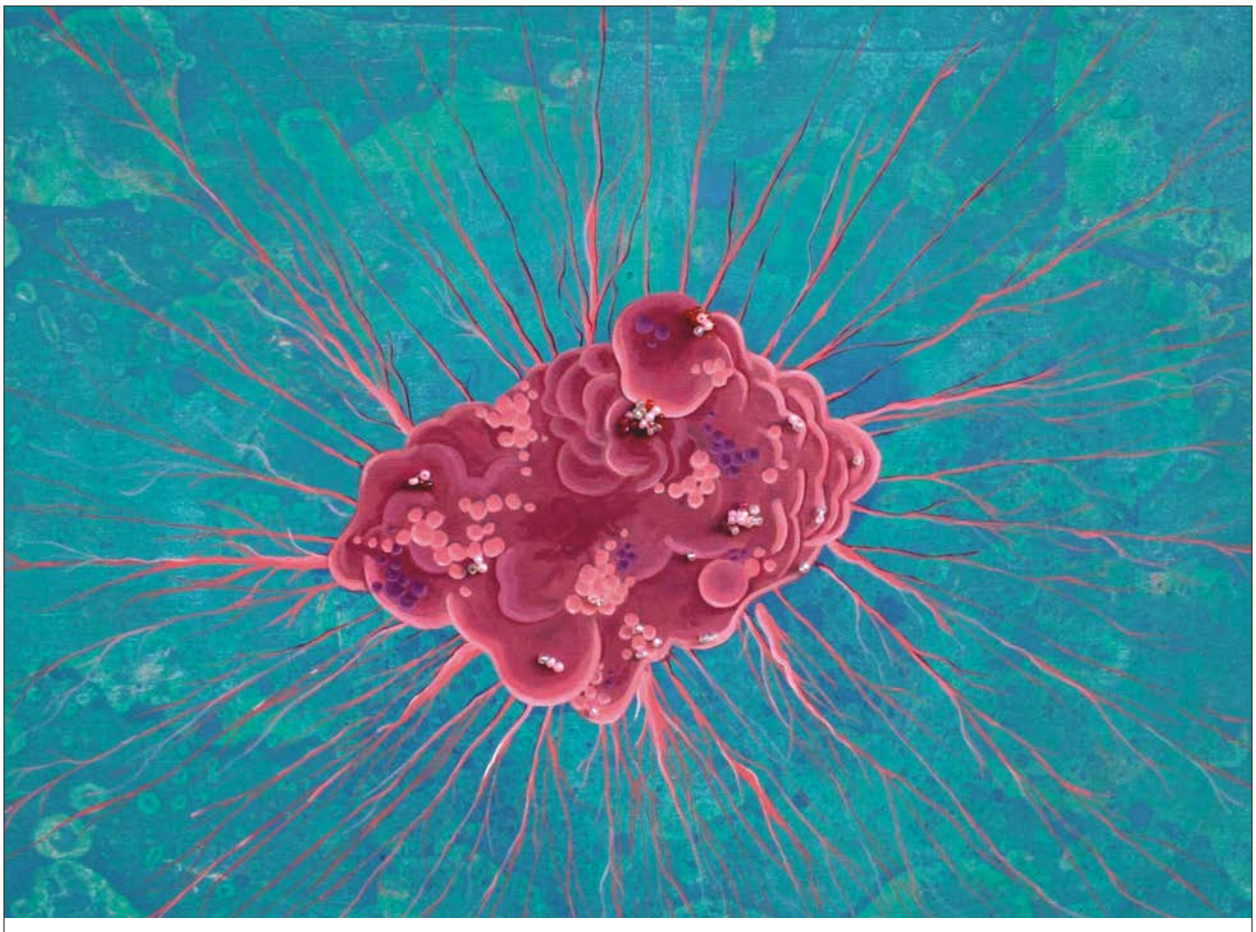

International Journal of Cancer Research

ISSN 1811-9727

Academic Journals Inc.

