RESEARCH ARTICLE

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Investigation of Genetic Polymorphisms Related *GSTM1*, *GSTT1*, *GSTP1* Genes and their Association with Radiotherapy Toxicity among Head and Neck Cancer Patients

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Abstract

Background: In this study we explored the association of polymorphisms of glutathione s transferase gene including *GSTM1*, *GSTT1* and *GSTP1* with adverse acute normal tissue reactions resulted from radiotherapy in HNC patients. We assessed the association of *GSTM1* and *GSTT1* null genotypes and Ile105Val of exon-5 and Ala114Val of exon-6 of GSTP1 gene polymorphisms with the risk of acute skin toxicity reactions after therapeutic radiotherapy in HNC patients. **Methods:** Four hundred HNC patients administered with Intensity modulated radiation therapy were enrolled in this study for the evaluation of radiotherapy associated toxicity reactions. The genotyping of *GSTM1* and *GSTT1* were performed by polymerase chain reaction (PCR). The *GSTP1* Ile/Val of exon-5 and Ala/Val of exon-6 polymorphism was determined by PCR followed by restriction fragment length polymorphism (PCR-RFLP). **Results:** The univariate logistic regression analysis showed that *GSTM1* and *GSTT1* null genotypes were not associated with either skin reaction or oral mucositis in response to radiotherapy induced after effects. When we studied, A313G polymorphism at exon 5 and *C341T* polymorphism at exon 6 of *GSTP1* gene, majority of genotypes were wild type A/A genotype for exon 5 showed non-significant association with Skin reactions whereas, C/T genotype of exon-6 showed significant negative association with skin reactions. **Conclusion:** The findings obtained from this study concluded that the null genotypes of *GSTM1* and *GSTT1* gene polymorphisms showed no association with radiotherapy induced acute toxicities such as dermatitis and oral mucositis. The results indicated negative association of heterozygous C/T genotype of exon-6 of *GSTP1* with acute skin reactions.

Keywords: Head and neck cancer- genetic polymorphism- *GSTM1- GSTP1- GSTT1-* radiotherapy toxicity

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Introduction

The carcinogens and toxins are metabolized via xenobiotic pathway, an important two phase defense mechanism. In the first phase xenobiotic substances are oxidized, restored and hydrolyzed by phase I metabolic enzymes i.e cytochrome p450 and the mutagenic electrophilic end products of this phase-I reaction are further metabolized by detoxifying process with the help of phase II metabolic enzymes i.e glutathione s-transferase (GSTs). Genetic polymorphisms of either phase I or II detoxification genes encoding metabolic enzymes may affect the metabolism of carcinogens which results in cancer susceptibility. GSTs are the most important class of phase II detoxifying enzymes that plays an important role in metabolism of carcinogens, environmental pollutants and therapeutic drugs. Among the several isoforms of *GSTs*, *GSTM1*, *GSTT1* and *GSTP1* play an important role in detoxification which is widely studied worldwide. The polymorphism of *GST* genes results into loss of enzyme activity thereby accumulating the carcinogens in cell and eventually leads to development of cancer. Glutathione S-transferase mu gene (*GSTM1*) gene encodes the enzyme glutathione S-transferase that belongs to mu class. One of most common gene polymorphism is homozygous null polymorphism (GSTM1) which does not express enzyme, due to deletion of both the alleles [1]. Glutathione S-transferase theta gene (*GSTT1*) gene encodes the enzyme glutathione S-transferase theta1, which is expressed in gastrointestinal tract, lungs, kidney, brain, skeletal muscles, heart, spleen and erythrocytes. The homozygous null deletion of *GSTT1* gene leads to total absence of enzyme [2]. The homozygous null polymorphism in *GSTM1* and *GSTT1* leads to increased DNA damage due to oxidative stress produced by carcinogens, promoting carcinogenesis in

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oesophagus, stomach, colorectum, lung, bladder and skin [2-3]. Glutathione S-transferase pi gene (*GSTP1*) found to play an important role in cancer susceptibility [4-5]. There are two common non-synonymous polymorphic sites found in *GSTP1* gene derived from amino acid substitution, one in exon-5 and other in exon-6. The SNP in exon-5 is characterized by substitution of Adenine (A) by Guanine(G) in the nucleotide 313, that results into replacement of isoleucine by valine at codon105, while the displacement of Cytosine(C) by Thymine(T) in the nucleotide position 341 resulting into substitution of Alanine(A) to Valine(V) in codon114 leading to SNP in exon-6 [6]. These SNPs in GSTP1 confer impaired detoxification and catalytic activity of the enzyme increasing the risk of cancer [7]. Thus the polymorphism in the genes encoding for GSTs (*GSTM1, GSTT1* and *GSTP1*) are associated with the lack of or reduced activity of GST enzymes and therefore increases risk of developing cancer.

