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A PHARMACOGENETIC STUDY OF CAPECITABINE-OXALIPLATIN THERAPY IN COLORECTAL CANCER PATIENTS AMONG SOUTH INDIAN POPULATION

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ABSTRACT

Capecitabine with oxaliplatin (CAPOX) is considered to be a standard treatment option in colorectal cancer (CRC) therapy. The limitation associated with the use of these anticancer drugs is the unpredictable interindividual variation in efficacy and toxicity. However, variability in treatment outcome for the multiple chemotherapeutic regimens has been attributed to genetic factors. Hence, this study analyzed the effect of *TS, MTHFR, DPD and GSTP1* gene polymorphism on toxicity and efficacy in CRC patients on CAPOX therapy. Sixteen patients between 18-75yrs of age on CAPOX therapy were included in the study. The genomic DNA was isolated from the peripheral blood and was genotyped using PCR RFLP method. No relationships between *TYMS, MTHFR, DPD, GSTP1* genotypes, and global toxicity were observed on univariate Fisher's exact tests. However, exon 14 skipping IV +1g>A which is related to DPD deficiency occurred in only 1of 16 patients with more toxic side effects. In patients carrying val/val GSTP1 Exon 5 genotype, Grade 2 cumulative peripheral neuropathy was observed relative to the other (1of 4 patients, 25%).Three patients with 2R/2R genotype were non responders. One patient was heterozygous for *TS, MTHFR677, MTHFR 1298* and *GSTP1* and had a poor prognosis .The present data indicates that the patients with 2R/2R were not good candidates for CAPOX therapy. Further, *DPD* deficiency and *GSTP1* polymorphism should be observed for patients receiving CAPOX therapy. A larger data set is required for confirmation as such information would allow individualization of chemotherapy and maintaining quality of life.

KEY WORDS: Pharmacogenomics, Colorectal cancer, Capecitabine. Oxaliplatin.

INTRODUCTION

Colorectal cancer is the fourth prime cause of cancer-related deaths worldwide. [1]In India rectal cancer is predominant than colon cancer[2] and Chennai has the age adjusted (world population) incidence rate of rectal cancer as 3.8/100,000 among males and 2.8/100,000 in females.[3] As most cases present in advanced stage, the prognosis for these patients is bleak, with a 5-year survival rate of 20%.[4]

Capecitabine is an oral pro-drug of 5FU that is selectively converted within cancer cells to the active drug 5-fluorouracil and a more selective substitute for 5 FU as first line treatment for MCRC [5] and as adjuvant therapy for stage III colon cancer, especially when there is a desire to avoid the use of indwelling venous catheters. In clinical practice, capecitabine is known for its good patient tolerability even in the elderly. [6] In addition patient prefer oral cytotoxic therapy to intravenous regimen. [7] Based on the reports of the clinical experience, capecitabine has proven to be a important stand in for 5FU in colorectal cancer and is now a standard treatment option both as monotherapy and in combination with oxaliplatin in a variety of different schedules. [8-9] Capecitabine plus oxaliplatin (CAPOX) is considered to be a standard treatment option in advanced colorectal cancer (CRC)and as an adjuvant therapy in colon cancer. [10-11] The standard dose of capecitabine is 2000 mg m^{-2} per day in divided dose (days 1–14) and oxaliplatin 130 mg m^{-2} (day 1) every 3 weeks (CAPOX).

As a single agent, it is well tolerated by patients, with the predominant grade 3 or higher toxicity being hand and

foot syndrome (HFS).[12-13] The Predominant dose limiting adverse effects reported with capecitabine are hyperbilirubinemia, diarrhea, hand and foot syndrome, myelosupression, fatigue and nausea [14] while Oxaliplatin displays a characteristic pattern of neurotoxicity. [15]

However these agents have variation in toxicity and anti tumor activity, making it difficult to select the optimal treatment for each individual patient. Germline gene polymorphism can state the part of interpatient pharmacodynamic variability of these cancer drugs. Studies have demonstrated that the gene encoding metabolising enzymes such as*(TS, MTHFR, DPD)*and transport enzyme *GSTP1*are associated with the response/toxicities with capecitabine and decreased risk of developing severe cumulative toxicity with oxaliplatin. [16] As a result, by utilizing such predictive markers, patients could be identified who are unlikely to respond and are at a high risk of excessive toxicity before drug administration. Such information would allow individualization of chemotherapy maintaining quality of life.