www.academicjournals.com

International Journal of Cancer Research 2 (4): 358-366, 2006
ISSN 1811-9727

Novel *CYP3A4* Gene Polymorphisms in Post Chemo Breast Cancer Patients

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Abstract: *CYP3A4* is the monooxygenase enzyme that interacts in the metabolism of great majority of drugs, especially in chemotherapeutic regimens which include combination of drugs. It is believed that by controlling a patient's *CYP3A4* expression level one could adjust the individual dose adjustments in therapies and may also be able to identify the subpopulation at increased risk for several common cancers. This belief prompted us to undertake this investigation. In this study we present the results of *CYP3A4* mutations in a large number of breast cancer patients undergoing chemotherapy after surgery. Blood samples collected from post chemo patients (n = 30) administered with 5-Fluorouracil, Doxorubicin and Cyclophosphamide (FAC) drugs were analyzed for *CYP3A4* polymorphisms. These samples were from among the first, third and sixth cycle regimen of chemotherapy. DNA extracted from all the blood samples was used for PCR amplification of *CYP3A4*, followed by sequencing in automated ABI 3770 sequencer. Biochemical tests performed on these blood samples included Liver Function Tests (LFT's). These investigations were also carried out on equal number (n = 30) of age matched individuals. We found 2 novel Single Nucleotide Polymorphisms (SNP's) in exon-10 (L338F and I334T) of *CYP3A4* and also found that these patients showed elevated Liver Function Tests (LFT) and ADR's such as alopecia, nausea, vomiting, neuro toxicity, GI toxicity, bone marrow suppression and leucopenia. It was also seen that a single base change in the 5' flanking region of the *CYP3A4* gene was associated with severe ADR's in breast cancer chemotherapy patients. It is concluded that mutations in *CYP3A4* gene influences drug metabolism leading to adverse drug reactions in post chemo breast cancer patients.

Key words: ADR'S, *CYP3A4,* drug metabolism, genotyping, LFT, SNP

Introduction

Breast cancer patients respond to a large number of chemotherapeutic agents and the highest response rates have been noted in clinical trails employing combinations of these agents (Bonadonna and Valagussa, 1988). The most common treatments are based on the drugs Fluorouracil, Doxorubicin (Adriamycin) and Cyclophosphamide, (FAC). When FAC based regimens are used for the treatment of metastatic breast cancer, response probability rates (partial remission plus complete remission) range from 30 to 70% depending on details of the therapeutic regimen and the patient's clinical history. Chemotherapeutic regimen often cause troublesome and occasionally life-threatening but reversible toxicities. Acute toxicities include GI toxicity, nephrotoxicity, cardiac toxicity, bone marrow suppression and adverse drug reactions include neutropenia, thrombocytopenia, alopecia, nausea, vomiting and general fatigue.

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FAC drugs are metabolized by Nifedipine oxidase enzyme which is also known as *CYP3A4*. *CYP3A* isoenzymes are the most abundantly expressed cytochrome P450s in human liver, accounting for up to 60% of the total hepatic cytochrome P450 activity in some individuals (Gonzalez, 1993). The expression and activity of the *CYP3A* isoenzymes show wide inter-individual variation, influencing both drug responses and disease susceptibility (Paris *et al*., 1999). *CYP3A4* is responsible for the oxidative metabolism of a wide variety of xenobiotics, including an estimated 60% of all clinically used drugs. There are only few references (Sata *et al*., 2000) on the impact of these mutations that showed *CYP3A4* allele (Ser222Pro) produces a lower *in vivo* intrinsic clearance of nifedipine, but no effect on wild type enzyme activity for testosterone 6-β-hydroxylation (Sata *et al*., 2000). Hsieh *et al*. (2001) have also reported that these mutations relate to 6-β-hydroxycortisol/cortisol ratio in heterozygotes. Eiselt *et al*. (2001) have reported 18 new *CYP3A4* variants of which several showed altered enzyme activity. All the recognized *CYP3A4* mutations/alleles are listed in the human Cytochrome P450 (CYP) Allele Nomenclature Committee Home Page. *CYP3A4* is considered the most important and is the most extensively studied member of the *CYP3A* sub-family (Eiselt *et al*., 2001). Thummel and Wilkinson (1998) have reported that some mutations contribute to harmful drug interactions frequently encountered during treatments.

