Association of nasopharyngeal cancer risk with genetic polymorphisms of drug-metabolizing enzyme genes *GSTM1*, *GSTT1* and *CYP1A1* (rs4646903 variant), in tobacco addicted patients of Pashtun ethnicity of Khyber Pakhtunkhwa Province of Pakistan

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Abstract: Associations of *GSTM1*, *GSTT1* and *CYP1A1* gene variants with risk of developing nasopharyngeal cancer were evaluated in this case-control study, in which 130 cases along with 151 population-based healthy controls of Pashtun ethnicity from Khyber Pakhtunkhwa province of Pakistan were recruited. Socio-demographic data were obtained and blood samples were collected with informed consent for analysis. Specific RT-PCR and conventional PCR methods were used to detect *CYP1A1* and *GSTs*, respectively, and results analyzed through SPSS version 20. Study showed that *CYP1A1* homozygous (C/C) had an almost 4-fold increased risk for nasopharyngeal cancer; while heterozygous (T/C) had an almost 2 times increased risk. Overall the C allele is significantly associated with nasopharyngeal cancer as compared to T allele. Null genotypes of *GSTM1* were having 3-fold increased risk; whereas null genotype of *GSTT1* was having 2 times increased risk. Similarly, GSTM/GSTT both null genotype was having more than 5 times increased association. Presence of all three gene variants showed strong and significant association. Findings of the study suggest that presence of *GSTM1* and/or *GSTT1* null genotypes along with variant alleles of *CYP1A1* are significant risk factors for nasopharyngeal cancer susceptibility in Pashtun population.

Keywords: Nasopharyngeal carcinoma risk - *GSTM1*, *GSTT1* and *CYP1A1* - Pashtun population.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is rear around the globe, with highest incidence rate in China and South East Asian region. (Ferlay *et al*., 2010) Annually, approximately 84,000 cases appear with the age standardized rate (ASR) of 1.2 per 100,000 for both genders. NPC is the 24th most commonly diagnosed cancer worldwide, while in developing countries it ranks 22nd. In Pakistan NPC is from moderate to high risk with an estimated annual incidence of less than 1000 cases. (Abbasi *et al*., 2011)

Multiple etiological factors are reportedly responsible for the development of NPC like viral, environmental influences, and heredity. (Zhang *et al*., 1999) Epstein-Barr virus has been suggested as the leading cause but research has shown that Epstein-Barr virus very rarely resulted in the development of cancer. This clearly indicates towards other influencing factors like, genetic susceptibility and consumption of food (particularly salted fish) containing carcinogenic nitrosamines (Chu *et al*., 2008). Variation is also seen in the association of tobacco use and NPC occurrence. Some have reported association with tobacco, while others have reported no association. (Fachiroh *et al*., 2012, Friborg *et al*., 2007, Guo *et al*., 2009, Sriamporn *et al*., 1992) The variation in incidence and mortality rate of NPC globally suggested the role of genetic and environmental factors in its pathogenesis. Hence genetic susceptibility, gene expression profiling and genetic polymorphism studies might help to aid in better understanding of the carcinogenesis of NPC in near future.

Keeping in view the mechanism of carcinogenesis of various tobacco related carcinogens, we have mainly focused on two families of genes responsible for the expression of CYP1A1 (phase I cytochromes P450), GSTM1 and GSTT1 (phase II glutathione-S-transferase) enzymes, involved in carcinogen processing and oxidative stress. CYP1A1 isoform (expressed by *CYP1A1* gene located on chromosome 15q22-q24) is known to be involved in the activation of tobacco related Poly Aromatic Hydrocarbons (PAHs), Tobacco Specific Nitrosamines (TSNAs) and aromatic amines into carcinogenic species; while GSTs are involved in their detoxification and excretion from the body (Khan *et al*., 2015). *GSTT1* locus has been mapped on chromosome 22q11.2 while *GSTM1* loci exist on chromosome 1p13.3 (Bin *et al*., 2013). Genetic polymorphism in genes encoding xenobiotic metabolizing enzymes may lead to inter individual differences in patients and susceptibility to particular cancer type can be determined by evaluating polymorphism in these genes (Nebert *et al*., 1996). Some individuals may be 'genetically more susceptible' that carry alleles with more functional activity of carcinogen processing enzymes and/or decreased activity of

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carcinogen detoxifying enzymes (Al-Dayel *et al*., 2008). This argument can be further strengthened by the fact that among the exposed individuals, under similar environmental conditions, only few develop cancer. Variation in alleles of different carcinogen metabolizing enzymes, along with environmental factors such as tobacco use, may highly influence an individual susceptibility towards different types of cancers (Zakiullah *et al*., 2014).

Genetic polymorphism in genes encoding xenobiotic metabolizing enzymes mostly leads to heterogeneity in patient's carcinogen processing. Two families of genes i-e CYPs (phase I cytochromes P450) and GSTs (phase II glutathione-S-transferase) are mainly focused in the current study. The enzymes encoded by these genes play vital role in carcinogen processing. Pro-carcinogens i-e Poly Aromatic Hydrocarbons (PAHs), Tobacco Specific Nitrosamines (TSNAs) and aromatic amines found in tobacco products are activated to carcinogenic species by CYPs, especially *CYP1A1* gene located on chromosome 15q22-q24; while GSTs (*GSTT1* and *GSTM1)* play role in their detoxification and excretion from the body. (Khan *et al*., 2015) The locus of *GSTT1* is present on chromosome 22q11.2 while that of *GSTM1* exists on chromosome 1p13.3 (Buch *et al*., 2001). Genetic susceptibility towards a particular cancer type can be evaluated by polymorphisms in these genes. Those individuals having active form of carcinogens activating enzymes (CYPs) are likely to be at high risk of cancer development while those having in-active form of it will be at low risk (Al-Dayel *et al*., 2008). Likewise, null genotypes of *GSTT1* and *GSTM1* may result in carcinogens amassing, leading towards cancer development (Zakiullah *et al*., 2014). Studies on the association of these genes with NPC have so far showed variable results. One study has reported association in Chinese population, while another study from Taiwan has shown no association, which points towards the differences in various ethnicities. (Junfeng., 2011, Cheng *et al*., 2003)

Association of genetic polymorphisms of the above mentioned genes with NPC has not been so far reported in Pakistani Pashtun population. Therefore, a case-control study was carried out in the said population of Khyber Pakhtunkhwa province of Pakistan. This will help to adopt pro-active approaches for early detection and preventive life style modification strategies to decrease the incidence of the disease in the target population.

