African Journal of Biotechnology Vol. 10(27), pp. 5273-5280, 15 June, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1743
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

A novel *CYP1A1* gene polymorphism and the risk of head and neck cancer in Pakistani population

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Accepted 4 March, 2011

Several polymorphisms in **the** *CYP1A1* **locus have been identified and their genotypes appear to exhibit population frequencies that depend on ethnicity. In this study, we assessed the role of** *CYP1A1* **genotype** in 388 **head and neck cancer patients in Pakistani population via a case-control study. Polymerase chain reaction (PCR) and single stranded conformational polymorphism assays were used. Most of the patients (51%) enrolled for the study, were from the age group of 40 to 60 years (±16.59). Mean age of the cancer patients involved in the study was 48 ± 16.59 years. Statistical analysis has shown that, tobacco users have more chances of head and neck cancer (P < 0.05) than non tobacco users, whereas male to female ratio is 1:1 (P > 0.05). Jobless persons are more prone to head and neck cancer (P < 0.01) compared with employers and housewives. After the genetic analysis, it was found that no already reported variants of *CYP1A1* gene were found in Pakistani population. A novel mutation in *CYP1A1* gene at exon 2 in 21 patients (P < 0.001, Odd Ratio (OR) = 9.4 and 95% confidence interval (CI) 1.3 to 70.8) has been found with a serine formation instead of tyrosine at amino acid 110. The patients showing this mutation have the mean age of 51.75 (±15.7). Therefore, mutation in *CYP1A1* gene may be one of several factors that increase the chance of developing head and neck cancer.**

Key words: Cytochrome P450 1A1 gene (*CYP1A1*), head and neck cancer (HNC), mutation, novel polymorphism, Pakistani population.

INTRODUCTION

Head and neck cancer (HNC) includes the cancers of oral cavity, larynx and pharynx and is the sixth most frequent cancer worldwide and is particularly, high in south East Asian countries (Johnson 1991; Parkin et al., 2002). In Pakistan, incidence of head and neck cancer is 40.1% of all the cancers (Hanif et al., 2009) and the second most frequent type of prevalent cancers (Faheem, 2007). In France, cancer of pharynx is more common in men, while cancer of oral cavity is more common in women in India. In Europe and Japan, (Makimoto et al., 2000) marked rise in incidence of HNC has been reported in last few decades (Sankaranarayanan et al., 1998; Morelatto and Lopez, 2006).

Multitudes of factors are responsible for occurrence of HNC like lifestyle, dietary habits and mental stress (Jensen et al., 2010). Workers of mines and nickel or wood industry are more prone to HNC (Tevfik et al., 2007). Environmental factors like chewing tobacco and alcohol are given special attention in relation to head and neck cancer (Sabitha et al., 2010). Exposure to synthetic or natural chemical compounds present in the environment is usually associated with HNC. Xenobiotics metabolizing enzymes are responsible for metabolism of many exogenous chemicals that are toxic, mutagenic or carcinogenic. Carcinogen detoxifying enzymes include the phase I enzymes involved in the detoxification of carcinogens and either neutralize them or change them into electrophilic compounds that are detoxified by the phase II enzymes (Rajani et al., 2003).

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Abbreviations: ***CYP1A1,*** Cytochrome P450 1A1 gene; **HNC,** head and neck cancer; PCR, polymerase chain reaction; GSTM1, glutathione-S-transferase isozyme M1; GSTP1, glutathione-S-transferase isozyme P1; GSTT1, glutathione-S-transferase isozyme T1; OR, odd ratio; CI, confidence interval.

The principal enzymes responsible for phase I reaction belong to cytochrome P-450 multigene family. The cytochrome P450 1A1 enzyme functions by the addition of oxygen atom into the toxic chemical and initiate detoxification and elimination by increasing hydrophilicity (Guengerich and Shimada 1991). Cytochrome P450 1A1 gene (*CYP1A1*) is located on chromosome 15q22-24 and encodes aromatic hydrocarbon, hydroxylase that converts polycyclic aromatic hydrocarbons (PAHs) (Shimada et al., 1989) to carcinogen and is predominantly expressed in extrahepatic tissues including lungs (Anttila et al., 1992).