Polymorphic studies of other genes which influence drug metabolism in cancers have been reviewed earlier by Chenna *et al*. (2004) and also reported by Haranatha and Jamil (2006) and Kumar and Jamil (2006). The inheritance of drug response has been elaborately reviewed by Weinshillouin (2006) and the factors that affect a patients response to drugs has been reported by Huang *et al*. (2006). In the light of the above theories, we designed our investigations to enumerate the possible role played by *CYP3A4* gene polymorphism which are known to alter the activity or level of expression of these enzymes. The levels of these enzymes can also be reflected in the blood of the individuals undergoing chemotherapy, hence we proposed to collect blood samples from cases and controls and determined the polymorphism of this gene (*CYP3A4*).

Materials and Methods

Subjects Included in the Study

The present study was performed in 30 South Indian women patients aged 40-70 years of age with confirmed carcinoma of breast. Patients necessitating chemotherapy up to six cycles were included in the study. Patients who were pregnant prior to chemotherapy or under any form of cytotoxic agent treatment in the recent past were excluded from this study. Equal numbers of age matched healthy females with their complete health profile were enrolled in the present study for comparing the results. Details regarding lifestyle habits as well as health status were obtained from all these women after personal counseling by the clinician. Informed written consent was obtained from all individuals. This study was approved by the Institutional Ethical Committee Review Board.

Collection of Samples

Blood samples from Invasive Ductal carcinoma (IDC) of breast cancer patients undergoing chemotherapy (FAC) at post first, third and sixth cycles were taken. Equal numbers of blood samples were collected from control group. All the samples collected were from M.N.J. Cancer Hospital, Hyderabad, AP, India. The experiments were carried out at Bhagawan Mahavir Medical Research Center, Hyderabad during 2004-05.

Liver Function Tests (LFT's)

LFT was performed by the Biochemist using standard kits from Bayer Diagnostics for Total Bilirubin, SGOT and SGPT.

Genotype Analysis (CYP3A4 Polymorphism Studies)

DNA Extraction and PCR Amplification

Genomic DNA was extracted from the blood samples of cases and controls by the Salting-out procedure (Miller *et al*., 1988). The primers at the coding region of 5'-flanking portion of the *CYP3A4* gene were used for *CYP3A4* genotyping. 5' CCTGTTGCATGCATAGAGG and 5' GATGATGGTCACACATATC (exon-7) and 5' CCAGTGTACCTCTGAATTGC and 5' CAGAGCCTTCCTACATAGAG (exon-10). Using these primers 366 and 430 bp fragments were obtained by amplification.

The PCR amplification was carried out in a total volume of 25 μL reaction volume. Each reaction contained 10 mmol L^{-1} Tris-HCl (pH 8.3), 2.5 mmol L^{-1} $MgCl_2$, 50 mmol L^{-1} KCl, 100 μmol L^{-1} each dNTP, 0.5 μmol L^{-1} each of the primer pair, 1.25 U of AmpliTaq Gold DNA polymerase (Roche Molecular Biochemicals) and 100 ng of template DNA. After pre-incubation at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. A final extension at 72°C for 10 min was carried out to complete extension of all DNA fragments.

DNA Sequencing

PCR samples were purified with the Ultra Clean™ 15 DNA purification kit (MO BIO, Solana beach, CA, USA). The purified samples were then used directly for DNA sequencing of both strands without any further treatment. Where a variation in DNA sequence from the wild-type was found, the original PCR product was also sequenced for confirmation. In the case of novel mutations, additional repeat amplification and sequencing was performed. In all samples where novel 5' regulatory region mutations were found, direct sequencing of PCR products from exon-7 and exon-10 of *CYP3A4* gene was also performed to identify any linked coding region mutations. Numbering of nucleotides has been carried out by assigning the figure +1 to the base A in the translation ATG initiation codon and -1 to the base before the A. Primer pair was sequenced with a Taq-Dye deoxyterminator cycle sequencing kit (Applied Biosystems) using an automated ABI 3770 sequencer. These were carried out at Centre for Cellular and Molecular Biology, Hyderabad, India.