Hence, this study was carried out to evaluate the possible association and influence of polymorphism of genes (TS, MTHFR, DPD, and GSTP1) involved with CAPOX therapy in colorectal cancer which could be a genetic markers to predict the response/ toxicities

METHODOLOGY

This prospective observational study was carried out in the department of medical oncology,Sri Ramachandra university, Chennai, India from Feb2013-June 2014. Patients with Histological or cytological confirmed colorectal cancer, Stage- II,III,IV,18-65yrs of either sex, Eastern cooperative oncology group performance status between 0-2,KARNOFSKY performance status >77%were included in the study and patients who are pregnant or lactating ,HIV positive , psychiatric illness were excluded from the study.

With the approval of institutional ethics committee and written consent of colorectal cancer patients on CAPOX therapy, data were collected in a data collection form which included data on demographic Characteristics such as age, sex, performance status; primary tumor location, involved metastatic sites, several pretreatment characteristics, objective tumor response, and clinical adverse reactions of the treatment and data regarding time to tumor progression and survival. blood investigations which includes complete blood count, liver and renal function tests were recorded. Reports of CT imaging of the chest, abdomen and pelvis were also noted. The evaluation of toxicities was carried out throughout the cycle according to Common Toxicity Criteria version 4. [17] Duration of response was accounted from the commencement of therapy until disease progression.

Analysis of genetic variants

DNA Isolation

Blood samples were collected from the patients in an EDTA tube and high molecular genomic DNA was isolated from it. DNA was isolated from all samples using the modified salting out method. The DNA in the samples were quantified with, NanoDrop ND-1000 Spectrophotometer (Thermo Scientific NanoDrop Technologies, Wilmington, DE). The ratio between OD values at 260nm and 280nm (260/280 ratio) was used to estimate the purity of the nucleic acid. Pure DNA preparations gave the ratio of 1.8 while the higher or lower values indicates either RNA or protein (or phenol) contaminations, respectively. Determination of the intactness of DNA samples was performed by 0.8% agarose gel electrophoresis using Medox horizontal agarose gel electrophoresis. The gel was run for 45 minutes and ethidium bromide-stained to visualize under UV light which was subsequently documented using gel documentation system.

Genotyping

Genotyping was performed using PCR RFLP for MTHFR (C677T and A1298C), DPD (IVSI14+1G>A) and GSTP1 (I105V). The TS (*2R*/*3R* repeat) genotyping was performed using PCR and gel electrophoresis. The details of primers and annealing temperatures are documented in table 1. All the reactions were set in a final volume of 25 µl consisting 10 µl of DNA (100 ng), 1 µl forward primer, 1 µl reverse primer and 13 µl of 2X PCR master mix. Except the annealing temperatures provided in table 1, the PCR cycling conditions were Initial denaturation at 95° C for 5 min, Denaturation at 95°C for 1 min, Extension at 72°C for 40 sec, Final extension at 72°C for 7 min which was common for all primers. All the amplifications were completed within 35 PCR cycles. The amplified PCR products were resolved on 2% agarose gels and visualised under UV light.

For TS alleles, amplified products was resolved and visualised under UV. The amplicons with size of 248 base pairs was considered as 2R/2R, 270 base pairs as 3R/3R and both of these products as 2R/3R.

For MTHFR C677T polymorphism, the wild type (677CC) showed a single band of 198 bp. The presence of the 'T' allele introduces a cut among heterozygous (677 CT) and 3 bands of 198, 175 and 23 bp were seen. The homozygous (677 TT) have two bands of 175 bp and 23bp (Fig. 1).