The polymorphisms of *GST* genes may also predict the radiotherapy treatment response and outcomes in the form of toxicities. However, there are limited studies addressing the polymorphisms of *GST* genes and their association with radiotherapy response in cancer patients. Accordingly some studies observed the polymorphisms of *GSTM1*, *GSTT1* genes and their association with increased acute skin reactions in breast cancer (BC) patients in relation with radiotherapy [8]. GSTP1 variant genotypes were associated with cutaneous adverse effects of radiotherapy in BC patients [9-10]. The variant genotype of GSTP1 (rs1695) was associated with radiotherapy response in glioblastoma patients treated with radiotherapy [11]. Another studies depicted an association of genetic variants of *GSTM1, GSTT1* and *GSTP1* genes with clinical toxicities in response to chemoradiotherapy in cervical [12], and head and neck cancer patients [13]. However, null genotypes of GSTM1, GSTT1 showed no association with development of acute toxicity reactions in BC [14]. The GSTP1 rs1695 polymorphic variant A>G, showed no risk of developing skin toxicity reactions after radiotherapy treatment in breast cancer patients [10]. Very recently another study failed to establish an association with GSTP1 (105G>C) polymorphism with radiotherapy induced normal tissue reactions in nasopharyngeal carcinoma patients [15-16]. Thus, number of studies has been performed to analyze the polymorphisms of phase II detoxification genes; however there remained further scope to explore the association of polymorphic variants of genes with radiotherapy induced toxicity effects on normal tissue of head and neck cancer patients. Therefore, in this study we explored the possible role of polymorphisms of *GST* genes including *GSTM1, GSTT1* and *GSTP1* with adverse acute normal tissue reactions resulted from radiotherapy in HNC patients. We assessed the association of *GSTM1* and *GSTT1* null genotypes and Ile105Val of exon 5 and Ala114Val of exon 6 of *GSTP1* gene polymorphisms with the risk of acute skin toxicity reactions after therapeutic radiotherapy in HNC patients.

Materials and Methods

Study Design

Cross sectional/ Observational/Analytical study was carried out at Krishna Hospital and Medical research center of Karad, Satara between, 2019-2022.

Patient enrollment and Clinical Information

Four hundred (400) patients histopathologically dignosed with HNC and visiting to Medical Oncology Out Patient Department (OPD) for the treatment at the Department of Oncology of Krishna Hospital and Medical Research Center, Krishna Vishwa Vidyapeeth (Deemed to be Univesrsity) Karad were enrolled based on predefined inclusion criterias; Patients with 22 to 85 years age diagnosed with HNC on histopathology; No metastasis at diagnosis; Clinically localised or locally advanced according to standard staging system, and normal skin and oral mucosa before the first radiotherapy fraction and exclusion criteria with no pathological diagnosis; relapsed disease or metastasis; severe co-morbidities; incomplete treatment taken; incomplete follow-up; associated severe co-morbidities.

Clinical and Demographic data

Detailed information of HNC patient characteristics including clinicopathological record and data on demographic factors, carcinogen exposure were collected with detailed clinical information with all examination findings were recorded in prescribed proforma and details of are depicted in Table 1. Radiation toxicity effects in the form of skin reactions and oral mucositis are recorded according to Radiation Therapy Oncology Group (RTOG) criteria. The clinical and radiological responses are documented as per Response Evaluation Criteria in Solid Tumors (RECIST) criteria at planned initial and end of treatment assessment. After giving radiation therapy patients are followed up at regular prespecified intervals for three months to assess for the clinical response such as complete response, partial response, stable disease, progressive disease, early death from disease or toxicity or any other cause. The patients were communicated regarding the purpose of their involvement in the study protocol.

