than the cost of extended hospitalization and irradiated single donor platelet transfusion.

Conclusion: 25–50 mg once daily dosing of Eltrombopag to enhance platelet recovery for post-hematopoietic stem cell transplantation thrombocytopenia is well tolerated, appears efficacious and offers transfusion independence.

Keywords: Eltrombopag,Post-hematopoietic stem cell transplantation, Thrombocytopenia

03. management of locally advanced cancers of thyroid

Joshipura Aditya
Surgical Oncology

Summary

Introduction

Locally advanced cancers of thyroid are a matter of surgical dilemma with decision to be made regarding the complete removal and the complications arising thereof compared to the incomplete resection.

Materials and methods: 2 patients of locally advanced well differentiated cancers of thyroid were operated upon with removal of larynx in one case and tracheal rings in one case. The post operative measures and follow up with adjuvant treatment studied.

Results: adequate removal of tumor and relatively less complications were seen with complete removal of the tumor. Both patients are on adjuvant therapy and will be followed up regularly

Conclusion: complete resection of locally advanced carcinoma of thyroid is better and leads to better post operative outcome and prognosis.

04. Pelvic Nodal Metastasis in Primary Malignant Sex-Cord Stromal Tumors of the Ovary: A Single Institution Experience

Modi Rahul Deepak
Gynecologic Oncology

Summary:

Aim: To evaluate the incidence of pelvic nodal metastasis in patients with primary malignant sex-cord stromal tumors(SCSTs) of the ovary and add to the limited data available on nodal metastasis in ovarian SCSTs.

Methods: A retrospective 5-year single institution review of patients with primary malignant ovarian

SCSTs at The Gujarat Cancer and Research Institute (GCRI) between 2009 and 2014 was done. Information was collected regarding patient and tumor characteristics from pathology and medical records.

Results: A total of 48 patients were reviewed, 36 (75%) had granulosa cell tumor followed by fibroma-thecoma group (14.6%). All the nodal tissues examined in these patients were negative. Stage I (85.4%) was most common at presentation.

Conclusion: Our study supports the recommendation of recent published data regarding lack of lymph node metastasis in SCSTs and abandonment of lymphadenectomy for staging procedures of these tumors in primary upfront surgeries.

Keywords: Ovarian malignancy; sex-cord stromal tumor; pelvic node metastasis.

05. Overview of Cancer Screening Camp Activity at GCRI.

Shah Janmesh
Community Oncology & Medical Records

Summary

Aim: This is to make aware all staff members about the types of camps and procedure of arranging any camp by GCRI. This is also to discuss work done in various camps in last year.

Method: Standard Operating Procedure (SOP) of camp is discussed in this presentation. The data of year 2014 has collected, compiled and analyzed. The data of year 2013 was also gathered and it is compared with year 2014. Problems in conducting camps is discussed with the camp coordinator and discussed here.

Results: After compiling data of year 2014 (January 2014- December 2014), I found 239 camps were organized compared to 184 camps in 2013. While number of camp beneficiaries have increased from 2842 in 2013 to 9980 in 2014. Almost 700 mammography were performed in the last year's camps. While 189 beneficiaries were referred to GCRI and from those only 42 came to GCRI and took treatment.

Conclusion: The camp activity is increasing gradually with increase in patient load. But there is a strong need of coordinated work and increase referral of patients from camp site.

Keywords: camp, Sanjivani Rath

06. A Comparative Study of the effect of Dexmedetomidine HCL and Lignocaine HCL on Hemodynamic Responses and Recovery following Tracheal Extubation in Patients undergoing Intracranial Surgery

Prajapati Dhvanan
Anesthesiology

Summery:

Introduction: The α2-adrenoreceptor agonist, dexmedetomidine, provides excellent sedation with cardiovascular stability and may be a useful adjunct to facilitate smooth tracheal extubation in intracranial surgery.

