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Genotype-phenotype association of *TGF-β1* and *GST* with chemo-radiotherapy induced toxicity

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► ***Original article***

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ABSTRACT

Background: Normal tissue toxicity continues to remain as a major challenge for radiation oncologists for delivering the total dose to the tumour cells in cancer patients. Cellular, molecular and plasma based early biomarkers to predict the overreactions and non-overreactions of normal tissue toxicity before the initiation of radiotherapy can be valuable for personalised treatment. The aim of the current study was to analyse the interrelationship between polymorphisms in Glutathione S- Transferases (*GSTs*) and Transforming Growth Factor-β1 (*TGF-β1*), the plasma level/activity of these proteins with the development of chemo-radiotherapy induced oral mucositis and skin reaction in head and neck cancer (HNC) patients. ***Materials and Methods***: We analysed polymorphisms in *TGF-β1* and *GST* by restriction digestion of the PCR amplified products and we also assessed circulating TGF-β1 levels and GST activity by Enzyme Linked Immunosorbent Assay (ELISA). ***Results:*** The results indicate that pre-radiotherapy plasma TGF-β1 levels and total GST activity has no correlation with radiation induced normal tissue skin reaction and oral mucositis in HNC patients. ***Conclusion:*** The selected polymorphisms in *TGF-β1* and *GST* had no influence on TGF-β1 levels and total GST activity. Plasma TGF-β1 and GST activity was not affected by the presence of selected polymorphisms and lacks significance in predicting skin reaction and oral mucositis prior to chemo-radiotherapy.

Keywords: *Single nucleotide polymorphism, TGF-β1, glutathione S- Transferase, oral mucositis, skin reaction, chemo-radiotherapy.*

INTRODUCTION

Radiotherapy is one of the widely used therapeutic modules for cancer treatment and more than 60% of cancer patients undergo radiotherapy either alone or as an adjuvant treatment. However, it is observed that radiotherapy invariably affects the normal tissue around the solid tumour contributing to various normal tissue adverse reactions [(1)]. It was previously thought that this was due to deterministic or stochastic variations. However, as extensively reviewed by Bentzen [(2)], there exist a genetic basis for the differences in risk of developing normal tissue toxicity among individuals. Furthermore, the severe radio-sensitivity observed in certain genetic syndromes like Ataxia-telangiectasia, Fanconi anemia, and Bloom syndrome, provides us with proof for the involvement of a genetic component behind these normal tissue acute reactions. Earlier findings have illustrated the role of cellular basis in determining normal tissue toxicity [(3,4)]. Also, efforts to find the role of

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genetic component in determining normal tissue toxicity have yielded positive as well as negative associations [5, 6]. Therefore, it has been a persistently sought after goal to discover an approach for predicting the radiotherapy induced acute effects. Biomarkers to determine normal tissue toxicity at a rapid rate are much appreciated to address the side effects arising due to radiation therapy. This may help radiation oncologists to tailor the radiation therapy for improved tumour control with reduced side effects.

Transforming growth factor-ß1 (*TGF-ß1*), a vital component in the regulation of cellular homeostasis [7], a member of *TGF-ß* family, which is composed of a number of cytokines functioning in cellular processes such as proliferation, migration, differentiation and adhesion [8]. *TGF-ß1* is a pro-fibrotic cytokine and has been linked with the predisposition to late radiotherapy toxicity. This gene has been found to be over-expressed in irradiated tissues after radiotherapy [8] and considered as an important biomarker for inflammatory as well as fibrotic response to radiotherapy [7]. Because of its role in the development of radiation-induced inflammatory processes and as a biomarker for radiation toxicity [9], it was analysed in plasma samples. It has also been shown that inhibition of *TGF-ß1* prevented the activity of Ataxia Telangiectasia Mutated protein (ATM) and thereby contributing to increased radiosensitivity [7].

Glutathione S-Transferase (GST) is a family of enzymes which catalyses the transfer of glutathione to the electrophilic centres of many compounds, resulting in the elimination of toxic substances and thereby acting as a detoxifying enzyme [10]. Glutathione is a gold standard biomarker for global oxidative stress in the body, a key indicator in cancer, especially in head and neck squamous cell carcinoma [11]. There have been at least five cytosolic types of GST enzymes; *GST-α* (alpha), *GST-μ* (Mu), *GST-θ* (Theta), *GST-σ* (Zeta) and *GST-π* (Pi), as well as membrane bound and microsomal GST enzymes [12]. Radiation enhances the expression of *GST* as a protective mode of response and implicates the host defence mechanism. This study aims to provide more insight into the relationship between the polymorphisms in *GST* and *TGF-β1* and their plasma levels with the development of chemoradiotherapy induced acute reactions in head and neck cancer patients.