The cytochrome P-450 that are known to exhibit polymorphism include *CYP1A1*, CYP1B1 (Arrind et al., 2008), CYP2A6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1 (Rajani et al., 2003). Polymorphism of *CYP1A1* gene has been studied with relation to different cancers including head and neck cancer (Sabitha et al., 2010). Four different sequence polymorphisms have been reported in *CYP1A1* gene, first known as *CYP1A1**2 involves a T_{6235} to C transition in the 3' noncoding region (Kawajiri et al., 1990; Jun et al., 2010), second known as *CYP1A1**3 involve a A_{4889} to G transition in exon 7 (Jun et al., 2010; Hayashi et al., 1991), third known as *CYP1A1**4 involves a T_{5639} to C transition in intron 7 (Crofts et al., 1993) and fourth known as *CYP1A1**5 involves a C_{4887} to A transition in exon 7 (Jun et al., 2010; Cascorbi et al., 1996). The present study is aimed at evaluating the role of environmental factors in head and neck cancer risk along with *CYP1A1* gene polymorphisms in Pakistani population.

MATERIALS AND METHODS

Identification of patients and normal controls

The present case-control study consisted of 388 cases with pathologically confirmed head and neck cancer along with age and sex matched 150 cancer free normal individuals as controls. They were recruited from National Oncology and Radiotherapy Institute (NORI) and Pakistan Institute of Medical Sciences (PIMS), Islamabad from March 2008 to September 2009 with a prior approval from Ethical Committees of both university and hospitals.

All study subjects participated on a volunteer basis. All subjects were personally interviewed according to a structured questionnaire. They were asked about area of cancer, age, tobacco addictions and occupational exposures. Blood was collected from subjects with their informed consent. Information concerning alcohol intake was found fairly unreliable and was disregarded due to Muslim community. However, our experiences with patients and control blood donors have shown that, majority are tobacco addicted in the form of betel quids and moist snuff. Subjects' blood was sampled before starting the therapy.

Sample collection and DNA isolation

Blood samples were collected in EDTA-containing tubes and stored at -20°C until further use. DNA was isolated, using organic protocol with phenol-chloroform extraction as previously described (Baumgartner-Parzer et al., 2001; Vierhapper et al., 2004).

Electrophoresis was performed on isolated DNA on 1% ethidium-bromide stained agarose gel and photographed (BioDocAnalyze Biometra). 5 ng dilutions were made of each DNA isolated and stored at 4°C until use.

Primer designing and polymerase chain reaction (PCR)

Primers for 7 exons of *CYP1A1* were synthesized by using primer 3 input software version 0.4.0 (Table 1) and BLAST using NCBI PRIMER BLAST. 2 µl DNA (10 ng/µl) was added to a 20 µl PCR mixture composed of 2 µl PCR buffer, 2 µl of each primer (10 mM), 0.24 µl deoxynucleotide triphosphate (25 mM) and 0.2 µl Taq polymerase (5 u/µl). The reaction mixture was placed in 9700 thermal cycler of ABI systems for 5 min at 94°C and subjected to 30 cycles at 94°C for 25 s, annealing temperature for 1 min and 72°C for 1 min, followed by a final step at 72°C for 10 min and held at 4°C.

Amplification products were resolved on a 2% ethidium bromide-stained agarose gel along with 100 bp DNA ladder. All the patients and control DNA was amplified for all the 7 exons of *CYP1A1* gene with exon specific primers. All of the photographs of gel electrophoresis were read by two technicians blind to each other's assessments.

Single strand conformational polymorphism

PCR product was analyzed by single stranded conformational polymorphism (SSCP) using the procedure described by Patricia et al. (2009) and Amalio et al. (1993) with some minor modifications. SSCP results were analyzed with gel documentation system (BioDocAnalyze Biometra) after ethidium bromide staining and photographed. The samples showing mobility shifts from the controls were then sequenced.

