RESEARCH ARTICLE

Association between *p53* Gene Variants and Oral Cancer Susceptibility in Population from Gujarat, West India

Kinjal R Patel[1,3], Bhairavi N Vajaria[1,3], Rasheedunnisa Begum[2], Franky D Shah[1,3], Jayendra B Patel[1,3], Shilin N Shukla[3], Prabhudas S Patel[1,3]*

Abstract

Background: *p53* gene variants i.e. 16 bp duplication in intron 3, Arg72Pro in exon 4 and G>A in intron 6 have been reported to modulate susceptibility to various malignancies. Therefore, the present study evaluated the role of these *p53* polymorphisms in oral cancer susceptibility in a population from Gujarat, West India. Method: Genotype frequencies at the three *p53* loci in 110 controls and 79 oral cancer cases were determined by the PCR-RFLP method. Results: Heterozygous individuals at exon 4 showed protection from developing oral cancer. Homozygous wild and heterozygous individuals at intron 3 and those heterozygous at exon 4 in combination appeared to be at lowered risk. Furthermore, carriers of the 16 bp duplication allele at intron 3, proline allele at exon 4 and G allele at intron 6 were protected from oral cancer development. Conclusion: *p53* polymorphisms, especially Arg72Pro in exon 4 could significantly modify the risk of oral cancer development in Gujarat, West Indian population.

Keywords: Arg72Pro polymorphism - genetic susceptibility - oral cancer - *p53* polymorphisms - PCR-RFLP

Asian Pacific J Cancer Prev, **14 (2)**, 1093-1100

Introduction

Oral cancer has emerged as a major health problem worldwide. In India, 69,820 new cases and 47,653 deaths were registered in 2008 (Ferlay et al., 2010). This high death rate is mainly due to problems in clinical management of oral cancer i.e. lack of early detection and high incidence of local regional recurrence. Epidemiological studies revealed that tobacco chewing is an important risk factor for oral cancer development in India. Moreover, differences in the clinico-pathological and molecular pathological profile in the tobacco (smoking) and alcohol associated oral cancers in the western countries and tobacco - chewing associated oral cancers, mainly in the Indian subcontinent have been reported (Paterson et al., 1996). Although, tobacco use plays a major role in the etiology of oral cancer, only a fraction of tobacco users develop this disease, suggesting that genetic susceptibility may contribute to carcinogenic mechanisms in the population. In addition, complex interactions between these genetic and environmental factors may play a major role in oral carcinogenesis (Imyanitov et al., 2004). This susceptibility is modulated by polymorphisms in genes encoding DNA repair proteins, cell cycle control proteins and metabolic enzymes (Wu et al., 2002).

p53 gene encode important tumor suppressor protein, plays a critical role in regulating cell cycle arrest, apoptosis and DNA repair. Inactivation of *p53* is frequently observed in human cancers. In addition, numerous sequence variations are present in the *p53* gene. Most of these variations are intronic and have no cancer related biological consequences (Whibley et al., 2009). However, two intronic polymorphisms in *p53* gene; 16 bp duplication in intron 3 and G>A transition in intron 6 have been suggested to affect the levels of *p53* gene expression as well as its function (Chumakov and Jenkins, 1991; Lazar et al., 1993). Introns were originally believed to be non functional because they do not code for proteins, still they have been implicated in regulation of gene expression and DNA – protein interactions (Beenken et al., 1991; Lozano and Levine, 1991; Mattick, 1994; Shamsher and Montano, 1996). Sequence variations in introns may affect function of proteins and hence cancer risk. Studies of the association between certain types of cancer and intron 3 and intron 6 polymorphisms have showed conflicting results (Wang-Gohrke et al., 1999; Gallì et al., 2009; Hu et al., 2010).

Of the 19 exonic polymorphisms, 11 polymorphisms in *p53* are non-synonymous, resulting in an amino-acid change and only four of these have been validated. Out of these, there are sufficient molecular evidences for two

[1]*Biochemistry Research Division,* [3]*The Gujarat Cancer and Research Institute, Ahmedabad,* [2]*Department of Biochemistry, The M. S. University of Baroda, Vadodara, Gujarat, India *For correspondence: prabhudas_p@hotmail.com*

polymorphisms (Pro47Ser and Arg72Pro) suggesting their role in functional change of the *p53* protein. The rest two polymorphisms (V217M and G360A) have not been associated with an altered cancer risk till date (Whibley et al., 2009). The codon 47 polymorphism results in proline to serine substitution is rare whereas arginine to proline substitution in codon 72 is common (Felley-Bosco et al., 1993; Beckman et al., 1994). The relationship between the polymorphism in codon 72 and oral cancer has been studied but the results are inconsistent (Zhuo et al., 2009).

It has been speculated that both ethnic back-ground and lifestyle differences contribute to the differences in predisposing genetic factors to cancer development (Chakrabarti et al., 2001). This fact is very crucial for country like, India where wide variations in ethnicity and life style exist. There is a single study from east region of India, analyzed association between these three polymorphisms and oral cancer till date (Mitra et al., 2005). It has also been reported that the frequencies of the polymorphisms tend to differ among Indians and this is mainly due to differences in ethnicity (Mitra et al., 2003). Therefore, the present study aimed to analyze whether the polymorphisms in exon 4, intron 3 and intron 6 of the *p53* gene are associated with differential oral cancer risk in Gujarati population from West India where incidence of oral cancer is dramatically high.

Materials and Methods

Subjects

The study was approved by the Institutional Review Board of The Gujarat Cancer and Research Institute, Ahmedabad, India and informed consent was obtained from all the subjects. Histopathologically confirmed and previously untreated oral cancer patients from the out patient's department of The Gujarat Cancer and Research Institute were enrolled. The controls were volunteer blood donors, genetically unrelated to patients and had no previous history of cancer. Both subjects should not have any major illness in past. A detailed questionnaire was administered to all participants for gathering information on age, sex, occupation and tobacco habits. Both patients and controls were found to have tobacco habits such as smoking and chewing in different forms. Habit of tobacco chewing was more prevalent in this population.

Methods

Five milliliters of blood was drawn by venipuncture from all the subjects. WBCs were separated and stored at -80^{0}C until analysis. DNA isolation was done using DNA blood mini isolation kit (Qiagen, USA) following manufacturer's instructions and stored at -20^{0}C until analysis.