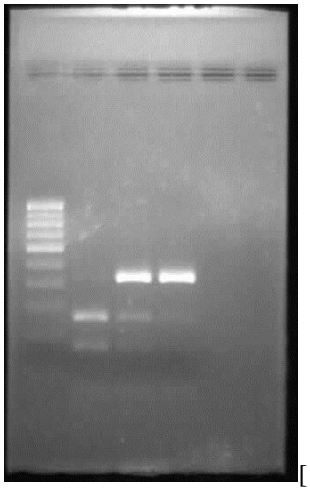

Fig. 1.MTHFR C677T polymorphism

For MTHFR1298A→C polymorphism, the Wild type (1298 AA) fragments of 56 bp, 30/31 bp, 28 bp and 18 bp. Heterozygous (1298 AC) produced fragments of 84, 56, 30/31, 28 and 18 bp, whereas homozygous for 1298 polymorphism (1298 CC) produced 84, 30/31 and 18 bp.

For DPD genotype, the fragments at 181 and 17 bp for the wild type allele an 154, 27, and 17 bp for the mutated allele was observed.

For GSTP1 genotype, electrophoresis of the digested PCR products showed individuals homozygous (Ile/Ile) for the *GSTP1* as one band of 176bp. Heterozygous (Ile/val, val/val) for the polymorphism showed three bands of 176, 91 and 85. Homozygotes (val/val) showed two bands of 91 and 85bp.

Allelic discrimination was done by RFLP using specific endonucleases provided in the table1.

Table 1: Specifications for RFLP

Gene	Name of primer	Sequence	Annealing temperature (^{0}C)	Restriction enzymes
TS	Forward	5’ GTGGCTCCTGCGTTTCCCCC3’	61	
	Reverse	5’ GCTCCGAGCCGGCCACAGGCA3’		
MTHFR	677-Forward	5’ TGA AGG AGA AGG TGT CTG CGG GA 3’	65	HinfI
	677-Reverse	5’ AGG ACG GTG CGG TGA GAG TG 3’		
MTHFR	1298-Forward	5’CTT TGG GGA GCT GAA GGA CTA CTA C-3’	64	MboII
	1298-Reverse	5’CAC TTT GTG ACC ATT CCG GTT TG-3’		
DPD	Forward	5'-ATCAGGACATTGTGACA**T**ATGTTTC-3'	58	NdeI
	Reverse	5'-CTTGTTTTAGATGTTAAATCACAC**A**TA-3'		
GSTP1	Forward	5′-ACCCCAGGGCTCTATGGGAA-3′	55	BSmA1
	Reverse	5′-TGAGGGCACAAGAAGCCCCT-3		

Statistical analysis

The overall toxicity was grouped as either mild (0-1) or moderate to severe (grade 2&3) for statistical analysis. Subgroup analysis was done on each adverse event individually and combined. The analysis of association between genotypes and the maximum observed grade of toxicity was carried out by χ^2 test to estimate the exact two-sided *P* value. Similarly the relationship between genotypes and the response were analyzed by means of χ^2 tests, using an Fischer’s exact test and a p value <0.05 was considered statistically significant. The Median follow-up of the study population of patients was 8 months. Statistics were done on SPSS software, version 13.1 (SPSS, Inc., Chicago, IL).

RESULTS

Patients’ characteristics

The study involved 16 colorectal cancer patients on CAPOX therapy between 18-75yrs of age and most of the patients were in the age group of 30-40yrs. The median number of cycles administered was eight**.** The patient characteristics are represented in table 2.

Table 2: Patient Characteristics

Patient characteristics	Frequency (n=16)	Percentage (%)
Gender		
Male	7	43.75
female	9	56.25
Age		
Mean	47.75± 12.37yr	
Range	30-65 yrs	
BSA		
Mean	1.49±0.13 Kg/m^2	
Range	1.29-1.74 Kg/m^2	

Location of tumour		
Colon	11	68.75
Rectum	5	31.25
Ko¨Hne Risk Index		
Low	0	0
intermediate	9	56.25
high	7	43.75

Majority of the patients had tumour located in colon and 5 patients had tumour located in the rectum. Among the female, 4 patients had tumour located in colon, 4 patients had tumour located in rectum and 1 patient had tumour located in both colon and rectum. Among the male, 6 patients had tumour located in colon and 1 patient had tumour located in rectum. Although technically better to describe by colonic segment, in practice the colon is often referred to in terms of right and left colon: Nine patients had the tumour located on the left side were as 7 patients had tumour located on the right side.