Sequencing

21 samples were screened out from SSCP and were sequenced from Macrogen (Korea). Reverse primer was used for sequencing. The sequenced results were made forward complementary before analysis using BioEdit v 7.0.5 software and analyzed. Statistical analysis was performed by using SPSS statistics 17.0 software and GraphPad Prism 5 Demo for calculating odd ratios, 95% confidence interval and standard error.

RESULTS

Highly significant difference in number of patient with cancer of oral cavity ($P < 0.01$) was found compared with pharyngeal and laryngeal cancers. In cancer of oral cavity, pharynx and larynx, difference in number of male patients compared with females was statistically non significant ($P > 0.05$).

Statistically significant increase in incidence of head and neck cancer was observed in age group of 40 to 60 years ($P < 0.05$). The mean age of cancer patients was 48 years (±16.59), whereas the mean age of controls was 46 (±17.69) years. A higher number of males had cancers of oral cavity and laryngeal cancer, whereas females had a higher frequency of pharyngeal cancer cases as shown in Figure 1. Surprisingly no statistical

Table 1. Primer sequences synthesized for different exons of *CYP1A1*.

Primer name	Primer sequence
CYP1A1 Exon1 Forward	TACAGGCACCGAGATGTGTC
CYP1A1 Exon1 Reverse	AGTCCTGGAGGCACCAAAAT
CYP1A1 Exon2a Forward	GTTTCCCCTTTCCCTGACAC
CYP1A1 Exon2a Reverse	CAGGTAGCAGGAGGTTGAGG
CYP1A1 Exon2b Forward	CCGACCTCTACACCTTCACC
CYP1A1 Exon2b Reverse	CCCATGCAGTTCCTCTTACC
CYP1A1 Exon3 Forward	GACCAGACCTGGATGGAGAG
CYP1A1 Exon3 Reverse	TGACTGTGTCAAACCCTGGA
CYP1A1 Exon4 Forward	TGTGTCCTTCCTGTGCTCAA
CYP1A1 Exon4 Reverse	AACACAGGGACAAGATGGATG
CYP1A1 Exon5 Forward	AGGTAGTGGCTCCCTTCAAA
CYP1A1 Exon5 Reverse	TGTCCCTCCCACTAACCCTA
CYP1A1 Exon6 Forward	GACACGGCATGGGAGACA
CYP1A1 Exon6 Reverse	ATGGACAGGAGGATCAATGC
CYP1A1 Exon7a Forward	GCATTGATCCTCCTGTCCAT
CYP1A1 Exon7a Reverse	CAGAGGCAAGTCCAGGGTAG
CYP1A1 Exon7b Forward	TGTCTACCTGGTCTGGTTGG
CYP1A1 Exon7b Reverse	CCTCCAGGACAGCAATAAGG
CYP1A1 Exon7c Forward	CTGCCAAGAGTGAAGGGAAG
CYP1A1 Exon7c Reverse	AACACAGAATGGGGTTCAGG