Genotyping was performed by Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) method. Intron 3 and exon 4 were amplified together within which the polymorphisms fall, using primers as mentioned previously (Mitra et al., 2003). The PCR products were resolved by 6% polyacrylamide gel electrophoresis (PAGE) and visualized after staining with ethidium bromide. 16 bp duplication in intron 3 was directly interpreted from 6% PAGE analysis of the PCR products. Resulting PCR products were either 432 or 448 bp DNA fragments depending on the absence or presence of 16 bp duplication in intron 3 in template genomic DNA. Homozygotes for the absence of duplication (A1/A1) produced band of 432 bp DNA fragment; heterozygotes produced both the bands (A1/A2); homozygotes for the presence of duplication (A2/A2) produced band of 448 bp DNA fragment (Figure 1).

Further, aliquots of the same PCR products were subjected to restriction digestion with BstUI (Fermetas, USA). The products were separated by 6% PAGE and visualized after staining with ethidium bromide. There were four DNA fragments of different sizes. Pro/Pro homozygotes produced band of 448 bp DNA fragment with the intron 3 duplication or produced band of 432 bp DNA fragment without the intron 3 duplication; Pro/Arg heterozygotes produced bands of 448 bp/432 bp, 246 bp/230 bp (depending upon the absence or presence of intron 3 duplication) and 202 bp DNA fragments. Arg/Arg homozygotes produced two bands of 246 bp and 202 bp DNA fragments with the intron 3 duplication or produced two bands of 230 bp and 202 bp DNA fragments without intron 3 duplication (Figure 2).

Intron 6 was amplified separately using primers as described previously (Mitra et al., 2003). The 913 bp PCR products were digested with NciI (Fermentas, USA), separated by 6% PAGE and visualized after staining with ethidium bromide. Homozygotes for the absence of NciI restriction site produced band of 563 bp DNA fragment (A/A); heterozygotes for the NciI restriction site produced bands of 563 bp, 286 bp and 277 bp DNA fragments (G/A); homozygotes for the presense of NciI restriction site produced two bands of 286 bp and 277 bp

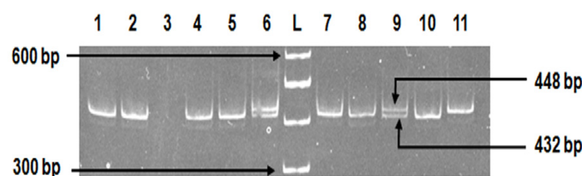

Figure 1. Representative Pattern for Three Genotypes of *p53* intron 3 (16 bp duplication) Polymorphism. Lane 1, 2, 4, 5, 7, 8, 10 represent homozygous for absence of 16 bp duplication (A1/A1); Lane 6, 9 represent heterozygous for 16 bp duplication (A1/A2); Lane 11 represents homozygous for presence of 16 bp duplication (A2/A2); Lane 3 represents Negative control; Lane L=100 bp ladder

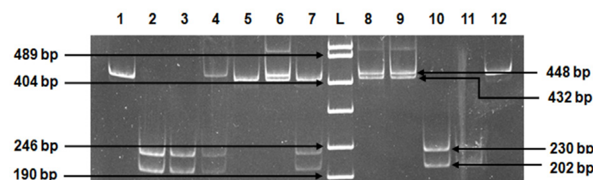

Figure 2. Representative Pattern for Three Genotypes of *p53* exon 4 (Arg72Pro) Polymorphism after BstUI Digestion. Lane 1, 5, 6, 8, 9, 12 represent Pro/Pro homozygous; Lane 4, 7 represent Arg/Pro heterozygous; Lane 2, 3, 10, 11 represent Arg/Arg homozygous; Lane L=PUC18/Msp1 digest DNA ladder

DNA fragments (G/G). All the three types of individuals contained band of 350 bp DNA fragment due to presence of a nonpolymorphic NciI site in the amplicon (Figure 3). Reproducibility of the assay was confirmed by repeating the samples.

Statistical analysis

The cases as well as controls were compared for genotype frequencies with the expected frequencies by the Hardy–Weinberg Equilibrium (HWE) by goodness-of-fit χ^2 test. Pearson's χ^2 test was performed to compare genotypic and allelic distributions between cases and controls. Odds ratios (ORs) and 95% Confidence Intervals (CIs) were calculated for the risk estimation of oral cancer development. The highest proportion of a homozygous genotype at any particular *p53* locus in the control group was considered as referent genotype to estimate risk associated with these polymorphisms (Mitra et al., 2005). Linkage disequilibrium analysis was performed for the study groups. The disequilibrium parameter (D) and the Pearson's χ^2 values for statistical significance were calculated. Statistical analysis was performed using SPSS (version 15). Frequencies of the pair-wise and extended haplotypes resulting from 3 polymorphisms in the *p53* gene were estimated using PHASE software (Stephens et al., 2001). p value<0.05 was considered to be statistically significant.

Results

Details of the subjects

The demographic characteristics of the subjects are summarized in Table 1. There were 68 (86.3%) males with age range 19-75 years (mean 44.7 years) and 11(13.7%) females with age range 22-62 years (mean 47.6 years) in oral cancer patients. In control group, there were 104 (95.3%) males with age range 19-54 years (mean 32.4 years) and 6 (4.7%) females with age range 24-46 years (mean 32.8 years). Tobacco habits were more prevalent in oral cancer cases (87.3%) as compared to the controls (48.2%) (p<0.0001). Tobacco habituates were more prone to develop oral cancer (OR=7.4, 95%CI:3.5-15.9; p<0.0001) as compared to tobacco non-habituates.

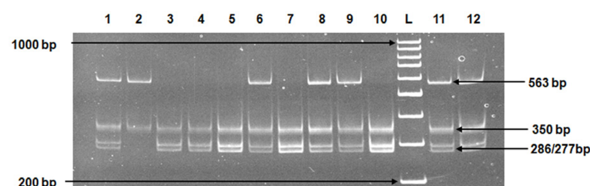

Figure 3. Representative Pattern for Three Genotypes of *p53* intron 6 (G>A) Polymorphism after NciI Digestion. Lane 2 represents homozygous for absence of NciI restriction site (A/A); Lane 1, 6, 8, 9, 11, 12 represent heterozygous for NciI restriction site (A/G); Lane 3, 4, 5, 7, 10 represent homozygous for presence of NciI restriction site (G/G); Lane L=100 bp ladder

Distribution of genotypes and allele frequencies among subjects

The genotype and allele frequency distribution of 16 bp duplication in intron 3, Arg72Pro in codon 72 of exon 4 and G>A transition in intron 6 among cases and controls is provided in Table 2. Controls and cases showed good fit to HWE for all three loci. There was marginal difference in the distribution of Arg72Pro genotypes at exon 4 locus between cases and controls (χ^2=5.19, p=0.075). However, no significant difference in the distribution of intron 3 and intron 6 genotypes between cases and controls was observed. Controls represent more Arg/Pro genotype compared to cases. The frequency of Arg and G allele was higher in cancer patients as compared to the controls.

