RESEARCH ARTICLE

Association of a p53 Codon 72 Gene Polymorphism with Environmental Factors and Risk of Lung Cancer: a Case Control Study in Mizoram and Manipur, a High Incidence Region in North East India

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Abstract

Background: A very high incidence of lung cancer is observed in Mizoram and Manipur, North East India. We conducted a population based case control study to establish associations of *p53* codon 72 polymorphisms and interactions with environmental factors for this high incidence. Material and Methods: A total of 272 lung cancer cases and 544 controls matched for age (±5 years), sex and ethnicity were collected and *p53* codon 72 polymorphism genotypes were analyzed using a polymerase chain based restriction fragment length polymorphism assay. We used conditional multiple logistic regression analysis to calculate adjusted odds ratios and 95% confidence intervals after adjusting for confounding factors. Results: *p53* Pro/Pro genotype was significantly associated with increased risk of lung cancer in the study population (adjusted OR=2.14, CI=1.35-3.38, p=0.001). Interactions of the *p53* Pro/Pro genotype with exposure to wood smoke (adjusted OR=3.60, CI=1.85-6.98, p<0.001) and cooking oil fumes (adjusted OR=3.27, CI=1.55-6.87, p=0.002), betel quid chewing (adjusted OR=3.85, CI=1.96-7.55, p<0.001), tobacco smoking (adjusted OR=4.42, CI=2.27-8.63, p<0.001) and alcohol consumption (adjusted OR=3.31, CI=1.10-10.03, p=0.034) were significant regarding the increased risk of lung cancer in the study population. Conclusions: The present study provided preliminary evidence that a *p53* codon 72 polymorphism may effect lung cancer risk in the study population, interacting synergistically with environmental factors.

Keywords: *p53*SNP - lung cancer - gene-environment interactions - North-East India

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Introduction

The p53 tumor suppressor gene plays a central role in modulating cellular process that governs the major defenses against tumor growth, maintenance of genetic integrity, cell cycle arrest and apoptosis (Whibley et al., 2009; Cheng et al., 2012; Azlin et al., 2014; Ren et al., 2013). p53 gene is one of the most studied genes in cancer research. The gene harbors high-frequency, functional single-nucleotide polymorphisms (SNPs) which may alter p53 protein function (Grochola et al., 2010). Several functional p53 SNPs have been reported to be associated with risk of developing different human cancers, including lung cancer (Yan et al., 2009; Francisco et al., 2011; Bellini et al., 2012; Karim et al., 2014; Zeichner et al., 2014). Among these the most common is codon 72 polymorphisms is a single-base substitution of cytosine for guanine, leading to arginine (A72) being replaced by proline (p72) (Pietsch et al., 2006) and has been associated by with several types of cancer (Ishan et al., 2011; Li et al., 2013; Malakar et al., 2014). However few studies examined if there are interaction between p53 codon 72 polymorphisms and smoking or other environmental factors on lung cancer risk.

Studies on p53 codon 72 polymorphisms reveals stricking difference on ethnicity (Sjalander et al., 1995). In a study conducted by Beckam et al (1994) have observed that the frequency of p53 variant allele varies with latitude as its increases in a linear trend as population near equator suggesting that ethnicity might be related to allelic distribution of the gene and its determinacy in disease involvement; however, some studies do refute the ethnicity–risk confounding relationship (Fan et al., 2000; Lin et al., 2014).

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Lung cancer is the most common cause of death from cancer worldwide, estimated to be responsible for nearly one in five (1.59 million deaths, 19.4% of the total) (Ferlay et al., 2012). India contributes 6.2% cases of lung cancer with approximately 58,000 incidence cases reported in 2008 (Ferlay et al., 2010; D'Souza et al., 2013). North Eastern parts of India represent a unique, strategic geographic location with demographically diverse population. These areas also shows similarities in occurrences of cancer incidence patterns for various cancer sites of North East India with South and East Asian region which reflect that genetic linkage between these regions (Sharma et al., 2014). Mizoram and Manipur are two states from North East parts of India where lung cancer is mostly predominant, with highest age-adjusted rate (AAR) in Mizoram (28.3 per 10^5 in males and 28.7 per 10^5 in females). Similarly, Manipur also contributes a very high incidence of lung cancer (with AAR of 14.1 per 10^5 in males and 11.9 per 10^5 in females) (NCRP, 2013).

