RESEARCH COMMUNICATION

The *p53* Codon 72 Polymorphism and Risk of Oral Cancer in Southern Thailand

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Abstract

The codon 72 polymorphism of the *p53* tumor suppressor gene has been investigated extensively for its association with various cancers around the world. However, its influence has not been elucidated in the Thai population. Therefore, a case-control study with 97 patients and 97 matched controls was conducted to elucidate the association between the polymorphic p53 and oral cancer risk in a Southern Thai population. The frequencies of the Arg/Arg, Arg/Pro, and Pro/Pro genotypes were 36%, 35%, and 29%, respectively in the controls and 33%, 45% and 22%, respectively in the patients. This study shows that there was no significant association between the p53 codon 72 polymorphism and oral cancer risk. There was also no link with respect to smoking or drinking habits. However, our data suggest that for individuals who were younger than 65 years old, the Pro/Pro genotype may offer some protection against oral cancer (OR = 0.13, 95%CI 0.04-1.10). This is the first report on p53 polymorphism and oral cancer in Thailand.

Key Words: p53 codon 72 polymorphism - oral cancer - tobacco smoking - alcohol consumption - betel chewing - genetic susceptibility

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Introduction

Oral cancer, mainly oral squamous cell carcinoma (OSCC), is one of the ten most common cancers in the world (Scully and Bedi, 2000) and the disease is predicted to increase in prevalence during the next several decades (Sciubba, 2001). In Thailand, the incidence for the disease is the highest in the southern part of the country where it is ranked the second in males and the sixth in females among all cancers (Srivatanakul et al., 1999). While the major risk factors for oral cancer are cigarette smoking and alcohol consumption in Western countries, the risk factors in Southern Thailand are alcohol drinking, tobacco smoking, betel quid chewing and the use of smokeless tobacco (Kerdpon et al., 2001). The latter practices are similar to those reported in other Asian countries (Ko et al., 1995; Balaram et al., 2002). Similar to many other malignancies, oral cancer is caused by the interactions between genetic and certain epigenic or environmental factors. The genetic component may influence an individual's susceptibility to cancer. This component includes polymorphic genes that modulate chemical metabolism, DNA repair, and cell cycle control (Sreelekha et al., 2001; Topcu et al., 2002). In our investigation of genetic susceptibility to oral cancer in Southern Thailand, we have reported that inheritance of the *GSTM1* null allele conferred an increased risk for oral cancer. The increased risk is particularly obvious among those who have had life-style risk habits such as alcohol drinking, tobacco smoking and betel chewing (Kietthubthew et al., 2001).

The *p53* tumor suppressor gene contributes to the maintenance of genomic stability by controlling cell cycle and facilitating DNA repair in response to DNA damage (Hollstein et al., 1991). It is a gatekeeper or guardian gene

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of cell division (Levine, 1997). Therefore, alterations in p53 function are critical events in carcinogenesis. In fact, mutations of p53 are frequently found in a variety of human cancers including oral cancer (Partridge et al., 2000; Oswald et al., 2000; Hsieh et al., 2001).