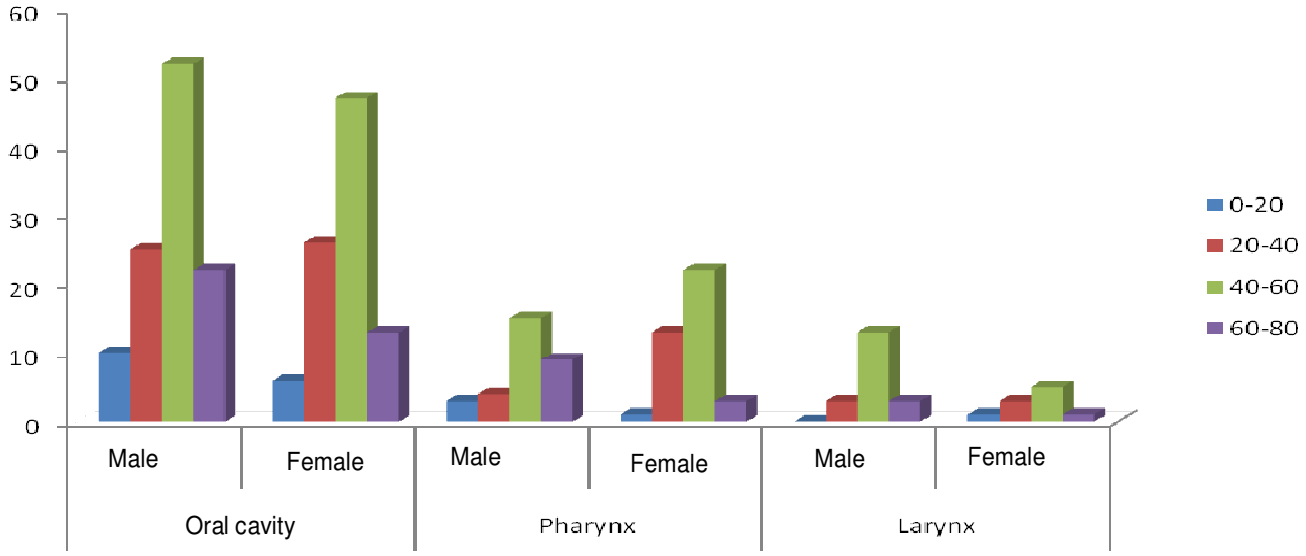

Figure 1. Frequency of head and neck cancer cases with reference to age bands, area of cancer and sex.

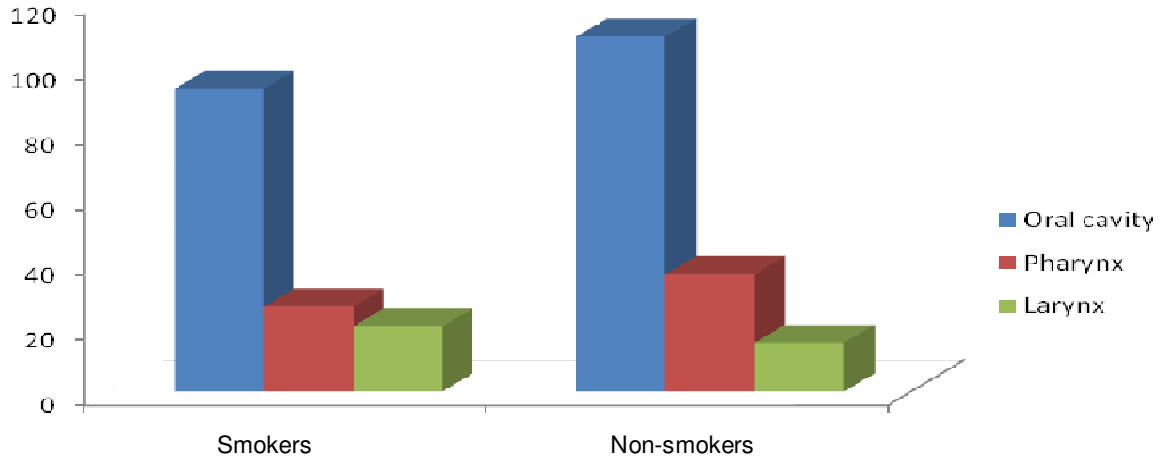

Figure 2. Comparison of different areas of cancer with respect to smoking status in head and neck cancer patients.

variations, with respect to gender were observed ($P > 0.05$) and male to female ratio was found to be 1:1 (Figure 1).

This study shows that persons using any form of tobacco (cigarettes, cigars, bidhi, moist snuff) and betel nuts are more affected by HNC ($P < 0.05$) compared with nontobacco users (Figure 2). In tobacco addicted patients, highest number of oral cancer cases were present (66%) compared with pharyngeal (24%) and laryngeal (10%) cancers.

In jobless persons, a statistically significant increase ($P < 0.01$) in incidence of head and neck cancer was found compared with housewives, businessman and labors. A lower incidence of head and neck cancer was observed in businessman and office employers (14%) when compared with labors (17%) and housewives (20%) shown in Figure 3.