The area also reports for a unique consumption of tobacco, betel quids and dietary habits that are different from other places (Phukan et al., 2001, 2005, 2006, 2014; Saikia et al., 2014). A high risk of lung cancer can be an outcome of either environmental and genetic risk factors or a complex interaction of both. Therefore the present study was an age, sex and ethnicity matched population based case control study carried out to find out any relevance of p53 codon 72 polymorphisms as well as their interaction with environmental factors for the increase risk of lung cancer in Mizoram and Manipur North East India.

Materials and Methods

Present study was an age (±5 years), sex and ethnicity matched population based case-control study. The study duration was from February 2010 - January 2014. The work was carried out at Regional Medical Research Centre (RMRC) North East Region, Indian Council of Medical Research (ICMR); India in collaboration with civil hospital Aizawl, Mizoram and Population Based Cancer Registry (PBCR), Imphal, Manipur. Incident cases and controls subjects willing to participate in the study were indigenous people of Manipur and Mizoram. Information of smoking, betel quid chewing, consumption of alcohol, exposure to household combustion were recorded in a structured pre-designed questionnaire. All subject provide written inform consent for participation in the study was done under a protocol approved by the institutional ethics committee of Regional Medical Research Centre (RMRC) North East Region, Indian Council of Medical Research (ICMR).

The inclusion criteria for the cases includes histopathologically or cytologically confirmed lung cancer cases with no evidence of pulmonary inflammation or benign lung tumours and were resident of Mizoram and Manipur. The exclusion criteria for the cases were patient's diagnosis reported by death certificate or at autopsy, diagnosed with stomach, colon, liver, pancreatic, breast or rectal cancers, cases too old or too debilitated to be interviewed elaborately and those who refuse to participate in the study. The inclusion criteria for controls were those who had no blood relation with cases, non malignant individual, residence of Mizoram and Manipur, age, sex and ethnicity matched with controls. A total of 272 lung cancer cases and 544 controls matched for age (±5 years), sex and ethnicity were enrolled in the study.

DNA extraction

Four ml. of blood sample was collected from all subjects in EDTA vials. DNA was isolated by using standard phenol chloroform method and stored at -80º C till further analysis (Landi et al., 2006).

Genotyping

Genotyping of p53 gene polymorphisms were ascertained by PCR amplification using gene specific primers followed by Restriction Fragment Length Polymorphisms (RFLP). PCR reaction for p53

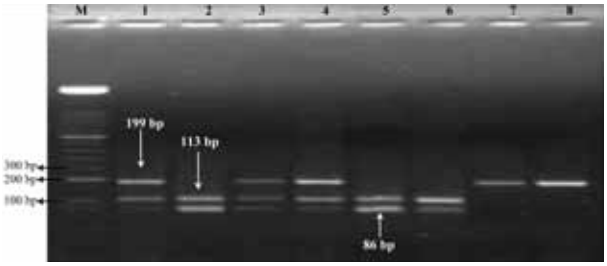

Figure 1. RFLP Photograph of 2% Agarose Gel Electrophoresis Representing p53 Genotype. Lame M represents 100bp DNA ladder. Lane 1, 3 & 4 were characterised by 199bp, 113bp and 86bp representing Arg/Pro genotype. Lane 2, 5 & 6 representing 113bp and 86bp represents Arg/Arg genotype. Lane 7 & 8 were characterised by single 199bp representing Pro/Pro genotype.