Material and Methods: 75 patients scheduled for elective intracranial surgery for intracranial space occupying lesions were randomly divided into three groups of 25 each. Balanced general anesthesia was given. At the end of the surgery after return of spontaneous respiration patient in Group D received injection dexmedetomidine 0.5 μg/kg intravenous (IV), Group X received injection lignocaine 1.5 mg/kg IV and Group P received 10 ml normal saline IV over 60 sec. Heart rate (HR), mean arterial pressure (MAP), quality of extubation were measured at 1, 3, 5, 10, 15 mins interval after extubation. Emergence time and extubation time were noted and quality of extubation was evaluated on cough grading.

Results: There was a significant decrease in MAPs and HR in Group D as compared to Group L and Group P ($p<0.05$) at all-time interval after extubation. Extubation quality score of the majority of patients was 1 in Group D, 2 in Group X, and 3 in Group P ($p<0.001$). The duration of emergence and extubation were comparable in all three groups. Sedation score of the most patient was 3 (44%) in Group D and 2 (56%) in Group X. Six patients in Group D and 1 patient in Group X had bradycardia.

Conclusion: Dexmedetomidine HCl 0.5 mcg/kg IV given before tracheal extubation effectively stabilize hemodynamics and facilitate smooth extubation.

Keywords: Dexmedetomidine HCL, Lignocaine HCL, Extubation, Intracranial surgery

Wherever the art of medicine is loved, there is also a love of humanity.
Hippocrates

Presentations at the Clinical Meetings

(Jan 2015 to June 2015)

Sr. No.	Date	Speaker/Department	Title
1.	10.01.2015	Shah Dwiti Radiology	Imaging predictors of severity assessment in patients with Pulmonary Embolism
2.	24.01.2015	Choudhary Mukesh Medical Unit-I	Safety and Efficacy of Eltrombopag in Post-hematopoietic Stem Cell Transplantation (HSCT) Thrombocytopenia
3.	14.02.2015	Joshipura Aditya P. Surgical Unit-I	Management of locally advanced cancers of thyroid
4.	25.04. 2015	Modi Rahul Deepak Gynic Oncology Unit-III	Pelvic nodal metastasis in primary malignant sex-cord stromal tumors of the ovary: A single institution experience
5.	23.05.2015	Shah Janmesh Community Oncology	Overview of cancer Screening Camp Activity at GCRI.
6.	27.06.2015	Prajapati Dhvanan Anesthesiology	A comparative study of the effect of dexmedetomidine HCL and lignocaine HCL on hemodynamic responses and recovery following tracheal extubation in patients undergoing intracranial surgery

Journal Club/Guest Lecture/ Review Lecture Presentations

(July 2014 to December 2014)

