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ORIGINAL ARTICLE

Hypermethylation of carcinogen metabolism genes, *CYP1A1*, *CYP2A13* and *GSTM1* genes in head and neck cancer

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OBJECTIVES: To investigate the role of aberrant hypermethylation of carcinogen metabolism pathway genes, *CYP1A1*, *CYP2A13* and *GSTM1* in head and neck cancer independently as well as its relation to tobacco and alcohol consumption and *CYP1A1* and *CYP2A13* polymorphisms in Indian population.

METHODS: Seventy-three histologically confirmed head and neck cancer patients undergoing treatment in Post-graduate Institute of Medical Education and Research, Chandigarh, India were recruited. Non-cancerous tissues were obtained from 19 trauma subjects undergoing maxillofacial surgery. Methylation-specific PCR was performed to determine the methylation status of selected genes.

RESULTS: The aberrant hypermethylation of *CYP1A1*, *CYP2A13* and *GSTM1* genes was found in cancer tissues with frequency of about 39.7%, 27.4%, and 58.1%, respectively, and in normal healthy tissues with a frequency of about 10.5%, 15.8%, and 20.0%, respectively. Hypermethylation of *CYP1A1* (*P* 0.027) and *GSTM1* (*P* 0.010) showed significant association with head and neck cancer. We also observed significant interaction between smoking and methylation status of *CYP1A1* (*P* 0.029) and *CYP2A13* (*P* -0.034) in head and neck cancer. No association was observed between methylation status and alcohol consumption, clinical features and genetic polymorphisms of *CYP1A1* and *CYP2A13*.

CONCLUSIONS: Hypermethylation of carcinogen metabolism pathway genes independently and in interaction with smoking is associated with increased risk of head and neck cancer.
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Keywords: methylation; head and neck cancer; *CYP1A1; CYP2A13; GSTM1;* smoking

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This is the first study to report that the genes of carcinogen metabolism pathway, namely *CYP1A1*, *CYP2A13* and *GSTM1* are aberrantly hypermethylated in head and neck cancer. Further, we showed association of hypermethylation of *CYP1A1* and *CYP2A13* genes with tobacco smoking in head and neck cancer. This manuscript provides an evidence for smoking related methylation changes that may contribute to head and neck carcinogenesis.
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Introduction

Head and neck cancer is the sixth most common type of cancer, representing about 6% of cases worldwide and is the commonest cancer in India (Parkin *et al*, 2005). The development of head and neck cancer is multifactorial in nature, associated with variety of risk factors which include tobacco and alcohol consumption (Choi and Kahyo, 1991). Despite the advancement in diagnosis and molecular mechanisms underlying head and neck cancer, the 5-year survival rate for patients with head and neck cancer has not changed significantly (Le and Giaccia, 2003). To improve this situation, it is necessary to understand the fundamental biological processes leading to the disease and its progression.

Progression of head and neck cancer is driven by genetic and epigenetic factors. Genetic polymorphisms of various genes belonging to carcinogen metabolism, DNA repair and cell cycle control and oncogenes have been reported to influence susceptibility to develop cancer. Carcinogen metabolism is an important facet of neoplastic process. This pathway includes activation and detoxification of carcinogens that involves phase I and phase II enzymes. Cytochrome P450, phase I enzymes are involved in metabolic activation of tobacco-derived procarcinogens such as polycyclic aromatic hydrocarbons and nitrosamines while Glutathione S-transferases, phase II enzymes detoxify many electrophilic substrates by conjugation with reduced glutathione. Imbalance between activation and detoxification of carcinogens

leads to DNA damage and consequently to carcinogenesis process (Smith *et al*, 1995; Geisler and Olshan, 2001). Promoter methylation has been found to be another important mechanism of gene inactivation during carcinogenesis besides mutations and deletions (Rodenhiser and Mann, 2006). Hypermethylation of regulatory sequences of various tumor suppressor genes has been observed to cause transcriptional silencing of these genes in various types of cancer. It has been proposed that hypermethylation could be used not only as biomarkers for the early detection of head and neck cancer but also to improve prevention strategies and therapy outcomes (Nephew and Huang, 2003). Although epigenetic alterations of tumor suppressor genes have been recently found to be associated with tobacco-related head and neck cancer (Puri *et al*, 2005; Chen *et al*, 2007), however, information on methylation status of genes involved in carcinogen metabolism pathway in tobacco-related head and neck cancer is lacking. Since both carcinogen metabolism as well as DNA methylation, are early events in tumorigenesis, they may facilitate not only the elucidation of biomarkers for the early detection of head and neck cancer but also the timely diagnosis and improved treatment. Moreover, there is no report available which has studied the association between epigenetic and genetic factors in head and neck cancer. Against this background, we have examined the association of genetic polymorphisms, environmental factors as well as promoter methylation status of carcinogen metabolism pathway genes with head and neck cancer in the present study.