Treatment of HNC patients with radiotherapy, follow up and toxicity assessment

All patients were treated using 3DCRT or Intensity modulated radiation therapy (IMRT) based on the CT-based planning, simulation, verification and quality assurance. Patients were treated using Linear accelerator (Model: Unique Performance, Make: Varian Medical System, USA) 6-Mega Volt (MV) (X-ray) with the total radiotherapy dose of 60- 66 Gy (2 Gy per fractions for 5 days a week) with volumetric modulated arc therapy (VMAT) technique. Patients after surgical resection having positive margins were given a dose of 66 Gy in 33 fractions. Chemotherapy was added if clinically indicated and the drug used was cisplatin at doses of 40 mg/m^2 every week given for 6 doses along with RT. The patients were followed up for three months post

Table 1. Details of Baseline Demographic, Clinico-Pathological Characteristics of Head and Neck Cancer Patients Enrolled in the Study.

Variables	Number/Percentage (%)
Total Number of patients	400
Age (Mean ± SD) years	56.04 ±13.42 (Range:22-85) Median:55
≤ 50	142 (35.50)
>50	258 (64.50)
Sex	
Male	298 (74.50)
Female	102 (25.50)
BMI Kg/m^2	
≤20	170 (42.50)
>20	230 (57.50)
Smoking/Tobacco chewing	
Users	354 (88.50)
Non-Users	46 (11.50)
Alcohol Consumption	
Drinkers	165 (41.25)
Non-Drinkers	235 (58.75)
Treatment	
Chemo-radiotherapy	261 (65.25)
Radiotherapy alone	102 (25.50)
Tumor size in cm	
≤ 2 cm	108(27.00)
> 2 cm	292 (73.00)
Tumor Staging	
T1	56 (14.00)
T2	62 (15.50)
T3	80 (20.00)
T4	183 (45.75)
Tx	19 (4.75)
Radiation Response	
Tumor Response	
Complete Response	328 (82.00)
Partial Response	72 (18.00)
Node Response	
Complete Response	319 (79.75)
Partial Response	81 (20.25)
Skin reaction (RTOG Grading)	
Grade 0	15 (3.75)
Grade 1	153 (38.25)
Grade 2	99 (24.75)
Grade 3	133 (33.25)
Mucositis (RTOG Grading)	
Grade 1	19 (04.75)
Grade 2	166 (41.50)
Grade 3	210 (52.50)
Grade 4	5 (01.25)

radiotherapy treatment to review the clinical response (Partial/complete/no response), stable, progressive disease, early death from disease, toxicity or any other cause. The skin over face and neck was selected as the observing area in patients with acute radiation induced acute skin reactions. The occurrence of acute side effects of radiotherapy was monitored and acute adverse effects (oral mucositis and skin reaction) were documented during and after completion of radiatiotherapy according to Radiation Therapy Oncology Group (RTOG) criteria. Acute radiation toxicity effects were defined as injuries appearing from the initial day of radiotherapy treatment until 3 months after the end of radiotherapy. The post-radiotherapy toxicity effects on normal skin and oral mucosa were recorded weekly. At the 1st and 3rd months after radiotherapy, the evaluation was performed again. The severity of acute radiation injury was determined and severity of acute radiation dermatitis and oral mucositis was graded according the RTOG grading system. For comparison of HNC patients with skin reactions such as severe skin reactions and oral mucositis (>2 grade) were considered as radiosensitive groups were compared to patients with ≤ 2 grade skin reactions.

Genomic DNA isolation from blood and Genotyping assays

Five milliliter (mL) of whole blood from patients was collected in sterile EDTA containing vacutainer after receiving informed consent. The blood sample from patients was collected before initiation of radiotherapy treatment. The genomic DNA extraction was carried out by salting out method where the whole blood was processed with lysis buffer-1 containing 10mM Tris-HCl pH-7.6, 320mM sucrose, 5mM $MgCl_2$, 1% triton X-100, pH 7.6 to lyse RBCs, thereafter the sample was treated with the lysis buffer 2 to lyse out WBCs (10mM Tris- HCl, 11.4mM sodium citrate, 1mM EDTA, 1% SDS, pH-8.0). The sample was further treated with Proteinase K (200µg/µl) to digest the proteins and subsequently RNase A (200µg/µl). The genomic DNA was precipitated by addition of twice the volume of ice cold ethanol and 1/10th volume of 3M Sodium acetate (pH-5.2). The precipitated DNA was aggregated together by centrifugation. The obtained pellet of DNA was then resuspended in T10E1 buffer and was checked on 1% agarose gel for its quality and quantity. This purified DNA was used for further genotyping assays after quantitative and qualitative analysis.

