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Original Article

Association of genetic polymorphism of glutathione S-transferases with colorectal cancer susceptibility in snuff (*Naswar*) addicts

Associação de polimorfismo genético de glutationa S-transferase com suscetibilidade ao câncer colorretal em viciados em rapé (Naswar)

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Abstract

The current study aimed to investigate the relationship between polymorphisms in detoxifying (GSTM1, GSTT1, and GSTP1) genes and their association with colorectal cancer (CRC) in tobacco addicts of Pashtun ethnicity. Polymorphisms in the selected genes were genotyped in a case-control study consisting of 100 histologically confirmed male CRC patients and 100 birth-year and gender-matched healthy controls using the PCR–RFLP method. The GSTM1 null, and GSTT1 null genotypes were significantly contributed to the risk of CRC in the cases (OR= 3.131, 95% CI: 1.451–6.758, *P* = 0.004, and OR= 3.541, 95% CI: 1.716–7.306, *P* = 0.001, respectively), whereas the association observed for GSTP1 Val/Val (1.139, 95% CI: 0.356–3.644, *P* = 0.826) did not show statistical significance. The combined GSTM1 null and GSTT1 null showed a 41-fold increased risk (95% CI: 4.945–351.950, *P* = 0.001), while, the combined GSTM1 null and GSTP1 Ile/Val or Val/Val variant genotypes exhibited about 3-fold (95% CI: 1.196–7.414, *P* = 0.019) increased risk to CRC. Similarly, the combined GSTT1 null and GSTP1 Ile/Val or Val/Val variant genotypes showed about a 3-fold (95% CI: 1.285–8.101, *P* = 0.013) increased risk of CRC. In the combination of three GST genotypes, the GSTM1 null, GSTT1 null, and GSTP1 Ile/Val or Val/Val variant genotypes demonstrated a more than a 22-fold (95% CI: 2.441–212.106, *P* = 0.006) increased risk of CRC. Our findings suggest that GSTM1 and GSTT1 polymorphism and its combination with GSTP1 may be associated with CRC susceptibility in the Naswar addicted Pashtun population of Khyber Pakhtunkhwa, Pakistan.

Keywords: genetic polymorphism, GSTM1, GSTT1, GSTP1, colorectal cancer, tobacco, Naswar.

Resumo

O presente estudo teve como objetivo investigar a relação entre polimorfismos em genes desintoxicantes (GSTM1, GSTT1 e GSTP1) e sua associação com câncer colorretal (CCR) em tabagistas da etnia pashtun. Os polimorfismos nos genes selecionados foram genotipados em um estudo de caso-controle composto por 100 pacientes do sexo masculino com CCR, confirmados histologicamente, e 100 controles saudáveis, pareados por ano de nascimento e sexo usando o método PCR-RFLP. Os genótipos GSTM1 nulo e GSTT1 nulo contribuíram significativamente para o risco de CCR nos casos (OR = 3,131, IC 95%: 1,451-6,758, P = 0,004; OR = 3,541, IC 95%: 1,716-7,306, P = 0,001, respectivamente), enquanto a associação observada para GSTP1 Val/Val (1,139, IC 95%: 0,356-3,644, P = 0,826) não apresentou significância estatística. O GSTM1 nulo e o GSTT1 nulo combinados mostraram um risco 41 vezes maior (IC 95%: 4,945-351,950, P = 0,001) para CCR, enquanto os genótipos GSTM1 nulo e GSTP1 Ile/Val ou Val/Val combinados apresentaram risco cerca de 3 vezes maior (IC 95%: 1,196-7,414, P = 0,019) para CCR. Da mesma forma, os genótipos combinados GSTT1 nulo e GSTP1 Ile/Val ou Val/Val tiveram um risco para CRC cerca de 3 vezes maior (95% CI: 1,285-8,101, P = 0,013). Na combinação de três genótipos GST, os genótipos GSTM1 nulo, GSTT1 nulo e GSTP1 Ile/Val ou Val/Val apresentaram um risco 22 vezes maior (IC 95%: 2,441-212,106, P = 0,006) para CRC. Nossos achados sugerem que o polimorfismo GSTM1 e GSTT1 e sua combinação com GSTP1 podem estar associados à suscetibilidade ao CRC da população pashtun de Khyber Pakhtunkhwa, Paquistão, viciada em Naswar.

Palavras-chave: polimorfismo genético, GSTM1, GSTT1, GSTP1, câncer colorretal, tabaco, Naswar.

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1. Introduction

Recent studies have reported a surge in colorectal cancer (CRC) cases for patients above the age of 50 years in Pakistan (Hasan et al., 2017). In Pakistan in 2020, CRC was the fourth most common cancer in males (5,107 cases, 5.8% of all cancers) and the fifth most common in both males and females (8,602 cases, 4.8% of all cancers) (GLOBOCAN, 2020). Globally, CRC was the third most common cancer among both males and females in 2020, occurring in 1.9 million cases (10% of all cancers) (GLOBOCAN, 2020). Worldwide 0.9 million (9.4%) deaths occurred due to CRC, which is the second most death toll after lung cancer (1.7 million, 18% of all cancer-related deaths) (GLOBOCAN, 2020).