Majority of the patients were in stage III (n=7), followed by 5 patients in stage IV, 2 patients in stage II and 2 patients in stage I. The histopathology of biopsy sample, adenocacinoma was observed in 10 patients, mucinous adenocarcinoma was observed in 3 patients, signet ring cell carcinoma was observed in 3 patients.

Only 3 patients among the study population had comorbidities with diabetes, hypertension and diabetes with MI respectively.

Most of them had an ECOG 2 and according to Ko¨hne classification based on ECOG, The tumor site, alkaline phosphate level and WBC count they were classified as low, intermediate and high risk patients and a low tumor burden was observed among them. [18]

Toxicity profile of the study population

The toxicity profile is listed in Table 3. The most common haematological toxicity was anaemia, observed in all patients. Grade 3 anaemia was observed in 2 patients and grade 3 neutropenia was observed in 2 patients.

Table 3: Distribution of toxicity

Adverse event	Grade1	Grade2	Grade 3	Total
Neutropenia	-	-	2	2
Anaemia	8	6	2	16
Nausea	1	1	-	2
Vomiting	1	-	-	1
Diarrhoea	1	-	2	3
Mucositis	1	2	-	3
Neurotoxicity	1	-	-	1
Asthenia	4	2	2	8
Hand Foot syndrome	2	-	-	2

Gastrointestinal disturbances were mild, although grade 3 diarrhoea was observed in 2 patients and grade 2 nausea was observed in 1 patient and grade 1 vomiting was observed in one patient. There was a delay in dose for two patients among the study population due to grade 3 diarrhoea. Among the other toxicities, 8 patients' complain fatigue. Grade 1 neurosensory toxicity was developed in 1 of the patients, grade 2 stomatitis was observed in Two patients. Hyperglycemia was observed in three patients. Among eight patients with hypoalbuminemia, 4 had grade 3 hypoalbuminemia. Grade 1 hand and foot syndrome was observed in 2 patients.

Genotype distribution

With genotyping of *TS* of the 28 bp tandem repeat 18.75% of the patients were with 2R2R, 81.25% with 2R3R, and none of the patients had 3R3R..The Distribution of TS *5′* genotype, *MTHFR* genotypes 677C>T and 1298A>C and IVS14 + 1 genotype in *DPD* is depicted in Table 3. Only 1 of 16 patients carried the mutation (heterozygous) with respect to *DPD* gene. The distribution of *GSTP1* polymorphism was 37.5% for Ile/Ile and Ile/Val respectively and 25% with Val/Val.

Table 4: Distribution of Genotypes

GENE	GENOTYPES	FREQUENCY (N=16)	PERCENTAGE (%)
TS-5-UTR	2R/2R	3	18.75
	2R/3R	13	81.25
	3R/3R	-	0

MTHFR-677	CC	11	68.75
	CT	5	31.25
	TT	-	
MTHFR-1298	AA	4	25
	AC	6	37.5
	CC	6	37.5
DPD	w/w	15	93.75
	w/mt	1	5.46
	Mt/mt	0	0

Genetic determinants of toxicity

For statistical analysis between the genotypes and toxicity the overall toxicity were categorised as either moderate (grades 1–2) or severe (grades 3–4). Further analyses were performed by grouping toxicity into three broad categories: haematological, gastrointestinal and others. No statistically significant differences with respect to toxicity were observed according to relevant clinical variables. This might be attributed to less number of sample size.

No relationships between TYMS, *MTHFR, DPD, GSTP1* genotypes, and global toxicity were observed on univariate Fisher's exact tests.

The incidence of grade 2/3 toxicity on analysis of patients with *MTHFR* 677 genotype Fig 2a and *MTHFR* 1298A>C is depicted in fig 2b.

