



## Association of polymorphism of Enzyme Thiopurine Methyl Transferase in Head & Neck Squamous Cell Cancer and treatment response to Concurrent Chemo Radiotherapy

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### Abstract

**Objectives:** The aim of the present study was to determine the frequency of the functional TPMT polymorphisms and their association with the treatment outcome in patients of HNSCC receiving cisplatin based chemoradiation.

**Methods:** 100 patients of locally advanced head & neck squamous cell carcinoma and 100 controls frequently matched with cases were enrolled in the study. Allelic variant of TPMT gene was done with the help of RFLP PCR analysis. All patients received radiation 70 Gy with concurrent cisplatin 35 mg/m<sup>2</sup> every weekly. Response assessment was done with help of World Health Organization (W.H.O.).

**Results:** Majority of Indian population had TPMT\*3B wild genotype. The patients carrying the wild type genotype of all four alleles responded to Chemoradiation, while TPMT\*3B heterotypes and TPMT \*3C homozygous mutant form were found among the non responder patients.

**Conclusion:** Genotyping of TPMT helps us to identify responders and non responders to standard treatment and enhances the precision regarding selection of treatment modality.

**Keywords:** Head and Neck Cancer, Thiopurine Methyltransferase gene, Treatment Response, Polymorphism.

### Introduction

Head and neck cancer is a major form of cancer in India accounting for 23% of all cancer in males and 6% in females. Most cases (80%) present in late stages and survival is poor with all modalities of treatment<sup>[1]</sup>.

Although tobacco and alcohol consumption are recognized as the major causes of head and neck cancer, geographic distribution of cancer incidence is not strongly correlated with areas of high tobacco and alcohol consumption; and only a small number of smokers actually develop head

and neck cancer, also there is wide inter individual variability in response and survival after treatment in same stage disease, which suggests a possibility of gene environmental interaction to have an influential role in the susceptibility to cancer and variation in treatment response.<sup>[2]</sup>

Pharmacogenomics aims to elucidate the genetic basis for inter individual differences in cancer susceptibility and treatment response and also to use this genetic information to predict the safety, toxicity, efficacy and individualization of treatment modality<sup>[3,4]</sup>

Thiopurine Methyl Transferase (TPMT) is known to be a drug metabolizing enzyme mainly considered to contribute phase-II biotransformation of xenobiotics<sup>[5,6]</sup>

It is responsible for activation, inactivation and detoxification of many chemotherapeutic agents<sup>[7,8]</sup>. Deficiencies of this enzymes play a vital role in various malignancies including head and neck causing variation in exposure to chemotherapeutic agents which sub sequentially influences the efficacy of treatment.<sup>[4]</sup> Since Thiopurine Methyl Transferase (TPMT) is mainly considered to contribute phase-II biotransformation of xenobiotics<sup>[5]</sup> its deficiency primarily leads to treatment related toxicity which has been documented in many of the studies; however there are no studies till date to document its association with treatment response.

Mounting literature evidences indicate that polymorphism of TPMT gene which have functional consequences on Thiopurine Methyl Transferase, give rise to important inter individual and interethnic variability in metabolism of carcinogens and anti cancer agents causing differences in susceptibility to cancer and its treatment outcome.<sup>[7]</sup>

Three common polymorphic alleles have been known to be associated with impaired activity of this enzyme. These are TPMT\*2, TPMT\*3A and TPMT\*3C.<sup>[4,16]</sup>

The aim of this study is to explore whether polymorphism of TPMT gene correlate with

treatment response. This will elicit the mechanism of altered response with genetic variations in HNSCC. The current study has hence been proposed to evaluate the frequency of the functional TPMT polymorphisms and their association with responders versus non-responders to chemo-radiotherapy.

### Material & Methods

A case control study was conducted. The study group comprised 100 histologically proven cases of locally advanced stage (III, IVa) with W.H.O. performance status of grade 0/1 attending radiotherapy O.P.D. in year 2008-2010 and equal number of healthy controls. Written informed consent according to institutional regulations was obtained from all patients before enrollment. These cases were assessed thoroughly (history, clinical examination and investigations) as given in Table 1.