Figure 4 shows that, no polymorphism in either of the reported variants *CYP1A1*2, *CYP1A1*3, *CYP1A1*4 and *CYP1A1*5 has been observed in this study consisting of 388 head and neck cancer cases and 150 controls. A novel substitution mutation of A_{2842} with C (Figure 5 and Table 2) was observed in 21 patients ($P < 0.001$, odd ratio (OR) = 9.4 and 95% confidence interval (CI) 1.3 to 70.8), whereas no control showed this mutation in exon 2. This A_{2842} to C mutation causes a change in DNA sequence from TAC to TCC and resulting UCC which codes serine, whereas wild type UAC codes for tyrosine. This tyrosine to serine mutation is in the conserved P450 domain and not in the transmembrane domain. In 21 patients with *CYP1A1* mutation in 2nd exon, 62% are female (OR 1.6, 95% CI 0.09 to 29.8) and 38% are males (OR 0.6, 95% CI 0.03 to 11.3), while 76% have cancer of oral cavity, 14% of pharynx and 10% of laryngeal cancer (OR 3.2, 0.17 and 0.11, respectively). The mean age of patients, showing A to C substitution mutation, was 51 ± 15.7 years. This mutation was present in more patients with a job (OR 0.75, 95% CI 0.04 to 13.7) when compared with jobless (OR 0.62, 95% CI 0.03 to 11.3) and house wives (OR 0.2, 95% CI 0.01 to 4.62).

DISCUSSION

The main aim of this case control study was to evaluate the key risk factors for head and neck cancer (HNC). The current study clearly demonstrates that, environmental factors along with genetic polymorphism of *CYP1A1* gene are the main cause of head and neck cancer. Oral cavity cancer is the most prevalent area of head and neck cancer followed by pharynx and then larynx in our data. These findings are in accordance with the previous findings by Llewellyn et al. (2004), Shiboski et al. (2000) and Ping et al. (2007) differing only in increased incidence of HNC. In Americans and Caucasians, incidence of oral cancer has increased in the past few years; however, pharyngeal cancer is not common in most of the world populations (Parkin et al., 2002).

The mean age of our study patients was 48 years and highest HNC cases were observed in age group of 40 to 60 years. The age is an uncontrollable variable with the increase in age particularly between 40 to 60 years the risk of head and neck cancer is increased (Shanmugaratnam et al., 1982). This study reveals that, age more than 45 years should be considered as a risk factor for HNC development as found in earlier studies (Llewellyn et al., 2004) and once HNC is developed, the survival, particularly >80 years of age, is reduced when compared with patients <65 years age (Clayman et al., 1998). Mean age of pharyngeal cancer patients in South East Asia is lower compared with Western countries (Yu and Yuan, 2002).

In the present study, 50% were males and equal females. This shows that the males and females are equally affected by head and neck cancer. This finding is in contrast with the findings from earlier studies which