Risk estimation of oral cancer associated with p53 genotypes

Table 3 depicts risk of oral cancer development associated with these polymorphisms. 16 bp duplication in intron 3 and G>A transition in intron 6 did not show any association with oral cancer risk. The Arg/Pro genotype at

Table 1. Demographic Details of Controls and Oral Cancer Patients

Characteristics	Controls (110) no. (%)	Oral Cancer Patients (79) no. (%)
Sex:		
Male	104.0 (95.3)	68.0 (86.3)
Female	6.0 (4.7)	11.0 (13.7)
Ratio: Male: Female	17.3:1	6.2:1
Age: Mean (Range)		
Male	32.4 (19 - 54)	44.7 (19 - 75)
Female	32.8 (24 - 46)	47.6 (22 - 62)
Both	32.4 (19 - 54)	45.0 (19 - 75)
Tobacco habits:		
Non-habituates	57.0 (51.8)	10.0 (12.7)
Habituates	53.0 (48.2)	69.0 (87.3)
Types of tobacco habit:		
Exclusive Chewers	46.0 (86.7)	47.0 (68.1)
Exclusive Smokers	4.0 (7.5)	7.0 (10.1)
Both Chewers and Smokers	3.0 (5.7)	15.0 (21.7)

Table 2. *p53* Genotype and Allele Frequencies in Controls and Oral Cancer Patients

Polymorphisms	Groups	T*P53* genotype 1-1 no. (%)	1-2 no. (%)	2-2 no. (%)	Pearson's χ^2	Allele Frequencie 1:2	χ^2 HWE	p value
16 bp duplication	Controls	74 (67.3)	31 (28.2)	5 (4.5)	0.29	0.82:0.18	0.649	0.723
(Intron 3)	Cases	56 (70.9)	20 (25.3)	3 (3.8)	p=0.866	0.84:0.16	0.549	0.760
Arg72Pro	Controls	22 (20.0)	58 (52.7)	30 (27.3)	5.19	0.46:0.54	0.411	0.841
(Exon 4)	Cases	18 (22.8)	29 (36.7)	32 (40.5)	p=0.075	0.41:0.59	4.638	0.098
G>A	Controls	5 (4.5)	32 (29.1)	73 (66.4)	2.13	0.19:0.81	0.378	0.828
(Intron 6)	Cases	1 (1.3)	20 (25.3)	58 (73.4)	p=0.345	0.14:0.86	0.248	0.883

*1= A1 at intron 3, Proline at exon 4, A at intron 6; 2= A2 at intron 3, Arginine at exon 4, G at intron 6

Table 3. Risk Associated with *p53* Genotypes in Oral Cancer Patients

Genotypes	Controls no. (%)	Cases no. (%)	OR (95%CI)	p value
16 bp duplication (Intron 3)				
A1/A1	74 (67.3)	56 (70.9)	1.0 (Referent)	
A1/A2	31 (28.2)	20 (25.3)	0.9 (0.4 - 1.7)	0.636
A2/A2	5 (4.5)	3 (3.8)	0.8 (0.2 - 3.5)	0.757
A1/A2 +A2/A2	36 (32.7)	23 (29.1)	0.8 (0.5 - 1.6)	0.597
Arg72Pro (Exon 4)				
Arg/Arg	30 (27.3)	32 (40.5)	1.0 (Referent)	
Arg/Pro	58 (52.7)	29 (36.7)	0.5 (0.2 - 0.9)	0.026
Pro/Pro	22 (20.0)	18 (22.8)	0.8 (0.4 - 1.7)	0.515
Arg/Pro + Pro/Pro	80 (72.7)	47 (59.5)	0.6 (0.3 - 1.0)	0.057
G>A (Intron 6)				
G/G	73 (66.4)	58 (73.4)	1.0 (Referent)	
G/A	32 (29.1)	20 (25.3)	0.8 (0.4 - 1.5)	0.474
A/A	5 (4.5)	1 (1.3)	0.3 (0.0 - 2.2)	0.214
G/A + A/A	37 (33.7)	21 (24.2)	0.7 (0.4 - 1.4)	0.301
Total	110	79		

codon 72 of exon 4 locus conferred protection from oral cancer development (OR=0.5; 95%CI:0.3-0.9; p=0.026). Combination of Pro/Pro and Arg/Pro genotypes at exon 4 locus showed marginal protection from oral cancer development (OR=0.6; 95%CI:0.3-1.0; p=0.057).

Determination of genotype combinations and risk of oral cancer

Present study also determined the genotype distribution of all the three polymorphisms in combination and risk associated with these combined genotypes (Table 4). Combination of genotypes for two loci of *p53* gene revealed that A1/A1 and A1/A2 genotypes of intron 3 polymorphism in combination with the Arg/Pro genotype at codon 72 significantly protect from oral cancer development (OR=0.5; 95%CI:0.2-1.0; p=0.044, OR=0.4; 95%CI:0.2-1.0; p=0.049, respectively). Individuals who were heterozygous at codon 72 and intron 6 locus showed marginal protection towards oral cancer development (OR=0.4; 95%CI:0.2-1.0; p=0.057). Also, combination of Arg/Pro and G/G genotypes at codon 72 and intron 6 locus, respectively showed marginal protection from oral cancer development (OR=0.5; 95%CI:0.2-1.1; p=0.070). Other combinations of intron 3 and intron 6 genotypes did not confer association with the risk of oral cancer development. When genotype combination was made for all the three polymorphisms of *p53* gene, heterozygous genotypes at all the three loci in combination was found to have protective effect from oral cancer development (OR=0.4; 95%CI:0.2-1.1; p=0.069). Further, Arg/Pro at codon 72, A1/A1 at intron 3 and G/G at intron 6 genotypes in combination also showed marginal protection from oral cancer development (OR=0.5; 95%CI:0.2-1.1; p=0.077). Individuals who were carriers of proline allele at codon 72 of exon 4 in combination with A1 and G allele at intron 3 and intron 6, respectively were significantly protected from oral cancer development (OR=0.5; 95%CI:0.3-1.0; p=0.047).