The genotyping of *GSTM1* and *GSTT1* were performed by polymerase chain reaction (PCR). The PCR amplification of *GSTM1* and *GSTT1* were carried out separately in 20 micro liter (µL) reaction mixtures containing 1X PCR buffer 0.2 mM each dNTP, 10 picomole (pmol) of each primers (IDT technologies) , 1U Taq DNA polymerase (GeNei, Merck Bioscience) and 100 nanogram (ng) of purified genomic DNA. The primer sequence used to amplify the *GSTM1* and *GSTT1* are shown in Table 2. The PCR conditions for amplification of 625 bp fragment of *GSTM1*: Initial denaturation at 95°C for 5 minutes (min) followed by 30 cycles of 95°C- 30 seconds (sec) , 56°C- 30 sec, 72°C- 30 sec and final extension at 72°C for 10 min. The conditions for GSTT1 of 480 bp: Initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C- 30 sec, 60°C- 30 sec, 72°C- 30 sec and final extension at 72°C- 10 min. The non-functional allele homozygous-null for *GSTM1* and *GSTT1* was evidenced by the absence of gene fragment, and presence

Table 3. Univariate Analysis of Polymorphism of *GSTM1, GSTM1, GSTT1* Genes and Radiation Induced Skin Reactions and Mucositis in Head and Neck Cancer Patients.

Gene Name	Genotype	Skin reaction ≤2 n=267	Skin reaction >2 n=133	OR 95% CI	p value	Oral mucositis ≤2 n=185	Oral mucositis >2 n=215	OR 95% CI	p value
GSTM1	*GSTM1*	177	83	1 (Reference)		126	134	1(Reference)	
	NULL	90	50	1.18 (0.76-1.82)	0.442	59	81	1.29 (0.85-1.95)	0.227
GSTT1	*GSTT1*	195	102	1 (Reference)		136	161	1(Reference)	
	NULL	72	31	0.82 (0.50-1.33)	0.43	49	54	0.93 (0.59-1.45)	0.754
GSTP1 A313G	A/A	158	74	1 (Reference)		112	120	1(Reference)	
	A/G	89	49	1.17 (0.75-1.83)	0.476	58	80	1.28 (0.84-1.96)	0.244
	G/G	20	10	1.06 (0.47-2.39)	0.873	15	15	0.93 (0.43-1.99)	0.858
	A/G +G/G	109	59	1.15 (0.75-1.75)	0.499	73	95	1.21 (0.81-1.81)	0.339
GSTP1 C341T	C/C	129	122	1 (Reference)		162	189	1 (Reference)	
	C/T	35	11	0.33 (0.16-0.68)	0.002*	23	23	0.85 (0.46-1.58)	0.623
	T/T	3	0	0.15 (0.007-2.95)	0.212	0	3	6.00 (0.30-117.07)	0.237
	C/T +T/T	38	11	0.30 (0.14-0.62)	0.001	23	26	0.96 (0.53-1.76)	0.917

SNP, Single nucleotide polymorphism; OR, Odds ratio, CI, Confidence interval; Significance $p< 0.05$; *, Indicates significant Odds Ratio ($p<0.05$), p value determined based on $\chi 2$

Table 2. The List of Candidate Genes with Details of PCR and RFLP Procedures Including Primers and Restriction Enzymes and Expected Products of Carcinogen Detoxifying Genes (*GSTM1, GSTP1, GSTT1*).