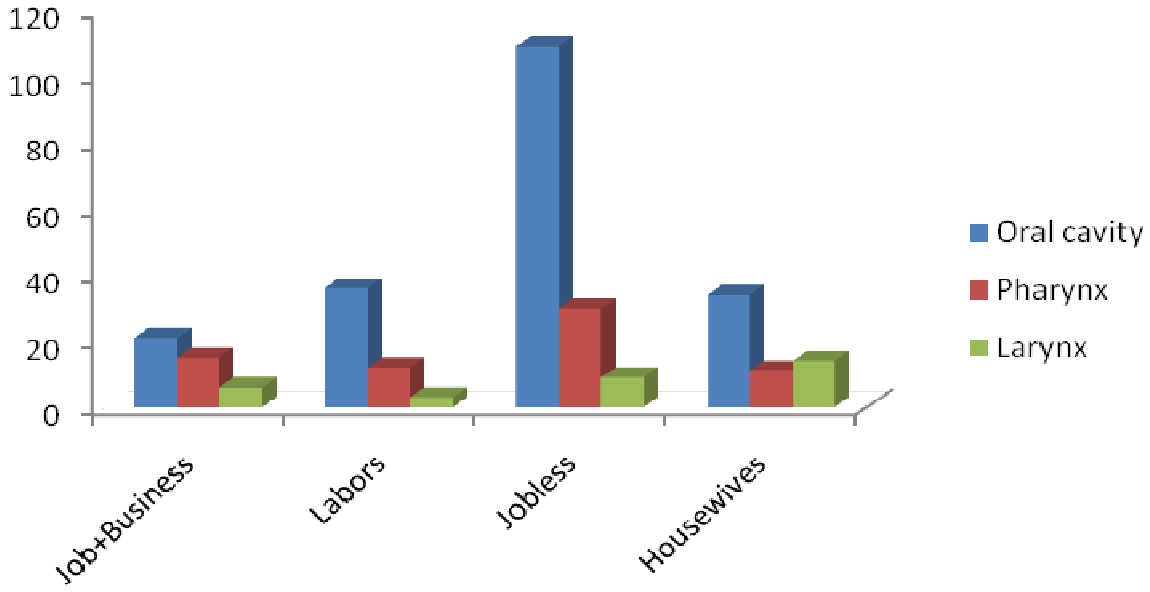

Figure 3. Comparison of number of patients with repect to area of cancer and occupation.

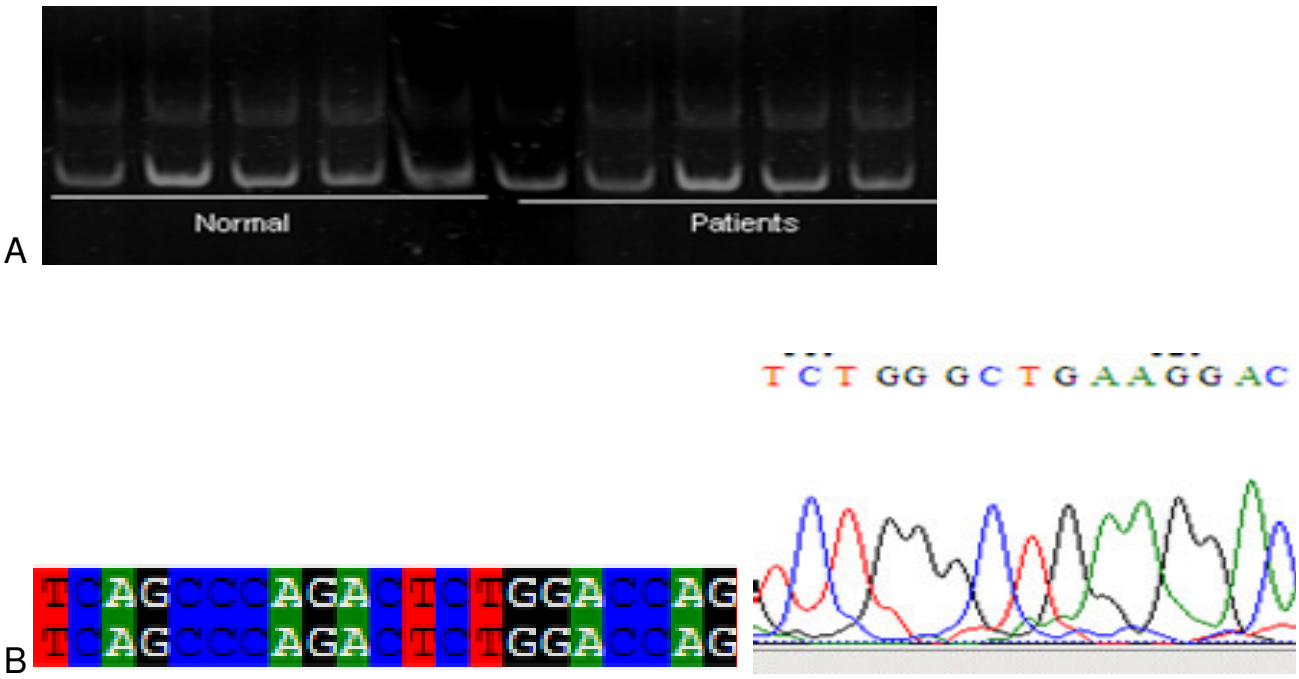

Figure 4. (A) No mobility shift in exon 7 *CYP1A1* gene of patients and normal controls in ethidium bromide stained PAGE; (B) no difference in sequencing resuls (upper sequence) compared with original sequence of *CYP1A1* gene exon 7 using