Distribution of p53 genotypes frequencies in controls and cases with respect to tobacco habits

Table 4. Distribution of Combined Genotypes among Subjects and Risk Estimation

	Controls	Cases	OR (95%CI)	p value
16 bp duplication (Intron 3)+Arg72Pro (Exon 4)				
A1/A1+Arg/Arg	29	32	1.0 (Referent)	
A1/A1+Pro/Pro	11	7	0.6 (0.2-1.7)	0.314
A1/A2+Pro/Pro	6	9	1.4 (0.4-4.3)	0.6
A2/A2+Pro/Pro	5	2	0.4 (0.1-2.0)	0.246
A1/A1+Arg/Pro	34	17	0.5 (0.2-1.0)	0.044
A1/A2+Arg/Pro	24	11	0.4 (0.2-1.0)	0.049
Arg72Pro (Exon 4)+G>A (Intron 6)				
Arg/Arg+G/G	30	32	1.0 (Referent)	
Pro/Pro+A/A	5	1	0.2 (0.0-1.7)	0.136
Pro/Pro+G/A	8	9	1.1 (0.4-3.1)	0.922
Pro/Pro+G/G	9	8	0.8 (0.3-2.4)	0.739
Arg/Pro+G/A	24	11	0.4 (0.2-1.0)	0.057
Arg/Pro+G/G	34	18	0.5 (0.2-1.1)	0.07
16 bp duplication (Intron 3)+G>A (Intron 6)				
A1/A1+G/G	69	56	1.0 (Referent)	
A1/A2+G/A	27	18	0.8 (0.4-1.6)	0.578
A1/A2+G/G	4	2	0.6 (0.1-3.5)	0.584
A2/A2+A/A	5	1	0.3 (0.0-2.2)	0.207
Arg72Pro+16 bp duplication+G>A				
Arg/Arg+A1/A1+G/G	29	32	1.0 (Referent)	
Pro/Pro+A1/A1+G/G	9	7	0.7 (0.2-2.1)	0.536
Pro/Pro+A1/A2+G/A	6	8	1.2 (0.4-3.9)	0.752
Pro/Pro+A2/A2+A/A	5	1	0.2 (0.0-1.6)	0.129
Arg/Pro+A1/A1+G/G	31	17	0.5 (0.2-1.1)	0.077
Arg/Pro+A1/A2+G/A	21	10	0.4 (0.2-1.1)	0.069
Arg/Pro+A1/A2+G/G	3	1	0.3 (0.0-3.1)	0.311
(Pro/Pro+Arg/Pro)+A1/A1+ G/G	40	24	0.5 (0.3-1.1)	0.094
(Pro/Pro+Arg/Pro)+(A1/A2+A2/A2)+(G/A+A/A)	32	21	0.6 (0.3-1.3)	0.172
(Pro/Pro+Arg/Pro)+(A1/A2+A1/A1)+(G/A+G/G)	75	44	0.5 (0.3-1.0)	0.047

Table 5. Distribution of *p53* Genotypes among Subjects with Respect to Tobacco Habit and Risk Estimation

Genotypes	Tobacco users Controls no. (%)	Tobacco users Cases no. (%)	OR (95%CI)
16 bp duplication (Intron 3)			p =0.55
A1/A1	35 (66.0)	49 (71.0)	1.0 (Referent)
A1/A2 +A2/A2	18 (34.0)	20 (29.0)	0.8 (0.4 - 1.7)
Arg72Pro (Exon 4)			p=0.16
Arg/Arg	15 (28.3)	28 (40.6)	1.0 (Referent)
Arg/Pro + Pro/Pro	38 (71.7)	41 (59.4)	0.6 (0.3 - 1.2)
G>A (Intron 6)			p=0.32
G/G	34 (64.2)	50 (72.5)	1.0 (Referent)
G/A + A/A	19 (35.8)	19 (27.5)	0.7 (0.3 - 1.5)
Total	53	69	

Table 6. Frequencies of Pair-wise Haplotypes and Linkage Disequilibrium Analysis

Groups	Pair-wise Haplotype freq. 1-1	1-2	2-1	2-2	Pearson's χ^2	D	Pearson's χ^2
16 bp duplication-Arg72Pro							
Controls	0.284	0.533	0.17	0.007	0.902	0.0886	p<0.0001
Cases	0.255	0.579	0.155	0.008	p= 0.825	0.0877	p=0.0044
Arg72Pro-G>A							
Controls	0.19	0.268	0	0.54	1.828	0.1026	p<0.0001
Cases	0.138	0.272	0	0.588	p=0.401	0.0811	p<0.0001
16 bp duplication-G>A							
Controls	0.023	0.79	0.167	0.018	4.531	0.1315	p<0.0001
Cases	0	0.835	0.139	0.025	p=0.209	0.1161	p<0.0001

*1=A1 at intron 3, Proline at exon 4, A at intron 6; 2=A2 at intron 3, Arginine at exon 4, G at intron 6

Table 7. Frequencies of Extended Haplotypes among Controls and Cases

Groups	Extended Haplotype frequencies						Pearson's
	1-2-02	1-1-02	1-1-01	2-2-02	2-1-02	2-1-01	χ^2
Controls	0.527	0.264	0.023	0.013	0.005	0.167	6.905
Cases	0.581	0.254	0	0.007	0.018	0.139	p=0.228

*1=A1 at intron 3, Proline at exon 4, A at intron 6; 2=A2 at intron 3, Arginine at exon 4, G at intron 6

Table 8. Distribution and Risk of Oral Cancer Associated with Haplotypes among Controls and Cases

		Controls (n=220)	Cases (n=158)	OR (95%CI)	p value
Haplotypes	1-2-02	116 (52.7)	92 (58.2)	1.0 (Referent)	
	1-1-02	58 (26.4)	40 (25.3)	0.87 (0.53-1.41)	0.574
	1-1-01	5 (2.3)	0	NA	
	2-2-02	3 (1.4)	1 (0.6)	0.31 (0.03-2.86)	0.305
	2-1-02	1 (0.4)	3 (1.9)	3.78 (0.38-36.97)	0.253
	2-1-01	37 (16.8)	22 (13.9)	0.74 (0.41-1.36)	0.342

*n=no of Chromosomes; 1=A1 at intron 3, Proline at exon 4, A at intron 6; 2=A2 at intron 3, Arginine at exon 4, G at intron 6; NA=Not Applicable

We also explored the role of genetic and environmental factors in combination which could further modify the risk of oral cancer. The results suggested that tobacco habituates with Pro/Pro and Pro/Arg genotypes at exon 4 locus were protected from oral cancer development (OR=0.6; 95%CI: 0.3-1.2; p=0.16) (Table 5).