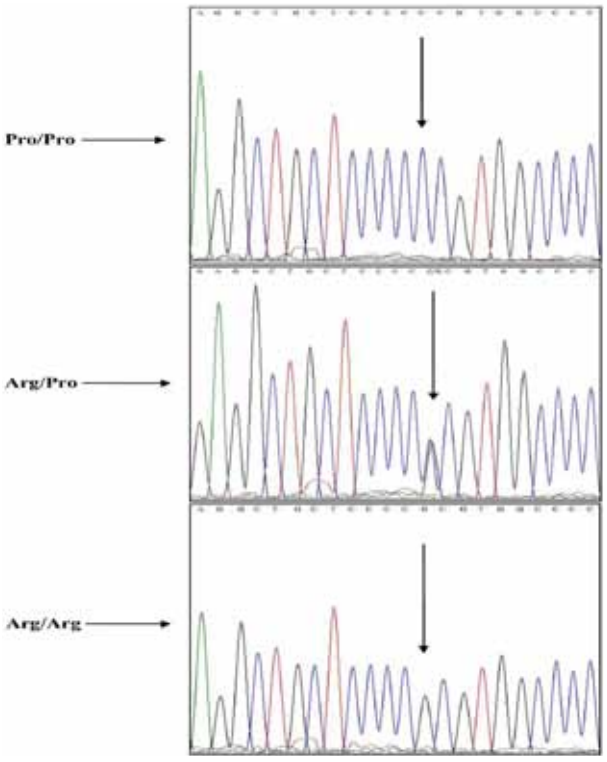

Figure 2. Representative Genotypes of p53 Codon 72 Polymorphisms by Sequencing

genotype were carried out by a Master cycler gradient thermo cycler (Bio-Rad, United States) in a final volume of 25 ul containing 150 ng of each primer (Sigma, United States), 50 ng genomic DNA, 2mM $MgCl_2$ (Roche, Germany), 200 ul dNTPs (Roche, Germany) and 1.5 unit of Taq DNA Polymerase (Roche). Primer sequences for PCR amplification were 5"TTGCCGTCCCAAGCAATGGATGA3"and 5"TCTGGGAAGGGACAGAAGATGAC3" which produced a 199 base-pair band (Ihsan et al., 2011). Amplifications for p53 genotype were performed under the following conditions: 96°C for 5 minutes; 30 cycles of 94°C for 1 minute (denaturation), 55°C for 1 minute (annealing) and 72°C for 1 minute (extension); followed by 72°C for 4 minutes. After amplification of the PCR product, it was digested at 55˚C for 3 hours with BstU1 restriction enzyme. The RFLP product were then electrophoresed in 2.5% agarose gel and then visualized in gel documentation system (Cell Biosciences). Arg/Arg genotype yields two small fragments of 113 bp and 86 bp. Pro/Pro genotype yield single 199bp. The heterozygote Arg/Pro genotype has three fragments of 199, 113 and 86 bp size (Figure 1). Genotyping of 10% of the randomly selected cases and controls were confirmed by sequencing and the results were observed 100% concordance was observed (Figure 2).

Statistical analysis

Multiple logistic regression analysis was used to analyze the data. The conditional maximum likelihood method was used to estimate the parameters of the regression model because of the matched design and significance was taken at $p \leq 0.05$ (two tailed). The crude measure of association between single putative risk factors and lung cancer was expressed as the odds ratio (OR) and its 95% confidence interval (CI) was calculated from the standard error of the regression coefficient. To control for confounding variables and other covariates, the data was analyzed by conditional multiple logistic regression to evaluate the extent of risk association. The statistical package used for the analysis was SPSS version 19.

Tests for Hardy-Weinberg equilibrium in both the subject were conducted using observed genotype frequencies and a chi-square test featuring one degree of freedom.

Results

Socio demographic characteristics of lung cancer cases and controls were presented in Table 1. No statistically significant difference in age and sex were observed between cases and controls. 66.5% of the cases are of non small squamous cell carcinoma, 26.5% are of non-small adenocarcinoma and 7.0% are of small cell carcinoma. Illiteracy (OR=2.10, CI=1.15-3.86, p=0.016) is significantly associated with increased risk of lung cancer of the study population. Among occupational status cultivators (OR=1.76, CI=1.13-2.75, p=0.013) and those of unskilled workers (OR=1.85, CI=1.09-3.16, p=0.024) were also significantly associated with increased risk of lung cancer in the study population.

Frequency distribution of Arg/Arg, Arg/Pro and Pro/Pro genotype among lung cancer cases were 34.9%, 46% and 19.1% while that of control were 41.4%, 47.8% and 10.8% respectively. The p53 Pro/Pro genotype were found to be significantly associated with increased risk of lung cancer of the study population (adjusted OR=2.14, CI=1.35-3.38, p=0.001). In gender wise stratified analysis, the p53 Pro/Pro genotype demonstrated slightly higher risk of lung cancer risk association among female (adjusted OR=2.02, CI=1.05-3.90, p=0.035) than that of male (adjusted OR=1.96, CI=1.01-3.82, p=0.048). The distribution of p53 genotype in both cases and controls were in Hardy-Weinberg Equilibrium (HWE) (Table 2).