Gene Genotype	Amino acid/ nucleotide change	Primer Sequence Forward/Reverse	PCR Conditions	PCR product size	Enzyme / Digestion conditions	Dominant (Wild type) Genotype	Heterozygous Genotype	Recessive (Mutant) Genotype
GSTM1		FP: 5'-CAA ATT CTG GAT TGT AGC AGA TCA TGC -3' RP: 5'- CAC AGC TCC TGA TTA TGA CAG AAG CC -3'	95^0C-5 min, 30 cycles of 95^0C-30 sec, 56^0C-30 sec, 72^0C-30 sec 72^0C-5 min.	625 bp		*GSTM1 PRESENT*		NULL
GSTT1		FP: 5'-TTC CTT ACT GGT CCT CAC ATC TC-3' RP: 5'-TCA CCG GAT CAT GGC CAG CA -3'	950C- 5 min, 30 cycles of 950C -30sec, 60^0C - 30 sec, 72^0C-30 sec, 72^0C -5 min	480 bp		*GSTT1 PRESENT*		NULL
GSTP1 Codon-105 Exon-5 A313G	Ile105Val (A>G)	FP: 5'-GTA GTT TGC CCA AGG TCA AG-3' RP: 5'-AGC CAC CTG AGG GGT AAG -3'.	95^0C- 5 min, 30 cycles of 95^0C- 20sec, 54°C-20 sec, 72^0C 20sec, 720C-5min.	433bp	1 U of BsmA1 37°C Incubation for 16 hrs	328bp, 105bp	328bp, 222bp, 106bp, 105bp	222bp, 106bp, 105bp.
GSTP1 Codon-114 Exon-6 C341T	Ala114Val (C>T)	FP: 5'-GGG AGC AAG CAG AGG AGA AT-3' RP: 5'- CAG GTT GTA GTC AGC GAA GGA G -3'	95^0C-5 min, 30 cycles of 95^0C-30sec, 570C-30 sec, 72^0C-30sec, 72^0C - 5 min.	420 bp	1 U of AciI 37°C Incubation for 16 hrs	246bp, 116bp, 58bp	362bp, 246bp, 116bp, 58bp	362bp, 58bp

Table 4. Association of Polymorphisms of *GSTM1, GSTP1, GSTT1* Genes with Risk of Skin Reaction after Radiotherapy in Head and Neck Cancer Patients

Gene/ SNP	Genotypes	All Patients	Radiosensitive patients	OR 95% CI	p value
GSTM1	*GSTM1*	260	83	1 (Reference)	
	NULL	140	50	1.11 (0.74-1.68)	0.588
GSTT1	*GSTT1*	297	102	1 (Reference)	
	NILL	103	31	0.87 (0.55-1.38)	0.574
GSTP1 (Exon-5)	A/A	232	74	1 (Reference)	
	A/G	138	49	1.11 (0.73-1.69)	0.615
	G/G	30	10	1.04 (0.48-2.23)	0.909
	A/G +G/G	168	59	1.10 (0.74-1.63)	0.633
GSTP1 (Exon-6)	C/C	351	122	1 (Reference)	
	C/T	46	11	0.68 (0.34-1.37)	0.287
	T/T	3	0	0.40 (0.02-7.99)	0.556
	C/T +T/T	49	11	0.64 (0.32-1.28)	0.211

SNP, Single nucleotide polymorphism; OR, Odds ratio, CI, Confidence interval; Significance p< 0.05; *, Indicates significant Odds Ratio (p<0.05), p value determined based on $\chi 2$

of gene was indicated by amplification gene fragment in the PCR. The *GSTP1* Ile/Val of exon 5 and Ala/Val of exon 6 polymorphism was determined by PCR followed by RFLP. The exon 5 and 6 of GSTP1 were amplified by using specific primers mentioned in Table 2. The PCR cycling conditions for the amplification of 433 bp fragment of GSTP1 Ile105Val: Initial denaturation at 95°C for 5 minutes (min) followed by 30 cycles of 95°C- 20 seconds (sec) , 55°C- 20 sec, 72°C- 20 sec and final extension at 72°C for 10 min) and 420 bp of GSTP1 Ala114Val : Initial denaturation at 95°C for 5 minutes (min) followed by 30 cycles of 95°C- 30 seconds (sec) , 57°C- 20 sec, 72°C- 30 sec and final extension at 72°C for 10 min) respectively.

Statistical Analysis

The relative risk and Odds Ratio (OR) and corresponding 95% confidence intervals (CI) were determined through unconditional multiple logistic regression. The OR estimated to test whether any association exists between the grade of acute toxicity and selected SNPs. The correlation between confounding factors and radiation toxicities were evaluated by the χ^2 test. The p values <0.05 were considered as statistically significant. The event of occurrence of clinical severity of post-radiotherapy adverse effects are defined as skin reactions and oral mucositis scored as grade >2. All statistical analyses were carried out using SPSS 11 Software.