All the patients were treated by chemoradiation total dose of 70 Gy in 35 fractions in 7-weeks by cobalt therapy to primary tumour site and neck along with weekly concurrent cisplatin 35mg/m<sup>2</sup>.

All the cases included in the study belonged to the same ethnic group (Indo-European community) of North India based on geographical location and linguistic basis. Controls were frequency-matched to cases by year of birth in 15-year classes. It was ensured that the controls also belonged to the same geographical location and socio-economic conditions. Based on medical check-up, controls were not found to suffer from any chronic disease. All study subjects completed a questionnaire covering medical, residential and occupational history. Information pertaining to dietary habits, family history of disease, smoking, tobacco chewing and alcohol drinking was also obtained in the questionnaire filled by the cases. Information pertaining to dietary habits, family history of disease, smoking, tobacco chewing and alcohol drinking was also obtained from the patients and controls as well.

The definitions of treatment response viz. complete response (CR), partial response (PR) and

no response (NR) [stable disease (SD) + progressive disease (PD)] were based on the standard definitions established by W.H.O. (1979).

End point was to evaluate genetic polymorphism of TPMT gene as an independent prognostic factor and its correlation with clinical benefits obtained with chemoradiation on response rate in patients of locally advanced head and neck cancer. Genomic DNA was isolated from blood samples collected from controls & patients. Polymorphism in genomic DNA was studied by PCR-RFLP technique.

### Procedure

The PCR process usually consists of a series of twenty to thirty-five cycles. Each cycle consists of denaturing, annealing and extension. The double-stranded DNA has to be heated to 94-96°C in order to separate the strands. This step is called denaturing; it breaks apart the hydrogen bonds that connect the two DNA strands. Prior to the first cycle, the DNA is often denatured for an extended time to ensure that both the template DNA and the primers have completely separated and is now single-strand only. Time duration is 1-2 minutes up to 5 minutes; also Taq-polymerase is activated by this step.

After separating the DNA strands, the temperature is lowered so the primers can attach themselves to the single DNA strands. This step is called annealing. The temperature of this stage depends on the primers and is usually 5°C below their melting temperature (45-60°C).

Finally, the DNA-Polymerase has to copy the DNA strands. It starts at the annealed primer and works its way along the DNA strand. This step is called extension. The extension temperature depends on the DNA-Polymerase. The time for this step depends both on the DNA-Polymerase itself and on the length of the DNA fragment to be amplified.

### Standardization of PCR conditions for TPMT\*2:

Allele specific PCR method was used for identifying TPMT\*2 polymorphisms<sup>[9]</sup>. DNA was amplified with primers P2C (5'-TAAATAAACCATCGGACAC-3') (reverse) and either P2W (5'-GTATGATTTTATAGGTTTG-3') or P2M (5'-GTATGATTTTACAGGTTTC-3') (forward) to identify the wild type specific or mutant specific amplification, respectively (Table2). The reaction mixture in 50 µl contained 1X buffer (10 m M-Tris-HcCl pH 8.3, 1.5 m M-MgCl<sub>2</sub>, 25 m M-KCl), 200 µM of each nucleotides, 200nM of each primers, 1.5 unit of Taq polymerase (MBI Fermentas, Germany), 100ng of genomic DNA and sterile milliQ water. Amplification was performed on a thermal cycler using the protocol: Initial denaturation was at 94°C for 5 min, followed by 35 cycles of amplifications at 94°C for 45 sec., annealing at 57 °C for 30 sec and extension at 72°C for 1 min. The PCR product of 254 base pair was separated on agarose gel (3%).

### Standardization of PCR condition for identifying allele variation of TPMT\*3A,

The primer sequences used for identifying polymorphic sites in TPMT\*3A is shown in Table 2. Amplification was performed on Gene Amp PCR system 9700 of Applied Biosystems using the following protocol: 94°C for 5 minutes for initial denaturation followed by 35 cycles of 94°C for 45 sec, 56°C for 45 sec, and 72°C for 1 min and a final elongation step of 72°C for 10 min. PCR reaction resulted in a 365 bp product, which was further digested with 10U of MwoI restriction enzyme (MBI Fermentas, Germany) at 37°C for 16 hrs. The digested PCR products were separated by electrophoresis using 2% agarose gel containing ethidium bromide and DNA bands were visualized on VERSA DOC Imaging System (Model 1000, Bio-Rad). Genotypes were determined based on the size of the respective DNA fragments. Digestion with MwoI resulted in two fragments of 267 and 98 bp which is indicative of wild type genotype. The digestion of