The two main modes of tobacco consumption around the globe are smoking and smokeless tobacco (SLT) (Kumar et al., 2017). Due to excessive legal restrictions and social constraints on smoking in communal areas and indoors, the popularity and use of SLT have been increased as an alternate means of nicotine addiction (Joshi et al., 2011). Betel quid, Gutkha, Khaini, Mawa, Pan Masala, Tombak, Maras Powder, and Naswar are some of the 40 types of SLT used globally (Benowitz, 1991; WHO, 2012). More than 350 million people use SLT around the globe, of which more than 90% live in South Asian countries including Pakistan (Sinha et al., 2015; Sohail et al., 2020). The global adult tobacco survey (2014) has reported that more than 17 million people in Pakistan are SLT users (Saqib et al., 2018). Gutkha, Paan (Betel quid with tobacco), and Naswar are some of the common types of SLT consumed in Pakistan (National Cancer Institute, 2014; Khan et al., 2017). Pashtun ethnicity of Khyber Pakhtunkhwa, Sindh, and Baluchistan provinces of Pakistan commonly consume Naswar as a major type of addiction (Basharat et al., 2012). The Pashtun population of Peshawar uses about 60% of tobacco in the form of Naswar (Ali et al., 2017). It is also considered a low-priced nicotine replacement remedy for people who wish to quit smoking (Ullah et al., 2011).

The major carcinogenic components of SLT products are tobacco-specific N-nitrosamine (TSNA), Volatile N-nitrosamines, N-nitrosamino acids, acetaldehyde, nicotine alkaloids, heavy metals like Polonium-210 (Po^{210}), and hydrocarbons, etc. (Kaur and Prasad, 2013). Tobacco is a heavily pesticide-dependent crop; as various types of pesticides are regularly used on tobacco for its better yield. These pesticides mostly include fungicides, insecticides, suckercides, and herbicides, which are regarded as essential to tobacco production. As these pesticides contain toxic (mutagenic and carcinogenic) chemicals which after treatment remain on tobacco leaves and bio-accumulate in their tissues (McDaniel et al., 2005). Regular and frequent use of Naswar adds remarkable harmful tobacco contents into the oral cavity, which not only stains the teeth and gums but also causes serious health hazards like malignancy. Data show that Naswar is associated with oral and esophageal cancer (Khan et al., 2017; Khan et al., 2019; Zakiullah et al., 2012).

GSTs are a multigene family of phase II metabolic enzymes and are important metabolic enzymes for all eukaryotes (Safarinejad et al., 2013). These enzymes perform a reaction by the attachment of reduced glutathione with different types of exogenous and endogenous electrophilic substances like carcinogenic substances, environmental compounds, and xenobiotics (Hayes et al., 2005; Safarinejad et al., 2013). In this way, they reduce the reactivity of toxic chemicals, make them water-soluble, and favor their safe excretion from the body, without harming the cells and tissues. Data show that based on sequence homology and substrate specificity human GST-superfamily is composed of almost 16 genes divided into eight GSTs i.e. GSTA (alpha), GSTM (mu), GSTP (Pi), GSTT (theta), GSTK (kappa), GSTS (sigma), GSTO (omega) and GSTZ (zeta) (Lo and Ali-Osman, 2007; Strange et al., 2001). GSTs show ethnicity-based polymorphisms, so it modifies the susceptibility to various diseases like cancers in different ethnicities around the globe. The most commonly studied polymorphisms amongst the GSTs are GSTT1, GSTM1, and GSTP1.

The GSTM1-null genotype results in the total absence of enzymatic activity, that's why it is of more interest to investigate (Campos et al., 2018). The literature reports that the GSTM1 null homozygous genotype has a significant predisposition to various kinds of tumors (Ateş et al., 2005; Benhamou et al., 2002; Singh et al., 2008; Smits et al., 2003). The frequency of the GSTT1 null genotype also shows variation in different ethnicities. As a consequence of reduced or lack of enzymatic activity, the detoxification process of carcinogens or toxins is halted resulting in the development of cancers (Bell et al., 1993; Lafuente et al., 1993). GSTT1 null genotype is also been related to a significantly increased risk of colorectal, renal, bladder, prostate cancers, etc (Abid et al., 2016; Grando et al., 2009; Kempkes et al., 1996). The common functional GSTP1 polymorphism at codon 105 change in the nucleotide from A to G results in an amino acid variation from isoleucine to valine i.e. Ile105Val, as a result, the catalytic activity of the GSTP1 enzyme is reduced (Ali-Osman et al., 1997). The mutant genotype Val105Val of GSTP1 has shown susceptibility to different types of cancers like CRC, pancreatic cancer, breast cancer, lymphoma, ovarian cancer, etc. (Tew et al., 2011).

The data about the incidence of smoking and smokeless tobacco-related cancers have been published around the globe but no data has been published about the interaction of GSTs, and tobacco (Naswar and Cigarette) addicted CRC patients from Khyber Pakhtunkhwa province of Pakistan. Hence a case-control study was performed in the Pashtun ethnicity of Khyber Pakhtunkhwa province, to find out the interaction of GSTM1, GSTT1, and GSTP1 gene variation and their association with CRC among tobacco addicted Pashtun population.