The *p53* gene was found to be polymorphic with a single nucleotide polymorphism in the exon 4 in codon 72 (Matlashewski et al., 1987). The substitution of G to C changes the amino acid from arginine to proline. It gives rise to three genotypes, Arg/Arg, Arg/Pro and Pro/Pro. More recently, the p53Arg and p53Pro proteins are reported to be biologically and biochemically different from each other (Thomas et al., 1999; Martin et al., 2000). For example, Storey et al demonstrated that p53Arg was more readily degraded by the E6 oncoprotein of the high-risk human papilloma virus strains (HPV16 and 18) than the p53Pro and the Arg/Arg genotype increased the risk of HPV-related cervical cancer (Storey et al., 1998). Several other studies in the association of the *p53* polymorphism on cancer susceptibility were reported recently, however, the results have not been consistent (Kuroda et al., 2003; McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Connor et al., 2001; Tsai et al., 2002; Lee et al, 2000; Guimaraes et al.,2001; Nagpal et al., 2002; Shen et al., 2002). So far, there were only few reports on the association of the *p53* codon 72 polymorphism and oral cancer (McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Nagpal et al., 2002; Shen et al., 2002). Summersgill et al (2000) reported that there was no association between the *p53* codon 72 polymorphism and the risk for oral cancer among Caucasians. Nagpal et al (2002) observed that the Arg/Arg genotype conferred susceptibility to HPV infection and oral carcinogenesis among an Eastern Indian population. Shen et al (2002) reported that although the *p53* polymorphism was not associated with head and neck cancer among a group of non-Hispanic white population; the Pro allele was associated with an early age of onset of the cancers, particularly oral cancer. The controversy could be due to differences in ethnic composition of the studied populations and to their associated risk factors as mentioned earlier. Since such an investigation has not been reported in Thailand, we have studied the *p53* codon 72 polymorphism and oral cancer with adjustment based on the presence of other significant risk factors.

Materials and Methods

Recruitment of study participants

In this case - control study, the criteria for recruitment of patients and controls were the same as those used in our previous study (Kietthubthew et al., 2001). The two groups were one-to-one matched by sex and by age ($\pm$ 5 years), smoking status and drinking status. The study subjects included 97cases and 97 controls. Briefly, patients with cancer in the oral cavity, histologically squamous cell carcinoma, were sequentially recruited from August 1998 to May 2001 from the Department of Radiology, Songkhlanagarind Hospital, before they had chemo- and/or radio-therapy. The controls were recruited from residents living in the similar geographic area (Songkhla Province and its vicinity). All individuals voluntarily participated in the study after they had provided informed consent. Each participant was personally interviewed with a questionnaire that had been approved by the university ethic committee. The information collected and used in this study were related to past history of individual's demographic background, tobacco smoking, use of smokeless tobacco, betel chewing, alcohol drinking, and other possible risk factors as occupational exposure, nutrition, oral hygiene as well as personal and family history of various cancers.

Blood collection and DNA extraction

Peripheral blood samples from the qualified participants were collected. Each blood sample was centrifuged at 2,000 rpm, blood cells were kept frozen (-20°C) until whole blood DNA extraction was performed. A non-organic DNA extraction procedure was used to isolate DNA specimens (Sambrook et al., 1989).

Analysis of the p53 codon 72 polymorphism

The exon 4 of the *p53* gene was amplified by the PCR procedure. The upper and the lower primer was 5' - CCCGGACGATATTGAACA- 3' and 5'- AGAAGCCCAGACGGAAAC- 3', respectively. The reaction conditions were: activation of polymerase at 95°C for 9 min, then followed by 40 cycles of 94°C for 1 min; 61°C for 1 min; 72°C for 1 min. The PCR product was a 203 base pair DNA fragment. The fragment was then digested for two hours by a restriction enzyme, using either of these enzymes: *ACCII* (Takara, Japan), at 37°C or *BstUI* (New England BioLabs, USA) at 60°C. The genotypes were determined by electrophoresis on 3% agarose gel and visualized with ethidium bromide under UV light. The enzymes cut the PCR product of the Arg allele into two fragments, 125 bp and 78 bp, while the PCR product of the Pro allele remained uncut (Fig 1) .

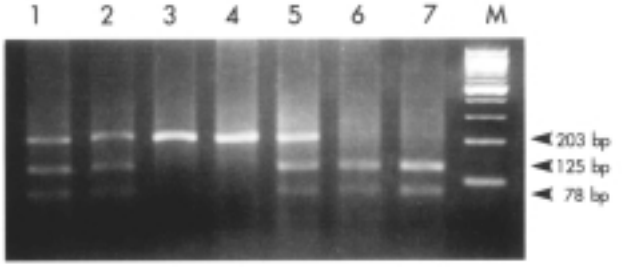

Figure 1. *p53* Polymorphism on 3% Agarose Gel Evaluated by the Restriction Enzyme Analysis. The Arg allele showing two small bands at 125 bp and 78 bp (lanes 6,7). The Pro allele showing a single band of 203 bp (lane 3,4). The heterozygous form has three bands at 203, 125 and 78 bp (lanes 1,2,5). M is the DNA 100 bp marker.