Estimation of haplotype frequency and risk of oral cancer

Pair-wise haplotype frequencies for all possible combinations of these three *p53* polymorphisms were also estimated. The frequencies of pair-wise haplotypes did not differ significantly between controls and cases (Table 6). Linkage disequilibrium analysis was also performed to examine the linkage among the three loci (intron 3-exon 4-intron 6). There was significant linkage disequilibrium between these three loci in both the studied groups (Table 6).

We also determined the extended *p53* haplotypes (3 loci haplotypes) in controls and cases which are reported in the order of intron 3-exon 4-intron 6 (Table 7). For haplotype analysis, absence of 16 bp duplication at intron 3, Pro at exon 4 and A at intron 6 is defined as allele 1 at each of the three loci, respectively. The predominant haplotype consisted of the highly prevalent alleles (1-2-2). It was followed by 1-1-2 and 2-1-1, respectively. 1-1-1 haplotype was completely absent in cases and present in controls whereas 2-1-2 haplotype was more frequent in cases compared to the controls. There was no significant difference in the distribution of haplotypes between cases and controls (Table 8). Risk associated with various extended *p53* haplotypes are presented in table VIII. 2-1-2 haplotype showed 3 times higher risk of oral cancer development.

Discussion

Present study evaluated the relationship of three polymorphisms i.e. 16 bp duplication in intron 3, Arg72Pro in exon 4 and G>A transition in intron 6 of *p53* gene in order to predict the oral cancer risk in West Indian population. The controls as well as the cases belonged to same ethnicity and were from the same geographic location. An allele frequency of Arg72Pro polymorphism has been reported to vary with respect to ethnicity and latitude (Nagpal et al., 2002). The allele frequency of proline at codon 72 varies from 0.12-0.69 worldwide (Francisco et al., 2011) whereas for the Indian population; it ranges from 0.42-0.72 (Nagpal et al., 2002; Tandle et al., 2001; Mitra et al., 2003; 2005; Mittal et al., 2011; Suresh et al., 2011). In our population, the frequency of proline was 0.46. A2 and A allele frequencies at intron 3 and intron 6 in our population were 0.18 and 0.19, respectively. The freque[illegible]f th[illegible]eles [illegible] from 0.10-0.23 for A2 allele [illegible]on 3[illegible].19 [illegible]or [illegible] of intron 6 among [illegible]ent [illegible]tion[illegible]ng-[illegible] et al., 1999; Wu et [illegible]02; [illegible]e et [illegible]07; [illegible] et al., 2009; Hu et [illegible]0).

Ris[illegible]ral [illegible] was [illegible]stim[illegible] association with *p*[illegible]noty[illegible] all [illegible]poly[illegible]ic loci. The Arg/Pr[illegible]otyp[illegible]*53* [illegible]72 s[illegible]as well as in combi[illegible] with [illegible]ro g[illegible]es [illegible]ed protection toward[illegible]anc[illegible]elop[illegible]Ther[illegible]vidences that the co[illegible]poly[illegible]nism[illegible] pro[illegible] effect on the primar[illegible]ture[illegible]*3* pr[illegible]nd [illegible]hemical and biolog[illegible]tivit[illegible]atla[illegible]i et[illegible]87; Ozeki et al., 20[illegible]has [illegible]how[illegible]the [illegible] form of *p53* has in[illegible]d tra[illegible]tion[illegible]ns-a[illegible]on capacity, induce[illegible]ghe[illegible]l of[illegible]rres[illegible] senescence compa[illegible]the [illegible] for[illegible]oma[illegible]., 1999; Pim and Ba[illegible]04; [illegible]et a[illegible]). It [illegible]ound that the Arg-72 [illegible]of t[illegible] pro[illegible]as [illegible]usceptible to human [illegible]oma[illegible] (HP[illegible]-me[illegible] degradation compa[illegible]the [illegible] for[illegible]rey [illegible]1998). Hsieh et al. (2005) found that oral squamous cell carcinoma (OSCC) patients with the Arg allele had a significantly higher frequency of *p53* mutation than those with Pro/Pro genotype among patients with common alleles of intron 3 and intron 6. In addition, *p53* mutant acts as a more potent inhibitor of p53, which is responsible for apoptosis when *p53* has Arg-72 rather than Pro-72 (Bergamaschi et al., 2003). In contrast, both Pro-72 and Arg-72 form of *p53* are capable of inducing equal levels of apoptosis but with different kinetics (Thomas et al., 1999). Dumont et al. (2003) observed that the Arg-72 form has a much stronger capacity to induce apoptosis than the Pro-72 form of *p53* in tumor cells but not in normal cells. Cell-line based studies suggest that the Arg-72 has superior proapoptotic function in human tumor cell-lines. This effect of Arg72Pro polymorphism may inhibit *p53* function in vitro but whether this effect also occur in vivo remains to be determined. Recently, studies on mouse model indicate that the Arg-72 variant induces increased apoptosis in mouse embryo fibroblast (MEF) and in the small intestines of mice along with decreased apoptosis in the thymus compared to Pro-72 (Zhu et al., 2010; Azzam et al., 2011). Thus, there is tissue specific influence of Arg72Pro polymorphism on apoptosis. However, in primary head and neck tumors, retention of the Arg allele in codon 72 correlates with poor apoptosis (Schneider-Stock et al., 2004). Recently Hu et al. (2008) also observed that Pro allele at codon 72 of *p53* gene has significantly higher level of benzo[a]pyrene-7,8-9,10-diol

epoxide (BPDE) induced apoptotic index compared to Arg allele in primary lymphocytes. Such tissue specific function of this polymorphism may explain why most of the epidemiological studies remain inconclusive.