2. Materials and Methods

2.1. Subjects

A case-control study was conducted to examine the association of functionally important polymorphisms in GSTs (GSTM1, GSTT1, and GSTP1), with colorectal cancer in tobacco addicted Pashtun population. The study was approved by the Advanced Study and Research Board

(ASRB) and Ethical Committee, University of Peshawar (No: 22-09/09/2016). Figure 1 shows the criteria for the selection of the subjects. The study subjects consisted of 100 healthy controls and 100 histopathologically confirmed CRC patients. Institute of Radiotherapy and Nuclear Medicine (IRNUM) Peshawar was selected for obtaining the blood samples from the cancer patients, as it is one of the major hospitals in the province and most of the cancer patients from various districts visit it. Similarly, control subjects were recruited from the representative districts and the friends and relatives of the cancer patients. The sampling period consisted of Six months (03/10/2016 to 01/04/2017). Each subject was given consent for becoming part of the study.

2.2. Inclusion and exclusion criteria

Cases and controls with Pashtun ethnicity, age range 30–70 years, having tobacco addiction of at least 15 years, and histopathologically confirmed colorectal cancer patients were included in the study. While the subjects were aged less than 30 years and more than 70 years, non-Pashtun ethnicity had tobacco addiction of fewer than 15 years, and the cases who were not histopathologically confirmed were excluded from the study.

2.3. Sample collection

Information from the patients was obtained by filing a properly designed questionnaire which had questions about the possible contributing factors and other demographic characteristics i.e. age, gender, address, occupation, education level, socioeconomic status, cancer type, etc. A similar questionnaire was used to obtain information from the control subjects, but it lacked questions related to cancer. Properly labeled EDTA tubes were used to obtain 3 ml of blood from each subject. The blood samples were shifted in a well-protected container to the Molecular and Toxicology Laboratory, Department of Zoology, University of Peshawar, and stored at -20ºC. The subjects were selected according to the following inclusion and exclusion criteria.

2.4. Genotyping

Genomic DNA Was extracted from the peripheral blood of the subjects using a GF-1 DNA extraction kit (*Vivantis* technologies) and according to the method described by Nosheen et al. (2010). The extracted DNA was quantified using gel electrophoresis (1% agarose gel) and spectrophotometer (Biowave DNA, WPA). For the GSTM1 gene (chromosome 1p13.3, exon 4) DNA samples were amplified with the primers: 5'–CATGTGACAGTATTCTTATTTC–3' and 5'-ACTCAATCTCAGCATCACAGC-3', while primers for GSTT1 (chromosome 22q11.2, exon 5) were 5'-ATCTGTGGTCCCCAAATCAG-3' and 5'-GGGGGTTGTCTTTTGCATAG-3' using PCR (XP cycler, BIOER). PCR was performed in a final volume of 25 µL, containing 17.6 µL Milli Q water, 1 µL genomic DNA (25-100ng/25µL), 2.5 µL reaction buffer (10X with KCl) (Thermo Scientific), 2 µL mixed primers (100 nM), 0.5 µL dNTPs (2.5 mM) (Thermo Scientific), 1 µL $MgCl_2$ (25 mM) (Thermo Scientific), 0.4 µL Taq polymerase (5 U/µL) (Thermo Scientific). The thermal conditions for the GSTM1 gene were: Initial denaturation at 95 ºC for 3 min, followed by 40 cycles of which each cycle having a denaturation at 95 ºC for 30 s, annealing at 52 ºC for 45 s, extension at 72 ºC for 45 s and the final extension step of2 ºC for 10 min. For the GSTT1 (Macrogen) genotyping the same

