

RESEARCH COMMUNICATION

Codon72 Polymorphism in the P53 Tumor Suppressor Gene in Oral Lichen Planus Lesions in a Thai Population

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Abstract

Background: Oral lichen planus (OLP) is a T-cell mediated autoimmune disease in which autotoxic CD8+ T cells (CTLs) trigger apoptosis of oral epithelial cells. Activated CTLs can produce Fas ligand and by binding to Fas lead to apoptosis. This Fas pathway and the action of p53 tumour suppressor gene are important in producing apoptosis. Current data demonstrate a link between these two factors at the transcriptional level. **Objective:** The purpose of this study was to investigate the p53 polymorphism at codon 72 which results in encoding of either proline or arginine. **Methods:** Our study used 97 OLP cases and 94 control blood samples from non-OLP individuals and performed PCR-RFLP. **Results:** Compared to control individuals, we found a significant increase in occurrence in OLP patients of the proline encoding cytosine allele (adjusted odd ratio (95% CI)=2.29 (1.42-3.70) and p=0.001). In addition, in individuals with the non-erosive type of OLP, the same situation was evident (OR=2.29, 95% CI (1.38-3.78), p=0.001). Furthermore, we noted a significantly higher prevalence of homozygosity [OR=3.17, 95% CI(1.58-7.25), p=0.001] for the p53 pro allele in the OLP group, which indicates a recessive mode of inheritance. **Conclusion:** Our data suggest a strong association between the pro/pro genotype and OLP, and that the process of apoptosis, in which p53 plays a role, is a factor in OLP pathogenesis.

Keywords: Oral lichen planus - polymorphism - p53 codon 72 - apoptosis

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Introduction

Oral lichen planus (OLP) is a chronic autoimmune inflammatory condition manifesting as small white or red patches, pain, and a burning sensation. The prevalence of OLP is 0.5-2.2% in a typical patient population, and is more common in women than in men (Axell and Rundquist, 1987). Generally, affected patients experience a feeling of burning and irritation on consumption of hot or spicy foods and have difficulty with swallowing and speech. These symptoms can lead to malnutrition and a low quality of life (Lopez-Jornet et al., 2009).

Histopathologically, the epithelium in OLP patients shows a degeneration of the basal cell layer. There is evidence suggesting that autotoxic CD8+ T cells trigger apoptosis of oral epithelial cells in OLP lesions (Porter et al., 1997). The apoptosis is primarily due to cytotoxic CD8+ T cells expressing both the Fas and the ligand (FasL)(Ni et al., 2001; Guillermo et al., 2007) that act against the epithelial cells. There is data showing that apoptosis from the expression of p53 protein in OLP is the causal factor in OLP(Acay et al., 2006; Gonzalez-

Moles et al., 2008).

The p53 tumour suppressor gene is now known to be involved in apoptosis (Yonish-Rouach et al., 1991; Meulmeester and Jochemsen, 2008; Nayak et al., 2009; Zuckerman et al., 2009). In DNA damaged cells, this gene responds by activating the downstream target (p21WAF1) to arrest the cell cycle and facilitate cell repair. However, if the cell damage is intense and beyond repair, p53 can induce apoptosis by activating transcription of genes such as PUMA (Vousden, 2005) Bax (Bouvard et al., 2000) and Fas (Lin et al., 2002b).

Single nucleotide polymorphisms (SNPs) of p53 codon 72 (SNP: P53 CODON 72) have been identified. SNP: P53 CODON 72 encodes two types of amino acid: Arginine (R) and Proline (P). This nucleotide has been reported to be involved in susceptibility to open angle glaucoma, which is a disease involving cell death by apoptosis(Lin et al., 2002a). Report of OLP and its relationship to SNP: P53 CODON 72 in an Iran population has already been published. But, there are studies show that there is a correlation between SNP: P53 CODON 72 and ethnicity (e.g. in Caucasian, Turkish and Japanese population, allele

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G is more prevalent than C; in Africans, allele G is less than C (Weston et al., 1992; Beckman et al., 1994; Jin et al., 1995; Ngan et al., 1999; Peixoto et al., 2001).

The aim of this study was to investigate SNP: P53 CODON 72 and OLP in Thai population.