We observed no association between intron 3 and intron 6 polymorphisms and oral cancer risk. Recent researches suggest that intronic polymorphisms may affect the function of wild type *p53* protein and hence cancer risk (Avigad et al., 1997; Lehman et al., 2000; Gemignani et al., 2004). However, in cell culture analysis, these two intronic polymorphisms (16 bp duplication in intron 3 and G>A in intron 6) did not seem to be sufficient to impair *p53* function during the neoplastic transformation but required an additional coding region mutation. They have also reported that there were no splicing errors linked to these polymorphisms (Wang-Gohrke et al., 1999). In contrast, Hu et al. (2008) observed that variant alleles of intron 3 and intron 6 (A2 and A allele respectively) have significantly higher level of BPDE induced apoptotic index compared to its homozygous wild-type genotypes. Wu et al. (2002) have reported low apoptotic index and less repair capacity of lymphoblastoid cell lines that harbor A2 and A alleles of intron 3 and intron 6 polymorphisms, respectively. Gemignani et al. (2004) have also found that 16 bp duplication in intron 3 reduced the mRNA level of *p53* but because of strong linkage disequilibrium between intron 3 and exon 4 polymorphism, it remains to be determined that whether intron 3 alone influence mRNA stability or need the presence of exon 4 polymorphism. Overall, the functional role of these 2 intronic polymorphisms of *p53* in cancer risk remains uncertain.

When genotypes of these three polymorphisms were assessed in combination for the association with oral cancer risk, it was found that Arg/Pro genotypes in combination with A1/A1 and A1/A2 genotypes were significantly protected from oral cancer development. Moreover combination of heterozygous genotypes of exon 4 and intron 6 loci were also marginally protected from oral cancer development and individuals who were heterozygous at all three loci were protected from oral cancer development. It may be noted from the genotype combination analysis that individual who were carriers of proline allele at codon 72 of exon 4 in combination with A1 and G allele at intron 3 and intron 6 respectively were significantly protected from oral cancer development. More interestingly, Wu et al. (2002) have observed that proline at exon 4 in conjugation with intron 3 and 6 variant alleles exert a protective effect rather than a detrimental effect for lung and colorectal cancers though they found significant risk of lung cancer associated with these variants. The present study found that proline at exon 4 in conjugation with intron 3 and 6 variant alleles was associated with lower risk but not have the statistical power. To the best of our knowledge, there are no reports on the association of these three genotypes combinations and oral cancer risk from India till date. As there was significant linkage disequilibrium between these 3 loci in our population, these intronic *p53* polymorphisms may confer protection from oral cancer risk through linkage disequilibrium with a functional variant of the *p53* gene.

There are only few reports which have assessed the role of these *p53* polymorphisms in oral cancer from India. Among them, most of reports are on Arg72Pro polymorphism of *p53* gene. There is only one study from eastern region of India, explored the role of these three polymorphisms in oral cancer (Mitra et al., 2005). They have observed that the Proline allele was more frequent in control population and no association was found between oral cancer risk and Arg72Pro polymorphism. Another study from east region of India noted that Arginine allele was more frequent in population (Nagpal et al., 2002). In the present study, Arginine allele was more frequent in this population. Other studies from India did not find any association between Arg72Pro polymorphism and oral cancer risk (Tandle et al., 2001; Nagpal et al., 2002; Katiyar et al., 2003). Meta-analysis by Zhuo et al. (2009) on this polymorphism revealed that there was no association between Arg72Pro polymorphism and oral cancer risk. When comparison was made for all other cancers from India, proline allele was at risk for colorectal cancer and bladder cancer but it was found to be protective in breast cancer and prostate cancer and no association was observed for head and neck cancer (Pandith et al., 2010; Sameer et al., 2010; Suresh et al., 2010; 2011; Mittal et al., 2011). Studies from other populations showed conflicting results for the association of Arg72Pro polymorphism with oral cancer risk (Kietthubthew et al., 2003; Kuroda et al., 2007; Saini et al., 2011). Francisco et al. (2011) suggested that ethnicity, allelic frequency, histological and anatomical sites may modulate the penetrance of Arg72Pro in cancer susceptibility. The results of intron 3 and intron 6 polymorphisms are also inconsistent and ranges from no association to increased risk in different population (Wang-Gohrke et al., 1999; Wu et al., 2002; Sprague et al., 2007; Gallì et al., 2009; Hrstka et al., 2009; Hu et al., 2010). From India, only one study from east region has analyzed the association between intron 3 and intron 6 polymorphisms in oral cancer risk, suggested no association between intron 3 polymorphism and oral cancer risk, however, A allele at intron 6 was protective for oral cancer development (Mitra et al., 2005). This is the first study from this region which has analyzed association of these intronic polymorphisms and oral cancer risk.

The rising numbers of oral cancer cases each year in India is alarming. Buccal mucosa represents the primary site for cancer development that may be due to the habit of keeping the tobacco in contact with cheek for a long time. In this cohort of study, tobacco habituates were significantly higher in cases compared to controls and they were at significant risk to develop oral cancer. It was also found that tobacco users who were carriers of Pro allele at exon 4 locus may be protected from oral cancer development. However, the only study from India failed to observe any association between Arg72Pro polymorphism and oral cancer development in tobacco users (Mitra et al., 2005). They have suggested that combination of A/A and A/G genotypes at intron 6 locus showed protective effect towards oral cancer development but at a low smoking dose. However in our study, most of the subjects were tobacco chewers.

As the haplotype structure of a population is indicative of its evolutionary history and different haplotypes are