MATERIALS AND METHODS

Patients and clinical data

The study was conducted on 94 HNC patients undergoing chemoradiotherapy at the Shirdi Saibaba Cancer Hospital and Research Centre (table 1). The investigation was approved by the Institutional Ethical Committee and a written informed consent was obtained from all the patients before collecting blood. Radiation (external beam) was delivered for all the patients using Linac 6-MV X-ray linear accelerator (Elekta Precise Digital, Stockholm, Sweden). Patients with recurrent tumour and distant metastasis were excluded. The total dose of radiation delivered to tumor was 60-70 Gy (2 Gy per day for 5 days a week until the total dose was reached). Patient treatments were planned by using computed tomography images. A conventional simulator was used to plan the mould before initiating radiotherapy. Patients were either treated with platinum based (Cisplatin/Carboplatin) concurrent chemo-radiotherapy or by radiotherapy alone. Acute adverse events (oral mucositis and skin reactions) were recorded according to Radiation Therapy Oncology Group (RTOG) criteria [13]. Blood samples were collected using sodium heparin vacutainers and plasma was collected after centrifugation at 1000 rpm for 10 minutes. All the plasma samples were stored in -80°C until further analysis. DNA was isolated from the remaining part of the blood by standard phenol chloroform extraction procedure and used for genotyping.

Genotype analysis

The genotyping of the selected candidate gene polymorphisms were done by Polymerase Chain Reaction based Restriction Fragment Length Polymorphism (PCR-RFLP) or multiplex PCR. The details of primers selected for

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amplification along with enzymes used for digestion of PCR products are provided in the table 2. The PCR conditions along with the number of cycles of amplifications are provided in table 3. After amplification, each PCR product was run in 1.2% agarose gel for the confirmation of amplified product. After the confirmation of PCR product, restriction digestion was carried out using specific restriction enzyme for each selected SNPs. Genotyping of *TGF-β1* polymorphisms (rs1800469 & rs1800471) and *GSTP1* rs1695 was done by RFLP whereas *GSTM1* and *GSTT1* genotypes were determined by multiplex PCR. 5 % of the samples were randomly selected and re-genotyped to assess the consistency in results.

Table 1. Clinical characteristics of HNC patients considered under this study

Clinical details of patients		
Number of patients	94	
Mean Age (Range)	55 years (26-80)	
Males	83	
Females	11	
Chemotherapy	Cisplatin + RT	60
	Carboplatin + RT	10
	RT alone	24
Skin reaction (RTOG Grading)	Grade 0	2
	Grade I	14
	Grade II	52
	Grade III	25
	Grade IV	1
Oral mucositis (RTOG Grading)	Grade 0	1
	Grade I	9
	Grade II	44
	Grade III	35
	Grade IV	5

Table 2. Details of the primer, PCR product and RFLP for selected polymorphisms.

Gene	rs number	Forward & Reverse primer	PCR product size (bp)	Enzyme	RFLP Fragments
TGF-β1	rs1800469	F: CCCGCCTCCATTTCCAGGTG R: CCAGGCGGAGAAGGCTTAATC	418	*Bsu36I*	Wild type: 418 Heterozygous: 418, 229, 189 Mutant: 229, 189
TGF-β1	rs1800471	F: TGTTCGCGCTCTCGGCAG R: GACCTCCTTGGCGTAGTAG	365	*BglI*	Wild type: 312,53 Heterozygous: 312, 252,60,53 Mutant: 252,60,53
GSTP1	rs1695	F: GTAGTTTGCCCAAGGTCAAG R: AGCCACCTGAGGGGTAAG	433	*BsmAI*	Wild type: 222, 105, 106 Heterozygous: 328, 222, 105,106 Mutant: 328, 105
GSTT1	-	F: TTCCTTACTGGTCCTCACATCTC R: TCACCGGATCATGGCCAGCA	480	-	-
GSTM1	-	F: GAACTCCCTGAAAA-GCTAAAGC R: GTTGGGCTCAAATATACGGTG	240	-	-

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Table 3. PCR conditions for the selected SNPs.