Materials and Methods

OLP Samples and DNA Extraction

Ninety seven oral mucosal samples from OLP cases were obtained from the Faculty of Dentistry, Mahidol University. All specimens were embedded in paraffin blocks and diagnosed OLP by an oral pathologist. Clinical data were collected from the patients' files. The samples were then divided into two groups. One group was fixed in formalin and submitted for routine histo-pathological examination, and the second was fixed and embedded in paraffin wax for use in further experiments. Ninety four control blood samples were collected from random donors. From OLP paraffin-embedded samples that contained 10% of lesion and 90% of connective tissue, DNA was extracted using a Megnesil Genomic fixed tissue system kit (Promega, Madison, WI). Briefly, incubation buffer/proteinase K solution was added to 2-micron-thick tissue sections and which were incubated overnight at 56°C. Lysis buffer and resin were added, vortexed, placed on a magnetic stand and the solution were discarded. The resins were washed in wash buffer, air-dried and then incubated at 65°C in elution buffer for 5 min. The extracted DNAs were transferred to clean tubes. After collection by centrifuging in buffy coat the Genomic DNAs from control blood cells were extracted in lysis buffer (0.075 M NaCl, 0.024 M EDTA, pH 8.0) that was mixed with 10% SDS and 20 mg/ml proteinase K, incubated at 50°C overnight and precipitated with phenol/chloroform and ethanol. After washing, the DNAs were air dried and resuspended in 50µL dH2O. The DNAs integrity was proved by gel-electrophoresis quantitative-analysis to ensure the samples were of high molecular weight.

Genotyping of the p53 Tumor Suppressor Gene.

DNA (50 ng/µl) from both the OLP and control blood samples underwent PCR-RFLP (Polymerase chain reaction - restriction fragment length polymorphism) in which the samples were amplified using specific primers and digested to detect the polymorphism in SNP: P53 CODON 72 (rs 1042522). The primers that were used for genotyping included 5'-CCGGACGATATTGAACA 3' (the forward primer) and 5' AGAAGCCCAGACGGAAAC 3' (the reverse primer). The DNA was initially denatured for 15 min at 95°C, followed by 35 cycles of 1 min at 95 °C, 1 min at 57°C, and 1 min at 72°C, and 72°C for 7 min. The resulting PCR products were digested with 40 units of BstUI, (a restriction enzyme recognizing nucleotide 5/-CG/CG- 3/) for 16 h as PCR-RFLP. The Allele at SNP: P53 CODON 72 contains CCC and encodes proline. It remains undigested. In contrast, CGC, which encodes arginine, is digested and gives two products of 79 and 125 base pairs (bp). The PCR products containing both alleles have 3 sites, the sequence encoding proline remains undigested (204bp), whereas the sequence encoding arginine is

digested into 79bp and 125bp fragments. The genotyping results were also confirmed by sequence analysis using reverse primer. Sequencing was performed using the ABI Prism 3100 DNA sequencer (Applied Biosystem, Foster City, CA, USA).

Statistical Analysis

All statistical analyses were carried out using Statcalc Program (AcaStat Software, Leesburge, VA, USA). A chi-square test was used to analyse the association between genotype and OLP samples. A value of p <0.05 was considered significant. ODDS ratio (OR) and 95% confidence interval (CI) were used as parameters to compare the frequency of SNP to be risk factor in OLP. If OR is >1, this would indicate a positive association or increase risk between the SNP allele and OLP and by contrast, <1 would be suggestive of a decrease susceptibility to disease outcome at the 5% significance level. Coefficient of variation (CV) was used to analyze the distribution of density of PCR band in each heterozygous sample to demonstrate that there was no mosaicism from epithelium and connective tissue. (A %CV <= 10 indicates there is no distribution of data.)

Results

Genotyping of SNP: P53 CODON 72

The genotypic data of SNP: P53 codon72 were summarized in Table 1. The distribution of the genotypes among the controls was in Hardy-Weinberg equilibrium (P= 0.9999999). The SNP: P53 CODON 72 distribution in our control group was similar to other previous studies in Thailand(Tiwaweche et al., 2003, Settheetham-Ishida et al., 2006), particularly when the control populations were derived from the same areas. From our studies, we were able to detect three genotypes of OLP and control blood samples. From 97 OLP cases, genotypes included Pro/Pro (46.4%), Pro/Arg (37.1%) and Arg/Arg (16.5%), while in control samples the frequency of polymorphisms were Pro/Pro (21.3%), Pro/Arg (50%) and Arg/Arg (28.7 %). Representative OLP PCR products of each of the three genotypes digested with BstUI are shown in Figure 1. Products containing C (encoding pro) at this SNP could not be digested whereas products containing G (encoding arg) could be digested. In this respect, a 204 bp fragment was

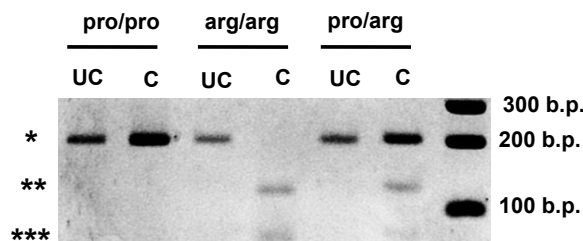


Figure 1. Genomic DNA (50 Ng/Ml) was Genotyping of Olp was Assessed by Pcr-Rflp and Compared Between Uncut (Uc) and Cut (C) with Bstu1. M Depicts a 100bp Ladder. Arg Allele was Cut with Bstu1 to Give a Product Size of 79 (*) and 125bp (). The same enzyme is unable to cut the pro allele which results in a product size of 204bp (***)**