Table 1. Distribution of Demographic Characteristics and Risk of Lung Cancer

Category	Case n (%)	Control n (%)	Crude OR (95% CI)	p-value	Multivariate# OR (95% CI)	p-value
Sample size	272 (100)	544 (100)				
Age (years)						
Range	24-84	23-86				
Means±SD	59.89±11.77	60.38±12.09		0.579†		
Histological type						
Non-small squamous	181 (66.5)					
Non-small adenocarcinoma	72 (26.5)					
Small cell carcinoma	19 (7.0)					
Educational status						
College and above	44 (16.2)	88 (16.2)	1.0 (Reference)		1.0 (Reference)	
Illiterate	37 (13.6)	41 (7.5)	1.81 (1.02-3.20)	0.043	2.10 (1.15-3.86)	0.016*
Primary-middle	138 (50.7)	262 (48.2)	1.05 (0.70-1.60)	0.806	1.07 (0.69-1.66)	0.763
Secondary	53 (19.5)	153 (28.1)	0.70 (0.43-1.12)	0.132	0.70 (0.43-1.14)	0.152
Occupational status						
Office workers	53 (19.5)	135 (24.8)	1.0 (Reference)		1.0 (Reference)	
Cultivators	82 (30.1)	106 (19.5)	1.97 (1.28-3.03)	0.002	1.76 (1.13-2.75)	0.013*
Skilled workers	9 (3.3)	17 (3.1)	1.35 (0.57-3.21)	0.500	1.48 (0.62-3.55)	0.380
House wife	91 (33.5)	235 (43.2)	0.99 (0.66-1.47)	0.946	0.84 (0.55-1.28)	0.409
Unskilled workers	37 (13.6)	51 (9.4)	1.85 (1.09-3.14)	0.023	1.85 (1.09-3.16)	0.024*

*Significant; †For independent samples T-test; #OR were estimated through conditional multiple logistic regression model

Table 2. Distributions of p53 Genotype and Risk of Lung Cancer

Genotypes	Case n (%)	Control n (%)	Crude OR (95% CI)	p-value	Adjusted OR (95% CI)†	p-value
Arg/Arg	95 (34.9)	225 (41.4)	1.0 (Reference)	0.428	1.0 (Reference)	
Arg/Pro (Overall)	125 (46.0)	260 (47.8)	1.14 (0.83-1.57)	1.08 (0.77-1.50)		0.662
Pro/Pro (Overall)	52 (19.1)	59 (10.8)	2.09 (1.34-3.25)	0.001	2.14 (1.35-3.38)	0.001*
Arg/Pro (Male)	66 (50.8)	132 (50.8)	1.21 (0.76-1.93)	0.431	1.20 (0.74-1.92)	0.460
Pro/Pro (Male)	23 (17.7)	29 (11.2)	1.91 (1.00-3.69)	0.050	1.96 (1.01-3.82)	0.048*
Arg/Pro (Female)	59 (41.5)	128 (45.1)	1.08 (0.69-1.68)	0.748	0.96 (0.60-1.54)	0.880
Pro/Pro (Female)	29 (20.4)	30 (10.6)	2.26 (1.24-4.12)	0.008	2.02 (1.05-3.90)	0.035*
Hardy-Weinberg equilibrium test ‡ χ^2 0.89		1.58 p-value	0.34	0.20		

*Significant; †Adjusted OR were estimated by adjusting exposure of wood combustion, cooking oil fumes, betel-quid chewing, tobacco smoking and alcohol consumption in conditional multiple logistic regression model; ‡ Hardy-Weinberg equilibrium test is calculated for 1 (one) degree of freedom and values rounded to two decimals