Results

Genotype Distribution of GSTM1, GSTT1, GSTP1 genes and radiotherapy toxicity in HNC patients

Phase II carcinogen detoxification genes including glutathione S Transferase (GSTs) were studied for their probable association with radiotherapy induced adverse normal skin toxicity effects in HNC patients exposed to radiotherapy. The univariate logistic regression analysis was used to study the genotype polymorphisms of *GSTM1, GSTT1*, and *GSTP1* genes and their association with radiation induced toxicities such as skin reactions

Table 5. Association of Polymorphisms of *GSTM1, GSTP1, GSTT1* Genes with Risk of Mucositis after Radiotherapy in Head and Neck Cancer Patients

Gene /SNP	Genotypes	All Patients	Radiosensitive patients	OR 95% CI	p value
GSTM1	*GSTM1*	260	134	1 (Reference)	
	NULL	140	81	1.12 (0.79-1.58)	0.509
GSTT1	*GSTT1*	297	161	1 (Reference)	
	NILL	103	54	0.96 (0.66-1.41)	0.863
GSTP1 (Exon-5)	A/A	232	120	1 (Reference)	
	A/G	138	80	1.12 (0.78-1.59)	0.526
	G/G	30	15	0.96 (0.50-1.86)	0.919
	A/G +G/G	168	95	1.09 (0.78-1.52)	0.601
GSTP1 (Exon-6)	C/C	351	189	1 (Reference)	
	C/T	46	23	0.92 (0.54-1.57)	0.784
	T/T	3	3	1.85 (0.37-9.29)	0.451
	C/T +T/T	49	26	0.98 (0.59-1.63)	0.954

SNP, Single nucleotide polymorphism; OR, Odds ratio; CI, Confidence interval; Significance p< 0.05; *: Indicates significant Odds Ratio (p<0.05); p value determined based on $\chi 2$

Table 6. Association between Genotypes of *GSTM1, GSTP1* and *GSTT1* Genes with Tumor and Node Response in Head and Neck Cancer Patients towards Radiotherapy.

Gene Name (SNP)	Genotype	Tumor Response		Risk Ratio 95% CI	p value	Node Response		Risk Ratio 95% CI	p value
		CR n=328	PR/NR n=72			CR n=319	PR/NR n=81		
GSTM1	*GSTM1*	213	47	1 (Reference)	0.956	211	49	1(Reference)	
	NULL	115	25	0.98 (0.57-1.68)		108	32	1.27 (0.77-2.10)	0.341
GSTT1	*GSTT1*	241	56	1 (Reference)	0.45	230	67	1(Reference)	
	NULL	87	16	0.79 (0.43-1.45)		89	14	0.54 (0.28-1.00)	0.053
GSTP1 A313G Exon-5	A/A	185	47	1 (Reference)		180	52	1(Reference)	
	A/G	115	23	0.78 (0.45-1.36)	0.394	112	26	0.80 (0.47-1.36)	0.415
	G/G	28	2	0.28 (0.06-1.22)	0.09	27	3	0.38 (0.11-1.31)	0.128
	A/G+G/G	143	25	0.68 (0.40-1.17)	0.168	139	29	0.72 (0.43-1.19)	0.206
GSTP1 C341T Exon-6	C/C	288	63	1 (Reference)		280	71	1 (Reference)	
	C/T	38	8	0.96 (0.42-2.16)	0.926	37	9	0.95 (0.44-2.07)	0.916
	T/T	2	1	2.28 (0.20-25.60)	0.502	2	1	1.97 (0.17-22.05)	0.581
	C/T +T/T	40	9	1.02 (0.47-2.22)	0.943	39	10	1.01 (0.48-2.12)	0.976

SNP, Single nucleotide polymorphism; CR, Complete Response; PR, Partial Response; NR, No Response; RR, Risk ratio; CI, Confidence interval; Significance p< 0.05; *, Indicates significant Odds Ratio (p<0.05), p value determined based on χ^2

as well as mucositis which are represented in Table 3. When we studied the association of *GSTM1* and *GSTT1* null genotypes, the results of univariate analysis depicted that none of the SNPs of *GSTM1* and *GSTT1* null genotypes were associated with radiotherapy toxicity effects. The GSTM1 null genotype was not associated with either skin reaction (OR=1.18, 95% CI: 0.76-1.82; p=0.442) or oral mucositis (OR=1.29, 95% CI: 0.85-1.95; p=0.227) in response to radiotherapy induced after effects. Similarly the ORs derived for the *GSTT1* null genotypes of the patients showed no association with skin reactions (OR=0.82, 95% CI: 0.50-1.33; p=0.430) or oral mucositis (OR=0.93, 95% CI: 0.59-1.45; p=0.754). When we studied, A313G polymorphism at exon 5 and C341T polymorphism at exon 6 of *GSTP1* gene, majority of genotypes were wild type A/A genotype for exon 5 showed non-significant association with skin reactions reactions (exon-5: whereas, C/T genotype of exon 6 showed significant negative association with skin reactions with OR=0.33, 95% CI: 0.16-0.68; p=0.002). Similarly, when polymorphism of GSTP1 at exon 5 and exon 6 was investigated for their association with oral mucositis, the results showed no statically significant difference between recessive or heterozygous genotypes of GSTP1 exon-5 (OR=0.93, 95% CI: 0.73-1.99; p=0.858; OR=1.28, 95% CI: 0.84-1.96; p=0.244) and exon-6 (OR=6.00, 95% CI: 0.30-117.07; p=0.1237; OR=0.85, 95% CI: 0.46-1.358; p=0.623) when considered with the degree of radiotherapy toxicity (Table 3).