MATERIALS AND METHODS

Sample collection

Study sample comprised of 130 NPC patients and 151 healthy control subjects between 30 and 70 years of age as per exclusion/inclusion criteria. Patients were registered at the Institute of Radiotherapy and Nuclear Medicine (IRNUM), Peshawar, Khyber Pakhtunkhwa; while eligible control samples were collected from various districts of the same province. All the patients were having histopathologically confirmed nasopharyngeal carcinoma.

Inclusion criteria (patients)

Histo-pathologically proven NPC patients having age between 30 and 70 years with Pashtun ethnicity, and not less than 20 years of tobacco exposure in any form.

Exclusion criteria (patients)

Patients with non-Pashtun ethnicity and/or having more than 70 years of age. Criteria for selection of control subjects: Normal healthy age-matched subjects of similar ethnicity with not less than 20 years of exposure to tobacco in any form, and free from cancer. Prior approval of the study was obtained from the Ethical Committee of the Department of Pharmacy, University of Peshawar (No. 440, dated 17.12.2011). Informed consent and thorough interview was taken by expert in the relevant field before blood collection on a carefully designed proforma that contained information regarding age, place, occupation, socioeconomic status, cancer type and tobacco use habits etc. Three milliliters of whole blood was collected from all subjects in properly labeled EDTA tubes and genomic DNA was subsequently extracted by using standard DNA Isolation kit **(**Purelink Genomic DNA kit Invitrogen, USA) as per manufacturer's recommendations. The DNA quality and quantity were determined using a double beam spectrophotometer (Perkin Elmer series 200 system, Norwalk, USA).

Genotyping of CYP1A1 (T>C, rs4646903)

The *CYP1A1* (T>C, rs4646903) polymorphisms were analyzed using a previously reported highly specific Real Time Polymerase Chain Reaction (RT-PCR). (Zakiullah *et al*., 2014) Light SNiP rs4646903 (primers and probes) and FastStart DNA Master Hyprobe kit were purchased from Tib-Molbiol (Germany) and Roche Diagnostics (Germany), respectively. Reaction was performed as per supplier's recommendation as follows. Reaction mix comprised of Reagent Mix (1µl), FastStart DNA Master (2µl), Magnesium chloride (25mm, 1.6µl), and water (14.4µl). Finally, DNA (1µl, 100-150ng) was added to the reaction mix to make the final volume 20µl. Thermocycler **(**MiniOpticon Model CFB-3120EDUUSA**)** conditions were as follows: Denaturation at 95ºC for 10 minutes; Cycling for 45 cycles of 95ºC for 10 seconds, 45ºC for 60 seconds and 72ºC for 15 seconds; followed by melting curves analysis at 95ºC for 10 seconds, 40ºC for 2 minutes through 75ºC for 0 seconds. Duplicate samples were used as control. Melting peaks at 51-52ºC represented wild type (T/T) allele; the one at 59-60ºC represented variant (C/C) allele, while samples giving two peaks at 51ºC and 59ºC were heterozygous (T/C) allele (fig. 1).

Table 1: Demographic characteristics of nasopharyngeal carcinoma subjects

S. No.	Variables	Control N (% within group)	NPC N (% within group)	*P-value*
1	Geographic Area (district)			
	Charsadda	15 (9.9 %)	0 (0 %)	
	Bannu DI Khan	12 (7.9 %)	18 (13. 8%)	
	Kohat	1 (0.6 %)	0 (0 %)	
	Mardan	15 (9.9%)	18 (13.8 %)	
	Swat malakand	23 (15.2 %)	19 (14.6 %)	*.9*
	Nowshera	11 (7.2 %)	0 (0 %)	
	Peshawar	19 (12.5%)	19 (14.6 %)	
	Dir chitral etc.	8 (5.2 %)	0 (0 %)	
	Swabi	9 (5.9 %)	19 (14.6 %)	
	Tribal	38 (25.16 %)	37 (28.4 %)	
2	Age (yrs)			
	20-40	24 (15.8 %)	18 (13.8 %)	
	41-50	42 (27.8 %)	37 (28.4 %)	*.9*
	51-60	41 (27.1 %)	38 (29.2 %)	
	60+	44 (29.1 %)	37 (28.4 %)	
3	Occupation			
	Coal labour	5 (3.3 %)	19 (14.6 %)	
	Driver	9 (5.9 %)	0 (0 %)	
	Farmer	44 (29.1 %)	0 (0 %)	
	Labour	36 (23.8 %)	37 (28.4 %)	*.0*
	Press	1 (0.6 %)	0 (0 %)	
	Odd jobs (laborious)	35 (23.1 %)	28 (21.5 %)	
	Farmer/labour (tobacco)	19 (12.5 %)	28 (21.5 %)	
	Teacher	2 (1.3 %)	18 (13.8 %)	
4	Tobacco type used			
	Smoker	68 (45.0 %)	74 (56.9 %)	*.6*
	Naswar user	132 (87.4 %)	130 (100 %)	*.6*
	Other tobacco users	53 (35.0 %)	74 (56.9 %)	*.8*
5	Age at 1st exposure (yrs)			
	10-15	66 (43.7 %)	37 (28.4 %)	
	16-20	57 (37.7 %)	56 (43.0 %)	*.3*
	21-25	11 (7.2 %)	18 (13.8 %)	
	25+	17 (11.2 %)	19 (14.6 %)	
6	Daily use			
	Mild	109 (72.1 %)	93 (71.5 %)	*.8*
	Moderate	12 (7.9 %)	0 (0 %)	
	Heavy	30 (19.8 %)	28.4 %)	