Gene	rs number	Denaturing temp (°C)/Time	Annealing temp (°C)/Time	Extension temp (°C)/Time	Cycles
TGF-β1	rs1800471	95/50sec	58/55sec	72/60sec	32
TGF-β1	rs1800469	95/40sec	53/20sec	72/60sec	33
GSTP1	rs1695	95/45sec	61/40sec	72/60sec	30
GSTM1	Null	95/30sec	65/35sec	72/60sec	32
GSTT1	Null				

Enzyme Linked Immunosorbent Assay (ELISA)

Plasma samples were thawed and centrifuged at 3000 rpm for 10 minutes before analysing the considered parameters. The amount of TGF-β1 was analysed using Human TGF-β1 ELISA kit (BD Biosciences, San Diego, USA). Total GST activity was estimated by Glutathione S-Transferase assay kit (Cayman chemical company, Ann Arbor, USA).

Statistical analysis

Genotype frequencies for each polymorphism were calculated and the deviations from Hardy-Weinberg equilibrium were analysed. Statistical significance was analysed by Fisher's exact test. Odds ratio was used to find any associations between the polymorphisms and grade of oral mucositis as well as skin reactions. The statistical significance between the RTOG graded acute reactions and plasma levels of TGF -β1/GST was evaluated by One-way Analysis of Variance and with Bonferroni's post-hoc test. All the statistical tests were performed using Prism v.5.0 (GraphPad Software, San Diego, California, USA). The criterion for statistical significance was $p < 0.05$.

RESULTS

Polymorphisms in TGF-β1 and GST and normal tissue toxicity

All the polymorphisms tested, were found to be in Hardy-Weinberg equilibrium. The patients experiencing lesser than or equal to grade 2 levels of toxicity were considered as non-overreactors while, patients with greater than grade 2 were considered as overreactors. None of the polymorphisms presented any significant association with either oral mucositis or skin reactions (table 4).

Plasma TGF-β1 levels and genotypes/normal tissue toxicity correlation

The plasma levels of TGF-β1 was checked for its correlation with the dominant, heterozygous and recessive genotypes of *TGF-β1* rs1800469 (509C>T) and rs1800471 (Arg25Pro). Plasma TGF-β1 levels were higher in dominant genotype (rs1800469) in comparison to heterozygous and recessive genotypes, but the statistical analysis showed no significance between the groups (figure 1A). Also, there was no statistically significant difference in TGF-β1 levels (figure 1B) observed in dominant and heterozygous genotypes of rs1800471 (Arg25Pro).

In addition, we compared the plasma TGF-β1 concentration with different grades of RTOG graded oral mucositis and skin reactions. There was no increase or decrease in plasma concentration with increase in RTOG grades of acute reactions (figure 2A and figure 2B).

Plasma GST activity and genotypes/ normal tissue toxicity correlation

Our analysis revealed that the presence or absence of *GSTM1* and *GSTT1* as well as *GSTP1* (Iso105Val) genotypes had no effect on the plasma GST activity (figure 3A and figure 3B). Also, there was no statistically significant variation in GST activity with the increasing grades of oral mucositis and skin reactions (figure 3C and figure 3D).

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Table 4. Association between candidate polymorphisms and radiation-induced skin reactions and oral mucositis in head and neck cancer patients.

Gene/rs number	Genotype	Skin reaction ≤2	Skin reaction >2	Odds ratio	CI%95	p-value	Oral mucositis ≤2	Oral mucositis >2	Odds ratio	CI%95	p-value
TGF-β1 rs1800469	CC	29	12				21	20			
	CT	32	8	0.604	0.216 1.68	0.441	27	13	0.505	0.205 1.246	0.176
	TT	7	6	2.071	0.575 7.46	0.318	5	8	1.680	0.469 6.009	0.530
TGF-β1 rs1800471	GG	58	21				45	34			
	GC	7	4	1.578	0.418 5.946	0.490	6	5	1.103	0.310 3.919	1
	CC	0	0				0	0			
GSTP1 rs1695	AA	34	12				27	19			
	AG	27	11	1.154	0.441 3.020	0.809	20	18	1.1279	0.537 3.041	0.660
	GG	7	3	1.214	0.269 5.467	1	6	4	0.947	0.234 3.823	1
GSTM1	1	46	20				36	30			
	0	22	6	0.627	0.220 1.783	0.455	17	11	0.776	0.315 1.910	0.652
GSTT1	1	56	21				43	34			
	0	12	5	1.111	0.349 3.537	1	10	7	0.885	0.305 2.570	1