Table 1. P53 Codon 72 Polymorphism in Oral Lichen Planus and Control Subjects

Groups	No. of samples	Ave. age	P53 genotypes (n(%))			Allele frequency (n(%))		Allelic test	
			Arg	Hetero	Pro	G	C	OR (95%CI)	P value
			G/G	G/C	C/C				
Controls	94	33.6	27(28.72%)	47(50.00%)	20(21.28%)	101(53.72%)	87(46.28%)	ref.	
Sex									
Male	55	32.6	16(29.09%)	29(52.73%)	10(18.18%)	61(55.45%)	49 (44.55%)	ref.	
Female	39	35.2	11(28.21%)	18(46.15%)	10(25.64%)	40(51.28%)	38 (48.72%)	ref.	
Oral lichen planus	97	36.2	16(16.49%)	36(37.11%)	45(46.39%)	68(35.05%)	126 (64.95%)	2.15 (1.40-3.32)	0.0004
Sex									
Male	18	53.16	1(5.55%)	6(33.33%)	11(61.11%)	8(22.22%)	28(77.78%)	4.36(1.70-11.47)	0.001
Female	79	49.54	15(18.99%)	30(37.97%)	34(43.04%)	60(37.97%)	98(62.03%)	1.72 (0.96-3.09)	0.07
Adjust OR								2.29 (1.42-3.70)	0.0005
Two main types of OLP									
Erosive type	16	46.69	2(12.5%)	6(37.5%)	8 (50%)	10(31.25%)	22(68.75%)	2.55 (1.08-6.15)	0.03
Sex									
Male	3	46.33	1(33.33%)	1(33.33%)	1(33.33%)	3 (50%)	3 (50%)	1.24 (0.19-8.18)	0.87
Female	13	46.80	1(7.69%)	5(38.46%)	7(53.85%)	7(26.92%)	19(73.08%)	2.86 (0.99-8.51)	0.05
Adjust OR								2.32 (0.95-5.79)	0.06
Non erosive type	81	51.64	14 (17.3)	30 (37.04)	37(45.68)	58(35.80)	104 (64.20)	2.08 (1.32-3.28)	0.001
Sex									
Male	15	54.53	- (0%)	5(33.33%)	10(66.67%)	5(16.67%)	25(83.33%)	6.22(2.06-20.13)	0.0003
Female	66	50.98	14(21.21%)	25(37.88%)	27(40.91%)	53(40.15%)	79(59.85%)	1.57 (0.86-2.87)	0.15
Adjust OR								2.29 (1.38-3.78)	0.001

OR; odd ratio, CI; confidence interval

Table 2. Risk of Oral Lichen Planus Associated with P53 Codon 72 Genotype According to Different Models of Inheritance

	OR (95%CI)	P value	Adjusted OR (95%CI)	P value
C dominance, G wild type				
GG	1.00		1.00	
CC or GC	2.04(0.96-4.35)	0.06	2.37(0.99-5.24)	0.05
C recessive, G wild type				
GG or GC	1.00		1.00	
CC	3.20(1.62-6.36)	0.0004*	3.17(1.58-7.25)	0.001*

*Fisher exact test: 2 tailed test

Table3: Density Average, Standard Deviation and CV of 36 Heterozygous Samples

	Density average (%)	Standard deviation (SD)	% CV
C band	58.87083	1.606584	2.73
G band at 125 bp.	28.35222	1.041019	3.67
G band at 79 bp.	12.77694	1.248434	9.77

the product of Pro/Pro ; 125 and 79 bp were the products of Arg/Arg; 125, 79 and 204 bp were the products of Pro/Arg.

SNP: P53 CODON 72 in OLP Case-Control Study

On comparison between the frequencies of these alleles in OLP patients and control group, a significant risk association with SNP: P53 CODON 72 was detected (Table 1). The OR (95% CI) was 2.15 (1.42-3.32) among OLP cases with C as the susceptibility allele. Using the Mantel-Haenszel stratification method to correct for sex matched controls increased this to 2.29 (1.42-3.70) with a p value of <0.001. The OR (95% CI) of erosive and non erosive type of OLP cases with C as susceptibility allele was 2.55(1.08-6.15) and 2.08(1.32-3.28), sex adjusted 2.32(0.95-5.79) and 2.29(1.38-3.78), respectively. We found that the OLP susceptibility conferred by SNP: P53 CODON 72 required homozygous C to increase the likelihood of OLP. When the mode of inheritance was recessive the OR (95% CI) of CC was 3.20 (1.62-6.36), sex

adjusted 3.17(1.58-7.25).When the mode of inheritance was dominant, OR (95% CI) of CC was 2.04 (0.96-4.35), sex adjusted 2.37(0.99-5.24). Our data suggested that C allele was a risk factor for OLP in recessive inheritance.