Genotyping assay for GSTM1 and GSTT1

For the determination of homozygous null polymorphisms of GSTM1 and GSTT1 previously reported method was used, as described over here. (Zakiullah *et al*., 2015) Primer sets used was 5′-CATGTGACAGTATTCTTATTTC- 3′, 5′-ACTCAATCTCAGCATCACAGC- 3′ and 5′- ATCT GTGGTCCCCAAATCAG-3′, 5′- GGGGGTTGTCTTTT GCATAG-3′, for GSTM1 and GSTT1 respectively. PCR was separately performed for both genes in a 25µl reaction mixture containing 20mM Tris-HCl pH 9.0, 50mM KCl, 2mM $MgCl_2$, 200µM dNTPs (Promega, USA), primers (Macrogen, South Korea) 10 pmol of each set individually, 0.5 units of Taq DNA polymerase (Bio-Labs, UK), and 50-100ng of genomic DNA. PCR was individually performed in the GeneAmp PCR system 9700 (Applied Biosystems, USA). After an initial denaturation at 95°C for 4 minutes, amplification was carried out for 40 cycles at 95°C for 30 seconds, 52°C for 45 seconds and 72°C for 1 minute for GSTM1 and 55°C for 45 seconds and 72°C for 1 minute for GSTT1, followed by final elongation at 72°C for 10 minutes. The PCR products were electrophoresed in a 1 % agarose gel for analysis. GSTM1 and GSTT1 genotype were identified by the presence of a band at 298 and 632 bp respectively (fig. 2 and 3).

STATISTICAL ANALYSIS

Chi-square ($\chi 2$) test was used to detect whether there were significant ($\alpha = 0.05$) differences in frequencies of genes. Odds Ratios (OR) for each polymorphism using binary logistic regression model were estimated with 95% confidence intervals (CIs), and the difference in genotype

prevalence and association between case and control group were assessed independently as well as adjusted for confounding factors. Age, gender, place of residence, tobacco type used, amount of tobacco used per day and age at first exposure were included as covariates as well as all the possible genotypes studied. *GSTM1* and *GSTT1* were categorized on the basis of presence and absence (null genotype) of the gene, while *CYP1A1* rs4646903 polymorphism was classified into homozygous wild type and variant allele containing genotypes. Wild type was used as reference group to assess the effects of the different alleles. Analyses were performed by SPSS (Version 20.0).

RESULTS

Subject Characteristics

Demographic and other subject characteristics of nasopharyngeal cancer patients are shown in table 1. Mean age of healthy subjects (controls) and cancer patients were 54.14±10.91 and 56.02±10.83 years, respectively. Almost equal distribution was found in the 5^{th}, 6^{th} and 7^{th} decades of life with twenty-eight percent (28.46 %) of the patients in the age range of 41-50 years; similar percentage (29.23 % in the age range of 51-60 years, while 28.46 % of the patients in the range of 60+ years of age. All the patients were male and no female patient during study period was observed. Highest incidence (28.46 %) of nasopharyngeal cancer was observed in tribal areas. Interestingly, equal distribution (14 %) was observed in Bannu DI Khan, Malakand, Mardan, Peshawar and Swabi districts; while no patient (0 %) was observed from Charsadda, Chitral, Dir, Kohat and Nowshera areas. Highest incidence (cumulative 71.52 %) was observed in patients having laborious jobs with a significant portion (21.53%) working in tobacco fields/industry. Interestingly, a significant portion (13.84 % and 14.61% each) was involved in teaching as profession and coal mining. Several aspects of tobacco use habit were noted including type of tobacco product used, age at 1^{st} exposure and amount of tobacco used per day. Hundred percent (100 %) of the patients were naswar addicts while 56.92 % each were also smokers or using other tobacco products specially huqqa (chillum). Forty-three percent patients started tobacco at the age of 16-20 years, while 28.46% started at the age of between 10-15 years, with mean age of 17 years. Majority (71.53%) of the patients were mild users with interestingly no patient (0%) having moderate use. About 28% were heavy users.

Genetic susceptibility to NPC due to CYP1A1 gene

The allele frequencies and genotype of CYP1A1 rs4646903 polymorphism among controls and cases are given in table 2. In control group they were not departure from Hardy-Weinberg equilibrium. The distributions of genotypes of *CYP1A1* rs4646903 were significantly different between the cases and controls groups (Pearson chi Square $\chi 2_{0.05,\ 2}$=0.003, P>0.05). The prevalence of CC and TC genotypes was more in cases (10.66 % and 33.33 % respectively) when compared to controls (3.57 % and 25 %). Homozygous variant type (C/C) had an almost 4-fold increased risk for pharyngeal cancer, compared with wild genotype (T/T) (OR: 3.810 (1.505-9.640), p-value=.005); while heterozygous variant (T/C) had an almost 2 times increased risk for oral cancer, compared with wild genotype (T/T) (OR: 1.701 (1.055-2.743) p-value = .029) at 95% CI, with almost no effect of confounding factors (Adjusted OR=3.260 (1.252-8.486), p-value= .016 and 1.556 (.944-2.5621.550, p-value= .083. respectively). Overall the C allele is significantly associated with the occurrence of pharyngeal cancer as compared to T allele (OR=3.224 (1.688-6.159), p-value= .000) which is slightly reduced in the presence of confounding factors (Adjusted OR=2.834 (1.454-5.523), p-value= .002). Allele carriage rate showed that the absence of T allele is associated with the susceptibility to nasopharyngeal cancer (Adjusted OR=3.224 (1.291-8.052), p-value= .012).