Materials and methods

Specimens

Seventy-three individuals diagnosed with primary head and neck cancer (consisting of oral cavity, pharynx and larynx) and nineteen controls were enrolled in this study. Biopsies were obtained from patients, prior to starting any treatment. The diagnosis of primary head and neck cancer was confirmed by the histopathological examination. All samples were also screened for human papillomavirus (HPV) infection and only samples negative for HPV were included in the present study. Noncancerous tissues (oral and pharyngeal) were obtained from trauma cases undergoing maxillofacial surgery. Absence of tumor cells in normal tissues was confirmed histopathologically. All the subjects were interviewed using a standard questionnaire regarding the demographic information, use of smoking and alcohol consumption, family history of cancer and medical history prior to diagnosis date of cancer. Smokers included individuals who smoked tobacco in form of cigarette and beedi (a form of smoking prevalent in India). The lifetime smoking consumption was expressed as pack years. Patients with chewing habit were not included in the present study as this habit was found to be absent in control group. The study was approved by Institute Research Ethics Committee, PGIMER and all subjects enrolled in this study provided informed consent.

DNA extraction and isolation

Genomic DNA was isolated from the frozen tissue using standard proteinase K digestion method and purified by phenol–chloroform extraction.

Bisulphite DNA modification and methylation-specific PCR

The CpG island methylation status of *CYP1A1*, *CYP2A13* and *GSTM1* genes was determined using methylation-specific PCR (MSP) (Herman *et al*, 1996). Bisulphite modification of genomic DNA was performed using EZ DNA GOLD kit following manufacturer's instructions. Primers for MSP were designed by using the online program Methprimer (http://www.urogene.org/methprimer). All the primer sequences were designed to contain atleast one CpG site at 3′ end to facilitate maximal discrimination between methylated and unmethylated DNA. Primer sequences and PCR conditions used for MSP are described in Table 1. Genomic DNA treated with SssI methylase and blood DNA samples were used as positive and negative controls for methylation respectively. Figure 1 shows the MSP for the selected genes. All the samples analyzed in this study were amplified in duplicates to ensure the specificity of MSP.

Table 1 Primer sequence and conditions for methylation specific PCR

Gene	*Primers*	*Amplicon (bp)*	*Ta (°C)*	*$MgCl_2$ (mM)*	*MSP target region (nt pos)*	*CpG sites in amplicon*
CYP1A1 M	F-TAATTTCGGGAAGGAGGTTATTACR-CACCCACTAAAACGCTAAACG	108	58.3	2.0	CpG island II 525–632	8
CYP1A1 U	F-TAATTTTGGGAAGGAGGTTATTATR-CACCCACTAAAACACTAAACATA	110	54.3	2.0		
CYP2A13 M	F-TTTCGCGTTTATTTTTTTAGGCR-AATCCCCTACTCACCGTACGTA	184	58.5	2.5	CpG island 1 1565–1748	21
CYP2A13 U	F-TTTTTGTGTTTATTTTTTTAGGTGTR-AAATCCCCTACTCACCATACATA	186	56.4	2.0		
GSTM1 M	F-GATATTGGGGTATTGGGATATTCR-CATACACAAACCCTTCTAAAACG	153	56.0	3.0	CpG island I 63–215	13
GSTM1 U	F-TGATATTGGGGTATTGGGATATTTR-TACACAAACCCTTCTAAAACAAA	154	56.6	2.5		

M, methylation; U, unmethylation; Ta, optimized annealing temperature; nt pos, nucleotide position.

