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Research Article

Association of *Glutathione S-transferase* (*GSTM1*, *GSTT1* and *GSTP1*) polymorphisms and passive smoking in cervical cancer cases from North India

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Abstract

Objectives: To evaluate association of genetic polymorphisms in *GSTM1*, *T1*, and *P1* genes with cervical cancer (CC) susceptibility and effect of passive smoking.

Methods: The polymorphisms in *GSTM1* and *T1* were analyzed by multiplex polymerase chain reaction (mPCR) and *GSTP1* by PCR - Restriction Fragment Length Polymorphism (PCR-RFLP) in 150 CC cases and 165 healthy controls.

Results: CC cases showed significant association with marriage age, parity and hemoglobin level ($p<0.0001$). *GSTM1* (-/-) genotype was prominent among cases (30.0 %) whereas on combined analysis, *GSTM1*(-/-) / *T1*(-/-) and *GSTM1*(-/-) / *P1*(A/G or G/G) genotypes showed 2.75 and 2.98 times higher susceptibility to CC respectively. Triple combinations of *GSTM1* (-/-), *T1* (+/+) and *P*1 (A/G or G/G) genotypes showed significant association ($P=0.02$) with a 3.04 times risk of developing CC. Further analysis of cervical cancer histological subtypes such as squamous cell carcinoma and adenocarcinoma revealed an association of *GSTM1* (-/-), *GSTP1* A/G and G/G genotypes in adenocarcinoma cases alone (4.01, 2.25 and 2.40 folds respectively). Out of all the CC cases, passive smokers with *GSTM1* (-/-) and *GSTP1* (G/G) genotypes showed 4.19 and 2.88 times respectively of increased risk of developing the disease.

Conclusions: This study confirms the possible interactions between different *GST* polymorphisms and passive smoking in cervical cancer cases from North India.

Keywords: Cervical Cancer, *GSTM1*, *GSTT1*, *GSTP1*, Passive smoker

1. Introduction

Carcinoma of cervix is the second most common cancer among women worldwide[1]. In India, about 130,000 new cases are diagnosed each year out of which >70,000 deaths occur annually[2]. Many previous epidemiologic studies have shown that cervical cancer is mainly caused by high-risk HPV e.g. HPV-16 and HPV-18[3]. However, all HPV infected women do not develop cervical cancer. This indicates the role of additional etiological co-factors such as marriage age, early and multiple child birth, low socio-economic status and heavy cigarette smoking[4]. HPV infected women exposed to kitchen

smoke from wood for 35 years or more were found to be at 5.69 times higher risk of developing cervical cancer[5]. The current interest of this study was the pathogenesis of cervical cancer on the interaction of environmental and genetic factors. Life style and environment are two kinds of acquired susceptibility factors, while inherited factors are those genetic variants which are related to cervical cancer carcinogenesis. Both active as well as passive smoking habits have been confirmed to be risk factors for cervical cancer[6]. The presence of cotinine and nicotine in cervical mucus of women exposed to passive smoking can contribute to carcinogenesis through the same pathways as active smoking including genotoxic and immunomodulatory effects[7,8]. Although tobacco smoking is a risk factor and well established cause for cervical precancerous and cancerous states, it has not been explored to a great extent[9]. Poppe *et al*[10] have shown immunohistochemically that in smokers there is a decrease in Langerhans cells as well as helper-T lymphocytes in the squamous epithelia of uterine cervix transformation zone which is the start site of cervical cancer. Another cause responsible for pathogenesis of cervical cancer may be the direct contact of seminal fluid of smoking sex partners with the mucus membrane of cervix during sexual intercourse[11]. Active smokers are those who inhale smoke directly but passive smokers do not smoke themselves but inhale it from others smoking around them.

Chemical carcinogens are detoxified by phase II metabolizing enzymes *e.g.* glutathione S-transferase (GST), N-acetyltransferase, epoxide hydroxylase and sulphotransferase[12]. GST supergene family plays a central role in the detoxification of toxic and carcinogenic electrophilic compounds. There are three families of GSTs *i.e.* cytosolic, mitochondrial and microsomal[13]. Cytosolic GST family consists of seven multigene classes designated as alpha, mu, pi, theta, zeta, sigma and omega[14]. They catalyze the conjugation of reduced glutathione (GSH) to electrophilic substances of endo- or exogenous compounds (*e.g.* dopamine, prostaglandins, products of lipid peroxidation, chemotherapeutical substances and environmental carcinogens) after which the compounds are easily excreted from the body. GSTs belonging to mu, theta, and pi classes (*GSTM1*, *GSTT1* and *GSTP1*) play important roles in detoxification of metabolites of carcinogens in tobacco smoke[15]. Polymorphisms in these enzymes have been associated with a high risk of bladder, colon, gastrointestinal and lung cancers[16,17].