Genetic susceptibility to NPC due to GSTT1 and GSTM1 gene

The allele frequencies and genotype of GSTs of both control and pharyngeal cancer cases are given in Table 3. The distribution of *GSTM1* and *GSTT1* genotypes were significantly different between the cases and controls groups (Pearson chi Square $\chi 2_{0.05,\ 2}$=0.000, P>0.05). When analyzed for individual genes the prevalence of null genotypes of *GSTM1* and *GSTT1* was more in cases (80.76% and 37.69% respectively) when compared to controls (56.95% and 23.17% respectively). Null genotypes of GSTM1 gene were having 3-fold increased risk of pharyngeal cancer compared with wild type, which increased when OR were adjusted for confounding factors such as tobacco use habits, age and area of residence etc. Whereas null genotype of GSTT1 gene was having 2 times increased risk with little or no effect of confounding factors. Similarly, when analyzed for two GST gene combinations the association of GSTM1 Null/GSTT1 positive combination was more and significant than GSTM1/GSTT1 Null, which was having non-significant association. GSTM/GSTT both null genotype was having more than 5 times increased association with pharyngeal cancer which further boosted up with adjustment for confounding factors.

Combined effect of all three genes on NPC

Combined effect of *GST* and *CYP1A1* gene variants on susceptibility to pharyngeal cancer was analyzed as given in table 4. Presence of all three gene variants showed very strong and significant association with nasopharyngeal cancer as compared to control. Presence of both null and GSTM1 null along with wild type CYP1A1 gene also showed strong and significant association with pharyngeal cancer, which further enhanced in the presence of confounding factors. Whereas presence of *GSTM1* or *GSTT1* null alone, along with *CYP1A1* variant showed weak and non-significant association.

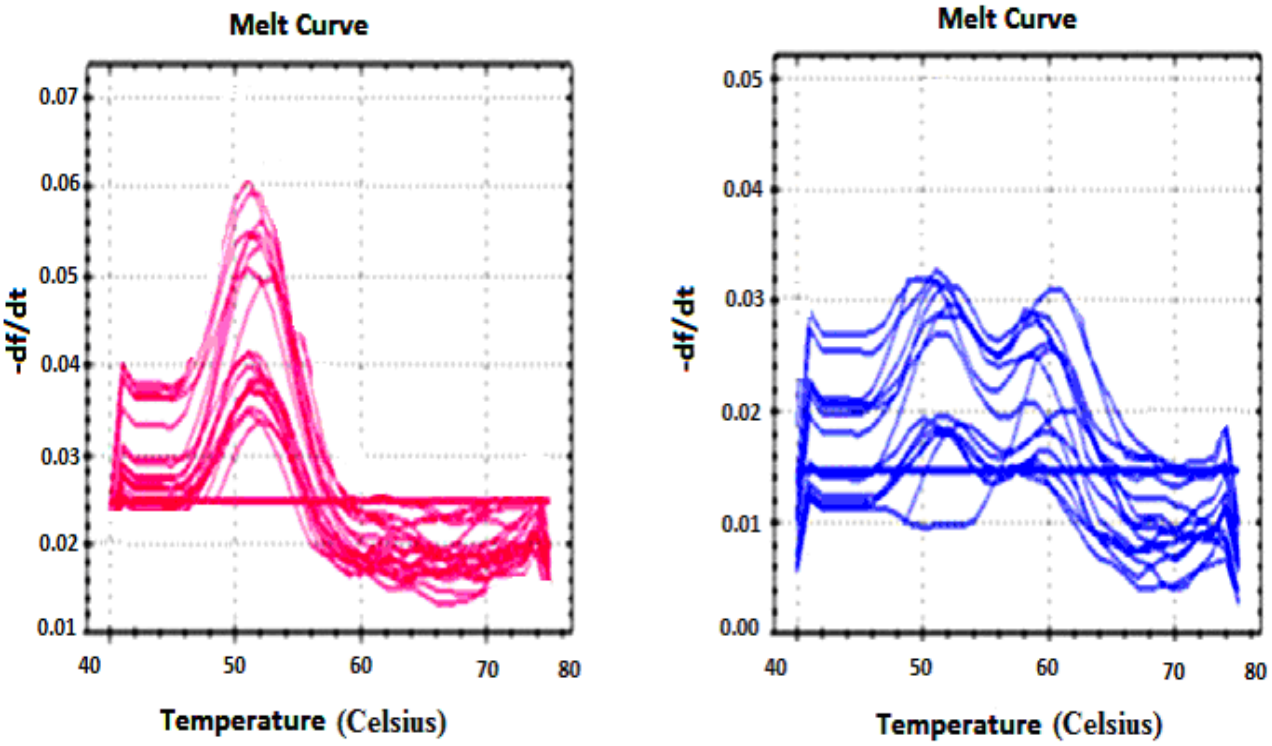

Fig. 1: Melting Peaks of RT-PCR of CYP1A1 rs4646903 polymorphism. Pink peaks represent CYP1A1 wild type (T/T), while blue peaks are of variant alleles (T/C) and C/C).

Table 2: Genotypic, allelic and carriage rate frequencies of CYP1A1 rs4646903 (T>C) gene polymorphism in controls and Nasopharyngeal carcinoma cases.