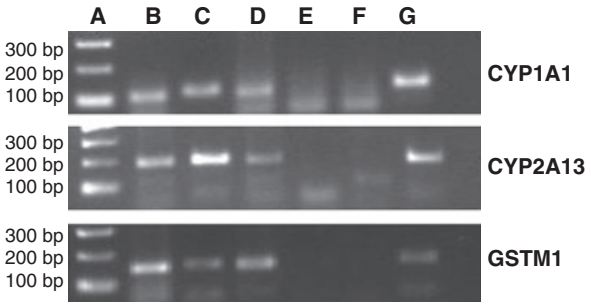

Figure 1 Representative results of methylation specific PCR analysis for three genes. Lane A 100 bp ladder, Lane B universal methylated DNA used as positive control for methylation, Lane C: bisulphite treated blood DNA used as positive control for unmethylation, Lanes D and F: tumor samples amplified methylated primers, Lanes E and G: tumor samples amplified unmethylated primers

Analysis of genetic polymorphisms

Polymorphisms in *CYP1A1* (T3801C, A2455G, and C2453A) were genotyped using PCR–RFLP method as described by Li *et al* (2004). In case of *GSTM1* gene, the samples were first screened for the presence of null deletion using the end point PCR method described by Anantharaman *et al* (2007) and promoter methylation was assessed only in the subjects carrying functional GSTM1 enzyme. The *GSTM1* null deletion and *CYP1A1* T3801C were detected using duplex PCR, whereby *CYP1A1* amplicon served as positive control in *GSTM1* amplification. C3375T and C7520G polymorphisms of *CYP2A13* were identified using PCR–RFLP as reported earlier by Cauffiez *et al* (2005).

Data analysis

Differences in promoter hypermethylation were analyzed between the cancerous and normal tissues using Fischer's exact test. Logistic regression analysis was performed to determine association between methylation status and environmental factors and genetic polymorphisms after adjusting for potential confounders. The statistical analysis was performed using the SPSS software (version 13). *P* values less than 0.05 were considered statistically significant.

Results

Demographic characteristics of study population are given in Table 2. All malignancies were histopathologically diagnosed as squamous cell carcinoma, with 61.4% of tumors belonging to stage IV according to TNM staging for head and neck cancer.

The results of methylation patterns of CYP1A1, CYP2A13 and GSTM1 observed in our study are shown in Table 3. 39.7% patients (29/73) showed CpG island methylation of *CYP1A1* gene as compared to 10.5% (2/19) controls. 27.4% patients (20/73) showed methylation of promoter region for *CYP2A13* as compared to 15.8% (3/19) controls. Promoter methylation of *GSTM1* was detected in 25 of 43 cases (58.1%) and 2 of 10 controls (20%). No significant correlation was observed between methylation status of any of genes with clinical features (age, gender, anatomic site and clinical staging) of the cancer patients (data not shown).

Table 2 General characteristics of our study population

	Cases, n *(%)*	*Controls,* n *(%)*
Gender		
Males	65 (89.0)	16 (84.2)
Females	8 (11.0)	3 (15.8)
Mean age (years ± SD)	50.53 ± 9.49	45.03 ± 8.16
Smoking status		
Smokers	64 (87.7)	12 (63.0)
Non-smokers	9 (12.3)	7 (37.0)
Alcohol consumption		
Drinkers	35 (47.9)	10 (52.6)
Non-drinkers	38 (52.1)	9 (47.4)

Table 3 Distribution of methylated CYP1A1, CYP2A13 and GSTM1 in patients and controls

	Methylation Patients, n *(%)*	*Methylation Controls,* n *(%)*	*OR (95% CI)*	*P* value
CYP1A1	29 (39.7)	2 (10.5)	5.6 (1.2–26.08)	0.027
CYP2A13	20 (27.4)	3 (15.8)	2.0 (0.53–7.65)	0.30
GSTM1	25 (58.1)	2 (20.0)	6.9 (1.35–35.60)	0.02

Table 4 Interaction between smoking and alcohol intake and gene hypermethylation in head and neck cancer

Gene	*Exposure*	*Methylation Patients,* n *(%)*	*Methylation Controls,* n *(%)*	*P* value
CYP1A1	Smokers	26 (40.6)	2 (16.6)	0.029
	Drinkers	12 (34.2)	1 (10.0)	0.75
CYP2A13	Smokers	18 (28.12)	2 (16.67)	0.024
	Drinkers	8 (22.86)	2 (20.0)	0.76
GSTM1	Smokers	17 (45.0)	2 (25.0)	0.34
	Drinkers	10 (48.0)	2 (33.0)	0.54

When we stratified our study population by smoking, methylation in *CYP1A1* and *CYP2A13* genes showed significant association with head and neck cancer among smokers (*CYP1A1*, *P* 0.029 and *CYP2A13*, *P* 0.034). No correlation was found between *GSTM1* promoter hypermethylation and smoking status. We did not observe any significant interaction between alcohol consumption and methylation status for all the three genes analyzed in present study (Table 4).