Sr. No.	Date	Presenter/ Department	Topic	Authors	Citation
1.	24.01.15	Rachh Swati Nuclear Medicine	PET/CT in patients with liver lesions of different nature	Rachh Swati, Basu Sandip	Clin Transl Imaging 2014 2:139-155
2.	28.02.15	Kubavat Rajdip Anaesthesia	Comparison of four techniques of nasogastric tube insertion in anaesthetised,intubated patients: A randomized controlled trial	Mandal Mohan Chandra, Dolai Sujata, Ghosh Santanu, Pallab Kumar, Mistri Rajiv Roy, Basu Sekhar Ranjan, Sabyasachi Das	Indian Journal of Anaesthesia 2014 /58:6 :714-718
3.	14.03.15	Anand Mridul Radiotherapy	Pre-operative Chemoradiotherapy for Esophageal or Junctional Cancer	Hagen P.Van, M.C.C.M Hulshof, JJB van Lanschot	N Engl J Med 2012;366 :2074-84
4.	28.03.15	Parwate Nikhil Gynec Oncology Unit-II	Views and Counter views: Role of pelvic and para-aortic lymph node dissection in surgical management of Endometrial Cancer	Holland Cathrine M.	The Obstetrician & Gynaecologist Volume 11, Issue 3, pages199-204, July 2009
5.	11.04.15	Patel Rutul Surgical Unit II	Frozen section examination may facilitate reconstructive surgery for mid and low rectal cancer	WisamKhoury, WisamAbboud, Dov Hershkovitz Simon D. Duek	Journal Of surgical Oncology 2014 ;110:997-1001
6.	25.04.15	Joshi Kshitij c. Medical Oncology Unit -II	Cisplatin and Etoposide Versus Carboplatin and Paclitaxel With Concurrent Radiotherapy For Stage III Non-small Cell Lung Cancer : An Analysis of Veterans Health Administration Data	Santana Rafael-Davila ,Kiran Devisetty,Aniko Szabo et al.	J Clin Oncol 2015; 33:567-574
7.	09.05.15	Trivedi Ruchira pain & palliative Care unit	Breaking bad news in cancer patients - study in general hospital, komotini, Greece	A postotos konstantis, Triada eciara	Indian journal of palliative care, Jan/April 2015, 21:1
8.	23.05.15	Rathod Maxesh R. Nursing	Malignant Fungating wounds: The meaning of living in an unbounded body.	Sebastianprobst, An Nearber	European Journal Of Oncology Nursing 17/13 3845
9.	23.06.15	Tadaiya Mahavir Surgical Unit-1A	Elective Versus Therapeutic Neck Dissection in Node-Negative Oral Cancer	D'Cruz Anil K., M.S., D.N.B.Richa Vaish, M.S.,Neeti Kapre, M.S., D.N.B.,MItali Dandekar, M.S., D.N.B ,Sudeep Gupta, M.D.,D.M., Rohini Hawaldar,, Et al	N Engl J Med, May 2015
10.	27.06.15	Kazi Mahnaz M. Division of Molecular Endocrinolog	The role of the Wnt signaling pathway in cancer stem cells: prospects for drug development	Yong-Mi Kim and Michael Kahn	Research and Reports in Biochemistry 2014:41–12

Case Presentations for Morbidity, Mortality at Clinical Meetings

(January 2015 to July 2015)

Sr No	Date	Presenter/Department	Case discussion
1	24.01.2015	Pol Dhiraj Anaesthesiology	Mortality and Morbidity Data presentation of Surgical and Medical Departments
2	24.01.2015	Tadiya Mahavir Surgical Oncology	Case presentation: Mortality of operated case of Radical cystectomy
3	28.03.2015	Makadia Gautam S Medical Oncology	Case presentation: Morbidity of a case of Acute Lymphoblastic Leukaemia with Rhino-orbital mucormycosis
4	28.03.2015	Pol Dhiraj Anaesthesiology	Mortality and Morbidity presentation of Surgical and Medical Departments
5	25.04.2015	Ravindar Manoj Anaesthesiology Kumari Rashmi Gynaecologiacl Oncology	Case presentation: Mortality of operated case of Ca Ovary
6	25.04.2015	Shah Nishita M Anaesthesiology	Mortality and Morbidity presentation of Surgical and Medical Departments
7	23.05.2015	Jha Raushan Kumar Anaesthesiology	Case presentation: Moratlity of operated case of Ca Thyroid and Ca Left Breast
8	23.05.2015	Pol Dhiraj Anaesthesiology	Mortality and Morbidity presentation of Surgical and Medical Departments
9	27.06.2015	Aagre Suhas Medical Oncology	Rituximab induced interstitial lung disease in a patient with follicular lymphoma
10	27.06.2015	Prajapati Dhavanan Anaesthesiology	A comparative study of the effect of Dexmedetomidine HCl and Lignocaine HCl on hemodynamic responses and recovery following Tracheal extubation in patients undergoing Intracranial surgery

About the Journal and Instructions to Author

Gujarat Cancer Society Research Journal is a biannually (April and October), ISSN 2320-1150, peer-reviewed journal published by the Gujarat Cancer Society. The journal is indexed with Directory of Open Access Journals (DOAJ), EBSCO,Googie Scholar, IC Journals Master List, Worldcat, ZBD. The journal's full text is available online at http://www.gcriindia.org