Statistical Analysis

The data were analysed by using Stata statistical software (Stata version 7.0). Chi-squared test was used for the comparison of proportion of categorical variables and t-test was used to compare the age. Due to the skewness of the data, the difference in alcohol consumption (gm-ethanol), tobacco smoking (pack-yr), and betel quid chewing (quid-yr) between cases and controls were tested by rank-sum (Mann-Whitney) test. This study was a matched design case-control, therefore, conditional logistic regression was applied appropriately to obtain odd ratios (ORs) of the association between oral cancer and the *p53* polymorphism, crude and adjusted for demographic and risk behavior variables and their 95% confidence intervals. Stratified analysis by smoking and drinking status with adjustment for levels of exposure cigarette, alcohol and betel chewing gave the risk of oral cancer for Arg/Pro and Pro/Pro genotypes of the *p53*, using the Arg/Arg genotype as a reference.

Results

As shown in Table 1, the study subjects including 97cases and 97 controls. They were well matched based on the selection criteria (age and gender) and the following results were obtained.

Table 2 shows the distribution of genotypes for the *p53* codon 72 polymorphism. The genotype frequency for Arg/Arg, Arg/Pro, and Pro/Pro were 33%, 45%, 22%, respectively in the patients, and were 36%, 35% and 29% in the controls, respectively. The distributions of genotype in both groups were in Hardy-Weinberg equilibrium. Furthermore, there was no significant difference ($p = 0.07$) in the allele frequencies between the control and case groups. Smoking and alcohol drinking behavior did not alter the risk for oral cancer based on the *p53* polymorphism. In subjects who consumed both tobacco and alcohol, the risk of oral cancer for Arg/Pro and Pro/Pro genotypes of *p53* was not significant (OR = 1.69, 95%CI = 0.61-4.71 and OR = 1.40, 95%CI = 0.41-4.73, respectively) using the Arg/Arg genotype as the reference. In subjects who neither smoked nor drank alcohol, the stratified analysis also demonstrated no association of oral cancer and the Arg/Pro or Pro/Pro genotypes (OR = 0.58, 95%CI = 0.09-3.81 and OR = 0.14, 95%CI = 0.01-2.17, respectively). Since there were few individuals who had a smoking or drinking habit alone, these two strata were excluded.

Table 1. Characteristics of the Subjects

		Case (97)	Control (97)	p-value
Sex	male	67	67	1.000[1]
	female	30	30	
Age	mean	67.4	67.8	0.812[2]
	SD	9.8	9.5	
Alcohol	Median	9.8	10.45	0.674[3]
(gm-ethanol)	Q1, Q3	0, 46.58	0, 29.00	
Smoking	Median	200	164	0.622[3]
(pack-yr)	Q1, Q3	0, 440	0, 410	
Betel Quid	Median	0	0	< 0.001[3]
(quid-yr)	Q1, Q3	0, 2880	0, 2640	

Q1 = first quatile , Q3 = third quatile, 1 = Chi-squared, 2 = t-test, 3 = rank-sum test

To assess the age-dependent development of oral cancer and the influence of the *p53* gene, we have subdivided the patient population into three age categories, irrespective of smoking and drinking habits: < 65, 66-75 and >75 years old, respectively. The data, as shown in Table 3, confirms that the *p53* variant genotype does not increase the risk for oral cancer. The Pro/Pro genotype seems to protect against oral cancer (using Arg/Arg as reference) in individuals younger than 65 years but statistical significance is not achieved (OR = 0.13, 95%CI = 0.02–1.10).