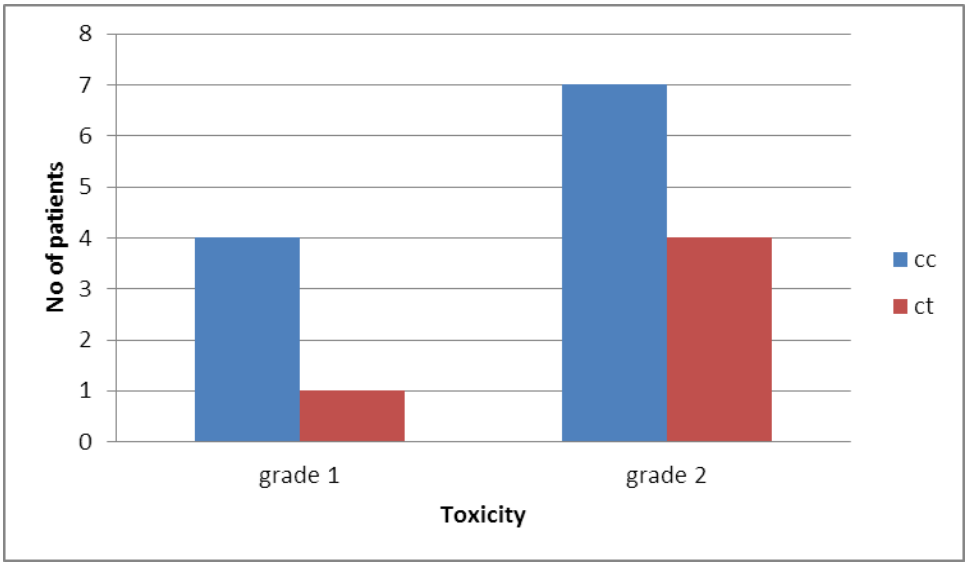

Fig2a:Incidence of toxicity with MTHFR677 genotype

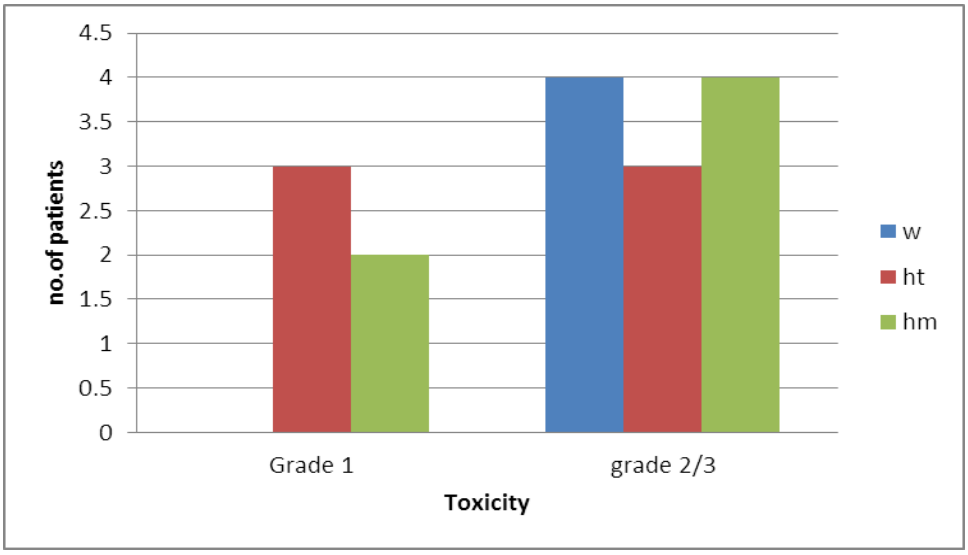

Fig2 b: Incidence of toxicity with MTHFR 1298 genotype

The well described exon 14 skipping IV +1g>A which is related to DPD deficiency occurred in only 1of 16 patients with more toxic side effects.