The Editorial Procss

A manuscript will be reviewed for possible publication with the understanding that it is being submitted to Gujarat Cancer Society Research Journal at that point in time and has not been published anywhere, simultaneously submitted, or already accepted for publication elsewhere. The journal expects that authors would authorize one of them to correspond with the journal for all matters related to the manuscript. On submission, editors review all submitted manuscripts initially for suitability for formal review. Manuscripts with insufficient originality, serious scientific or technical flaws, or lack of a significant message are rejected before proceeding for formal peer-review. Manuscripts that are unlikely to be of interest to the Gujarat Cancer Society Research Journal readers are also liable to be rejected at this stage itself.

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The following documents are required for each submission: (Font: Times New Roman)

- Title Page (Font size: 12)
- Title of manuscript (Font size: 16)
- Summary and Keywords (Font size: 9)
- Text (Introduction, Aims and Objectives, Materials and Methods, Results and Analysis, Discussion with Conclusions; Font size: 12).
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- Legends to Figures and Illustration: Present the legends for illustrations separate page using double-spacing, with Arabic numerals corresponding to the Illustrations. (Font size: 12)
- References (separate page, Number references consecutively in the order in which they are first mentioned in the text. Identify references in the text in numerals in superscript and parenthesis; Font size: 12).
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Units and abbreviations

Avoid abbreviations in the title and abstract. All unusual abbreviations should be fully explained at their first occurrence in the text. All measurements should be expressed in SI units. Drug names Generic drug names should be used.

Abbreviations of units should conform to those shown below:

Decilitre	dl	Kilogram	kg
Milligram	mg	Hours	h
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Molar	mol/L	Mililitre	ml
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The title page should include

1. Type of manuscript (article/case report)
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- Uniformly American English
- Abbreviations spelt out in full for the first time

- Numerals from 1 to 10 spelt out
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Materials and Methods: Describe precisely your selection of the observational or experimental subjects (patients, including controls). Identify the methods, apparatus (including manufacturer's name and address in parenthesis), and procedures in sufficient detail to allow others to reproduce the method. Give references to established methods, including statistical methods; provide references and brief descriptions for methods that have been published but are not well-known. For new or substantially-modified methods, describe and give reasons for using them and evaluate their limitations.

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Results: Present your results in a logical sequence in the text, Tables, and Illustrations. Do not repeat in the text all the data in the Tables or Illustrations. Emphasize or summaries only important observations. Specify the statistical methods used to analyze the data. Restrict Tables and Illustrations to those needed to explain the argument of the paper and to assess its support. Where possible, use Graphs as an alternative to Tables with many entries. Do not duplicate data in Graphs and Tables.

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Acknowledgements: State contributions that need to be acknowledged.

References

A list of all the references cited in the text should be given at the end of the manuscript and should be numbered consecutively in the order in which they are first mentioned in the text. Identify references in the text by Arabic numerals in superscript. Omit month and issue number. List all authors, but if the number is six or more, list first three followed by et al. The references should be cited according to the Vancouver agreement. Authors must check and ensure the accuracy of all references cited. Abbreviations of titles of medical periodicals should conform to the latest edition of Index Medicus. Some examples are shown below:

Standard Journal

You CH, Lee KY, Chey RY et al: Electrogastrographic study of patients with unexplained nausea, bloating, and vomiting. Gastroenterology 1980; 79:311-314

Online journal article

Miyamoto O, Auer RN. Hypoxia, hyperoxia, ischemia and brain necrosis. Neurology [serial online] 2000; 54:362-71. Available at: www.neurology.org. Accessed February 23, 2000.

Chapter in a book

Weinstein L, Swartz MN. Pathogenic properties of invading microorganisms. In: Sodeman WA Jr, Sodeman WA, eds. Pathologic Physiology: Mechanisms of Disease. Philadelphia: Saunders, 1974: 457-472

Online book or website

Garrow A, Weinhouse GL. Anoxic brain injury: assessment and prognosis. In: Up To Date Cardiovascular Medicine [online] Available at: www.UpToDateInc.com/card. Accessed February 22, 2000.