In this hospital-based case-control study, we examined the association of individual and combined genotypes of *GSTM1*, *T1* and *P1* gene polymorphisms with cervical cancer in general and also with the smoking status of affected women.

2. Materials and Methods

2.1. Sample Collection and DNA Isolation

The study was performed in 150 cases and 165 healthy controls, all between 30-70 years of age. The histopathologically confirmed patients of cervical cancer were from the eastern part of Uttar Pradesh in North India enrolled in the Radiotherapy Department of King George's Medical University (KGMU), Lucknow, India. Each patient was interviewed extensively regarding age, marriage age, parity, smoking status *etc.* The study was approved by the Institutional Ethics Committee (No. 274/R.Cell-10 dated 10th May, 2010) of KGMU, Lucknow.

Five ml blood samples were taken in EDTA vials from both controls and cases after their informed consent. Genomic DNA was isolated from peripheral blood leucocytes by standard salting out method with slight modifications [18,19]. DNA was checked on 1% agarose gel and used for genotyping by polymerase chain reaction (PCR) and PCR-Restriction Fragment Length Polymorphism (PCR-RFLP).

2.2. Genotyping of *GSTM1*, *GSTT1* and *GSTP1*

The *GSTM1* and *GSTT1* gene polymorphisms were analyzed by using multiplex polymerase chain reaction (mPCR). The PCR primers and annealing temperatures used for analysis are shown in Table 1. Amplification was performed in a 25 µl reaction mixture containing genomic DNA (100-150 ng), 5 pmol of each primer (IDT, USA), 200 µM dNTPs (Merck, India), and 0.5U of Taq DNA polymerase (MBI-Fermentas, USA) per tube using a gradient Master Cycler (Eppendorf, USA). The PCR products were checked on ethidium bromide (EtBr) stained 2% agarose gels and visualized in gel documentation system (Vilber Lourmat, France). *CD36* and *CYP1A1* genes were co-amplified and used as positive controls for *GSTM1* and *GSTT1* respectively.

The *GSTP1* A313G polymorphism was analysed using PCR-RFLP. A PCR reaction mixture of 25 µl was prepared as described above by using primers shown in Table 1. The PCR products were digested with 2.0 units of restriction enzyme *Alw26*I (MBI-Fermentas, USA) at 37°C for 16 hrs. The digested products were electrophoresed on 12% polyacrylamide gel (PAGE) and visualized after staining with ethidium bromide (EtBr).

2.3. Statistical Analysis

The continuous variables of each group were summarized as mean±SD and compared by Student's t-test after ascertaining the normality by Kolmogorov–Smirmov Z test. Dichotomous (categorical) variables were compared by chi-square ($\chi 2$) test. Genotype frequencies in both groups were compared using a 2x2 contingency table by Fisher's exact test. All *P* values were two-sided and differences were considered statistically significant for $P<0.05$. Odds ratio (OR) at 95% confidence intervals (CI) was determined to describe the strength of association by Logistic Regression Model. All analyses were performed on SPSS (Version 15.0).

3. Results

3.1. Clinical Stage, Histopathology and Demographic Characterization of Cases

Most of the patients were in stage II (50.0%) while 42.7 and 7.3% cases were in the stages III/IV and I respectively. All the 150 cases of cervical cancer were histopathologically confirmed in which 7.3% (11 out of 150) were adenocarcinoma and remaining 92.7% (139 out of 150) were squamous cell carcinoma. Squamous cell carcinoma was further differentiated according to cell types into well differentiated (37.4%), moderately differentiated (33.8%) and poorly differentiated (5.8%), while 23.0% cases had no differentiation specified in histopathological report.

Table.1: Primer sequences, PCR conditions, amplicon sizes and restriction enzyme with product sizes of different genes.