Statistical Analysis

The differences in frequencies between the case and control groups was analysed for statistical significance at the 95% confidence interval using Fisher's two-tailed test. Odds ratios (ORs) were calculated and reported within the 95% confidence limits. Unconditional multinomial Correlation analysis was performed with different factors as well as genotype as the independent factors, with the risk of ADR's being the dependent variable. Pearson Correlation was performed and significance was checked by the p-value. Factors which do not possess a significant Wald statistic, but tend to increase the p-value of the model, were selectively eliminated to obtain the final model. The statistical analyses were performed using SPSS for Windows (version 11.0) software. A p-value of <0.05 was considered as significant in all the analysis.

Results

Characteristics of the Study Group

Characteristics of the case-control groups included in the study are presented in Table 1.

It is seen that there were some smokers, alcohol consumers, beetle nut chewers and tobacco chewers in the study. The age group ranged between 40-70 years.

Studies on SNP 's in CYP3A4 Gene

DNA samples obtained from 30 Invasive Ductal Carcinoma of breast cancer women who were administered with FAC combination of drugs and 30 females who were healthy and age matched were

Table 1: Characteristics of the women included in the study

Parameters		Cases (n = 30)	Controls (n = 30)
Mean age in years (SD)	-	55±6.23	52±7.12
Chemotherapy Cycle	-	Post I III and VI	-
Complete Blood Picture (CBP)	-	Normal	Normal
Smokers	-	4 nos	0
Alcohol consumers	-	1 nos	0
Habits (Pan, Pan masala, Tobacco Chewing, Zarda)	-	19 nos	4 (Pan)

Table 2: Distribution of *CYP3A4* SNP frequencies in the studied individuals (Casecount.Exe)

	Total	No. (Frequency) (%)	Crude OR	95% CI	p-value
CYP3A4 L338F I334T	cases wt. n=30 mutant	24 (80) 6 (20)			
			0	0.0-0.21	0**
	controls wt. n=30 mutant	30 (100) 0 (0)			
Familial	8	2 (25)			
			1.51	0.728-3.204	0.302*
Sporadic	22	4 (18.18)			

n, Number of cases; **Significance at 99% level; * p-value is not significant at 95% level; Wt, Wild type

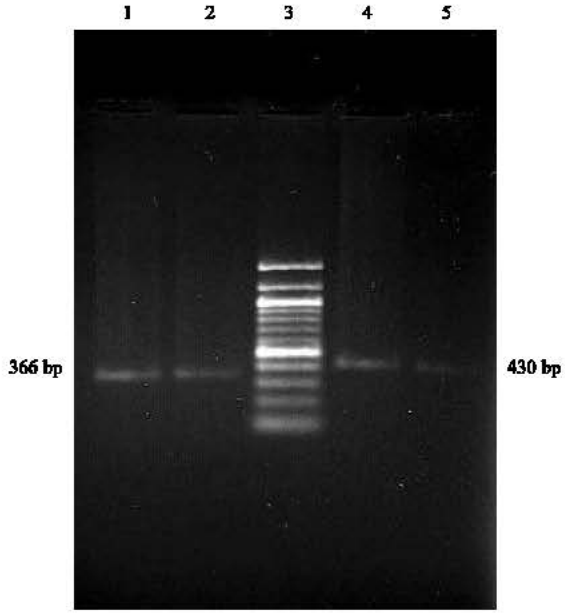

Fig. 1: 1.5% Agarose gel showing the amplified *CYP3A4* gene loci of exon-7 and exon-10 along with molecular weight marker. Where, Lane-1 and 2-Exon-7 amplified product of the *CYP3A4* gene. Lane-3-100 bp molecular weight marker. Lane-4 and 5-Exon-10 amplified product of the *CYP3A4* gene

analyzed for the variant single nucleotide polymorphisms (SNP's) in *CYP3A4*. DNA was isolated from both control and patient samples and by using respective PCR primers of exon-7 and exon-10 of the *CYP3A4* gene. These amplified products are shown in Fig. 1. To all the PCR products, sequencing was done and chromatograms recorded in Fig. 2A and B. This study revealed two novel *CYP3A4* SNP's (L338F and I334T), (Fig. 2A and B).