Discussion

The *p53* codon 72 polymorphism has been extensively investigated for its association in many diseases, including

Table 2. Association of *p53* Codon 72 Polymorphism and Risk Behavior and Oral Cancer

p53	Case	Control	OR	95%CI	Adj.OR*	95% CI
Arg/Arg(%)	32(33)	35(36)	ref.		ref.	
Arg/Pro(%)	44(45)	34(35)	1.37	0.74-2.53	1.29	0.58-2.86
Pro/Pro(%)	21(22)	28(29)	0.81	0.40-1.66	0.93	0.37-2.36
both smoke and drink						
Arg/Arg (%)	16(31)	20(39)	ref.		ref.	
Arg/Pro (%)	25(48)	19(37)	1.48	0.65-3.37	1.69	0.61-4.71
Pro/Pro (%)	11(21)	12(24)	1.11	0.43-2.91	1.40	0.41-4.73
never smoke never drink						
Arg/Arg (%)	11(37)	9(30)	ref.		ref.	
Arg/Pro (%)	13(43)	11(37)	1.00	0.31-3.24	0.58	0.09-3.81
Pro/Pro (%)	6(20)	10 (33)	0.43	0.10-1.87	0.14	0.01-2.17

* adjusted for past exposure (10 years before) to amount of cigarette smoking, ethanol consumption, and betel chewing.

Table 3. Association of *p53* and Age of Onset of Oral Cancer

p53 polymorphism	Age group		
	< = 65	66 - 75	> 75
Arg/Arg	ref	ref	ref
Arg/Pro	0.66 (0.17-2.58)	1.86 (0.48-7.33)	1.29 (0.23-7.33)
Pro/Pro	0.13 (0.02-1.10)	3.59 (0.67-19.17)	1.23 (0.26-5.92)

oral cancer (Kuroda et al., 2003; McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Connor et al., 2001; Tsai et al., 2002; Lee et al, 2000; Guimaraes et al.,2001; Nagpal et al., 2002; Shen et al., 2002), however, the results are inconsistent. The discrepancy could be caused by the different ethnic composition of the studied populations (McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Connor et al., 2001; Tsai et al., 2002; Lee et al, 2000; Guimaraes et al.,2001; Nagpal et al., 2002; Shen et al., 2002) and by the different risk habits in various regions (Summersgill et al., 2000; Guimaraes et al.,2001; Nagpal et al., 2002; Shen et al., 2002). The present study on oral cancer of the Southern Thai population indicates that there is no significant association between the *p53* codon 72 polymorphism and oral cancer. The *p53* polymorphism does not show evidence of interaction with any frequently practiced risk habits on oral cancer risk. This finding is concordant with some previous reports (McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Shen et al., 2002). Although this is a negative study of *p53* polymorphism and oral cancer, this is the first report in Southeast Asian region, where the risks exposure and modifying factors for oral carcinogenesis may be different from the previous studies.