Genotype/allele	Case N (%)	Control N (%)	Unadjusted OR (95% CI)	*P-value*	Adjusted OR (95% CI)	*P-value*
T/T	84 (56)	140 (71.4)	Ref.			
C/C	16 (10.6)	7 (3.6)	3.8 (1.5-9.6)	*.0*	3.260 (1.252-8.486)	*.0*
T/C	50 (33.3)	49 (25)	1.7 (1.05-2.7)	*.0*	1.556 (.944-2.562	*.0*
*T allele	218 (72.6)	329 (83.9)	Ref.			
*C allele	82 (27.3)	63 (16)	3.2 (1.7-6.1)	*.0*	2.834 (1.454-5.523)	*.0*
Allele carriage rate						
T (+)	134 (89.3)	189 (96.4)	Ref.			
T (-)	16 (10.6)	7 (3.6)	3.2 (1.3-8.05)	*.0*	2.834 (1.103-7.282)	*.0*
C (+)	66 (4)	56 (28.6)	Ref.			
C (-)	84 (56)	140 (71.4)	.50 (.3-.7)	*.0*	.934 (.731-1.194)	*.5*
*Alleles, total number of chromosomes in control and cases						

Table 3: Crude and adjusted Odds Ratios (OR) of GSTs for Nasopharyngeal carcinoma

Genotype/allele	Genetic polymorphism	Cases N (%)	Control N (%)	Crude OR (95% CI)	*P-value*	Adjusted OR (95% CI)	*P-value*
GSTM1	Wild type	25 (19.2)	65 (43)	Ref.			
	Null	105 (80.7)	86 (57)	3.1 (1.8-5.4)	*.0*	3.9 (2.1-7.3)	*.0*
GSTT1	Wild type	81 (62.3)	116 (76.8)	Ref.			
	Null	49 (37.7)	35 (23.1)	2. (1.1-3.3)	*.0*	. 2.0 (1.1-3.5)	*.0*
Combinations 2 genes	GSTM/GSTT both wild type	19 (14.6)	51 (33.8)	Ref.			
	GSTM1 Null/GSTT1	62 (47.7)	65 (43)	2.5 (1.3-4.8)	*.0*	3.0 (1.4-6.2)	*.0*
	GSTM1/GSTT1Null	6 (4.6)	14 (9.2)	1.4 (.6-3.3)	*.3*	1.0 (0.3-3.6)	*.9*
	GSTM/GSTT both null	43 (33)	21 (14)	5.4 (2.6-11.5)	*.0*	6.4 (2.7-15.0)	*.0*

Table 4: Crude and adjusted Odds Ratios (OR) of GSTs and CYP1A1 gene combination for Nasopharyngeal carcinoma

Genetic polymorphism	Control N (%)	Cases N %)	Crude OR (95% CI)	*P-value*	Adjusted OR (95% CI)	*P-value*
GSTM GSTT and CYP1A1 wild type	40	13	Ref.			
All 3 gene polymorphisms	7	24	10.5 (3.6-30.1)	*.0*	10.0 (3.2-31.1)	*.0*
GSTM1 Null and CYP1A1	33	23	2.1 (0.9-4.8)	*.0*	1.9 (0.7-4.9)	*.1*
GSTT null and CYP1A1	4	4	3.0 (0.6-14.0)	*.1*	1.8 (0.3-10.2)	*.4*
Both GSTs and CYP1A1	11	6	1.6 (0.5-5.4)	*.3*	1.354 (0.3-4.9)	*.6*
GST both null and CYP1A1 wild type	14	19	4.1 (1.6-10.6)	*.0*	4.8 (1.6-14.2)	*.0*
GSTM null and CYP1A1 wild type	32	39	3.7 (1.7-8.1)	*.0*	4.7 (1.9-11.5)	*.0*
GSTT null and CYP1A1 wild type	10	2	0.6 (0.1-3.1)	*.5*	0.5 (0.0-3.4)	*.5*

Fig. 2: Representative Electropherogram of GSTMI analysis: L is molecular weight marker (1kb); N represent negative control; 1-32 are NPC sample; Duplicate sample was used as positive control (shown in rectangles); GSTMI is obtained at 298bp; GSTMI null samples are encircled.

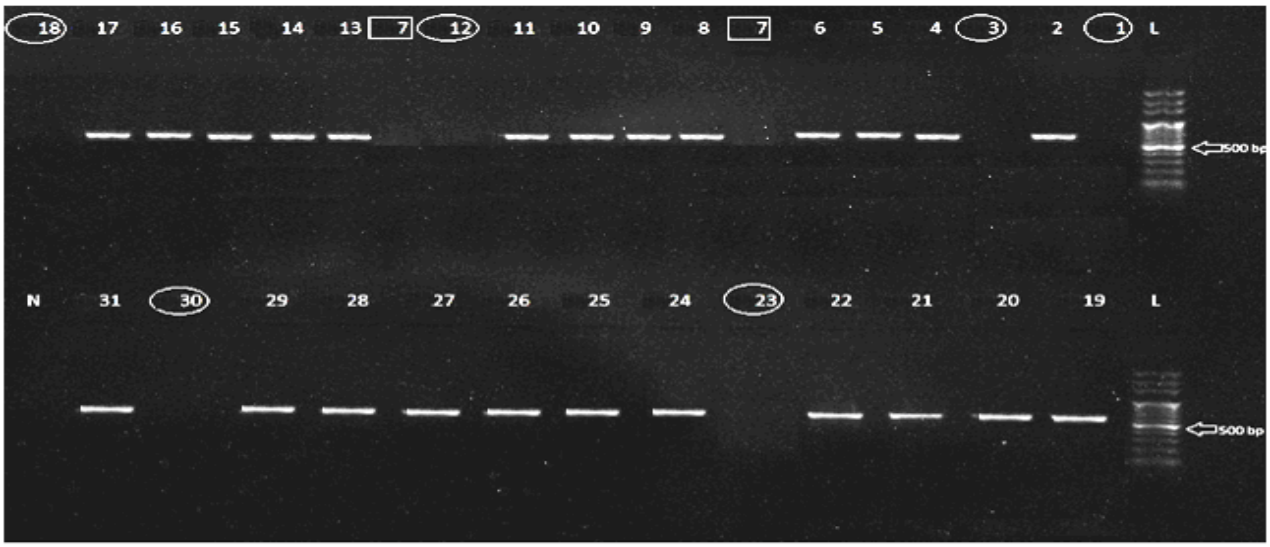

Fig. 3: Representative Electropherogram of GSTMI analysis: L is molecular weight marker (100bp); N represent negative control; 1-31 are NPC sample; Duplicate sample 7 was used as positive control (shown in rectangles); GSTMI in NPC samples has been shown at 632bp; GSTMI null samples are encircled.