associated with cancer in different ethnic population. There are many studies which showed positive association with one or more haplotypes constructed from these three polymorphisms with cancer risk. Hu et al. (2008). observed that individuals who were carrier of 0 and 2 copies of 1-2-2 and 2-1-1 haplotypes, respectively have significantly higher level of BPDE induced apoptotic index. 1-2-2 haplotype was more common in Caucasian population and 2-1-1 haplotype was associated with breast cancer risk in Caucasian population (Weston et al., 1998; Wu et al., 2002). Whereas haplotype 1-2-2 was found more frequent in breast cancer patients in Pakistani ethnic groups (Khaliq et al., 2000). They also observed that 1-1-2 haplotype was most common in the Makrani, Punjabis, and Sindhis. Significant differences in haplotype distribution among three Indian caste populations were also observed (Mitra et al., 2003). Thus, distributions of haplotype frequencies also tend to differ due to differences in the ethnicity among Indians. There is only one study from eastern India which has performed haplotype analysis in oral cancer (Mitra et al., 2005). In present study, all pair-wise haplotypes showed significant linkage disequilibrium. We did not find significant difference in the frequencies of haplotypes between cases and controls. On the other part, haplotype 1-2-2 and 2-1-2 were more prevalent in cases compared to controls while 1-1-1 was completely absent in cases. 1-2-1 and 2-2-1 haplotypes were completely absent in our population whereas in the population from eastern India, 1-2-1 haplotype was present and 2-2-2 and 2-2-1 haplotypes were absent (Mitra et al., 2005). We did not find any significant association between haplotypes and oral cancer risk. Mitra et al. (2005) found that individuals who were carrier of haplotype 1-2-2 were at risk of developing oral cancer that was also observed to be more prevalent among our population especially in cases. Present study also observed that 2-1-2 haplotype was more frequent in cases compared to controls. Earlier study from India has also found that 2-1-2 haplotype was more prominent in head and neck cancer patients compared to controls (Mitra et al., 2003).

In conclusion, this is the first study on Gujarati population from west India. Our data suggest that the *p53* polymorphisms especially Arg72Pro in exon 4 and its combination with 16 bp duplication in intron 3 and G>A transition in intron 6 could significantly modify the risk of oral cancer development in this population. Moreover, *p53* codon 72 genotypes might be associated with tobacco associated oral cancer development. Both alleles at each locus and haplotype analysis give important information. Further larger sample size will give more valuable information which may be used for the detection of individuals who will be at risk for this malignancy. Identification of such candidates is very crucial for screening and prevention programs and also may help in early detection of this dreaded disease.

References

Avigad S, Barel D, Blau O, et al (1997). A novel germ line *p53* mutation in intron 6 in diverse childhood malignancies. *Oncogene*, **14**, 1541-5.

Azzam GA, Frank AK, Hollstein M, Murphy ME (2011). Tissue-specific apoptotic effects of the *p53* codon 72 polymophism in a mouse model. *Cell Cycle*, **10**, 1352-5.

Beckman G, Birgander R, Själander A, et al (1994). Is *p53* polymorphism maintained by natural selection? *Hum Hered*, **44**, 266-70.

Beenken SW, Karsenty G, Raycroft L, Lozano G (1991). An intron binding protein is required for transformation ability of *p53*. *Nucleic Acids Res*, **19**, 4747-52.

Bergamaschi D, Gasco M, Hiller L, et al (2003). *p53* polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis. *Cancer Cell*, **3**, 387-402.

Chakrabarti S, Dasgupta S, Roy S, et al (2001). Microsatellite instability in squamous cell carcinoma of head and neck from the Indian patient population. *Int J Cancer*, **92**, 555-61.

Chumakov PM, Jenkins JR (1991). BstNI/NciI polymorphism of the human *p53* gene (T*P53*). *Nucleic Acids Res*, **19**, 6969.

Dumont P, Leu JI, Della Pietra AC 3rd, George DL, Murphy M (2003). The codon 72 polymorphic variants of *p53* have markedly different apoptotic potential. *Nat Genet*, **33**, 357-65.

Felley-Bosco E, Weston A, Cawley HM, Bennett WP, Harris CC (1993). Functional studies of a germ-line polymorphism at codon 47 within the *p53* gene. *Am J Hum Genet*, **53**, 752-9.

Ferlay J, Shin HR, Bray F, et al (2010). Estimates of worldwide burden of cancer in 2008: Globocan 2008. *Int J Cancer*, **127**, 2893-917.

Francisco G, Menezes PR, Eluf-Neto J, Chammas R (2011). Arg72Pro *P53* polymorphism and cancer susceptibility: a comprehensive meta-analysis of 302 case-control studies. *Int J Cancer*, **129**, 920-30.

Frank AK, Leu JI, Zhou Y, et al (2011). The codon 72 polymorphism of *p53* regulates interaction with NF-{kappa} B and transactivation of genes involved in immunity and inflammation. *Mol Cell Biol*, **31**, 1201-13.

Gallì P, Cadoni G, Volante M, et al (2009). A case-control study on the combined effects of *p53* and p73 polymorphisms on head and neck cancer risk in an Italian population. *BMC Cancer*, **9**, 137.

Gemignani F, Moreno V, Landi S, et al (2004). A T*P53* polymorphism is associated with increased risk of colorectal cancer and with reduced levels of T*P53* mRNA. *Oncogene*, **23**, 1954-6.

Hrstka R, Beranek M, Klocova K, Nenutil R, Vojtesek B (2009). Intronic polymorphisms in T*P53* indicate lymph node metastasis in breast cancer. *Oncol Rep*, **22**, 1205-11.

Hsieh LL, Huang TH, Chen IH, et al (2005). *p53* polymorphisms associated with mutations in and loss of heterozygosity of the *p53* gene in male oral squamous cell carcinomas in Taiwan. *Br J Cancer*, **92**, 30-5.

Hu Z, Li C, Chen K, et al (2008). Single nucleotide polymorphisms in selected apoptotic genes and BPDE-induced apoptotic capacity in apparently normal primary lymphocytes: a genotype-phenotype correlation analysis. *J Cancer Epidemiol*, **2008**, 147905.

Hu Z, Li X, Qu X, et al (2010). Intron 3 16 bp duplication polymorphism of T*P53* contributes to cancer susceptibility: a meta-analysis. *Carcinogenesis*, **31**, 643-7.

Imyanitov EN, Togo AV, Hanson KP (2004). Searching for cancer-associated gene polymorphisms: promises and obstacles. *Cancer Lett*, **204**, 3-14.

Katiyar S, Thelma BK, Murthy NS, et al (2003). Polymorphism of the *p53* codon 72 Arg/Pro and the risk of HPV type 16/18-associated cervical and oral cancer in India. *Mol Cell Biochem*, **252**, 117-24.

Khaliq S, Hameed A, Khaliq T, et al (2000). *P53* mutations, polymorphisms, and haplotypes in Pakistani ethnic groups and breast cancer patients. *Genet Test*, **4**, 23-9.

Kietthubthew S, Sriplung H, Au WW, Ishida T (2003). The *p53* Codon 72 polymorphism and risk of oral cancer in Southern Thailand. *Asian Pac J Cancer Prev*, **4**, 209-14.