PCR product into three fragments of 365, 267 and 98 bp, suggested the presence of heterozygous genotype<sup>[10]</sup>. In case of homozygous mutant, no digestion occurred and single band of 365bp persisted after overnight digestion with MwoI restriction enzyme digestion (Figure 1).

#### **Standardization of PCR condition for TPMT\*3B:**

The primer sequences used for identifying TPMT\*3B polymorphism is shown in Table 2. Amplification was performed on a thermal cycler using the following protocol: 94°C for 5 minutes for initial denaturation followed by 35 cycles of 94°C for 45 seconds, 56°C for 45 seconds and 72°C for 1 minutes and a final elongation step of 72°C for 10 minutes. PCR amplification resulted in the formation of a 694 bp product. 20µl of the PCR product was then digested with 10 U of MwoI (MBI Fermentas, Germany) in a final volume of 30 µl. This reaction mixture was incubated overnight at 37°C. Electrophoresis was subsequently carried out in a 3% agarose gel containing ethidium bromide and analyzed on VERSA DOC Imaging System (Model 1000, Bio-Rad).<sup>[9]</sup> Digestion of 694 bp PCR product into two fragments of 443bp & 251bp indicated the presence of wild type genotype. In case of homozygous mutant, no digestion occurred and single band of 694bp was visible when analyzed on imaging system (Figure 2).

#### **Standardization of PCR conditions for TPMT\*3C:**

The primer sequences used for identifying TPMT\*3C polymorphism is shown in Table 2. Amplification was performed on a thermal cycler using the following protocol: 94°C for 5 minutes for initial denaturation followed by 35 cycles of 94°C for 45 seconds, 59°C for 30 seconds and 72°C for 1 minutes and a final elongation step of 72°C for 10 minutes. PCR reaction resulted in the formation of a 373 bp product. 20µl of the PCR product was digested with 10 U of AccI (MBI Fermentas, Germa Digestion of 373 bp PCR product into two fragments of 283bp & 90bp indicated the presence of wild type genotype. An

undigested 373 bp PCR fragment was indicative of mutant type genotype<sup>[10]</sup>. The presence of three bands of 373 bp, 283bp & 90-bp would be indicative of the heterozygous type genotype (Figure 3).

Study end point was taken to be response assessment to treatment by the cases and identify the genotypes of TPMT gene not responding to treatment and to identify alleles of TPMT gene prevalent in patients and in normal controls.

#### **Results**

The results are discussed with reference to the site of involvement, age of presentation, method of detection, stage at presentation, demographic distribution, frequency of polymorphism, polymorphism and its association with head and neck carcinoma, association of use of tobacco and alcohol with head and neck carcinoma, association of polymorphism and tobacco and alcohol use and finally association of polymorphism and response to treatment.

Statistical Analysis was done with SPSS (statistical package for social sciences) software version 15.0 was used for data analysis. Mean and standard deviation were estimates of quantitative data. Allele frequency was calculated by counting the alleles. The odds ratio and 95% confidence level (CI) was calculated for all proportions of alleles and genotypes. The Chi-Square test was performed to test for deviation from Hardy-Weinberg equilibrium for each locus. Significant P value was set at 0.05.

The main characteristic of the study population are summarized in Table 1. The mean ages of the cases and controls were 46±9 and 45±11 years respectively. By tumor site, a majority of cases were suffering from oropharynx followed by Oral Cavity and Larynx (Table 2). The genotypic distribution of polymorphic TPMT in controls and cases were done for four major type of variation prevalent (\*2,\*3A,\*3B,\*3C) of genotype.