In press

Lillywhite HB, Donald JA. Pulmonary blood flow regulation in an aquatic snake. Science. In press.

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Department of Neuro-Oncology

Department of neuro-oncology is one of its kind first in India, a specialized branch in neurosurgery and oncology, managing and operating complex neuro-oncology and spinal cases.

The neuro-oncology is the first department to start modern stereotactic neurosurgery in Gujarat and also prides itself for possessing the only X-kinfe for radiosurgery in the state. The neuro-oncology work is backed by excellent surgical, medical and radiation oncology to provide patient care under one roof.

Foundation / Formation:

Advance Neuro-oncology department was established since 1999 with tremendous efforts by Ex-director Dr. Devendra Patel, late senior neurosurgeon Dr. S.N. Bhagvati - Bombay Hospital, Dr. Mahendra Parikh-senior neurosurgeon, Ahmedabad and Dr. Dipak D. Patel, H.O.D. and consultant neurosurgeon of neuro-onco department. It was funded by GMDC (Gujarat Mineral Development Corporation). It is separate department at 3rd and 4th floor of special room building. At 3rd floor, we have neuro-oncology wards. (male ward, female ward, pediatric ward and isolation ward), special rooms, nursing station and CSSD.

On 4th floor, we have neuro ICU and neuro post-operative care unit with two Isolation beds. ICU with 4 dragger ventilators, multipara monitoring system. Two state of art exclusive Neurosurgery operation theatres with two stann OT tables, Karl zeiss vario 700 microscope, CUSA, aesculep high speed drill, C-Arm, neuro endoscope, spinal endoscope system, linac laser target locator frame (stereotactic frame), CRW stereotactic system.

We have total 40 beds (23 in General, 06 in Special rooms and 10 in ICU/ Post Operative ward)

Services:

The department is running OPD on Monday and Thursday every week where new and old cases are examined, and the follow up patients are regularly attended in the OPD. but, also we attend & encourage patients any time of day also admit patients in emergency sunday and holiday. In each OPD we have average 50-60 patients (average 750-800 patients/month)

The indoor references are also attended regularly. The services to the poor patients for neurosurgical disease are offered.

Surgeries:

Daily we perform average 3-4 major, Supra major or selective surgeries (average 60-70 per month) Majority are craniotomies for malignant, benign and metastatic intracranial tumors. Laminectomies for benign, malignant and metastatic spinal tumors. Spinal instrumentation for cranio vertebral anomalies and pedicle screw fixation and fusion for spinal metastatic and vertebral body tumors.

Endoscopic transnasal surgeries for pituitary and other seller tumors, clivus tumors and nasopharyngeal tumors and endoscopic spinal surgery have been performed regularly.

Microvascular decompression for trigeminal neuralgia, radiofrequency lesion for trigeminal neuralgia stereotactic surgery for biopsy, aspiration of cystic tumor and stereotactic craniotomies.

Department started with 248 neurosurgical operations in 1999. Last year we performed 620 brain and spine surgery were performed.

Our Team:

There are 03 visiting neurosurgeons: Dr. Dipak D. Patel - Founder and H.O.D., Dr. Navin A. Patel, Dr. Tejas G. Patel, 01 fulltime neurosurgeon: Dr. Paresh G. Mody and 03 medical officers: Dr. K. G. Patel, Dr. Minesh S. Patel, Dr. Nimisha D. Gajera in the department.

Backup by Neuro OT staffs, Neuro ICU staffs and Neuro ward staffs.

Training Education & Academic Activities:

1) All neurosurgeons are regularly attending and participating in conferences and workshops to remain update in neurosurgery.
2) The conference and workshops has also been organized by neuro-oncology team. 5th National conference of Indian society of stereotactic and functional neurosurgery October, 2001 at Ahmedabad, National neurospine conference September, 2014 at Ahmedabad.
3) The faculties are presenting clinical papers in national and international conferences regularly.