DISCUSSION

Socio-demographic characteristics of cancer patients:

Age distribution pattern clearly demonstrates it as contributing factor, and ≥ 45 years of age should be considered as risk factor for above NPC, as has been reported in previous studies. (Llewellyn *et al.,* 2004) As far as gender is concerned we found only male patients fulfilling our criteria during hole of our study period. The reason may be that in Pashtun population tobacco consumption is socially an unacceptable practice for females and secondly the female patients may have provided us inaccurate information, as our main stay regarding tobacco habits were through questionnaire. Similarly address of the patients revealed that NPC was more prevalent in Tribal belt and adjacent districts. An observation worth further exploration was noted. Differential distribution of pharyngeal cancer among different districts was observed. For example, Bannu DI Khan and Swabi districts were having highest incidence of pharyngeal cancer; while Dir and Chitral districts were no NPC cases**.** This distribution points towards certain specific etiologic factors that need to be explored. For example, one of the reasons may be the intake of hot tea, as residence of tribal belt and adjacent areas are accustomed to it. Similarly, increased exposure to tobacco in these districts due to tobacco farming (especially in Swabi) and naswar consumption may be another factor. Regarding occupation (that reflects socioeconomic status) it was observed that generally the patients were very poor having laborious jobs. Interestingly, significant portion of the patients were having teaching and coal mining as profession or were working in tobacco fields/industry. Some of these findings points towards occupational exposure to certain etiologic factors, for example, teaching and coal mining. These observations may be explained by continuous irritation of the pharynx. For example, teaching involves rigorous use of the pharynx especially at primary and secondary level teaching, where teachers use to take classes for whole of the working day. Teaching style (e.g., very loud teaching) may also be a contributing factor. Tobacco use pattern shows that majority of the patients were exposed for more than 30 years to tobacco carcinogens. Use of chillum (huqqa) as a contributing factor can be explained with fact that the huqqa is forcefully inhaled through pharynx, thus exposing it more than the oral cavity. A significant portion of pharyngeal cancer cases were found to be chillum addicts. Previous studies have reported tobacco use including cigarette smoking, naswar intake and paan chewing as the main risk factors for upper aero digestive tract cancers.

Genetic susceptibility

It is well recognized fact that some individuals are more susceptible to certain types of cancers within similar environmental conditions. Different factors are involved in the initiation of carcinogenesis. These include, but not limited to, polymorphisms in genes responsible for the expression of carcinogen metabolizing enzymes, environmental exposure (such as tobacco use and alcohol consumption) and dietary and life style habits of the individuals. GSTs and CYP1A1 are among the most important enzymes involved in the processing of tobacco related carcinogens.

The ubiquitous family of enzymes cytochrome P450 plays an important role in the metabolic activation of major classes of tobacco related carcinogens. These include phase I enzyme CYP1A1 that is responsible for the activation of benzo[a]pyrene and nitrosamines thus affecting the metabolism of the environmental carcinogens and altering the susceptibility to cancer. Variation in CYP1A1 gene may alter function of its enzymes in such a way that toxicity of the extraneous stimulating factors that directly influence tissues may be enhanced, thus increasing susceptibility to cancer. (Hecht *et al.,* 1993, Bartsch *et al.,* 2000) Two major relevant polymorphic sites of the *CYP1A1* gene have been studied in several types of cancer that lead to large inter-individual differences in the activity of Aryl hydrocarbon Hydroxylase (AHH) enzyme involved in the processing of PAH and nitrosamines. One site (known as Msp1 or rs4646903) is located in the 3'-flanking region of the gene (T6235C position) in which the presence of C has been considered important risk factor. While the other (known as Ile/Val) (A4889G) is located in exon 7 (at codon 462) that alters the protein structure by replacing an isoleucine for a valine, making the carriers more susceptible to some tobacco-associated cancers (Wang *et al.,* 2002). Similarly, GSTs like GSTT1 and GSTM1 are involved in the phase II metabolism that causes detoxification of the electrophilic/toxic species generated in phase I metabolism. Homozygous deletions in *GSTM1* occur in gene cluster at chromosome 1p13.3 resulting in loss of *GSTM1* gene expression and loss of GSTM1 enzyme activity (Strange *et al.,*1998, Xu *et al.,* 1998). Deletion of *GSTM1* gene has been reported in many cancer patient populations, especially HNC (Hiyama *et al.,* 2008). Extensive polymorphism for *GSTM1* locus has been reported in Caucasians, with the presence of *GSTM1a*, *GSTM1b* alleles and deletion genotypes (Seidegård *et al.,* 1988, Hayes *et al.,*1995). Almost 50% of the Caucasians lack this gene and its deletion has shown increased risk for tobacco related cancers in this population (Smith *et al.,* 1994; Strange *et al.,* 1998)**.** Same type of deletion has been reported in a population based study in Chinese and Koreans (Kim *et al.,* 2000, Park *et al.,* 2000, Setiawan *et al.,* 2000)**.** Different ethnicities have different patterns of deletions that have been reported in Asians, Caucasians (Chen *et al.,* 1999, Cheng *et al.,* 1999, Crump *et al.,* 2000), European and Hispanic (Cotton *et al.,* 2000), and Frech (Stücker *et al.,* 1999) populations. Similarly, *GSTT1* gene deletion has been studied in different

populations. Some populations have shown association while other has shown weak or no association (Casson *et al*., 2003, Wang *et al*., 2004, Guo., 2005). Asians are reportedly possessing highest *GSTT1* deletion genotype. One study has shown that more than half of the Chinese and forty percent of Malaysians have *GSTT1* null genotype (Lee *et al*., 1995), and beside HNC cancers are also associated with increased incidence of laryngeal cancers (Cheng *et al*., 1999).