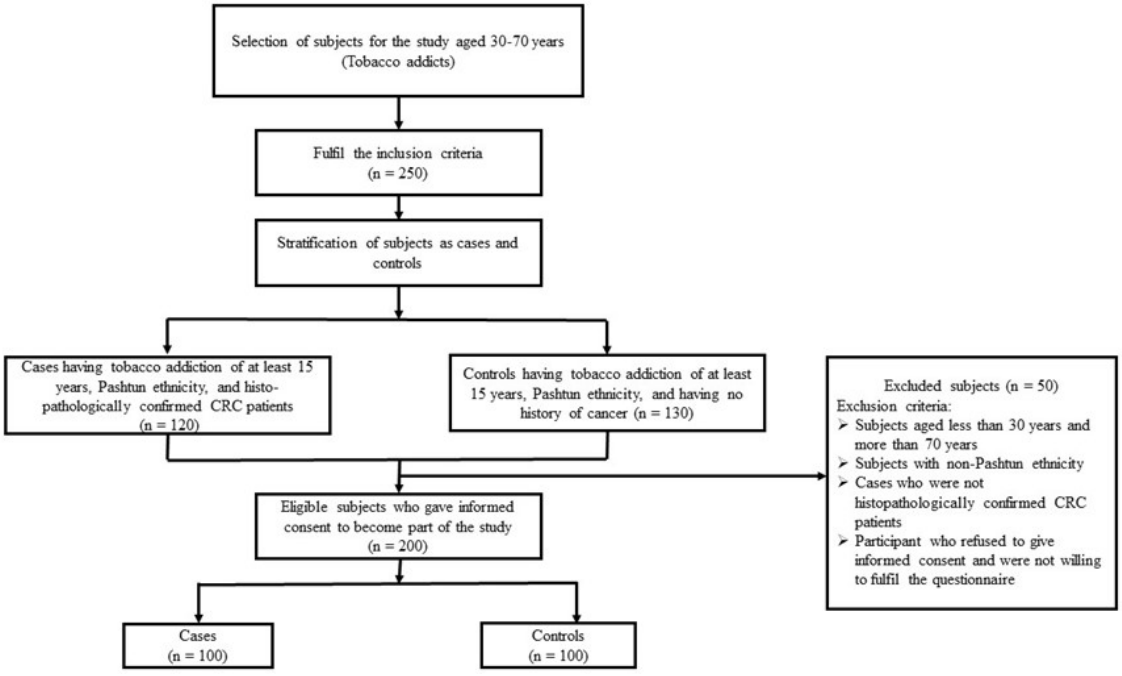

Figure 1. Flowchart for selection of the subjects.

reagents concentration and thermal conditions were used as for GSTM1 (Macrogen) except for the annealing temperature of 55 ºC. The GSTP1 (Macrogen) genotyping was performed as described by Safarinejad et al. (2013). A 189 bp fragment of the GSTP1 gene containing Ile to Val substitution in exon 5 (11q13 chromosome) was amplified using the primers 5'-CCAGTGACTGTGTGTTGATC-3' and 5'-CAACCCTGGTGCAGATGCTC-3'. The reagents and thermal conditions for the GSTP1 gene were the same as those used for GSTM1 and GSTT1 except for the annealing temperature which was 57 ºC. The PCR products were analyzed with 2% agarose gel (Sigma-Aldrich). A 100 bp DNA ladder (INtRON Biotechnology) was loaded as a reference. The electrophoresis was carried out at 100V for 1 hour. For visualization of the gel, a gel documentation AlphaImager MINI was used. The sample bands on the gel were compared with the 100 bp DNA marker (INtRON Biotechnology). The samples of GSTM1 and GSTT1 which showed bands at 298bp and 632bp, respectively, were considered normal genotypes, while those which lacked bands at these positions were considered null genotypes (deleted genes) (Figures 2-3).

The GSTP1 gene showed PCR products of 189 bp on agarose gel (Figure 4).

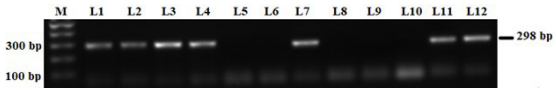

Figure 2. Representative gel image of GSTM1 polymorphism. M is the molecular weight DNA marker (100 bp). Sample no L6, and L8-L10 are null genotypes having no bands, while L1-L4, L7, and L11 are positive genotypes with an amplicon size of 298 bp. Sample L5 was used as a negative control, which had all the reagents except the DNA sample, while a duplicate sample L12 was used as a positive control.

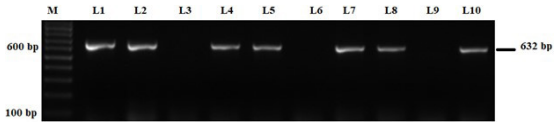

Figure 3. Representative gel image of GSTT1 polymorphism. M is the 100 bp DNA marker. Sample no L3, and L6 are null genotypes having no bands, while L1, L2, L4, L5, L7, and L8 are positive genotypes with a band size of 632 bp. Sample L9 was used as a negative control, having all the reagents except genomic DNA, while a duplicate sample L10 was used as a positive control.

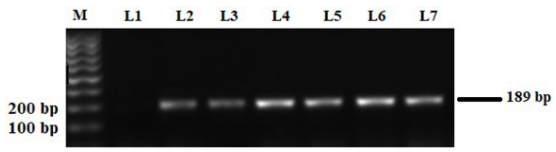

Figure 4. Represent the gel image of the GSTP1 gene PCR product. M is the 100 bp DNA marker, while sample no L1 was negative control, and L2 was a positive control. Samples no L3-L7 represent the required PCR product, with an amplicon size of 189 bp.

After confirming the GSTP1 PCR product in the gel, the products were further processed for restriction fragment length polymorphism (RFLP). A 10 µL PCR product of GSTP1 was digested with 5 U of restriction enzyme (*BsmA1*) (Thermo Scientific) at 37ºC overnight. The digested products were analyzed in 3% agarose gel. Three genotypic variants were observed based on band sizes. A single band of 189 bp (completely undigested) represented wild-type genotype (Ile/Ile). Two bands of 41 and 148 bp (completely digested) showed the mutant genotype (Val/Val), while the heterozygous genotype (Ile/Val) was represented by all three bands of 41, 148, and 189 bp. Genotyping was repeated with approximately 10% of randomly selected samples (Figure 5).