No significant association was observed between *CYP1A1* (T3801C, A2455G, C2453A and T3205C) and *CYP2A13* (C3375T and C7520G) polymorphisms and methylation status in head and neck cancer (Table 5).

Discussion

In the present study, we report that hypermethylation of two genes (*CYP1A1* and *GSTM1*), involved in metabolic activation and detoxification of tobacco-derived

Table 5 Association of CYP1A1 and CYP2A13 genotypes with methylation status of selected genes in head and neck cancer patients

Gene/ polymorphism	*CYP1A1 methylation* n *(%)*	*CYP2A13 methylation* n *(%)*	*GSTM1 methylation* n *(%)*
CYP1A1			
T3801C			
TT	18 (62.1)	9 (45.0)	17 (68.0)
TC	10 (34.5)	7 (35.0)	7 (28.0)
CC	1 (3.4)	4 (20.0)	1(4.0)
	P 0.274	*P* 0.079	*P* 0.22
A2455G			
AA	23 (79.3)		18 (90.0)
AG	6 (20.7)		2 (10.0)
GG	–	–	–
	P 0.64	*P* 0.197	*P* 0.063
C2453A			
CC	28 (96.6)	20 (100.0)	23 (92.0)
AC	1 (3.4)	–	2 (8.0)
AA	–	–	–
	P 0.23	–	*P* 0.059
CYP2A13			
C3375T			
CC	27 (93.1)	19 (95.0)	23 (92.0)
CT	2 (6.9)	1 (5.0)	2 (8.0)
TT	–	–	–
	P 1.0	*P* 1.0	*P* 0.065
C7520G			
CC	28 (96.6)	20	24 (96.0)
CG	1 (3.4)	–	1 (4.0)
GG	–	–	–
	P 0.39	–	*P* 0.223

carcinogens was associated with the risk of head and neck cancer. We further found the significant interaction between promoter methylation and smoking status in head and neck cancer. Numerous studies have investigated the occurrence of hypermethylation in genes involved in pathways such as cell cycle control, apoptosis, DNA repair, angiogenesis, cell signaling, metastasis in head and neck cancer (Sanchez-Cespedes *et al*, 2000), this is the first study to report hypermethylation of promoter region within metabolic genes in association with polymorphisms in head and neck cancer.

CYP1A1 belongs to phase I enzymes that metabolize pro-carcinogens present in tobacco and result in their activation into highly reactive mutagenic intermediates (Kawajiri, 1999). It is an important enzyme in initiation of tobacco-related carcinogenesis and a tissue-specific regulation of its expression in response to tobacco exposure has been reported (Czekaj *et al*, 2005). We observed increased methylation of *CYP1A1* gene, in head and neck cancer patients as compared to controls. Methylation of *CYP1A1* gene has been found to result in an altered inaccessible chromatin structure that suppresses its transcriptional responsiveness to mutagens (Okino *et al*, 2006). Thus, aberrant hypermethylation of *CYP1A1* seen in our patients may lead to transcriptional inactivation of *CYP1A1* and result in decreased metabolic activation of procarcinogens present in tobacco along with their decreased detoxification, resulting in increased exposure to tobacco carcinogens and increased risk of cancer. Hypermethylation of *CYP1A1* has been reported in human prostrate cancer (Okino *et al*, 2006), but no change in *CYP1A1* methylation status was found in lung cancer (Anttila *et al*, 2003). These observed differences may reflect tissue-specific regulation of methylation of this gene (Takahashi *et al*, 1998; Nakajima *et al*, 2003; Czekaj *et al*, 2005).

We further studied the methylation of phase II enzyme, *GSTM1* which contributes to elimination of wide variety of carcinogens present in tobacco. Numerous studies have provided an evidence for association of *GSTM1* null deletion and cancer risk (Jourenkova-Mironova *et al*, 1999; Olshan *et al*, 2000). The null deletion of *GSTM1* caused by homozygous deletion of the respective gene, leads to absence of *GSTM1* enzyme activity for detoxification process and subsequent elevated risk of head and neck cancer. In order to identify the mechanism responsible for carcinogenesis in individuals carrying functional *GSTM1* enzyme, we first screened our tumor samples for *GSTM1* null deletion, then analyzed *GSTM1* promoter methylation in the samples negative for null deletion. We observed significantly higher methylation in *GSTM1* gene in head and neck cancer patients as compared to controls. A previous report had suggested that *GSTM1* promoter is hypermethylated in prostate cancer (Lodygin *et al*, 2005); however, this is the first study reporting *GSTM1* methylation in head and neck tumors. Methylation of *GSTM1* suggests that its transcriptional repression leading to compromised detoxification, may be a mechanism for head and neck cancer pathogenesis in patients not harboring null deletion.