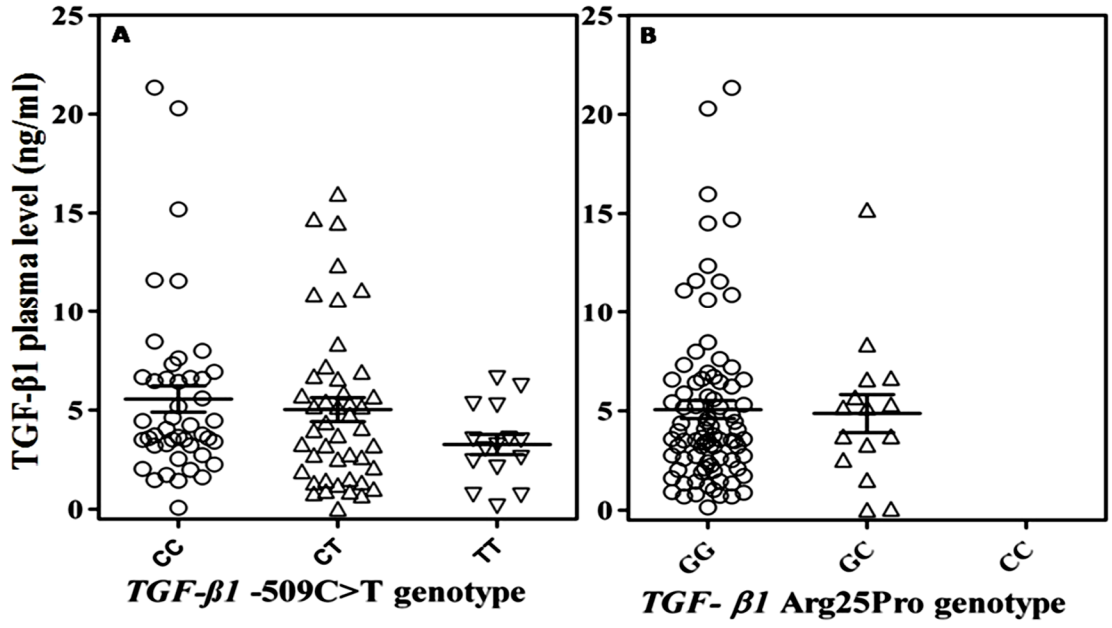

Figure 1. Plasma TGF-β1 levels with the dominant, heterozygous and recessive genotypes of *TGF-β1* **1A.** rs1800469 (-509C>T) **1B.** rs1800471 (Arg25Pro). Error bar indicate Mean ± SEM.

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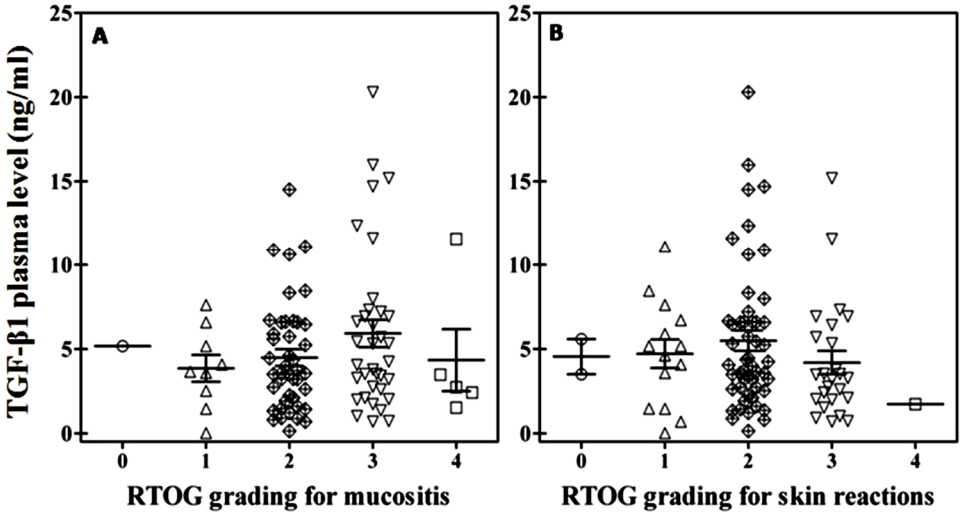

Figure 2. Plasma TGF-β1 levels with the RTOG grades **A.** oral mucositis **B.** skin reactions. Error bar indicate Mean ± SEM.