Kuroda Y, Nakao H, Ikemura K, Katoh T (2007). Association between the T*P53* codon72 polymorphism and oral cancer risk and prognosis. *Oral Oncol*, **43**, 1043-8.

Lazar V, Hazard F, Bertin F, et al (1993). Simple sequence repeat polymorphism within the *p53* gene. *Oncogene*, **8**, 1703-5.

Lehman TA, Haffty BG, Carbone CJ, et al (2000). Elevated frequency and functional activity of a specific germ-line *p53* intron mutation in familial breast cancer. *Cancer Res*, **60**, 1062-9.

Lozano G, Levine AJ (1991). Tissue-specific expression of *p53* in transgenic mice is regulated by intron sequences. *Mol Carcinog*, **4**, 3-9.

Matlashewski GJ, Tuck S, Pim D, et al (1987). Primary structure polymorphism at amino acid residue 72 of human *p53*. *Mol Cell Biol*, **7**, 961-3.

Mattick JS (1994). Introns: evolution and function. *Curr Opin Genet Dev*, **4**, 823-31.

Mitra S, Chatterjee S, Panda CK, et al (2003). Haplotype structure of T*P53* locus in Indian population and possible association with head and neck cancer. *Ann Hum Genet*, **67**, 26-34.

Mitra S, Sikdar N, Misra C, et al (2005). Risk assessment of *p53* genotypes and haplotypes in tobacco-associated leukoplakia and oral cancer patients from eastern India. *Int J Cancer*, **117**, 786-93.

Mittal RD, George GP, Mishra J, Mittal T, Kapoor R (2011). Role of functional polymorphisms of *p53* and p73 genes with the risk of prostate cancer in a case-control study from Northern India. *Arch Med Res*, **42**, 122-7.

Nagpal JK, Patnaik S, Das BR (2002). Prevalence of high-risk human papilloma virus types and its association with *p53* codon 72 polymorphism in tobacco addicted oral squamous cell carcinoma (OSCC) patients of Eastern India. *Int J Cancer*, **97**, 649-53.

Ozeki C, Sawai Y, Shibata T, et al (2011). Cancer susceptibility polymorphism of *p53* at codon 72 affects phosphorylation and degradation of *p53* protein. *J Biol Chem*, **286**, 18251-60.

Pandith AA, Shah ZA, Khan NP, et al (2010). Role of T*P53* Arg72Pro polymorphism in urinary bladder cancer predisposition and predictive impact of proline related genotype in advanced tumors in an ethnic Kashmiri population. *Cancer Genet Cytogenet*, **203**, 263-8.

Paterson IC, Eveson JW, Prime SS (1996). Molecular changes in oral cancer may reflect aetiology and ethnic origin. *Eur J Cancer B Oral Oncol*, **32**, 150-3.

Pim D, Banks L (2004). *p53* polymorphic variants at codon 72 exert different effects on cell cycle progression. *Int J Cancer*, **108**, 196-9.

Saini R, Tang TH, Zain RB, et al (2011). Significant association of high-risk human papillomavirus (HPV) but not of *p53* polymorphisms with oral squamous cell carcinomas in Malaysia. *J Cancer Res Clin Oncol*, **137**, 311-20.

Sameer S, Shah ZA, Syeed N, et al (2010). T*P53* Pro47Ser and Arg72Pro polymorphisms and colorectal cancer predispostion in an ethinic Kashmiri population. *Genet Mol Res*, **9**, 651-60.

Schneider-Stock R, Mawrin C, Motsch C, et al (2004). Retention of the arginine allele in codon 72 of the *p53* gene correlates with poor apoptosis in head and neck cancer. *Am J Pathol*, **164**, 1233-41.

Shamsher M, Montano X (1996). Analysis of intron 4 of the *p53* gene in human cutaneous melanoma. *Gene*, **176**, 259-62.

Sprague BL, Trentham-Dietz A, Garcia-Closas M, et al (2007). Genetic variation in T*P53* and risk of breast cancer in a population-based case control study. *Carcinogenesis*, **28**, 1680-6.

Stephens M, Smith NJ, Donnelly P (2001). A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet*, **68**, 978-89.

Storey A, Thomas M, Kalita A, et al (1998). Role of a *p53* polymorphism in the development of human papillomavirus-associated cancer. *Nature*, **393**, 229-34.

Suresh K, Chandiraasekar R, Kumar BL, Venkatesan R, Sasikala K (2010). No association between the T*P53* codon 72 polymorphism and Head and Neck Cancer: A case –control study in a south Indian population. *Asian Pac J Cancer Prev*, **11**, 1749-53.

Suresh K, Venkatesan R, Chandirasekar R, Kumar BL, Sasikala K (2011). Association of T*P53* Arg72Pro polymorphic variants with breast cancer - a case control study in south Indian population. *Biology and Medicine*, **3**, 15-22.

Tandle AT, Sanghvi V, Saranath D (2001). Determination of *p53* genotypes in oral cancer patients from India. *Br J Cancer*, **84**, 739-42.

Thomas M, Kalita A, Labrecque S, et al (1999). Two polymorphic variants of wild-type *p53* differ biochemically and biologically. *Mol Cell Biol*, **19**, 1092-100.

Wang-Gohrke S, Weikel W, Risch H, et al (1999). Intron variants of the *p53* gene are associated with increased risk for ovarian cancer but not in carriers of BRCA1 or BRCA2 germline mutations. *Br J Cancer*, **81**, 179-83.

Weston A, Wolff MS, Morabia A, et al (1998). True extended haplotypes of *p53*: indicators of breast cancer risk. *Cancer Genet Cytogenet*, **102**, 153-4.

Whibley C, Pharoah PD, Hollstein M (2009). *p53* polymorphisms: cancer implications. *Nat Rev Cancer*, **9**, 95-107.

Wu X, Zhao H, Amos CI, et al (2002). *p53* Genotypes and haplotypes associated with lung cancer susceptibility and ethnicity. *J Natl Cancer Inst*, **94**, 681-90.

Zhu F, Dollé ME, Berton TR, et al (2010). Mouse models for the *p53* R72P polymorphism mimic human phenotypes. *Cancer Res*, **70**, 5851-9.

Zhuo XL, Li Q, Zhou Y, et al (2009). Study on T*P53* codon 72 polymorphisms with oral carcinoma susceptibility. *Arch Med Res*, **40**, 625-34.