Table 3. Distributions of p53 Genotype and Risk of Lung Cancer

Model	Interaction Parameters		Case n (%)	Control n (%)	Crude OR (95% CI)	p-value	Adjusted# OR (95% CI)	p-value
1†	p53 Gene	Exposure of wood combustion						
	Arg/Arg	No	49 (18.0)	145 (26.7)	1.0 (Reference)		1.0 (Reference)	
	Arg/Arg	Yes	46 (16.9)	80 (40.7)	1.70 (1.05-2.77)	0.032	1.55 (0.93-2.57)	0.092
	Arg/Pro	No	64 (23.5)	163 (30.0)	1.16 (0.75-1.79)	0.498	1.12 (0.72-1.74)	0.626
	Arg/Pro	Yes	61 (22.5)	97 (17.8)	1.86 (1.18-2.93)	0.008	1.59 (0.99-2.56)	0.055
	Pro/Pro	No	23 (8.5)	38 (7.0)	1.79 (0.97-3.29)	0.061	1.99 (1.06-3.71)	0.032*
	Pro/Pro	Yes	29 (10.7)	21 (3.9)	4.09 (2.14-7.81)	<0.001	3.60 (1.85-6.98)	<0.001*
2‡	p53 Gene	Exposure of cooking oil emission						
	Arg/Arg	No	48 (17.6)	132(24.3)	1.0 (Reference)		1.0 (Reference)	
	Arg/Arg	Yes	47 (17.3)	93(17.1)	1.39 (0.86-2.25)	0.181	1.15 (0.70-1.91)	0.581
	Arg/Pro	No	60 (22.1)	146(26.8)	1.13 (0.72-1.77)	0.591	1.06 (0.67-1.67)	0.807
	Arg/Pro	Yes	65 (23.9)	114(21.0)	1.57 (1.00-2.46)	0.050	1.27 (0.79-2.03)	0.328
	Pro/Pro	No	28 (10.3)	43(7.9)	1.79 (1.00-3.20)	0.049	1.79 (0.99-3.25)	0.054
	Pro/Pro	Yes	24 (8.8)	16(2.9)	4.13 (2.02-8.42)	<0.001	3.27 (1.55-6.87)	0.002*
3§	p53 Gene	Betel-quid chewing						
	Arg/Arg	Nonchewer	24 (8.8)	82 (15.1)	1.0 (Reference)		1.0 (Reference)	
	Arg/Arg	Chewer	71 (26.1)	143 (26.3)	1.70 (0.99-2.90)	0.054	1.66 (0.96-2.88)	0.070
	Arg/Pro	Nonchewer	47 (27.3)	98 (18.0)	1.64 (0.92-2.91)	0.091	1.50 (0.84-2.70)	0.171
	Arg/Pro	Chewer	78 (28.7)	162 (29.8)	1.65 (0.97-2.80)	0.065	1.52 (0.88-2.61)	0.133
	Pro/Pro	Nonchewer	14 (5.1)	28 (5.1)	1.71 (0.78-3.75)	0.182	1.93 (0.86-4.32)	0.111
	Pro/Pro	Chewer	38 (14.0)	31 (5.7)	4.19 (2.17-8.08)	<0.001	3.85 (1.96-7.55)	<0.001*
4¶	p53 Gene	Tobacco smoking						
	Arg/Arg	Nonsmoker	21 (7.7)	96 (17.6)	1.0 (Reference)		1.0 (Reference)	
	Arg/Arg	Smoker	74 (27.2)	129 (23.7)	2.62 (1.51-4.55)	0.001	2.67 (1.53-4.69)	0.001*
	Arg/Pro	Nonsmoker	22 (8.1)	85 (15.6)	1.83 (0.61-2.30)	0.620	1.21 (0.62-2.36)	0.586
	Arg/Pro	Smoker	103 (37.9)	175 (32.2)	2.69 (1.58-4.58)	<0.001	2.74 (1.60-4.71)	<0.001*
	Pro/Pro	Nonsmoker	16 (5.9)	22 (4.0)	3.33 (1.50-7.39)	0.003	3.73 (1.66-8.41)	0.001*
	Pro/Pro	Smoker	36 (13.2)	37 (6.9)	4.45 (2.30-8.60)	<0.001	4.42 (2.27-8.63)	<0.001*
5¥	p53 Gene	Alcohol consumption						
	Arg/Arg	Nonalcoholic	84 (30.9)	193 (35.5)	1.0 (Reference)		1.0 (Reference)	
	Arg/Arg	Alcoholic	11 (4.0)	32 (5.9)	0.79 (0.38-1.64)	0.527	0.65 (0.31-1.37)	0.256
	Arg/Pro	Nonalcoholic	106 (39.0)	221 (40.6)	1.10 (0.78-1.56)	0.581	1.03 (0.72-1.47)	0.862
	Arg/Pro	Alcoholic	19 (7.0)	39 (7.2)	1.12 (0.61-2.05)	0.715	0.91 (0.49-1.69)	0.760
	Pro/Pro	Nonalcoholic	43 (15.8)	53 (9.7)	1.86 (1.16-3.00)	0.010	1.87 (1.14-3.06)	0.013*
	Pro/Pro	Alcoholic	9 (3.3)	6 (1.1)	3.45 (1.19-9.99)	0.023	3.31 (1.10-10.03)	0.034*

*Significant; #Adjusted OR were estimated through conditional multiple logistic regression model; †Exposure of cooking oil fumes, betel-quid chewing, tobacco smoking and alcohol consumption were adjusted to estimate adjusted OR in the model; ‡Exposure of wood combustion, betel-quid chewing, tobacco smoking and alcohol consumption were adjusted to estimate adjusted OR in the model; §Exposure of wood combustion, exposure of cooking oil fumes, tobacco smoking and alcohol consumption were adjusted to estimate adjusted OR in the model; ¶Exposure of wood combustion, exposure of cooking oil fumes, betel-quid chewing and alcohol consumption were adjusted to estimate adjusted OR in the model; ¥Exposure of wood combustion, exposure of cooking oil fumes, betel-quid chewing and tobacco smoking were adjusted to estimate adjusted OR in each model.