2.5. Statistical analysis

To assess the difference between the categorical variables, the Chi-Square test (χ^2) was used, while for the analysis of quantitative variables the *t*-test was used. Quantitative variable data are given as mean and standard deviation (SD), while data for categorical variables are shown as numbers and percentages. Binary logistic regression was used to estimate the association between the studied genotypes and cancers. Odds ratios at a 95% confidence interval were calculated. The classification of GSTT1 and GSTM1 was based on the absence (null genotypes) or presence of the genes, while GSTP1 polymorphism was categorized as homozygous wild, heterozygous and mutant genotypes. The wild genotype was regarded as the reference category. $P < 0.05$ was regarded as significant. The data was statistically analyzed by using IBM SPSS v.26.

3. Results

The demographic characteristics of the studied population are given in Table 1. The mean ages of the cases and controls were 58.6 ± 10.8 years and 58.2 ± 9.9 years, respectively. The distribution of age in patients and controls are not statistically different ($P = 0.776$) and the same applies to the pattern of tobacco use ($P = 0.920$). No significant difference ($P > 0.05$) was found regarding other studied variables.

Table 2 summarizes the distribution of genotype frequency of GSTs in colorectal cancer patients and controls.

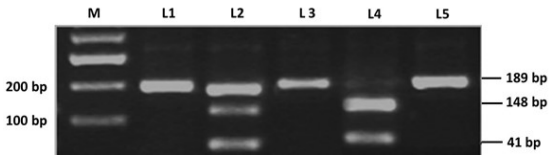

Figure 5. Representative gel image of the intended GSTP1 gene RFLP product. M is the 100 bp DNA marker. L1, L3, and L5 represent wild-type genotype (Ile/Ile) having one fragment with a size of 189 bp. L2 represents heterozygous genotype (Ile/Val) having three fragments with 41 bp, 148 bp, and 189 bp sizes. L4 represents mutant genotype (Val/Val) having two fragments with 41 bp and 148 bp sizes.

Table 1. Demographic characteristics of colorectal cancer cases and healthy controls.

No.	Variable	Cases N (%)	Control N (%)	*P* value
1.	**Age (Years)**			
	Mean	58.58 ± 10.87	58.16 ± 9.96	0.776[a]
	≤ 40	09 (09.0)	08 (08.0)	0.833[b]
	41–50	15 (15.0)	19 (19.0)	
	51–60	26 (26.0)	28 (28.0)	
	≥ 61	50 (50.0)	45 (45.0)	
2.	**Locality**			
	Peshawar	10 (10.0)	10 (10.0)	1.000[b]
	Swabi	09 (09.0)	09 (09.0)	
	Charsadda	18 (18.0)	16 (16.0)	
	Mardan	13 (13.0)	15 (15.0)	
	Southern districts (Kohat, Bannu, Karak, etc)	09 (09.0)	09 (09.0)	
	Northern districts (Malakand, Swat, Dir etc)	13 (13.0)	14 (14.0)	
	FATA (NWA, Bajaur etc)	17 (17.0)	16 (16.0)	
	Afghanistan	11 (11.0)	11 (11.0)	
3.	**Education**			
	None	51 (51.0)	50 (50.0)	0.998[b]
	Primary School	21 (21.0)	22 (22.0)	
	High School	13 (13.0)	13 (13.0)	
	Graduate	15 (15.0)	15 (15.0)	
4.	**Occupation**			
	Jobless	10 (10.0)	10 (10.0)	
	Laborer	22 (22.0)	23 (23.0)	1.000[b]
	Farmer	17 (17.0)	15 (15.0)	
	Shopkeeper	09 (09.0)	09 (09.0)	
	Carpenter	07 (07.0)	07 (07.0)	
	Driver	12 (12.0)	12 (12.0)	
	Teacher	07 (07.0)	07 (07.0)	
	Watchman	05 (5.0)	05 (05.0)	
	Mechanic	06 (06.0)	06 (06.0)	
	Businessman	05 (05.0)	06 (06.0)	
5.	**The pattern of tobacco use**			
	Naswar	69 (69.0)	70 (70.0)	0.920[b]
	Cigarette	17 (17.0)	15 (15.0)	
	Both	14 (14.0)	15 (15.0)	
6.	**Duration of addiction**			
	Mean	24.16 ± 12.16	24.17 ± 12.21	0.896[a]
	15–20	24 (24.0)	23 (23.0)	0.375[b]
	21–30	30 (30.0)	39 (39.0)	
	>31	46 (46.0)	38 (38.0)	
7.	**Daily use of tobacco**			
	Mild	21 (21.0)	21 (21.0)	0.987[a]
	Moderate	33 (33.0)	32 (32.0)	
	Heavy	46 (46.0)	47 (47.0)	

a: *t*-test. b: chi-square test. $P < 0.05$ is considered significant.