The information on the interaction of the *p53* codon 72 polymorphism and oral cancer remains obscure. There were six publications in the literature on the relationship between the *p53* polymorphism and oral cancer (McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Nagpal et al., 2002; Shen et al., 2002). McWilliam et al (2000), Hamel et al (2000) and more recently Shen et al (2002) did not find that the p53 codon 72 polymorphism plays a role in the risk for squamous cell carcinoma of the head and neck (SCCHN) among Caucasians. Although, Shen et al (2002) suggested that the Pro/Pro genotype was associated with an early onset in oral cancer (p=0.046), they commented that the observation needed to be confirmed by a larger sample size. In another study in a Caucasian population, Summersgill et al (2000) also failed to demonstrate the relationship between the *p53* polymorphism and oral cancer either with or without HPV infection. There were two reports concerning interaction of the *p53* polymorphism and oral cancer from Indian population. Tandle et al (2001) did not observe an association of the *p53* genotype and oral cancer susceptibility whereas Nagpal et al (2002) indicated that the Arg/Arg genotype increased susceptibility to HPV infection and associated with oral carcinogenesis. The latter study was investigated in 110 patients who were highly addicted to tobacco and betel quid chewing. As mentioned earlier, the major differences in the prevalence of the variant alleles and the associated risk factors may affect the risk association. The genotypes frequency of the normal control in many studies showed that there were significant differences in the prevalence of the *p53* codon 72 polymorphism among different ethnic populations (Kuroda et al., 2003; McWilliams et al., 2000; Hamel et al., 2000; Summersgill et al., 2000; Tandle et al., 2001; Connor et al., 2001; Tsai et al., 2002; Lee et al, 2000; Guimaraes et al.,2001; Nagpal et al., 2002; Shen et al., 2002). Our current analysis reveals that in the Southern Thai population, the frequencies of the *p53* codon 72 polymorphism (Arg/Arg, Arg/Pro, and Pro/Pro) are 36%, 35%, and 29%, respectively. The distribution pattern is similar to those reported in most Asian populations [Japanese 36%, 44% and 20 % (Kuroda et al., 2003); Indian 14%, 65% and 20% (Tandle et al., 2001); Taiwanese 37.0%, 45.7% and 17.3% (Lee et al, 2000)]. In one Chinese study (Guimaraes et al.,2001), the frequency of the p53 Pro/Pro genotypes was extremely high (42.1%) while a report of the Eastern Indian (Nagpal et al., 2002), very low Pro/Pro genotype was observed However, the sample sizes of these two studies were very small, i.e. 57 and 26, respectively. In Asian populations, the distribution of the heterozygous form (Arg/Pro) was more common than the homozygous genotypes. This distribution pattern was different from the Caucasian populations which showed that the Arg/Arg was the most common genotype, with frequencies ranging from 53% (Shen et al., 2002) to 79% (Connor et al., 2001) whereas the frequencies of the homozygous Pro/Pro genotype were very low 3.5% (Connor et al., 2001), 7.2% (Shen et al., 2002). Such major differences in the distribution of the variant alleles in different ethnic populations may impact their contribution to the susceptibility to environmental related cancers, like oral cancer.

Infection with human papilloma virus (HPV), especially the high risk types (HPV16/18), is involved in oral carcinogenesis (Nagpal et al., 2002; Miller et al., 1989; Schwart et al., 1998). This relationship has support from mechanistic observation which indicated that the E6 protein of the HPV16/18 binds to *p53*, thus facilitating the development of cancer (Storey et al., 1998). Recently, Nagpal et al (2002) revealed that the p53 polymorphism increased the susceptibility of the HPV-related oral carcinogenesis of Eastern Indians who were highly addicted to chewing tobacco and betel quid. They indicated that Arg/

Arg genotype caused more susceptible to oral cancer than Pro/Pro genotype. This data was contradictory to an earlier published data from a Caucasian study (Summersgill et al., 2000). In our study, we did not investigate the infection with HPV in our patients and controls. However, the interaction between the E6 and the p53 proteins may contribute to a significant influence on the risk for oral cancer. Of particular relevance is the observation that the p53Arg protein is more readily degraded by E6 than the p53Pro. Under this scenario, inactivation of the Arg/Arg genotype by E6 becomes the major risk factor, leaving the Arg/Pro and the Pro/Pro genotypes to interact with other risk factors for their contribution to oral cancer. With this consideration, our observed interactions between smokers/drinkers and the Arg/Pro and Pro/Pro genotypes for risk for oral cancer are possible. Furthermore, the protective effect of the Pro/Pro genotype is also possible.

In conclusion, the *p53* codon 72 polymorphism is not associated with oral cancer in Southern Thailand. The Pro/Pro genotype may offer some protective effect on oral cancer. The interactions between the HPV E6 protein and the p53 proteins from the different alleles need to be considered to understand more precisely the risk factors for oral cancer. Furthermore, the relationship between the *p53* polymorphism and polymorphisms in xenobiotics metabolizing genes will need to be considered when the sample size is large enough for such interactive analysis.

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