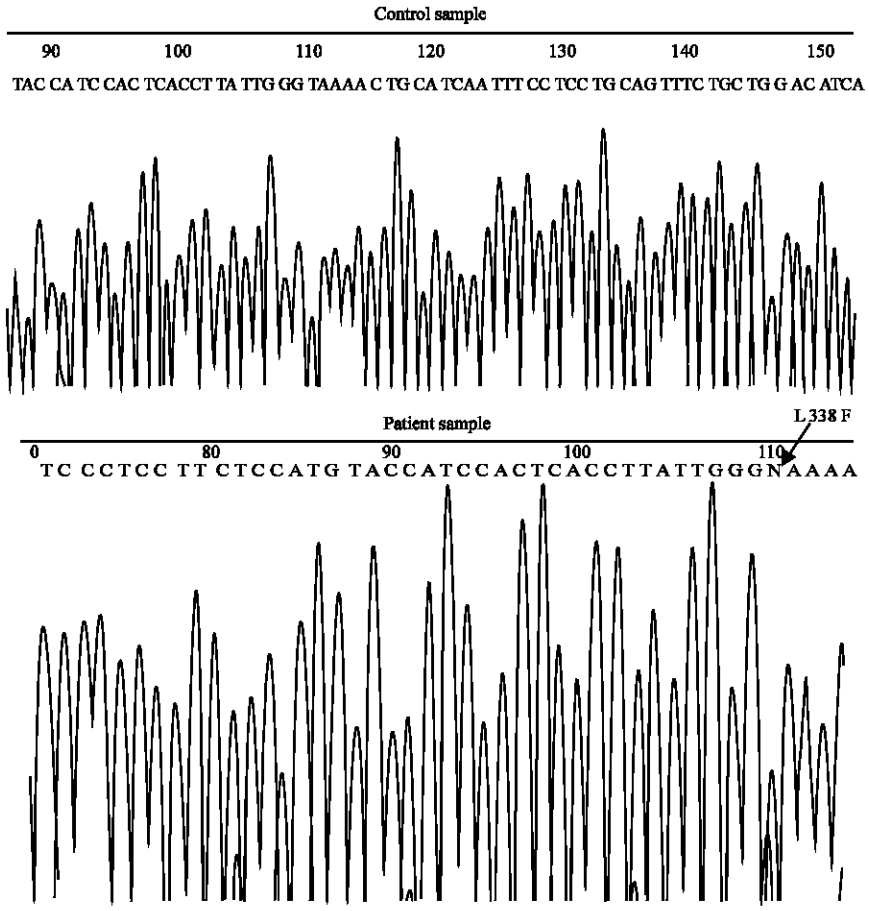

Fig. 2A: Indicates the heterozygous T to A mutant overlap in exon-10 of *CYP3A4*. Visualizing software used for control sequence-GENE TOOLS and for Patient sequence-CHROMAS

Distribution of CYP3A4-SNP Frequencies in the Cases

The frequencies of various polymorphisms of *CYP3A4* genotype studied along with the relevant statistical parameters are presented in Table 2. None of the polymorphisms showed a correlation with age, as revealed by a low Pearson's correlation coefficient ($p>0.05$; data not shown).

Correlation Analysis of the Data on ADR's, LFT's and SNP's

Correlation analysis was done by comparing ADR's and Liver Function Tests (LFT's) with the SNP's and presented in Table 3. From the p-values corresponding to the observed Pearson correlation coefficient between the variable ADR's, LFT's, SNP status, it is clear that a strong correlation exits among the variables. In addition, the p-value of zero observed for correlation between SNP status and LFT strongly suggests the importance of these mutations towards a change in enzyme activity. CYP enzymes are functional in liver to a great extent (Tirona *et al*., 2003) and hence an alteration in their activity can result in an abnormal LFT. Results of LFT's were shown in Table 4.