Table 3 summarizes the distribution of genotype and allele frequency of TPMT\*2 polymorphism of TPMT gene in the head and neck squamous cell

carcinoma patients and controls. As evident from Table 3, the proportion of individuals with homozygous mutant genotype of TPMT\*2 was found to be higher in the HNSCC patients (40%) when compared with the controls (28%). Thus the homozygous mutant genotype of TPMT\*2 in HNSCC patients conferred an increased HNSCC risk (O.R.1.7; 95% C.I, 0.95 – 3.1) when compared with control. However this increase risk in HNSCC patients was not found to be statistically significant

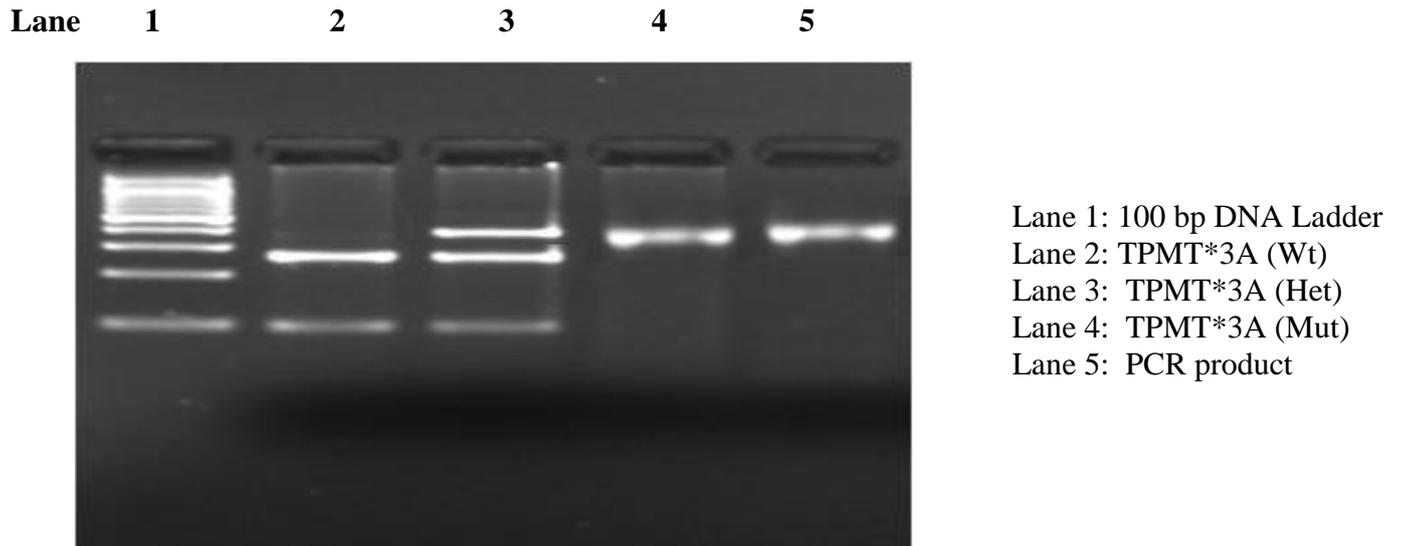
Genotype and allele frequency of TPMT\*3A polymorphism in HNSCC patients and controls are summarized in Table 4. As evident from Table 4, homozygous mutant genotypes of TPMT\*3A amongst HNSCC patients (20%) and controls (20%) exhibited equal distribution thereby exhibiting no increase in the risk in the patients carrying mutant genotype of TPMT\*3A (O.R.1.1; 95% C.I, 0.58 – 2.10). Similarly, similar distribution of heterozygous genotype of TPMT\*3A was also found in HNSCC patients (32) and controls (30) thereby exhibiting no increase in the risk (O.R.0.5; 95% C.I, 0.49 – 2.17) in HNSCC patients carrying heterozygous genotype.

Table 5 summarizes the distribution of genotype and Allele frequency of TPMT\*3B polymorphism in the head and neck squamous cell carcinoma patients and controls. As evident from Table 5, TPMT\*3B polymorphism was found to be present in healthy controls as well as in the head and neck squamous cell carcinoma patients. Wild type genotype was found to be present in 38% of the controls and 32% of the HNSCC patients carried this wild type genotype. However not much difference was observed in the distribution of the frequency of heterozygous genotype in controls (62%) and HNSCC patients (68%) due to which no risk OR:1.3; 95% CI: 0.72 – 2.33) was observed in the HNSCC patients carrying heterozygous genotype of TPMT\*3B polymorphism. As observed in Table 5 homozygous mutant forms for TPMT\*3B could not be detected in the present study due to small sample size.