Gene	Primer sequences (5'-3')	Annealing temp. (°C)	Product size (bp)	Restriction enzyme/ allele sizes
GSTM1	F:GAACTCCCTGAAAAGCTAAAGC R:GTTGGGCTCAAATATACGGTGG	64	230	No Enzyme/ Null/Present
GSTT1	F:TCCTTACTGGTCCTCACATCTC R:TCACCGGATCATGGCCAGCA	66	458	No Enzyme/ Null/Present
GSTP1	F:ACCCCAGGGCTCTATGGGAA R:TGAGGGCACAAGAAGCCCCT	66	176	*Alw*26I A/A 176 A/G 176, 93, 83 G/G 93, 83
CD36	F:ACTCACCCTGAACCCCTTC R:GCCTCTGAGTAGTTGGGGCC	64	401	Positive Controls
CYP1A1	F:ACTCACCCTGAACCCCTTC R:AGCCTCTGAGTAGTTGGGGCC	66	196	

The demographic variables of the study population such as age, marriage age and parity were recorded for squamous cell carcinoma and adenocarcinoma cervical cancer histological subtypes (Table 2). There was no difference in age distribution between controls and cases, the mean age being 49.21±8.70 and 47.78±9.54 years respectively ($P=0.12$). It was observed that cervical cancer patients were married at a younger age (17.64±2.00) and had more number of children (4.77). Marriage age, parity and hemoglobin level in cases showed a highly significant association when compared to controls ($P<0.0001$). Significant association was found between passive smokers among controls and cases ($P<0.0001$).

Table.2: Comparison of demographic characteristics in controls and cervical cancer cases.

Characteristics	Controls (n=165)	Cases (n=150)	P-value
	Mean ± SD	Mean ± SD	
Age (years)	49.21±8.70	47.78±9.54	0.125
Marriage age (years)	20.12±2.37	17.64±2.00	<0.0001
Parity	3.15±0.99	4.77±1.70	<0.0001
Hemoglobin (g/ml)	11.43±0.77	10.40±1.17	<0.0001
Smoking history Never smoker Active smoker Passive smoker	 126 (82.42)* 0 39 (17.58)	 61 (40.7) 8 (5.3) 81 (54.0)	<0.0001

Significance < 0.05, *1.0 (Reference)

3.2. Genotyping of *GSTM1*, *T1* and *P1* Gene Polymorphisms

Distribution of *GSTM1*, *T1*, and *P1* (A/G) genotypes in controls and cases is shown in Table 3. The frequency of *GSTM1* null (-/-) genotype was higher among cases than in controls (30.0 vs. 23.0%) thus corresponding to a marginal increase in risk for cervical cancer (OR=1.43, 95% CI= 0.86- 2.36). But in case of *GSTT1* null (-/-) genotype as well as *GSTP1* variants, none were found to be risk factors for cervical cancer. The *GSTM1* showed significant association with adenocarcinoma cases (*P*=0.02) with null (-/-) genotype increasing the risk by 4.01-folds. *GSTP1* A/G and G/G genotypes also showed higher risk of adenocarcinoma (2.25 and 2.40 folds respectively). None of the three genes showed any association with squamous cell carcinoma.

Table.3: Genotype frequencies of *GSTM1*, *T1* and *P1* and their association with cervical cancer.

Population	Genotype number (%)										
	GSTM1			***GSTT1***			***GSTP1***				
	(+/+)	(-/-)	P-value OR (95%CI)	(+/+)	(-/-)	P-value OR (95%CI)	A/A	A/G	P-value OR (95%CI)	G/G	P-value OR (95%CI)
Controls (n=165)	127* (77.0)	38 (23.0)	0.16 1.43 (0.86-2.36)	134* (81.2)	31 (18.8)	0.97 0.99 (0.56-1.74)	108* (65.4)	48 (29.1)	0.44 1.21 (0.74-1.96)	9 (5.5)	0.84 0.90 (0.32-2.52)
Cases (n=150)	105 (70.0)	45 (30.0)		122 (81.3)	28 (18.7)		93 (62.0)	50 (33.3)		7 (4.7)	
SCC (n=139)	100 (66.7)	39 (26.0)	0.31 1.30 (0.77-2.18)	112 (74.6)	27 (18.0)	0.89 1.04 (0.58-1.85)	88 (58.7)	44 (29.3)	0.64 1.12 (0.68-1.84)	7 (4.7)	0.92 0.96 (0.34-2.66)
AC (n=11)	5 (3.3)	6 (4.0)	0.02 4.01 (1.16-13.87)	10 (6.7)	1 (0.7)	0.43 0.43 (0.05-3.50)	5 (3.3)	5 (3.3)	0.21 2.25 (0.62-8.13)	1 (0.7)	0.44 2.40 (0.25-22.81)

AC= Adenocarcinoma; CI = Confidence Interval; OR= Odds Ratio; (+/+) = Present; (-/-) = Null; SCC= Squamous cell carcinoma; Significance < 0.05, *1.0 (Reference).