Platinum derivatives are commonly detoxified by the isoenzyme *GSTP*1 and are also an important mediator of both intrinsic and acquired resistance to platinum. Grade 2 peripheral neuropathy was observed in individuals carrying Val/Val *GSTP1* at Exon 5 genotype (1-of 4 patients, 25%).

Genetic determinants of Response

On an intent-to-treat basis we observed a 43.75% overall response rate (ORR), with 4 (25%) Partial response and 3 (18.75%) Complete Response. Clinical response (CR, PR, and SD > 6 months) was achieved in 62.5% of the patients after a median follow-up of 8 months.

Distribution of genotypes and response to chemotherapy are summarised in Table 4.The patients were grouped for statistical analysis as responders with CR, PR, or SD lasting 6 months, whereas patients with SD less than 6 months were referred to as non-responders. Statistical analyses indicate no significant relationship as the sample size was less. But if seen individually, all the 3 patients with TS 2R/2R were non responders.

Table 5: Distribution of genotypes and response to chemotherapy

Gene	Genotype	Responder	Non Responder	Total	P-Value
TS	2R/2R	0	3	3	0.250
	2R/3R	6	7	13	
	Total	6	10	16	
MTHFR677	CC	4	7	11	1.000
	CT	2	3	5	
	Total	6	10	16	
MTHFR 1298	AA	2	2	4	0.356
	AC	2	4	6	
	CC	2	4	6	
	Total	6	10	16	
DPD	AA	0	1	1	0.319
	GA	1	0	1	
	GG	5	9	14	
	Total	6	10	16	
GSTP1	Ile/ Ile	2	4	6	0.837
	Ile /val	2	4	6	
	val/val	2	2	4	
	Total	6	10	16	

Effect of palliative CAPOX and effect of polymorphism

At the time of analysis 3 of the 16 patients died. The reason for death in all patients was disease progression. One patient was heterozygous for *TS*, *MTHFR677*, *MTHFR 1298* and *GSTP1* and had a poor prognosis with the overall survival of 15 months.

DISCUSSION

Ethnic diversity in drug response or toxicity is becoming increasingly recognized as an important factor accounting for inter-individual variation in anticancer drug responsiveness.This study examined the association of *TS, MTHFR, DPD and GSTP1* gene polymorphisms in 16 successive colorectal cancer patients receiving CAPOX therapy in our settings.

The present study, suggests that patients bearing 2R/2R *TS* genotype were not good responders for the therapy and a study by Remy Largilllier et al reports a rapid disease progression with higher TS expression (i.e., 3R3R) relative to 2R2R in patients with capecitabine. [19] It is noticed that high intracellular TS expression is related to 5-FU resistance an indicator of unfavourable prognosis as a result of tumour aggressiveness. [20, 21]

The activity of the *MTHFR* gene is diminished by two commonly reported polymorphisms namely 1298A>C (rs1801131) and 677C>T (rs1801133). Even though the impact of MTFHR genotype on tumoral CH2FH4 concentrations has not been clearly established, deficient MTHFR genotypes may theoretically favour an increase in intracellular CH2FH4 concentrations. Several studies have reported the association of 677 variant in MTHFR with decreased risk of CRC which is concordant to our study wherein none of the patients were MTHFR677 variant .[22] This is implicated by the protective effect of the MTHFR variant due to the an increase in the MTHFR substrate, 5, 10-MTHF as a result of which it enhances the action of TS, which ultimately provide adequate amounts of thymidine for appropriate DNA synthesis and repair. However the results of Sunil Chandy et al indicates that, the homozygous state for 1298A>C polymorphism was associated with lower risk of CRC. [23] Hence this needs to be confirmed with a large sample size. The study data demonstrates that the

MTHFR 677 C→T allele was linked with the clinical response to capecitabine-based chemotherapy. Response rates were higher for those with mutant allele in comparison to those with patients carrying only the wild-type allele.