We found no significant difference in methylation status of *CYP2A13* between patients and controls. The prevalence of methylation of *CYP2A13* has been studied for the first time and does not appear to be a significant contributor to head and neck carcinogenesis.

We also observed significant interaction between smoking, DNA methylation and head and neck cancer. Hypermethylation in *CYP1A1* and *CYP2A13* genes was found to be significantly associated with head and neck cancer among smokers as compared to non-smokers and was observed in smokers only, irrespective of their disease status in the present study. It is speculated that tobacco smoking induces aberrant methylation of these genes in tumor cells of head and neck region. Hypermethylation in normal oral mucosa of smokers has also been reported by von Zeidler *et al* (2004). The association of hypermethylation with tobacco smoking has also been reported in various other genes in head and neck cancer including *p15* and *E cad* (Hasegawa *et al*, 2002; Wong *et al*, 2003). Tobacco smoke contains an array of carcinogens including nitrosamines, polycyclic aromatic hydrocarbons, chromium, cadmium, plutonium and nickel. In addition, tobacco smoke is a mucosal irritant which induces inflammation resulting in generation of reactive oxygen species (Hecht, 2001). Furthermore, smoking increases the activity of DNA methyltransferase which drives the de novo hypermethylation of susceptible loci (Belinsky *et al*, 1996; Vertino *et al*, 1996). These direct and indirect

mechanisms could be involved in the enhanced methylation in smokers in head and neck cancer.

We did not observe any significant association between promoter methylation of studied genes and age, gender, site, tumor size and nodal status in head and neck cancer. These results are in agreement with the earlier studies performed in head and neck cancer with other genes including *p16, hMLH1* and *MGMT* (Chen *et al*, 2007). We also did not observe any association between methylation, alcohol exposure and risk of head and neck cancer in our study.

Various epidemiological studies have investigated the association of genetic polymorphisms with promoter methylation status in various diseases (Anttila *et al*, 2003; Nan *et al*, 2005, Kawakami *et al*, 2006; Siraj *et al*, 2007; Kang *et al*, 2008). For example, Kang *et al*, found polymorphisms of *p14* gene to be associated with its methylation status among individuals with colorectal cancer (Kang *et al*, 2008). Although, several studies have shown significant association of genetic polymorphisms of *CYP1A1* and *CYP2A13* with risk of cancer (Park *et al*, 1997; Cauffiez *et al*, 2004; Jiang *et al*, 2004; Gattas *et al*, 2006; Cha *et al*, 2007), we did not observe any interaction between polymorphisms of *CYP1A1* and *CYP2A13* and the methylation status of *CYP1A1, CYP2A13* and *GSTM1* genes in our study. Our results are consistent with a previous study in lung cancer, in which none of the variant genotypes of *CYP1A1* was found to be associated with methylation of *CYP1A1* in lung (Anttila *et al*, 2003). These findings suggest that genetic polymorphisms of *CYP1A1* and *CYP2A13* genes may act as independent risk factors but do not play any role in modulating the risk of methylation of candidate genes in head and neck cancer tissues. This is the first report analyzing the effect of polymorphisms in *CYP1A1* and *CYP2A13* genes and methylation of the *CYP1A1, CYP2A13* and *GSTM1* genes. Genetic variants and DNA methylation in tobacco metabolism are interesting candidates for further study as it may lead to better understanding of susceptibility factors that predispose tobacco consumers to acquisition of multiple epigenetic alterations in key cellular regulatory genes and are currently being investigated in large cohorts of head and neck cancer patients.

A potential limitation of our study is the relatively small sample size of controls. It is difficult to obtain normal healthy tissues even from tertiary hospital like ours due to ethical considerations. Most of the earlier studies have taken tumor margin tissues as controls; however, we have used normal tissue from healthy controls for the comparison of methylation status of selected genes. As margins of tumor tend to show same methylation patterns, a comparison between normal tissues from healthy unrelated individuals would provide better comparison of methylation status.

In conclusion, our study showed that hypermethylation of carcinogen metabolism pathway genes is a frequent event in head and neck cancer. Further, the aberrant hypermethylation of these genes may result in altered exposure to tobacco carcinogens and subsequently elevated risk to head and neck cancer. These results may be useful for the future study of smoking-related epigenetic alterations in head and neck carcinogenesis.

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