reported a 2:1 male to female ratio (Toefil et al., 2007) and that of Abdulamir et al. (2008) who found no association of age with sex ratio and areas of head and neck cancer in Asian patients.

The smoking status is always expected to be a key risk factor in development of cancer of any origin particularly HNC. In this study, 63.79% were smokers and 36.21% were non-smokers. The P value with Fishers exact test is ($P < 0.05$) which show that incidence of HNC in smokers is approximately two fold compared with non-smokers. Such results have frequently been reported in other parts of the world (Jun et al., 2010; Hecht et al., 1993). A study in the USA have shown that, heavy smokers die due to carcinoma of larynx 32 folds earlier than age-adjusted non-smokers and of oral cavity cancer, more than 24 folds earlier compared with non-smokers (Berrino and

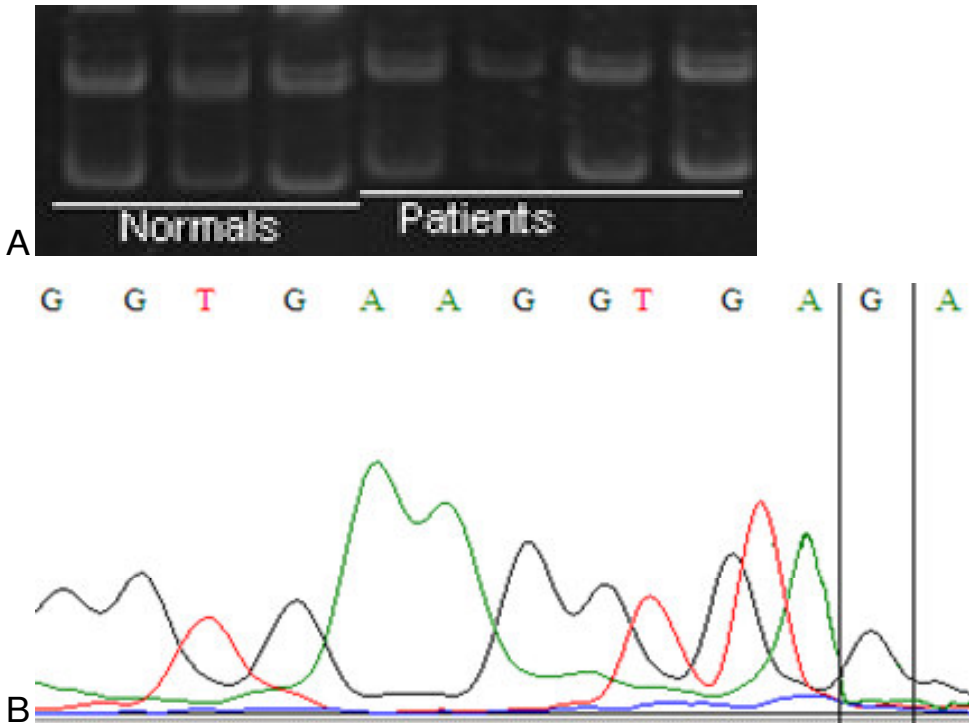

Figure 5. (A) Difference in mobility of exon 2, *CYP1A1* gene, between patients and normal controls in ethidium bromide stained PAGE; (B) G instead of T in sequencing compared with original sequence of *CYP1A1* gene exon 2 using BioEdit software v 0.7.0.

Table 2. Statistical data of patients having *CYP1A1* exon 2 mutation at aminoacid 110.