Both the cases and controls were in Hardy Weinberg equilibrium for GSTs. Regarding GSTM1, 40% of the cases showed null genotypes as compared to controls (20%) (OR=3.131, 95% CI: 1.451–6.758, P = 0.004). Among the patients, 45% showed null GSTT1 genotype compared with 25% in controls (OR = 3.541, 95% CI: 1.716–7.306, P = 0.001). Hence, a comparison between colorectal cancer patients and the control group using a logistic regression model exhibited a significant trend for GSTM1 and GSTT1 null genotypes. So, the GSTM1 and GSTT1 null genotypes were linked with more than a threefold risk of colorectal cancer. The three genotypes of GSTP1 i.e. Ile/Ile, Ile/Val, and Val/Val had a frequency distribution of 41, 50, 9%, and 43, 48, and 9% in cases and controls, respectively. The GSTP1 genotypes had no significant difference between the cases and controls (P > 0.05). To investigate the association of the variant GSTP1 genotypes with colorectal cancer we combined the frequency of Ile/Val, Val/Val, but the combined effect of this association was also insignificant (P > 0.05) (Table 2).

As null genotypes alone (GSTM1 or GSTT1) showed marked risk factors, therefore associations between the mutant genotypes (null genotypes) were also assessed. Table 3 shows the combined effects of double GST genotypes among the study subjects. The cases that had either null genotypes for both GSTM1 and GSTT1 showed significant effects for developing colorectal cancer (OR = 2.685, 95% CI: 1.374–5.246, P = 0.004) than those who had both positive genotypes. The individuals who had both the null genotypes of GSTM1 and GSTT1 showed a significantly higher risk for developing cancer (OR = 41.717, 95% CI: 4.945–351.950, P = 0.001) than cases who had both the positive genotypes. The individuals who had GSTM1 null and GSTP1 Ile/Ile

Table 2. GSTM1, GSTT1, and GSTP1 genotypes distribution in the colorectal cancer patients and controls.

Genotype	Cases, N (%)	Controls, N (%)	OR[a] (95% CI)	*P* value
GSTM1				
Present	60 (60.0)	80 (80.0)	Ref	
Null	40 (40.0)	20 (20.0)	3.131 (1.451–6.758)	0.004
GSTT1				
Present	55 (55.0)	75 (75.0)	Ref	
Null	45 (45.0)	25 (25.0)	3.541 (1.716–7.306)	0.001
GSTP1				
Ile/Ile	41 (41.0)	43 (43.0)	Ref	
Ile/Val	50 (50.0)	48 (48.0)	0.832 (0.400–1.731)	0.623
Val/Val	09 (09.0)	09 (09.0)	1.139 (0.356–3.644)	0.826
Ile/Val, Val/Val	59 (59.0)	57 (57.0)	1.077 (0.580–1.999)	0.815

[a]Adjusted odd ratios for occupation, age, occupation, education, and tobacco use.

Table 3. Double GST genotype distribution among cases and controls.

Double GST genotypes	Cases, N (%)	Controls, N (%)	OR[a] (95% CI)	*P* value
GSTM1 and GSTT1				
Both present	30 (30.0)	56 (56.0)	Ref	
Either Null	54 (54.0)	43 (43.0)	2.685 (1.374–5.246)	0.004
Both Null	16 (16.0)	01 (01.0)	41.717 (4.945–351.950)	0.001
GSTM1 and GSTP1				
M1(+/+) and P1(Ile/Ile)	26 (26.0)	36 (36.0)	Ref	
M1(+/+) and P1(Ile/Val or Val/Val)	34 (34.0)	44 (44.0)	1.017 (0.487–2.126)	0.964
M1(–/–) and P1(Ile/Ile)	15 (15.0)	07 (07.0)	3.239 (1.077–9.742)	0.036
M1(–/–) and P1(Ile/Val or Val/Val)	25 (25.0)	13 (13.0)	2.977 (1.196–7.414)	0.019
GSTT1 and GSTP1				
T1(+/+) and P1(Ile/Ile)	30 (30.0)	29 (29.0)	Ref	
T1(+/+) and P1(Ile/Val or Val/Val)	25 (25.0)	45 (45.0)	0.512 (0.239–1.098)	0.086
T1(–/–) and P1(Ile/Ile)	11 (11.0)	14 (14.0)	0.764 (0.276–2.112)	0.603
T1(–/–) and P1(Ile/Val or Val/Val)	34 (34.0)	12 (12.0)	3.226 (1.285–8.101)	0.013

[a]Adjusted odd ratios for age, education, occupation, and tobacco use.

genotypes had an elevated risk of developing colorectal cancer (OR = 3.239, 95% CI: 1.077–9.742, *P* = 0.036). Similarly, those individuals who had GSTM1 null and GSTP1 Ile/Val, Val/Val genotypes also had an elevated risk of developing colorectal cancer (OR = 2.977, 95% CI: 1.196–7.414, *P* = 0.019) as compared to those who had both the genes present of GSTM1 and GSTP1 Ile/Ile. The cases that had GSTT1 null and GSTP1 Ile/Val, Val/Val genotypes had significantly higher odds ratios (OR = 3.226, 95% CI: 1.285–8.101, *P* = 0.013).