Combination of two genotypes *GSTM1* (-/-) / *T1* (-/-) showed a 2.75-folds higher risk of developing cervical cancer (Table 4). Individuals with *GSTP1* genotype (A/G or G/G) in combination with *GSTM1* (-/-) showed of 2.98-folds risk (Table 4).

Table.4: Distribution of double and triple combinations of *GST* genotypes and their association with cervical cancer.

Genotyping	Controls (n=165); no (%)	Cases (n=150); no (%)	P- value	OR (95%CI)
Double				
GSTM1 and GSTT1 Both present (+/+) T1 null (+/-) M1 null (-/+) Both null (-/-)`	 99 (60.0)* 28 (17.0) 35 (21.2) 3 (1.8)	 84 (56.0) 21 (14.0) 38 (25.3) 7 (4.7)	 0.70 0.37 0.15	 1.0 (Reference) 0.88 (0.46-1.67) 1.28 (0.74-2.20) 2.75 (0.68-10.96)
GSTM1 and *GSTP1* M1{+/+} ,P1{A/A} M1{+/+} ,P1{A/G or G/G} M1{-/-},P1{A/A} M1{-/-},P1{A/G or G/G}	 78 (47.3)* 49 (29.7) 30 (18.2) 8 (4.8)	 69 (46.0) 36 (24.0) 24 (16.0) 21 (14.0)	 0.49 0.75 0.15	 1.0 (Reference) 0.83 (0.48-1.42) 0.90 (0.48-1.69) 2.98 (1.23-7.12)
GSTT1 and *GSTP1* T1{+/+},P1{A/A} T1{+/+},P1{A/G or G/G} T1{-/-},P1{A/A} T1{-/-},P1{A/G/or G/G}	 93 (56.4)* 41 (24.8) 15 (9.1) 16 (9.7)	 75 (50.0) 47 (31.3) 18 (12.0) 10 (6.7)	 0.18 0.29 0.55	 1.0 (Reference) 1.42 (0.84- 2.38) 1.48 (0.70-3.14) 0.77 (0.33- 1.80)
Triple				
GSTM1, GSTT1 and *GSTP1* M1{+/+},T1{+/+},P1{A/A} M1{+/+},T1{+/+},P1{A/G or G/G} M1{-/-},T1{+/+},P1{A/A} M1{-/-},T1{+/+},P1{A/G or G/G} M1{+/+},T1{-/-},P1{A/A} M1{+/+},T1{-/-},P1{A/G or G/G} M1{-/-},T1{-/-},P1{A/A} M1{-/-},T1{-/-},P1{A/G or G/G}	 65 (39.4)* 34 (20.6) 28 (17.0) 7 (4.2) 13 (7.9) 15 (9.1) 2 (1.2) 1 (0.6)	 55 (36.7) 29 (19.3) 20 (13.3) 18 (12.0) 14 (9.3) 7 (4.7) 4 (2.7) 3 (2.0)	 0.98 0.62 0.02 0.57 0.22 0.33 0.27	 1.0 (Reference) 1.00 (0.46 - 2.16) 0.84 (0.36 - 1.95) 3.04 (1.18 - 7.81) 1.27 (0.55 - 2.93) 0.55 (0.21 - 1.45) 2.36 (0.41 - 13.39) 3.55 (3.55 - 35.06)

Significance < 0.05, *1.0 (Reference)

In case of triple combinations, *GSTM1* (-/-), *T1* (+/+) and *P1* (A/G or G/G) showed significant association (*P*=0.02) with a high risk of 3.04-folds (Table 4). However, genotypic combinations of *GSTM1* (-/-), *T1* (-/-), *P1* (A/A) and *GSTM1* (-/-), *T1* (-/-), *P1* (A/G or G/G) showed higher risk (2.36 and 3.55-folds respectively) without any significant association.