An increased toxicity was reported by Capitain *et al.* in patients with 1298A>C rather than 677C>T in patients with metastatic CRC (mCRC) on 5-FU/leucovorin [24] and similarly Tsunoda *et al.* showed no association with 677C>T SNP however, the patients with AA carriers suffered less fatigue than AC with 1298A>C SNP. [25] In another study patients bearing AA genotype for 1298A>C experienced a higher ADRs when treated with capecitabine. [19]. Contradictorily, some studies report 677C>T SNP associated with grade 3–4 toxicity and diarrhea compared to 1298A>C. [26-28] Further, some authors report 677C>T CC carriers with higher rates of toxicity, while others report less toxicity with CC genotypes .[26, 29] Furthermore, several studies show no association between any of these polymorphisms and toxicity.[30,31] Our study also showed no statistical association between the 677C>T and 1298A>C SNPs with toxicity. Hence, with these results pharmacogenetics cannot be recommended to predict toxicity associated with *MTHFR* polymorphisms.

Dihydropyrimidine dehydrogenase (DPD) is the rate limiting step in the catalytic pathway of capecitabine. In DPD deficiency, the pathway for metabolism of 5-FU does not function normally, resulting in accumulation of toxic compounds and prolonged exposure to 5-FU. The most frequent inactivating mutation is IVS14+1G>A leading to skipping exon 14 and therefore missing 165 nucleotide in mRNA and the corresponding 55 aminoacids in the protein product. In our study, IVS14+1G>A mutation was found in only one case in heterozygote state, with Grade 3 global toxicity and poor response. A higher rates of severe toxicities are reported following exposure to capecitabine, in patients who were heterozygous (possessing two different forms of the gene) for the mutant *DPYD* allele, compared with patients with homozygous (possessing two identical forms of the gene) for the wild-type, or unmutated allele.[32,33]

Neurotoxicity is an important dose limiting toxicity of oxaliplatin with two distinct syndromes namely acute neurosensory toxicity and sensory neuropathy. Acute neurosensory toxicity is generally triggered by exposure to cold and is characterized by peripheral-nerve hyperexcitability.[34-35] Secondly, sensory neuropathy with loss of sensation and dysesthesias in the distal extremities is associated with the long-term administration of oxaliplatin. It is correlated with the cumulative dose of oxaliplatin and commonly occurs in patients who have received total doses ≥540 mg/m^2.[36] In a study, grade 3 neurotoxicity was observed in 18% of patients with a dose of 85 mg/m^2 per cycle,10% after three and nine cycles, 25% after 12 cycles, and 50% after 14 cycles.[37] In our study, the incidence of grade 2 oxaliplatin-related neuropathy was observed in only one patient after eight cycles. On cessation of drug, the chronic neurotoxicity was gradually resolved.

Platinum derivatives are detoxified by the isoenzyme GSTP1. The conjugation of glutathione to electrophilic xenobiotics to inactivate form are catalysed by Glutathione *S*-transferases (GST) a multigene family of enzymes (cytosolic and membrane-bound), and facilitates their excretion from the body [38]. Several Studies reveal that the Ile^{105}Val substitution modifies the substrate affinity of the GSTP1 enzyme. An altered catalytic activity was obsereved in Individuals homozygous for the *GSTP1* ^{105}Val genotype compared with individuals heterozygous for the *GSTP1* ^{105}Ile allele. [39] In a study by Gothery et al *GSTP1* Ile^{105}Val polymorphism was associated with neurotoxicity in a group of 299 patients receiving oxaliplatin.[40] In our study, the *GSTP1* val/val allele was observed in a patient with grade 2 peripheral neuropathy after fifth cycle of chemotherapy.

Presently, further studies on large number of patients are needed to confirm the role of *TS, MTHFR, DPD, GSTP1* gene polymorphism on CAPOX toxicity and efficacy due to limited number of patients.

CONCLUSION

From the study findings of our population it can be concluded that patients bearing TS 2R/2R genotypes are not good responders for the CAPOX therapy. In addition DPD deficiency should be looked in for association of toxicity in patients receiving CAPOX therapy. However, these data has to be confirmed over a large set of patients. Even though this is a small study the information generated may be important. Further, Pharmacokinetic measurement of individual 5FU and its metabolite would give a clear picture of the factors that might influence the response /toxicity in patients receiving CAPOX therapy.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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