Regarding the distribution of triple GST genotypes (Table 4), the results exhibited that the presence of 3 potentially risky genotypes i.e. GSTM1 null, GSTT1 null, and GSTP1 Ile/Val, Val/Val had more than twenty-two-fold risk of colorectal cancer (OR = 22.753, 95% CI: 2.441–212.106, *P* = 0.006).

4. Discussion

The role of GSTs polymorphism was investigated in colorectal cancer in a population-based case-control study. The cases and controls both were tobacco addicts. Mixed results have been yielded by previous case-control studies of GSTs polymorphism and CRC. This study is the first of its kind, on tobacco-addicted CRC patients and controls from the Pashtun ethnicity of Khyber Pakhtunkhwa Province. Naswar is also consumed by Pashtun living in other provinces of Pakistan.

An increased risk of colorectal cancer was found in the GSTM1 null genotype of our studied population. The results of our study are in agreement with the studies conducted by Ates *et al.*, who reported a 1.6-fold increase in the risk of colorectal cancer development of GSTM1 null genotype (Ateş et al., 2005). Likewise, the increased risk associated with GSTM1 null genotypes in colorectal cancer patients was reported by other case-control studies (Huang et al., 2006; Katoh et al., 1996; Little et al., 2006; Rodrigues-Fleming et al., 2018; Slattery et al., 2003). A meta-analysis of 13 studies was performed to assess the strength of association between GSTM1 genotypes and the risk of colorectal cancer. Their meta-analysis suggested that GSTM1 null genotype was significantly associated (*P* = 0.002) with the risk of colorectal cancer in the Chinese population (Teng et al., 2014). Likewise, a recent meta-analysis reported a significant risk association of GSTM1 null genotype with colorectal cancer in Asians (OR = 1.19, 95% CI: 1.08–1.32) and Caucasians (OR = 1.14, 95% CI: 1.05–1.23). Contrary to our results, some studies have reported no association between GSTM1 null genotype and risk of colorectal cancer (Hamachi et al., 2013; Klusek et al., 2018; Lalosevic et al., 2019; Little et al., 2006; Waś et al., 2018).

In the current study, it was reported that the GSTT1 null genotype in the cases was significantly higher as compared to controls (Table 2). The GSTT1 null genotype had a 3.5-times elevated risk of developing colorectal cancer (95% CI, 1.716–7.306, *P* = 0.001). In the same way, few studies (Ateş et al., 2005; Lalosevic et al., 2019; Song et al., 2020) have stated a highly significant association of GSTT1 null genotype with colorectal cancer risk, while on contrary some studies (Hamachi et al., 2013; Hezova et al., 2012; Little et al., 2006; Waś et al., 2018) have reported no remarkable association.

Polymorphisms in the GSTP1 gene are probably one of the most widely studied GST (Lalosevic et al., 2019). The data show that GSTP1 is commonly expressed in various cancers, including colorectal cancer, thus suggesting its involvement in the metabolism of various toxic carcinogens (Doğru-Abbasoğlu et al., 2002; Hezova et al., 2012; Lalosevic et al., 2019). Like other GSTs, there is the inconsistency of GSTP1 genotypes distribution among different world populations, with diverse ethnic and geographical backgrounds, hence it leads to conflicting results related to the role of GSTP1 genotypes in colorectal cancer progression and development. Some previous analyses did not show any association between GSTP1 variant genotypes and the risk of colorectal cancer, which are in agreement with our results (Ateş et al., 2005; Economopoulos and Sergentanis, 2010; Tan et al., 2013; Welfare et al., 1999). We found no association of GSTP1 variant genotypes with colorectal cancer risk (OR=1.077, 95% CI: 0.580–1.999). On contrary, other studies reported a significant association between GSTP1 variant genotypes and colorectal cancer risk (Kassab et al., 2014; Lalosevic et al., 2019; Matakova et al., 2009; Senthilkumar and Thirumurugan, 2012; Wang et al., 2011). While Hezova et al. (2012) have reported a decreased risk of GSTP1 heterozygote genotype (Ile105Val) and association with colorectal cancer (Hezova et al., 2012).

Table 4. Triple GST genotypes distribution among cases and controls.

Triple GST genotypes	Cases, N (%)	Controls, N (%)	OR (95% CI)	*P* value
M1 (+/+) and T1(+/+) and P1(Ile/Ile)	17 (17.0)	23 (23.0)	1.0 (Ref)	
M1 (+/+) and T1(+/+) and P1(Ile/Val or Val/Val)	13 (13.0)	32 (32.0)	0.429 (0.161–1.144)	0.091
M1(–/–), T1(+/+), and P1(Ile/Ile)	12 (12.0)	06 (06.0)	2.692 (0.753–9.626)	0.128
M1(–/–), T1(+/+), and P1(Ile/Val or Val/Val)	12 (12.0)	13 (13.0)	1.384 (0.461–4.158)	0.563
M1(+/+), T1(–/–), and P1(Ile/Ile)	09 (09.0)	12 (12.0)	0.946 (0.295–3.035)	0.926
M1(+/+), T1(–/–), and P1(Ile/Val or Val/Val)	21 (21.0)	12 (12.0)	2.702 (0.948–7.697)	0.063
M1(–/–), T1(–/–), and P1(Ile/Ile)	04 (04.0)	01 (01.0)	5.298 (0.468–59.971)	0.178
M1(–/–), T1(–/–), and P1(Ile/Val or Val/Val)	12 (12.0)	01 (01.0)	22.753 (2.441–212.106)	0.006

N=100.