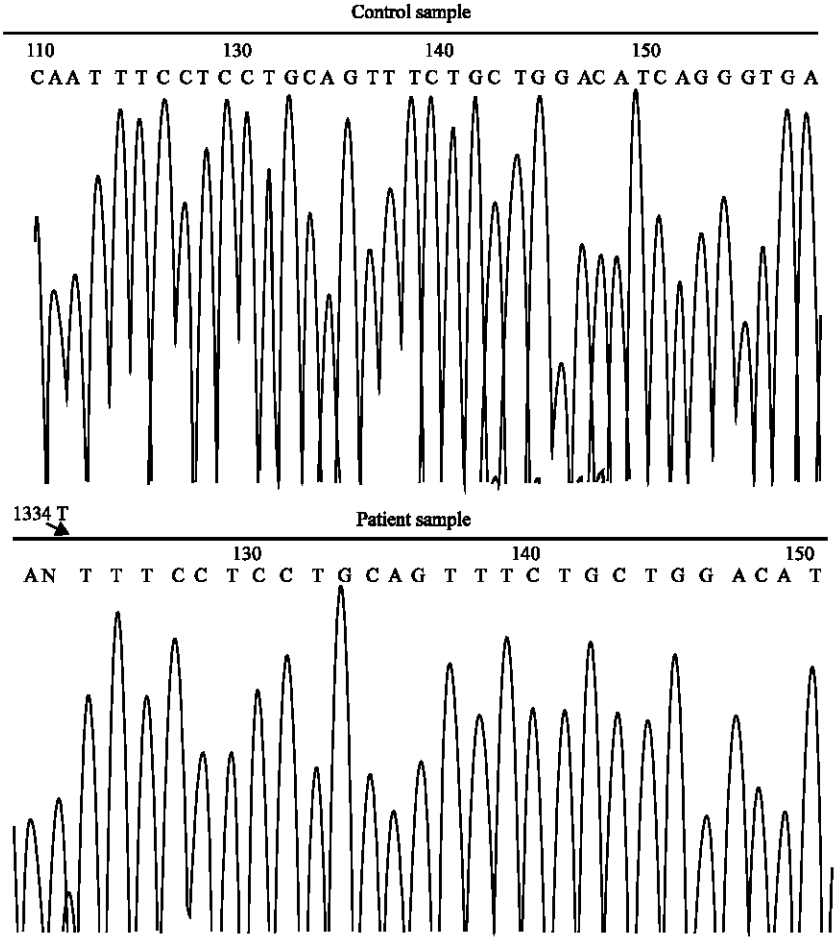

Fig. 2B: Indicates the heterozygous A to G mutant overlap in exon-10 of *CYP3A4*. Visualizing software used for control sequence-GENE TOOLS and for Patient sequence-CHROMAS

Table 3: Correlation analysis

	ADR's	LFT	SNP
ADR's	Pearson correlation 1	0.51	0.484
	p-value	0.007*	0.007*
LFT	0.51	1	0.735
	0.007*		0.0*
SNP	0.484	0.735	1
	0.007*	0.0*	

ADR: Neurotoxicity, GI Toxicity, Bone marrow suppression were considered as the factors representing severity of ADR's, LFT: Abnormal S.G.O.T, S.G.P.T, Total bilirubin were considered to categorize the subjects under study, * Significant at the 99% level (2-Tailed test)

Table 4: Details of the liver function tests from the patients

		Liver function tests		
Subjects	SNP's in CYP3A4	Total Bilirubin	SGOT	SGPT
Normal	-	0.2-1.2 mg dL^{-1}	5-35U	8-40U
Case-1	L338F	2 mg dL^{-1}	60 U	18 U
Case-2	L338F	2.2 mg dL^{-1}	45 U	40 U
Case-3	L338F	1.8 mg dL^{-1}	40 U	38 U
Case-4	I334T	2 mg dL^{-1}	45 U	45 U
Case-5	I334T	3 mg dL^{-1}	65 U	52 U
Case-6	I334T	3.5 mg dL^{-1}	50 U	40 U

Discussion

The demographic data collected from the cases showed that 19 cases among 30 women were having various habits like smoking, alcohol consumption, Pan Masala, Zarda and Tobacco chewing habits; it indicates that the lifestyle habits might also influence the remission of the treatment. Some of these factors could contribute to the risks involved in disease progression.

Apart from the established risk factors, detoxification mechanisms also play an important role in influencing the success of chemotherapy outcome. Studying the effect of *CYP3A4* gene polymorphisms in the context of cancer chemotherapy and ADR's, alteration of enzyme levels in the liver is gaining importance in recent years. Many reports indicate the importance of CYP enzyme system in drug metabolism (Hadfield *et al*., 2001). No such study has been reported so far in cancer chemotherapy cases from the Indian subcontinent. Hence, we tried to study the relationship of SNP's and ADR's with LFT levels which includes Total Bilirubin (Jendrassik and Grof, 1938), SGPT (Schellong and Wende, 1960) and SGOT (Gambino, 1965).