Future Plan and Scope:

1) Proposal for purchase of neuro navigation system, Intra-operative ultrasound and doppler and intraoperative neuromonitoring system which have become essential part for any neuro-oncology centre. This armaments will help as in total removal of tumors with decreased mobility and mortality and ultimately benefit patients quality of life.
2) Plan to start DNB neurosurgery.
3) Good scope for Neurosurgeons interested in neuro-oncology with clinical and research fellowship.

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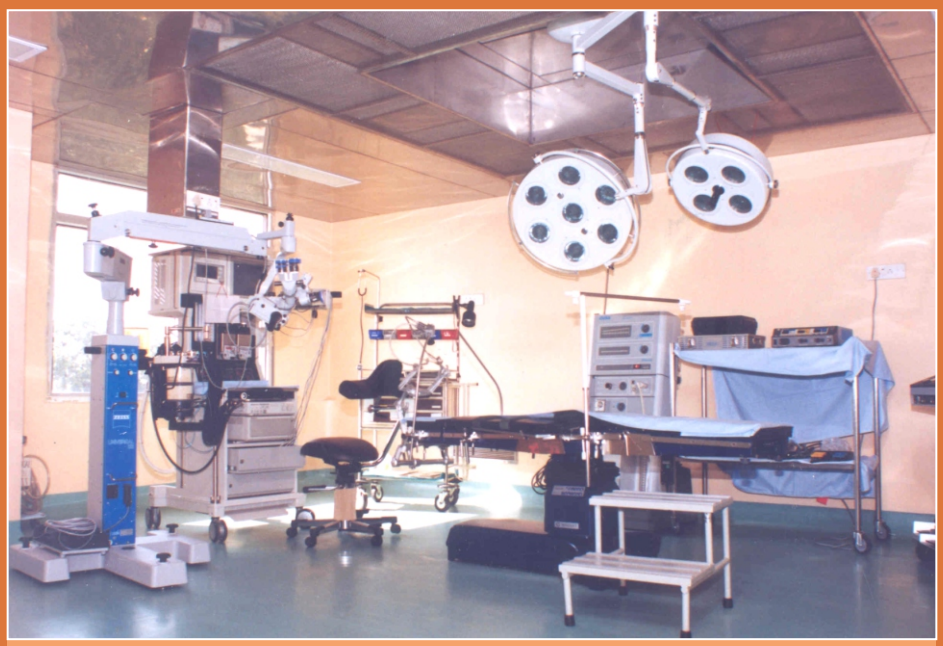
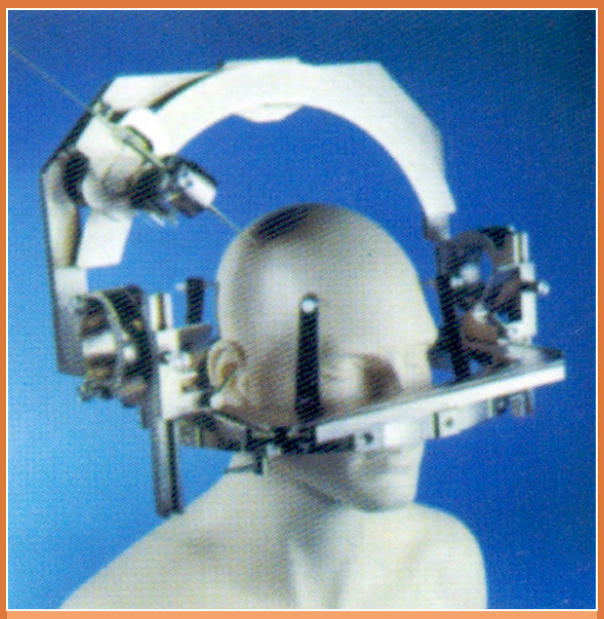


Neuro - Oncology Operation Theatre Set-up

Microscope by Zeiss in Neuro - Oncology Operation Theatre



Linac Laser Target Localizer Frame