Association of GSTM1, GSTT1, GSTP1 gene polymorphisms with risk of toxicity effects of radiotherapy in HNC patients

The logistic regression analysis of current study showed no significant association between genetic variants of *GSTM1* null genotypes and *GSTT1* null genotypes and the development of increased acute skin toxicity after radiotherapy when compared the genotypes of radiosensitive patients to the patients analyzed in this study (Table 4). The Odds ratios with 95% confidence intervals of the patients with skin reactions like acute dermatitis with null genotype of GSTM1 (OR=1.11 95% CI: 0.74-1.68; p=0.588) and null genotype of GSTT1 (OR=0.87 95% CI: 0.55-1.38; p=0.574). The ORs with 95% CI of variant genotypes of GSTP1 exon 6 (OR=1.04 95% CI: 0.48-2.23; p=0.909, and exon 6 (OR=0.40 95% CI: 0.02-7.99; p=0.4556) when compared with skin reactions which showed no association. Similar results were noted for the after effects of oral mucositis where no association of either GSTM1 null genotype (OR=1.12 95% CI: 0.79-1.58; p=0.509), null genotype of neither GSTT1 (OR=0.96 95% CI: 0.66-1.41; p=0.863) nor variant genotypes of GSTP1 exon-5 (OR=0.96 95% CI: 0.50-1.86; p=0.919) and GSTP1 exon 6 (OR=1.85 95% CI: 0.37-9.29; p=0.451) were noted in the HNC patients treated with radiotherapy (Table 5).

Association of GSTM1, GSTT1, GSTP1 gene polymorphisms with tumor and node response towards radiotherapy in HNC patients

The results of logistic regression analysis was carried out to find out the association of *GSTM1*, *GSTT1* and *GSTP1* genes with tumor and node response to radiotherapy shown in 6. The relationships between genotypes of GSTM1 null genotype, GSTT1 null genotype and GSTP1 polymorphisms and the response towards radiotherapy was studied where results demonstrated no association of polymorphisms of GSTM1 and GSTT1 null genotypes with tumor as well as node response towards radiotherapy. The GSTP1 313 A>G polymorphism with combined genotype of A/G + G/G showed no association with either tumor OR=0.68 95% CI: 0.40-1.17; p=0.168) or node response (OR=0.72 95% CI: 0.43-1.19; p=0.206) in HNC patients treated with radiotherapy. Similarly, GSTP1 341C>T polymorphism with combined genotypes of C/T +T/T were not associated with either tumor

response (OR=1.02 95% CI: 0.47-2.22; p=0.943) or node (OR=1.01 95% CI: 0.48-2.12; p=0.976) in HNC patients treated with radiotherapy (Table 6).