Variable	A to C mutation	OR (CI 95%)	P value
Total patient	**21**	**9.4 (1.3 to 70.8)**	**0.007**
Gender			
Female	62%	1.6 (0.09- 29.8)	0.4
Male	38%	0.6 (0.03-11.3)	0.4
Age			
Mean	51.75	(±15.7)	
<48	52%	1.1 (0.06- 20.0)	1
>48	48%	0.9 (0.05- 16.5)	1
Smoking			
Yes	62%	1.6 (0.09- 29.8)	0.4
No	38%	0.6 (0.03-11.3)	0.4
Occupation			
Job	9	0.75 (0.04- 13.7)	1
Jobless	8	0.62 (0.03- 11.3)	1
house wives	4	0.2 (0.01- 4.62)	1
Area of cancer			
Oral Cavity	76%	3.2 (0.17- 61.02)	0.03
Pharynx	14%	0.17 (0.01- 3.4)	1
Larynx	10%	0.11 (0.005- 2.4)	1

Gatta, 1998). These findings are also in agreement with earlier studies (Eaden et al., 2000; Lee et al., 2004; Llewellyn et al., 2004) in different populations where it has been established that, smoking plays a significant role in pathogenesis of HNC.

Interestingly, HNC is more common in jobless individuals due to increased tobacco addictions and mental stress when compared with on job individuals. This might be the reason that, house wives have least percentage of head and neck cancer (Abdulamir et al., 2008). Increased incidence of HNC in jobless individuals may also be attributed to increased mental stress (Jensen et al., 2010) as in Pakistan it has increased significantly (Pope et al., 1983; Bhugra, 2004). The results suggested that, the occurrence of head and neck cancer (HNC) is related to age, smoking and the occupation status.

CYP1A1 is involved in the activation of many polycyclic aromatic hydrocarbon and aromatic amines by enzyme aryl hydrocarbon hydrolase (Bartsch et al., 2000). In the present case-control study, none of the already reported mutations were observed. However, a novel mutation in exon 2 of *CYP1A1* gene was observed which causes tyrosine to change in serine at amino acid number 110 of *CYP1A1* gene. Tyrosine to serine substitution mutation causes a change in conserved domain of cytochrome P450. This mutation cause, a change in the protein structure as an aromatic amino acid is changed into a non aromatic amino acid and subsequently, gene function is also altered. Aside from being a proteogenic amino acid, tyrosine has a special role by virtue of the phenol functionality. It occurs in proteins that are part of signal transduction processes. Tyrosine is a precursor to neurotransmitters and increases plasma neurotransmitter levels (particularly dopamine and norepinephrine). Therefore, this mutation might lead to imbalanced functional activity in detoxification process as *CYP1A1* is a key enzyme that converts PAHs into active carcinogens (Hecht et al., 1993; Bartsch et al., 2000). PAHs present in tobacco smoke activate transcription of the *CYP1A1* gene and increase pulmonary *CYP1A1* activity several fold (Omiecinski et al., 1990). Mutated *CYP1A1* gene can not convert carcinogen into a hydrophilized form required for phase II enzyme activation (glutathione-S-transferase isozymes GSTM1, GSTP1 and GSTT1) for the process of detoxification.

The current mutation of *CYP1A1* gene along with environmental factors may be one of the several factors causing head and neck cancer. Polymorphisms in *CYP1A1* gene and alterations in their expression and function, may increase or decrease carcinogen activation/ detoxification followed by a variation of cancer risk (Shen et al., 2002; Jourenkova et al., 1998; Olshan et al., 2000; Lea et al., 2007; Matullo et al., 2001; Park et al., 2002). However, the ultimate phenotypic effect reflects a complicated network of interactions between genes and environmental factors. This study focuses on only one gene *CYP1A1* with environmental factors in HNC; however, determining the role of genetic polymorphisms as cancer risk factor would require studies aiming at the integrated analysis of many genes involved in cancer development.

ACKNOWLEDGEMENTS

All authors would like to acknowledge the patients and normal individual who contributed in this research work and also the hospital and staff for their cooperation. We would like to thank COMSATS and HEC for financial assistance.

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