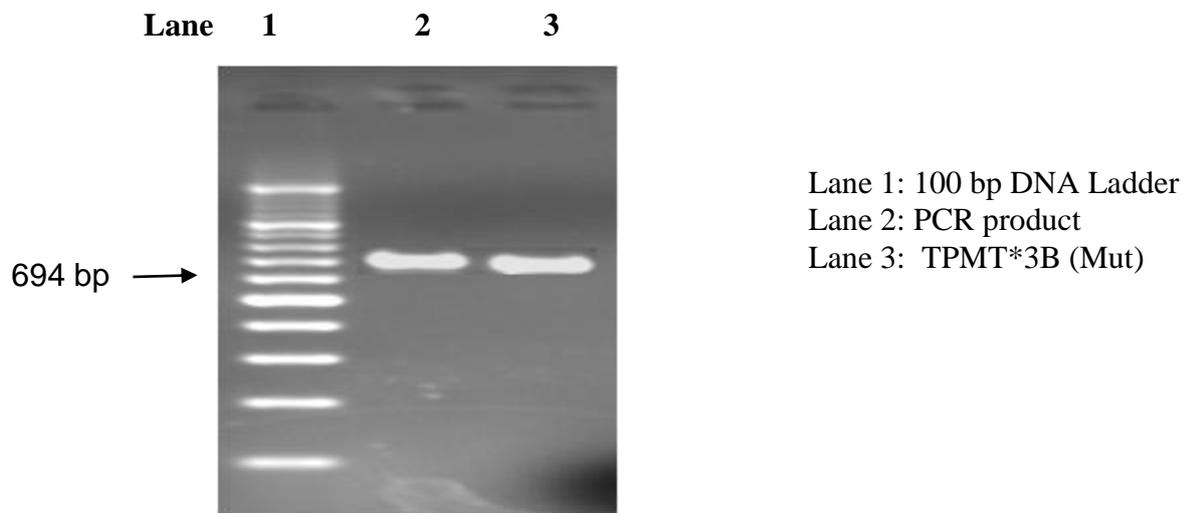
Distribution of genotype and Allele frequency of TPMT\*3C polymorphisms of TPMT gene in the head and neck squamous cell carcinoma patients and controls are shown in Table 6. Wild type genotype was found to be present in 80% of the controls, and 62% of HNSCC patients. The prevalence of the heterozygous as well as homozygous mutant genotype of TPMT\*3C polymorphism increased in the HNSCC patients (23% and 15%) when compared to the controls (11% and 9%) respectively. Our data showed that the increase in the frequency of the heterozygous genotype of TPMT\*3C polymorphism in the HNSCC patients when compared with controls was found to be associated with the increase risk in the HNSCC patients (OR: 2.7; 95% CI: 1.22 – 5.95) and this increase in risk was found to be statically significant. The OR was also found to increase with the increase in the frequency of the homozygous mutant genotype of TPMT\*3C polymorphism in the HNSCC patient when compared with controls (OR: 2.5; 95%CI: 0.88 – 5.23). However this increase in risk was not found to be statically significant.

The patients carrying the wild type genotype of all the four alleles responded to treatment regimen of chemoradiotherapy with cisplatin. While the TPMT\*3B heterotypes (p 0.01) and \*3C homozygous (p 0.13) mutant were among the non responders (Table 7).