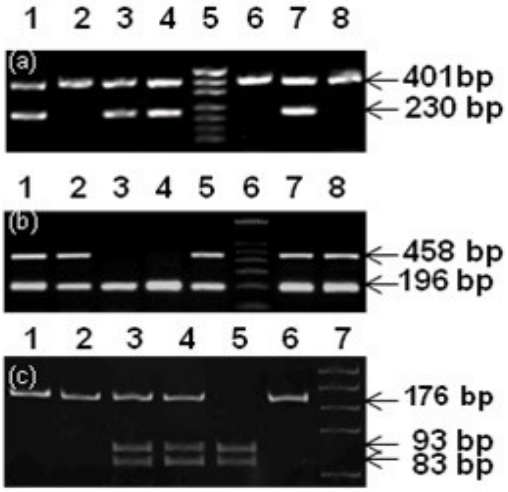
The association studies with passive smoking showed that *GSTM1* (-/-) genotype had significant association (*P*=0.004) with a risk of 4.19-folds while *T1* (-/-) genotype showed marginal risk of 1.81-folds in developing cervical cancer (Table 5). In case of *GSTP1* the G/G genotype increased risk of cervical cancer in passive smokers by 2.88-folds (Table 5).

Table.5: *GSTM1*, *T1* and *P1* genotypes and their association with smoking status in controls and cervical cancer cases.

Population (Smoking Status)	Genotype Number (%)										
	GSTM1			*GSTT1*			*GSTP1*				
	(+/+)	(-/-)	P-value OR (95%CI)	(+/+)	(-/-)	P-value OR (95%CI)	AA	AG	P-value OR (95%CI)	GG	P-value OR (95%CI)
Never											
Controls	94(57.0) *	32(19.4)	0.59 0.44 (0.19-1.03)	100 (60.6)*	26(15.8)	0.49 0.75 (0.33-1.68)	85(51.5)*	33(20.0)	0.25 0.64 (0.30-1.36)	8(4.9)	0.16 0.21 (0.02-1.82)
Cases	53(35.3)	8(5.3)		51 (34.0)	10(6.7)		48(32.0)	12(8.0)		1 (0.7)	
Active											
Controls	0	0		0	0		0	0		0	
Cases	6(4.0)	2(1.4)		7(4.7)	1(0.6)		5(3.3)	1(0.7)		2(1.3)	
Passive											
Controls	33(20.0) *	6(3.6)	0.004 4.19 (1.57-11.09)	34(20.6) *	5(3.0)	0.28 1.81 (0.61-5.32)	23(13.9)*	15(9.1)	0.42 1.38 (0.62-3.04)	1(0.6)	0.34 2.88 (0.31-26.14)
Cases	46(30.7)	35(23.3)		64(42.7)	17(11.3)		40(26.7)	36(24.0)		5(3.3)	

CI = Confidence Interval; OR= Odds Ratio; (+/+) =Present; (-/-) =Null; Significance < 0.05, *1.0 (Reference)

Fig 1. Ethidium bromide (EtBr) stained agarose and polyacrylamide gels showing different genotypes of *GSTM1, T1* and *P1* genes.



(a) Lanes 1, 3, 4 and 7:*GSTM1* (+/+); Lanes 2,6 and 8: *GSTM1* (-/-) ; Lane 5: pUC19DNA/*MspI* digest; Positive control *CD36* gene (401bp)
(b) Lanes 1, 2, 5, 7 and 8:*GSTT1* (+/+); Lanes 3 and 4: *GSTT1* (-/-); Lane 6: 100bp ladder, positive control *CYP1A1* (196bp)
(c) Lanes 1, 2 and 6: *GSTP1* (A/A); Lanes 3, 4; *GSTP1* (A/G); Lane 5: *GSTP1* (G/G) Lane 5: *GSTP1* (G/G) ; Lane 7: pUC19DNA/ *MspI* digest.