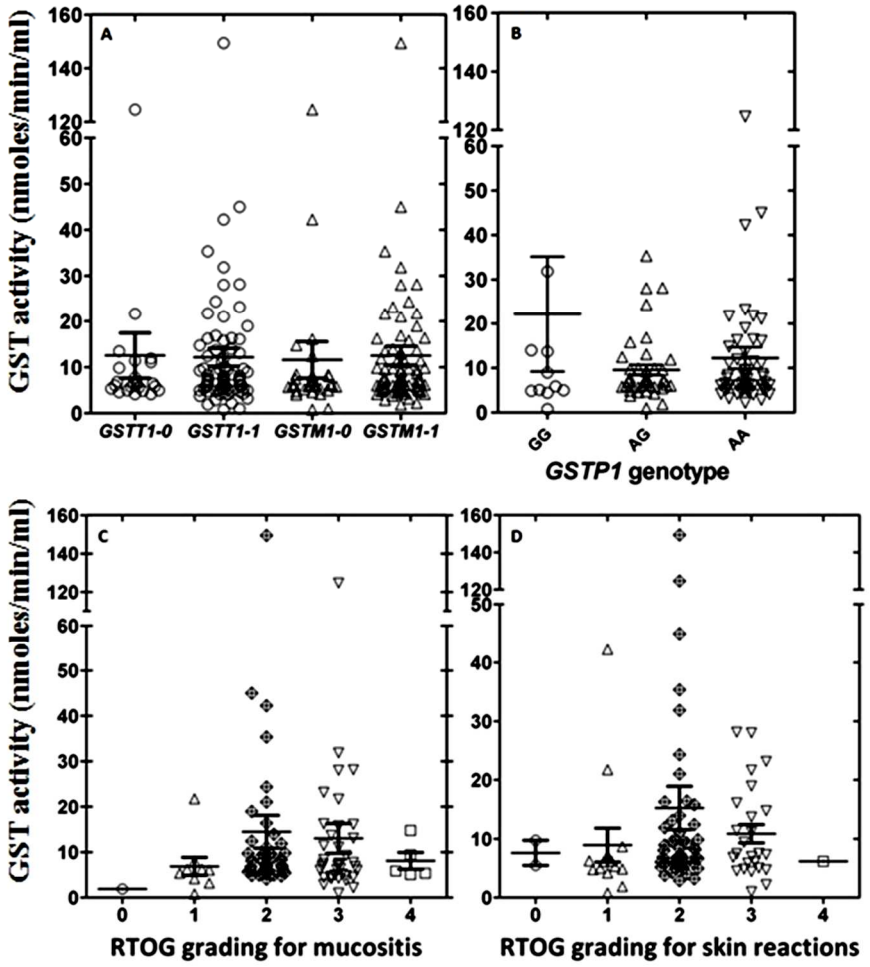

Figure 3. Plasma GST activity with **A.** *GSTM1* and *GSTT1* Null (0)/ presence (1) **B.** *GSTP1* genotype (Iso105Val). **C.** Plasma GST activity with the RTOG grades of oral mucositis **D.** Skin reactions. Error bar indicate Mean ± SEM.

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DISCUSSION

The human genome project clearly indicated an immense amount of genetic diversity, and now we are just beginning to understand how such changes influence the specific phenotypic expression. The Enormous genetic variations exist among populations and its understanding will help us to tailor the therapy with a personalised approach for safer and effective treatment outcome. Radiation induced normal tissue toxicity is dependent upon the levels of expression of numerous antioxidant enzymes and irradiation increases endogenous expression of many such antioxidants [14]. Also, radiation is known to induce the expression of several inflammatory cytokines which play an important role in radiation induced wound healing [2]. However, it is not clearly understood about the role of SNPs and the level of expression. It was hypothesized that overexpression of antioxidant enzymes and cytokines would be radioprotective and can help in reducing normal tissue toxicity to a greater extent. With this concept, the current study was undertaken to validate whether activity/ levels of an antioxidant enzyme (GST) and an inflammatory cytokine (TGF-β1) had any role in variation of normal tissue radio-responsiveness and thereby influencing the toxicity outcome.