Discussion

Glutathione S transferase plays an important role in detoxification and protection against radiation induced oxidative damage in biological molecules. Genetic polymorphisms are responsible for decreased activity of antioxidant GST enzyme genes may increase the susceptibility of oxidative damage and thus considered to be associated with normal tissue toxicity in response to radiation. However, to the best of our knowledge there are no distinct reports available on the relationship of genetic polymorphisms of *GST* genes and the association of GST activity with radiation therapy side effects in HNC patients. The association studies on genetic polymorphisms of *GSTM1*, *GSTT1*, *GSTP1* have been reported to be associated with range of cancers including bladder [17], liver [18], lung [19-20], breast [21-22], gastric [23-24], and cervix cancer [25-26].Similarly, extensive efforts have been made to understand the association of genetic polymorphisms of *GST* genes with increased susceptibility of HNC [27-29], but several other studies depicted contradictory results which failed to prove any association of any of the polymorphisms of either *GSTM1*, *GSTT1* or *GSTP1* with risk of cancer [30-33]. However, very limited information dealing with the correlation of genetic polymorphisms of GSTs genes with radiotherapy response in cancer patients is available in breast [10, 34], bladder [35], lung [36], and nasopharyngeal cancer [13]. But the results on the possible association of GST polymorphisms and the response towards radiotherapy in many cancers is still controversial [12, 37-38]. Thus the data of genetic background of phase II drug detoxification genes and the role of genetic variants in predicting radiotherapy response in HNC patients is limited. Therefore in this study we intended to investigate the association of *GSTM1*, *GSTT1* and *GSTP1* gene polymorphisms and to understand their response to radiotherapy toxicity in HNC patients. In this study we acknowledged, the polymorphisms of *GSTM1*, *GSTT1* and *GSTP1* gene with HNC patients. The results obtained after regression analysis for the association of *GST* gene polymorphisms stated that none of the SNPs of *GSTM1* and *GSTT1* null genotypes were associated with radiotherapy toxicity effects. Similarly, A313G polymorphism at exon 5 of *GSTP1* gene, showed non-significant association with skin reactions reactions as well as oral mucositis when considered with the degree of radiotherapy toxicity. The C/T polymorphism of *C341T* genotype of exon 6 showed significant negative association with skin reactions with OR=0.33, 95% CI: 0.616-0.68; p=0.002). The results of univariate analysis of GSTM1 showed that *GSTM1* null genotype showed that neither of heterozygous nor homozygous variant genotype of GSTP1 exon 5 and GSTP1 exon 6 showed any association with either tumor or node response in HNC patients treated with radiotherapy. Similarly, various studies have demonstrated that the Val/Val homozygous genotype of the Ile105Val polymorphism in the *GSTP1* gene correlates with heightened sensitivity to radiotherapy-induced toxicities, particularly in lung cancer. This heightened vulnerability is attributed to the compromised detoxification capacity of the GSTP1 enzyme in individuals carrying this variant, which results in a diminished ability to neutralize reactive oxygen species generated during radiotherapy [39]. Thus, the results obtained in current study clearly indicated that none of the gene polymorphisms had significant effect on clinoopathological features or radiotherapy response which was in accordance with recent studies carried out in HNC patients [15-16, 40]. Thus our results failed to establish significant association between the functional polymorphism of *GSTM1*, *GSTP1* and *GSTT1* within HNC patients in response to radiotherapy. Therefore, further studies with larger sample size is required in future to demonstrate the interaction between the polymorphisms of *GST* genes and their response towards radiotherapy.

In conclusion, the findings obtained from this study concluded that the null genotypes of *GSTM1* and *GSTT1* gene polymorphisms showed no association with radiotherapy induced acute toxicities such as skin reactions and oral mucositis. The results indicated no association of GSTP1 polymorphism with radiotherapy induced acute toxicity reaction except the heterozygous C/T genotype of exon 6 of GSTP1 showed negative association of with acute skin reactions.

Author Contribution Statement

Concept: AKG, SJB, KDD, RAG, Design: KDD, SJB, AKG, Experimental Studies: KDD, Clinical studies: AKG, RAG, Data analysis: KDD, AKG, Statistical analysis: KDD, Manuscript preparation: KDD, SJB, AKG, RAG. All authors read and approved the final manuscript.

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The study protocol was approved by protocol committee of Krishna Vishwa Vidyapeeth (Deemed to be University).

Ethics Committee Approval

The study protocol was approved by Institutional Ethics Committee of Krishna Vishwa Vidyapeeth 'Deemed to be University', Karad.

Availability of data

Not Applicable.

Declaration of Conflict of interest

The authors declare that they have no competing financial or any other conflict of interests that could have appeared to influence the work reported in this paper.

Abbreviations

HNC: Head and Neck Cancer
OPD: Out patient Department
GST: Glutathione S-Transferase
RT: Radiotherapy
Gy: Gray
VMAT: Volumetric Modulated Arc Therapy
RECIST: Response Evaluation Criteria in Solid Tumors
RTOG: Radiation Therapy Oncology Group
IMRT: Intensity modulated radiation therapy
PCR-RFLP: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
SNP: Single Nucleotide Polymorphism
OR: Odds Ratio
CI: Confidence Interval
SD: Standard deviation
µL: Microliter
µg: Microgram
DNA: Deoxyribose Nucleic acid
EDTA: Ethelyne Diamine Tetra Acetate

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