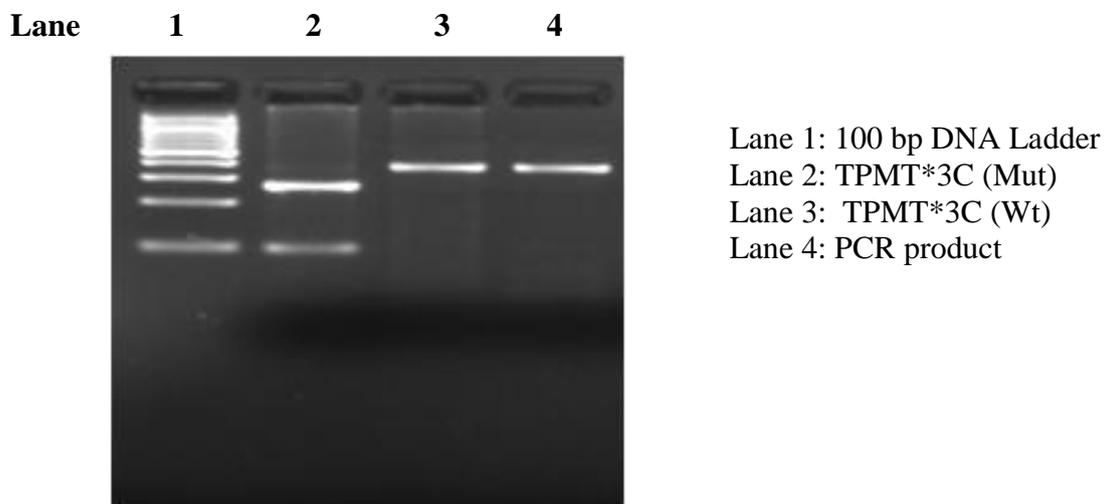
**Figure 1:** Ethidium bromide-stained agarose gel showing PCR & digested products of gene TPMT\*3A with Mwo I restriction enzyme



**Figure 2:** Ethidium bromide-stained agarose gel showing PCR & digested products of gene TPMT\*3B with Mwo I restriction enzyme



**Figure 3:** Ethidium bromide-stained agarose gel showing PCR & digested products of gene TPMT\*3C with Acc I restriction enzyme



**Table 1 :** Distribution of Demographic Variables and Putative Risk Factors of Hnscc Cases

Characteristics	Control n (%)	Cases n (%)
Subjects	100	100
Age (mean±S.D.)	45±11	46±9
Non-smokers	63(63%)	28(28%)
Smokers	37(37%)	72 (72%)
Nontobacco chewers	55(55%)	33 (33%)
Tobacco Chewers	45 (45%)	67 (67 %)
Non alcohol Users	62 (62%)	54(54%)
Alcohol Users	38 (38%)	46(46%)

**Table 2:** Site Wise Distribution

SITE	CASES (%)
Oral Cavity	26(26 %)
Hypopharynx	14 (14 %)
Oropharynx	28 (28 %)
Larynx	24(24 %)
Others	8 (8 %)

**Table 3:** Distribution of TPMT\*2 Genotypes among cases and controls

Genotype	Control n=100 (%)	Patients n=100 (%)	OR (95% CI)	p-value	Allele frequency			
					Control		Patients	
					Major	Minor	Major	Minor
Wild	72	60	1 (Ref.)		0.72	0.28	0.60	0.40
Hetro	00	00	-					
Mutant	28	40	1.7 (0.95 - 3.1)	0.07				

**Table 4:** TPMT\*3A Genotypes among cases and controls

Genotype	Control n=100 (%)	Patients n=100 (%)	OR (95% CI)	p-value	Allele frequency			
					Control		Patients	
					Major	Minor	Major	Minor
Wild	50	48	1 (Ref.)		0.65	0.35	0.64	0.36
Hetro	30	32	1.1 (0.58- 2.10)	0.75				
Mutant	20	20	1.05 (0.49 - 2.17)	0.91				

**Table 5:** Distribution of TPMT\*3B Genotypes among cases and controls

Genotype	Control n=100 (%)	Patients n=100 (%)	OR (95% CI)	p-value	Allele frequency			
					Control		Patients	
					Major	Minor	Major	Minor
Wild	38	32	1 (Ref)		0.69	0.31	0.66	0.39
Hetro	62	68	1.3 (0.72– 2.33)	0.37				
Mutant	00	00	-					

**Table 6:** Distribution of TPMT\*3C Genotypes among cases and controls

Genotype	Control n=100 (%)	Patients n=100 (%)	OR (95% CI)	p-value	Allele frequency				
					Control		Patients		
					Major	Minor	Major	Minor	
Wild	80	62	1 (Ref.)			0.89	0.15	0.74	0.27
Hetro	11	23	2.7 (1.22– 5.95)	0.01					
Mutant	09	15	2.15 (0.88– 5.23)	0.08					