Increased levels of antioxidant before the initiation of radiotherapy may improve the tolerability of normal cells to cope up with the radiation stress and may serve as biomarkers in HNSCC patients who may undergo radiotherapy [15]. Polymorphisms in these antioxidant and detoxification genes can increase or, more often, decrease the activity of the particular enzyme. Increased phase I clearance of toxic agents can exert pressure on phase II activity. In turn, reduced phase II activity can lead to the toxic intermediates. Similarly, reduced phase I activity can cause accumulation of toxins. Adverse reactions seen in case of radiation/chemotherapy are often due to a decreased capacity for clearing the toxins as well as intermediates from the system [15].

Levels of TGF-β1 in plasma have been used to predict the treatment outcome in breast cancer patients [16] and also as a serological marker for small hepatocellular carcinoma [17]. Plasma TGF-β1 level after the treatment has predictive potential to determine late morbidity in advanced HNC patients [18]. Changes in plasma TGF-β1 levels during radiotherapy have also been used to predict the risk of developing radiation pneumonitis [19]. It has also been used as a biomarker to predict the risk of developing radiation induced fibrosis in breast cancer patients [20]. Further, Zhao and co-workers [21] analysed plasma TGF-β1 is levels from twenty six lung cancer patients during radiotherapy and found that TGF-β1 is elevated during the fourth week of treatment and has predictive value in determining radiation induced lung toxicity. This study also suggested the need for further studies to predict radiation induced toxicity. In the present study, we evaluated the levels of TGF-β1 in rendering normal tissue toxicity in HNC patients. However, our results demonstrate that TGF-β1 levels before the initiation of radiotherapy had no predictive value to determine normal tissue overreactors/non-overreactors of radiotherapy. Also, our results are in line with several studies reporting the lack of correlation between *TGF-β1* gene expression and normal tissue toxicity [18, 22, 23]. Unlike the earlier report [24], we did not observe any statistically significant changes in plasma TGF-β1 levels with the different genotypes of rs1800469 (509C>T) and rs1800471 (Arg25Pro) polymorphisms. Our data is in concordance with the findings from Reuther and co-workers' [25] that *TGF-β1* promoter polymorphism has no effect on either gene or protein expression or cellular radio-sensitivity. A recent meta-analysis has confirmed no such statistically significant associations between late toxicity and fibrosis and rs1800469 genotype [26]. Our findings also identify that rs1800471 has no association with the risk for developing acute reactions in HNC patients.

It is well known that glutathione and glutathione dependent enzymes play a major role in defence against oxidative stress [27]. Earlier studies from our laboratory have demonstrated that glutathione levels can be a predictive and prognostic marker in cervical cancer patients undergoing radiotherapy [28]. Plasma glutathione is also associated with the outcome of HNC post radiotherapy treatment [11]. *GSTP1* has been found to have a significant association with a higher risk of radiation-induced fibrosis in breast cancer patients as well as acute skin toxicity [12, 29]. A study by Ambrosone and co-workers' demonstrated a significant association between the *GSTT1* and *GSTM1* null genotypes with skin toxicity post-radiation therapy [30]. Another study found no association between the absence of *GSTT1* and *GSTM1* with skin toxicity in breast cancer patients [31]. In the present study we estimated the total GST activity and compared it with RTOG graded acute reactions and SNPs in *GSTP1,* deletion of *GSTM1* and *GSTT1* genes. Similar to earlier findings, GST activity did not correlate with normal tissue acute effects and the polymorphisms had no effect on GST activity. Two

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important limitations of the study could be attributed to the SNPs selected and the sample size.

In summary, this study suggests that genetic variants in *TGF-β1* and *GST* have no association with either the acute reactions or with its protein plasma levels/activity. This study indicates that plasma TGF-β1 levels and total GST activity before the initiation of radiotherapy has no predictive implications in determining normal tissue acute effects in HNC patients. However this study has two important limitations viz, the SNPs selected for analysis and the sample size which could have possibly impacted for no association. Further studies with large samples are necessary to confirm the findings.

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Conflict of interest: *none to declare.*

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