Since, there is a positive correlation between ADR and SNP status; genotyping of these SNP's will be useful in predicting the individuals prone towards ADR during the chemotherapy. No association was observed between the familial natures of the cancer and the SNP incidence (2 out of 8 familial cases were found to contain SNP). Among the cases, the familial nature of mutation inheritance was checked using a 2×2 contingency table as shown in Table 2. From the Odds ratio, 95% Confidence interval and p-value it is clear that there is no association between the two mutation occurrence and the familial history of cancer.

The dominant role of *CYP3A4* in the metabolism of numerous clinically useful drugs has been described by Hirota *et al*. (2004). Their results present an insight the individualized *CYP3A4*-dependent pharmacotherapy and the importance of expression imbalance to human phenotype diversity. Thus it is clear that the potential of Pharmacogenomics which deals with the role of inheritance in the individual variation in drug response lies in the identification of genetic variations and drug response. The genetic basis for polymorphic *CYP3A4* expression was demonstrated by some workers. Felix *et al*. (1998) investigated genetic variation in drug metabolism as a potential host risk factor for carcinomas induced by DNA topoisomerase-II inhibitors.

Two unique polymorphisms of *CYP3A4* were found in the six post chemo patients suffering from cancer; these were identified using the sequencing analysis. It is also important to realize that genetic factors play an important role in inter individual variability in *CYP3A* activity. Since detection of such variant alleles and knowledge about their allelic frequency in diverse populations are important to individualize drug dosing and improved therapeutics.

The sequencing results for exon-10 of the *CYP3A4* are presented in Fig. 2A, B. It is evident that in the coding region of the 5'-flanking region, T to A transition with three samples and A to G transition in yet another three samples, was found creating a protein change L338F (Leu → Phe) and I334T (Iso → Thr). The correlation analysis revealed that these mutations identified in the samples were located on the exon-10 showed linkages between ADR's, LFT's and SNP's. The goal of this investigation was to identify novel mutations and determine their frequencies. The discovery of these SNP's showed marked influence on the ADR's and LFT's in breast cancer patients. The transmission T to A was reported in cancerous subjects (20%) with prostate cancers (Hamzeiy *et al*., 2002). The same was found in less percentage in Iranian population (18%). Whereas in the Indian population it was found to be 10% with T to A transition and 10% with A to G transition.

The patients with *CYP3A4* polymorphisms were found among women between the age of 50-65 undergoing post third and sixth cycle of chemotherapy regime. The structural variation in *CYP3A4* protein in our studies may explain the large inter individual variability in *CYP3A4* metabolic function. These findings are of considerable clinical importance.

We report here for the first time of two novel SNP's in *CYP3A4* gene. The present study revealed an association of the novel *CYP3A4* SNP's (L338F and I334T) and their association with the risk of adverse drug reactions in breast cancer chemotherapy patients. L338F-found in 3 post VI chemo patients and I334T-found in 3 post VI chemo patients. All the six patients with SNP's showed abnormal LFT's and suffered from adverse drug reactions. This study demonstrates the importance of CYP-gene polymorphisms in the context of ADR's. Polymorphisms in the drug metabolizing enzyme may lead to the improper metabolism of the drugs and induce ADR's. All the SNP data obtained from cases were statistically significant according to the statistical parameters performed like Crude Odds Ratio, 95% confidence interval and Fisher's two tailed p-value. This is the first report from the Indian subcontinent regarding the involvement of detoxification gene (CYP) polymorphisms in Cancer Chemotherapy.

Acknowledgments

We are grateful to all the participants without whose cooperation we could not have carried out this study. We are particularly grateful to MNJ Cancer Hospital and Indo American Cancer Institute, Hyderabad, for their help and cooperation in sample collection and to the Physicians who encouraged us to take up these studies. We are grateful to Bhagwan Mahavir Hospital and Research Center and CCMB, Hyderabad for the facilities provided. At present we have joined the IACI and RC.

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