We have addressed genetic, ethnic and demographic factors associated with the incidence of NPC in Pashtun population. Present data shows that genetic polymorphisms in *GSTM1*, *GSTT1* and *CYP1A1* genes have very important contribution towards the occurrence of pharyngeal cancer in Pashtun population. *CYP1A1* have shown a 4-fold independent association with oral cancer, followed by *GSTM1* and *GSTT1* that have, 3-fold and 2-fold independent association, respectively. Combined effect of both GST genes is interesting. *GSTM1* null in the presence of GSTT1 have shown association; while *GSTT1* null in the presence of *GSTM1* have no effect. This observation is predictable as *GSTM1* is the main detoxifying enzyme as discussed previously. But independent association of *GSTT1* shows that it also has significant contribution in the detoxification of carcinogens, although to a lesser extent than GSTM1. Stated otherwise GSTM1 can compensate for GSTT1 but not vice versa. Presence of both null genotypes has an almost 6-fold association, which further strengthens the contribution of *GSTT1* null towards the increased incidence of pharyngeal cancer. Similarly, null GSTs in combination with CYP1A1 variant allele have almost the effect of an etiological factor (OR = 10.549 (3.695-30.116) at 95% CI). Similarly, when adjusted for age, gender, place of residence, occupation, tobacco type used, amount of tobacco used per day and age at first exposure the risk further increased showing the importance of these environmental and demographic factors. All these findings are consistent with previous studies that have shown association of *GSTM1*, *GSTT1* and *CYP1A1* genes, tobacco consumption (like naswar and cigarettes) and other risk factors with pharyngeal cancer as discussed above. In conclusion, our study shows that the *CYP1A1*, *GSTM1* and *GSTT1* genes may be associated with the risk of pharyngeal cancer, especially in the presence of tobacco use.

Conclusion and recommendation

Based on above mentioned findings, reducing consumption of tobacco (especially naswar in Pashtun population) and elevating the socioeconomic status must be regarded as the primary preventive strategies for the control of nasopharyngeal cancer in Khyber Pakhtunkhwa province. Similarly, projects should be designed by governmental agencies to screen for genetically susceptible individuals and awareness campaigns regarding genetic susceptibility and environmental risk factors be initiated in general public.

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REFERENCES

Abbasi AN, Zahid S and Bhurgri Y (2011). Nasopharyngeal carcinoma-an update of treatment and acute radiation induced reactions from a tertiary-care hospital in Pakistan. *Asian Pac. J. Cancer Prev.,* **12**(3): 735-738.

Al-Dayel F, Al-Rasheed M and Ibrahim M (2008). Polymorphisms of drug-metabolizing enzymes CYP1A1, GSTT and GSTP contribute to the development of diffuse large B-cell lymphoma risk in the Saudi Arabian population. *Leukemia lymphoma.,* **49**(1): 122-129.

Bartsch H, Nair U and Risch A (2000). Genetic polymorphism of CYP genes, alone or in combination, as a risk modifier of tobacco-related cancers. *Cancer Epidemiol. Biomarkers Prev.,* **9**(1): 3-28.

Bin Q and Luo J (2013). Role of polymorphisms of GSTM1, GSTT1 and GSTP1 Ile105Val in Hodgkin and non-Hodgkin lymphoma risk: A Human Genome Epidemiology (HuGE) review. *Leukemia lymphoma.,* **54**(1): 14-20.

Buch S, Kotekar A and Kawle D (2001). Polymorphisms at CYP and GST gene loci. *Eur. J. Clin. Pharmacol.,* **57**: 553-555.

Casson A, Zheng Z and Chiasson D (2003). Associations between genetic polymorphisms of Phase I and II metabolizing enzymes, p53 and susceptibility to esophageal adenocarcinoma. *Cancer Detect. Prev.*, **27**(2): 139-146.

Chen C, Madelelne MM and Weiss NS (1999). Glutathlone S-Transferase M1 Genotypes and the Rismk of Vulvar Cancer: A Population-based Case-Control Study. *Am J Epidemiol,* **150**(5): 437-442.

Cheng L, Sturgis EM and Eicher SA (1999). Glutathione-S-transferase polymorphisms and risk of squamous-cell carcinoma of the head and neck. *Int J Cancer.,* **84**(3): 220-224.

Cheng YJ, Chien YC and Hildesheim A (2003). No association between genetic polymorphisms of CYP1A1, GSTM1, GSTT1, GSTP1, NAT2 and

nasopharyngeal carcinoma in Taiwan. *Cancer Epidemiol. Biomarkers Prev.*, **12**(2): 179-180.

Chu EA, Wu JM and Tunkel DE (2008). Nasopharyngeal carcinoma: The role of the Epstein-Barr virus. *Medscape J Med.*, **10**(7): 165.

Cotton S, Sharp L and Little J (2000). Glutathione S-transferase polymorphisms and colorectal cancer: A HuGE review. *Am J Epidemiol*, **151**(1): 7-32.

Crump C, Chen C and Appelbaum FR (2000). Glutathione S-transferase theta 1 gene deletion and risk of acute myeloid leukemia. *Cancer Epidemiol Biomarkers Prev.*, **9**(5): 457-460.

Fachiroh J, Sangrajrang S and Johansson M (2012). Tobacco consumption and genetic susceptibility to nasopharyngeal carcinoma (NPC) in Thailand. *Cancer Causes Control*, **23**(12): 1995-2002.