We also assessed the combined effects of two or three putative risk genotypes i.e. GSTM1 null, GSTT1 null, and GSTP1 Ile105Val compared to low-risk genotypes i.e. GSTM1 non-null, GSTT1 non-null, and GSTP1 Ile/Ile (Table 3). The combination of GSTM1 null with GTT1 null showed a 41.7-fold increased risk of colorectal cancer (95% CI: 4.945–351.950, P = 0.001). The cumulative effect of GSTM1 null and GSTP1 Ile/Val and GATP1 Val/Val genotypes on the risk of colorectal cancer were also significant (OR= 2.777, 95% CI: 1.196–7.414, P = 0.019). In the same way, the combination of GSTT1 null and GSTP1 Ile/Val and Val/Val genotypes showed a significant association with the risk of colorectal cancer (OR= 3.226, 95% CI: 1.285-8.101, P = 0.013). A similar cumulative effect of putative risk genotypes in colorectal cancer was reported by other studies. Wang et al. (2011) showed a 2.98-fold increase in colorectal cancer by combining GSTM1 null and GSTT1 null genotypes; a 2.14-fold increase by combining GSTM1 null and GSTP1 Val/Val genotypes; a 1.89-fold increase by combining GSTT1 null and GSTP1 Val/Val genotypes (Wang et al., 2011). Similarly, in other studies, the combined GSTM1 null and GSTT1 null genotypes reported an increased risk of colorectal cancer (Song et al., 2020). About a 2-fold increase in the risk of colorectal cancer was displayed when combined the GSTT1 null/GSTP1-variant and GSTM1 null/GSTP1-variant genotypes (Lalosevic et al., 2019). Unlike our study, some studies (Hamachi et al., 2013; Kassab et al., 2014) have reported no risk while combining the presumed risk genotypes (GSTM1/GSTT1-combined null genotypes).

We also analyzed three presumed risk GSTs genotypes concerning colorectal cancer. A combination of GSTM1 null, GSTT1 null, and GSTP1 Ile/Ile and Val/Val genotypes showed a 22.753-fold increase in colorectal cancer risk (95% CI: 2.441–212.106, P = 0.006), which is in parallel to the previous reports (Ateş et al., 2005; Lalosevic et al., 2019; Wang et al., 2011).

GSTs being the detoxifying enzymes have a key role in the cellular defense mechanism. GSTM1 is involved in the detoxification of active metabolites of PAH found in tobacco (Hayes et al., 2005), while the GSTT1 is known to detoxify various xenobiotic, environmental, and tobacco carcinogens like ethylene oxide and 1,3-butadiene (Landi, 2000). Whereas GTP1 is known to be broadly expressed in normal epithelial tissues, especially in colorectal cancer (Moscow et al., 1989; Terrier et al., 1990), where it metabolizes many tobacco carcinogens like benzo[a] pyrene (Saarikoski et al., 1998). So, due to the null or inactive GSTM1 or GSTT1 and variant GSTP1 genotypes, their ability to detoxify the carcinogens is reduced, causing cancer progression.

5. Conclusions and Recommendations

the GSTM1 null, and GSTT1 null genotypes individually significantly contributed to the risk of cancer in the cases. The combined GSTM1 null and GSTT1 null showed significant risk, similarly, the combined GSTM1 null and GSTP1 Ile/Val or Val/Val genotypes as well as GSTT1 null and GSTP1 Ile/Val or Val/Val genotypes significantly increased the individuals' susceptibility to cancer. The combination of three GST genotypes i.e. GSTM1 null, GSTT1 null, and GSTP1 Ile/Val or Val/Val genotypes also demonstrated gene-gene interaction and further contributed to the increased risk of colorectal cancer. Thus we found that the presence of GSTs null genotypes is associated with CRC risk because the null or missing genotype can't detoxify the tobacco carcinogens. However, this study should be considered a preliminary one from the Pashtun ethnic group, and further replicative studies with a large sample size should be carried out from the same belt. This study also suggests that the authorities should take strict measures to ban/discourage the use of Naswar (snuff) and other forms of tobacco to control tobacco-related cancers in the region.

6. Limitations of the Study

Our research work has some study gaps which should be considered in future studies. First, the sample size is relatively small, so further studies on large sample sizes and investigation of other detoxifying genes may substantially enrich our knowledge on the reason for the unusually high prevalence of tobacco-related cancers in the Khyber Pakhtunkhwa province of Pakistan. Second, our study only included male subjects, so female tobacco addicts need to be included in future studies. Finally, since the findings from the present study were only from Pashtun ethnicity, it is uncertain whether these results are relevant to other ethnic groups, so other ethnic populations should be considered in future studies.

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