Distribution of PCR density band of heterozygous samples

To prove that there was no mosaicism from epithelium and connective tissue, the % density of 3 PCR bands in 36 heterozygous samples (C, G at 125 bp and G at 79 bp) were measured. The density average, standard deviation (SD) and % CV of each band are shown in Table 3. The % CV of C band, G band at 125 bp. and G band at 79 bp are 2.73%, 3.67% and 9.77, respectively.

Discussion

Apoptotic cell death may be a contributory cause of the cell destruction that is commonly seen in OLP. Several studies have now revealed that in the basal epithelium cells

of OLP, there are proteins related to apoptosis (Fas and FasL) at elevated levels (Tanda et al., 2000; Neppelberg et al., 2001; Shen et al., 2004; Tobon-Arroyave et al., 2004). P53 was also readily detected in these cells (Acay et al., 2006; Gonzalez-Moles et al., 2008). Fas is now known to be a p53 mediated apoptotic protein and thus there is likely to be a correlation between p53 and Fas expression (Lin et al., 2002b).

SNP: P53 CODON 72 has been reported in many diseases including cancers (Brenna et al., 2004; Kuroda et al., 2007). It is also seen in primary open angle glaucoma (POAG), a disease which is associated with cell death by apoptosis (Lin et al., 2002a) and the polymorphism at SNP: P53 CODON 72 has been reported to be associated with apoptosis (Bonafe et al., 2002). Dumont et al., reported that tumours containing the arg form, which have the ability to localize to the mitochondria and release cytochrome c into the cytosol, induced apoptosis markedly better than those of the pro form (Dumont et al., 2003). However, there are several studies where genotype at SNP: P53 CODON 72 is associated with a particular disease in one ethnic group and not in another (Lin et al., 2002a; Acharya et al., 2002; Silva et al., 2009). For instance, a study found SNP: P53 CODON 72 Pro/Pro form was the risk factor of developing POAG in Chinese people (Lin et al., 2002a) whereas there was no association in Brazilian and Indian population (Acharya et al., 2002; Silva et al., 2009). We found that pro form of SNP: P53 CODON 72 was a risk factor for developing OLP in our Thai sample. Contrasting this, there was evidence show no association between this polymorphic site and OLP in an Iranian population (Ghabanchi et al., 2009). However, there is the same trend of Pro/Pro frequency in patients and control. Perhaps the differing result is due to the small number of cases in the Iranian study.

We collected OLP samples from oral biopsies which contained epithelium and mostly connective tissue, and control samples from blood. Because the genomic changes are at the DNA level and the input can be collected from any cells including those from the oral epithelium, connective tissue or blood.

To confirm that there was no somatic cell mutation in epithelial cell of OLP samples we used coefficient of variation (CV) to investigate the distribution of PCR band density (C and G band). We measured density of each band in 36 heterozygous samples calculated the % CV and found that it was less than 10%, indicating that there was no density variation of C and G band. In addition, DNA from OLP samples contained only 10% of epithelium and 90% of connective tissue. These two factors assured us that there was neither mosaicism from epithelium and connective tissue nor somatic cell mutation of epithelial cell in OLP sample.

If POAG and OLP are associated with cell death from the apoptotic pathway, why then, in these diseases, were the pro form and not the arg form found at SNP: P53 CODON 72? The answer perhaps lies in the results of Schneider-Stock study which observed SNP: P53 CODON 72 in squamous cell carcinoma of the head and neck (SCCHN) (Schneider-Stock et al., 2004). This study found that apoptosis was not observed in tumours

carrying the arg allele but present in lesions carrying the pro allele. Moreover, it was found that the Pro/Pro status of the tumour significantly correlated with a high Fas/FasL expression while no evidence was seen in samples with Pro/Arg and Arg/Arg form. Perhaps the homozygous Pro form at this polymorphic site is an important regulator of apoptosis via the Fas/FasL pathway in SCCHN. A previous study showed Fas/FasL pathway controlled apoptosis in OLP (Shen et al., 2004). Taken together with the Schneider-Stock study this suggests that perhaps PRO at SNP: P53 CODON 72 could play a role in inducing apoptosis in OLP via Fas/FasL pathway.

Our study suggests that PRO at SNP: P53 CODON 72 is one of the genetic risk factors for OLP and that this polymorphism may be useful as one of the genetic markers for predicting the occurrence of this disease. Moreover, our data may provide genetic evidence to support the importance of P53 protein in OLP development.

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