**Table 7:** Treatment response in cases of HNSCC with TPMT genotypes

Genotypes	cases	responders	nonresponders	p-value
TPMT*2WT	54	40	14	
TPMT*2HT	00	00	00	
TPMT*2MT	36	20	16	0.16
TPMT*3AWT	48	32	13	
TPMT*3AHT	29	13	16	
TPMT*3AMT	18	8	10	0.07
TPMT*3BWT	28	20	8	
TPMT*3BHT	62	20	42	0.01
TPMT*3MT	00	00	00	
TPMT*3CWT	56	40	16	
TPMT*3CHT	21	13	8	
TPMT*3CMT	13	3	10	0.13

WT – Wide Type

HT – Heterozygous Mutant Type

MT- Homozygous Mutant Type

**Discussion**

Thiopurine methyl transferase catalyzes the S-methylation of thiopurine drugs like 6-mercaptopurine, 6-thioguanine, etoposide and cisplatin<sup>[7,8]</sup>. Cisplatin is commonly used concurrently with radiotherapy for locally advanced HNSCC. Thus any variation in metabolism of this drug is bound to have effect on treatment response.

The Indian population has a unique structure and is subdivided into large endogenous ethnic groups which are genetically more homogenous than a conglomerate population and also show more similarity in life style and dietary studies. Genetic factors are estimated to account for 15% to 30% of inter individual differences in drug metabolism and response. But for certain drugs or classes of drugs genetic factors are utmost important and can

account for up to 95% of inter individual variations<sup>[11]</sup>.

TPMT is one of the two valid biomarkers for pharmacogenetics and pharmacogenomics in the FDA draft guidance for pharmacogenomics data substitution<sup>[12]</sup>. The best known recognized examples (TPMT, CYP2D6) are genetic polymorphisms of drug metabolizing enzymes which affect about 30% of all drugs[13]. TPMT activity in humans is inherited as an autosomal codominant trait<sup>[14]</sup>. TPMT illustrates the potential clinical importance of genetic polymorphisms in drug metabolism, determinants of toxicity and efficacy of treatment modality. Characterization of molecular mechanisms of this inherited trait has made it possible to accurately identify patients who are at high risk of toxicity there by providing

a rationale way of selecting therapeutic intervention and its dosage schedule. The TPMT genetic polymorphism represents a good example of the well established importance of pharmacogenetic variations of a drug metabolizing enzyme. The prevalence of various TPMT alleles in Caucasian population is reported to be 0.3% which occurs as homozygous mutants. 11% occurs as heterozygous and 89% as homozygous wild type<sup>[15,16]</sup>. At present a total of 25 TPMT genetic polymorphism mostly SNP's (single nucleotide polymorphism) have been identified<sup>[14]</sup>.

Decrease TPMT enzyme activity in 80% to 95% of patients has been attributed to three of nine variant alleles. TPMT\*2, \*3A is prevalent in Caucasians while TPMT \*3C is prevalent in Asians, Africans & African American populations<sup>[15]</sup>. TPMT gene exhibits a significant genetic polymorphism. Homozygous mutated individuals are at increased risk of life threatening toxicities while heterozygous are associated with some enzymatic activity influencing clinical efficacy and drug toxicity<sup>[16]</sup>.

Among Indian studies done on TPMT most were found to be homozygous for TPMT\*1/\*1 while TPMT\*3C was common among the alleles found in Indian population. TPMT\*3C allele had a frequency of 0.76%, representing 55.6% of mutated alleles among Indians<sup>[16]</sup>.

Currently there are no studies that have found a definitive association of polymorphism of head and neck cancer and response to chemoradiotherapy. Our study has found that most of the responders had wild variant. The hetero types \*3B and homozygous \*3C mutant allele were among the non responders and may warrant alternative mode of treatment for better therapeutic gain.

### Conclusion

Thus genotyping ultimately aids in predicting and individualizing treatment intervention protocols with enhanced precision at the cost of minimal toxicity.

**Sources of Support:** None

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