Ferlay J, Shin H and Bray F (2010). Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]. *IARC*.

Friborg JT, Yuan JM and Wang R (2007). A prospective study of tobacco and alcohol use as risk factors for pharyngeal carcinomas in Singapore Chinese. *Cancer*, **109**(6): 1183-1191.

Guo S-W (2005). Glutathione S-transferases M1/T1 gene polymorphisms and endometriosis: A meta-analysis of genetic association studies. *Mol Hum Reprod.*, **11**(10): 729-743.

Guo X, Johnson RC and Deng H (2009). Evaluation of nonviral risk factors for nasopharyngeal carcinoma in a high-risk population of Southern China. *Int. J. Cancer*, **124**(12): 2942-2947.

Hayes JD and Pulford DJ (1995). The Glut athione S-Transferase Supergene Family: Regulation of GST and the Contribution of the lsoenzymes to Cancer Chemoprotection and Drug Resistance Part II. *Crit. Rev, Biochem. Mol. Biol.*, **30**: 521-600.

Hecht SS, Carmella SG and Murphy SE (1993). Carcinogen biomarkers related to smoking and upper aerodigestive tract cancer. *J Cell Biochem.*, **53**: 27-35.

Hiyama T, Yoshihara M and Tanaka S (2008). Genetic polymorphisms and head and neck cancer risk (Review). *Int J Oncol.*, **32**(5): 945-973.

Junfeng CM (2011). Polymorphisms in GSTM1, GSTTI and GSTP1 and nasopharyngeal cancer in the east of China: A case-control study. *Asian Pac. J. Cancer Prev.*, **12**: 3097-3100.

Zakiullah, Ahmadullah, Khisroon M and Saeed M (2015). Genetic Susceptibility to Oral Cancer due to Combined Effects of GSTT1, GSTM1 and CYP1A1 Gene Variants in Tobacco Addicted Patients of Pashtun Ethnicity of Khyber Pakhtunkhwa Province of Pakistan. *Asian Pac. J. Cancer Prev.*, **16**(3): 1145-1150.

Kim WJ, Lee HL and Lee SC (2000). Polymorphisms of N-acetyltransferase 2, glutathione S-transferase mu and theta genes as risk factors of bladder cancer in relation to asthma and tuberculosis. *J Urol.*, **164**(5): 209-213.

Lee EJ, Wong JY and Yeoh PN (1995). Glutathione S transferase-[theta](GSTT1) genetic polymorphism among Chinese, Malays and Indians in Singapore. *Pharmacogenet Genomics.*, **5**(5): 332-334.

Llewellyn C, Johnson NW and Warnakulasuriya K (2004). Risk factors for oral cancer in newly diagnosed patients aged 45 years and younger: A case-control study in Southern England. *J Oral Pathol Med.*, **33**(9): 525-532.

Nebert DW, McKinnon RA and Puga A (1996). Human drug-metabolizing enzyme polymorphisms: Effects on risk of toxicity and cancer. *DNA Cel Biol.*, **15**(4): 273-280.

Park SK, Yoo KY and Lee SJ (2000). Alcohol consumption, glutathione S-transferase M1 and T1 genetic polymorphisms and breast cancer risk. *Pharmacogenet Genomics.*, **10**(4): 301-309.

Seidegard J, Vorachek WR and Pero RW (1988). Hereditary differences in the expression of the human glutathione transferase active on trans-stilbene oxide are due to a gene deletion. *Proc Natl Acad Sci.*, **85**(19): 7293-7297.

Setiawan VW, Zhang ZF and Yu GP (2000). GSTT1 and GSTM1 null genotypes and the risk of gastric cancer: a case-control study in a Chinese population. *Cancer Epidemiol Biomarkers Prev.*, **9**(1): 73-80.

Smith G, Stanley L and Sim E (1994). Metabolic polymorphisms and cancer susceptibility. *Cancer Surveys*, **25**: 27-65.

Sriamporn S, Vatanasapt V and Pisani P (1992). Environmental risk factors for nasopharyngeal carcinoma: A case-control study in northeastern Thailand. *Cancer Epidemiol Biomarkers Prev.*, **1**(5): 345-348.

Strange R and Fryer A (1998). The glutathione S-transferases: Influence of polymorphism on cancer susceptibility. *IARC Scientific Publications*, **148**: 231-249.

Stücker I, De Waziers I and Cenee S (1999). GSTM1, smoking and lung cancer: A case-control study. *Int. J. Epi.*, **28**(5): 829-835.

Wang L, Rodriguez M and Kim ES (2004). A novel C/T polymorphism in the core promoter of human de novo cytosine DNA methyltransferase 3B6 is associated with prognosis in head and neck cancer. *Int. J. Oncol.*, **25**(4): 993-1002.

Wang X, Zuckerman B and Pearson C (2002). Maternal cigarette smoking, metabolic gene polymorphism and infant birth weight. *Jama.*, **287**(2): 195-202.

Xu S, Wang Yp and Roe B (1998). Characterization of the Human Class Mu GlutathioneS-Transferase Gene Cluster and the GSTM1 Deletion. *J. Biol. Chem.*, **273**(6): 3517-3527.

Zakiullah MS, Ali S and Javed N (2014). Genetic Susceptibility to Esophageal Cancer due to CYP1A1 Gene Variant rs4646903 in Tobacco Addicted Patients of Pashtun Ethnicity: A Case Control Study in Khyber

Pakhtunkhwa Province of Pakistan. *Asian Pac. J. Cancer Prev.*, **15**(16): 6715-6720.

Zhang F and Zhang J (1999). Clinical hereditary characteristics in nasopharyngeal carcinoma through Ye-Liang's family cluster. *Chin. Med. J.*, **112**(2): 185-187.