4. Discussion

Genetic variations in genes encoding antioxidant enzymes (CYP and GST) have been observed amongst individuals which lead to susceptibility and development of various cancers[20]. Homozygous null genotypes of *GSTM1* and *T1* genes are frequently observed in lung and bladder cancers[21-23]. Polycyclic aromatic hydrocarbons and many other carcinogens require metabolic activation by phase I enzymes such as CYP1A1 which are detoxified by phase II enzymes such as GSTM1 and T1[24]. Those reactive metabolites which are not detoxified might react with DNA and lead to mutations. Low levels of phase II enzyme activity would therefore result in higher levels of active metabolites and consequently more DNA damage. Null genotype of *GSTM1* was found to be significantly associated with an increased risk of cervical cancer in Korean women with high risk HPV infection[25,26] including bladder, skin, lung, breast and ovary as well[27]. Environmental carcinogens such as 1, 3 butadiene in air and tobacco smoke were detoxified by *GSTT1*[28]. Null genotypes of *GSTT1* have an increased risk of cervical cancer in Korean women and ~15-20% of Caucasians[25]. An A/G polymorphism at the nucleotide level located within the substrate binding domain of *GSTP1* leads to an amino acid variation of isoleucine to valine at codon 105 (Ile 105 Val) in the protein which decreases enzyme activity[29].

Cigarette smoking either active or passive has been linked to the secretion of tumor specific metabolites in cervical mucus. This mucus maintains cervical HPV infection longer and decreases potential of clearing an oncogenic infection[30]. Women having *GSTP1* A/A (homozygous for the variant allele) genotype have an increased risk of invasive cervical cancer which is higher among active smokers[31]. In Indian population, women are rarely smokers however, exposure to smoke is passive which has been found to increase the risk of cervical cancer[32]. Due to absence of active smokers in the control group we did not find any association between active smokers and cervical cancer cases but significant association was found with cervical cancer in case of passive smokers (P<0.0001) (Table 2).

In the present study, influence of *GSTM1*, *T1* and *P1* gene polymorphisms in susceptibility to cervical cancer was evaluated. Like other studies, in the present study population individuals with null genotypes of *GSTM1* and *T1* were not found to be at risk of cervical cancer (Table 3)[33,34]. The association of *GST* polymorphisms with histological subtypes, squamous cell carcinoma and adenocarcinoma in Table 3 showed that *GSTM1* (-/-) genotype was significantly (P=0.02) associated with adenocarcinoma and the risk of developing cervical cancer was 4.01-folds. *GSTP1* genotypes did not show significant association with adenocarcinoma but individuals with A/G and G/G genotypes respectively were at 2.25 and 2.40 times higher risk of developing adenocarcinoma of the cervix.

Sobti *et al*[4] did not show any risk of developing cervical cancer with *GSTM1* (-/-) and *T1* (-/-) genotypes taken together. Although the P-value was not significant, the present study showed a 2.75-times higher risk of developing cervical cancer with the *GSTM1/T1* (-/-) double combination (Table 4). Individuals with *GSTM1* (-/-) and *P1* (A/G or G/G) genotypes in double combination showed an increased risk of 2.98-folds while *GSTM1* (-/-), *T1* (+/+) and *P1* (A/G or G/G) revealed 3.04-folds increased risk and a significant association in triple combination (P=0.02). *GSTM1* (-/-), *T1* (-/-) and *P1* (A/A) showed non-significant association (P=0.33) with cervical cancer but a risk of 2.36-folds. Similar result was obtained in case of *GSTM1* (-/-), *T1* (-/-) and *P1* (A/G or G/G) combination with a 3.55-folds increased risk (Table 4).

The association of gene variants with smoking status was also analyzed. The association of null genotypes of *GSTM1* (-/-) in passive smokers showed significant association (P=0.004) with an increased risk of 4.19-folds of developing cervical cancer (Table 5). In a previous study, *GSTP1* (A/G) genotype showed a risk of 6.4-folds and significant association in case of passive smokers[3] but our study showed an increased risk of 2.88-folds in passive smokers with *GSTP1* (G/G) genotype while a marginal risk of 1.81-folds was observed in *GSTT1* (-/-) individuals (Table 5).

5. Conclusion

It is evident from this study that risk of cervical cancer significantly increases in passive smokers with *GSTM1* (-/-) genotype while *GSTT1* (-/-) and *GSTP1* (G/G) genotypes showed 2-3 times higher risk although these associations were not statistically significant. However, larger sample sizes are required to confirm the possible interactions between different *GST* polymorphisms and passive smoking in cervical cancer cases from North India.

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