Interaction analysis of p53 codon 72 polymorphisms with environmental factors was analysed. Significantly higher risk association for lung cancer was observed when p53 Pro/Pro genotype interact with exposure of wood combustion (adjusted OR=3.60, CI=1.85-6.98, p<0.001) and exposure of cooking oil fumes (adjusted OR=3.27, CI=1.55-6.87, p=0.002). Interaction combination of p53 Pro/Pro genotype with that of betel quid chewing (adjusted OR=3.85, CI=1.96-7.55, p<0.001), tobacco smoking (adjusted OR=4.42, CI=2.27-8.63, p<0.001) and that of alcohol consumption (adjusted OR=3.31, CI=1.10-10.03, p=0.034) were also reveals highly significant association for increased risk of lung cancer in the study population (Table 3).

Discussion

p53 genes act as a central regulatory node of multiple cellular response pathways to endogenous or exogenous stresses (Robles et al., 2002). p53 protein has the capacity to regulate activity of key effectors of cellular processes, such as senescence, DNA repair, cell cycle arrest and apoptosis (Levine et al., 1997; Riley et al., 2008, Ren et al., 2013). Functional inactivation of p53 pathways affects p53 signaling and further alters cancer risk (Robles et al., 2002; Roy et al., 2012).

In this case control study we had examined whether association of p53 codon 72 polymorphisms and their interaction with environmental factors are associated with increased risk of lung cancer in the study population. Present study reveals significant association of p53 codon 72 polymorphisms and risk of lung cancer from high incidence region of North East India. Moreover we found synergistic effects of p53 gene with exposure of wood combustion, exposure of cooking oil fumes, tobacco smoking, betel quid chewing, tobacco smoking and alcohol consumption.

Tobacco smoking and environmental air pollution have been linked with high frequency of p53 polymorphisms (Pfeifer et al., 2002; Dagher et al., 2006; Patel et al., 2013). Tobacco smoking, betel quid and wood smoke constituents contains large number of polycyclic aromatic hydrocarbons, alkaloids and other carcinogens (Lissowska et al., 2005; Sellappa et al., 2009; Hosgood et al., 2010; Ishan et al., 2011). The positive interactions observed between p53 codon 72 polymorphisms with tobacco smoking, betel-quid chewing and other environmental factors or exposure in the present might suggest that individuals carrying the variant p53 Pro/Pro genotype may have compromised p53 function and may have been respond poorly to the adverse effects of smoking and environmental factors or exposure and thus have an elevated risk of developing lung cancer.

Association of p53 codon 72 polymorphisms and risk of lung cancer also varies across ethnicity (Wang et al., 2013). Significantly increased risks for lung cancer were found among Asians, but not in Africans for both p53 Pro/Pro and the Pro allele carriers in a previous meta-analysis by Li et al (2009). These inconsistencies ethnicities different may be attributed to different genetic backgrounds and environments. Furthermore, the effect of p53 codon 72 Arg/Pro polymorphisms on lung cancer risk differs in Asians. In a study conducted by Jung et al (2008), demonstrated that the p53 codon 72 Arg/Pro polymorphism was not significantly associated with lung cancer susceptibility in a Korean population (Jung et al., 2008). However in a study conducted by Piao et al (2011), the p53 codon 72 polymorphism was associated with an increase risk of lung cancer in another Korean population (Piao et al., 2011). Different study design, sample size, genotyping method, and source of controls may be responsible for the conflicting findings among individual studies.

In conclusion, the present study provides preliminary evidence that p53 codon 72 polymorphisms is novel single nucleotide polymorphisms that may effect lung cancer risk, especially among smokers and indoor environmental exposure. Taking into account, other confounding variables such as dietary habits, and infectious agents such as H. pylori status, HPV, etc will give us more probable factors for increased risk of lung cancer in the study population.

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