

## RESEARCH ARTICLE

# P53 Polymorphism at Codon 72 is Associated with Keratocystic Odontogenic Tumors in the Thai Population

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### Abstract

**Objective:** To clarify the association between the *p53* polymorphism at codon 72 and susceptibility to the sporadic keratocystic odontogenic tumor (KCOT). **Design:** One hundred KCOTs and 160 match-group healthy controls were genotyped to ascertain the frequency of the *p53* codon 72 polymorphism using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), confirmed by direct sequencing. **Results:** The frequencies of the Pro/Pro, Arg/Pro, and Arg/Arg genotypes were 23.8%, 49.4%, and 26.9%, respectively, in the controls, while the KCOT cohort demonstrated 43.0%, 39.0%, and 18.0%, respectively. Further analysis suggested that *p53* Pro could be a KCOT-susceptible allele (OR (95% CI)=1.77 (1.22 to 2.59),  $p=0.0024$ ), with a sex-adjusted OR (95% CI) of 1.71 (1.17-2.50),  $p=0.0046$ . Moreover, the results indicated that *p53* codon 72 Pro homozygous was associated with a two-fold risk of developing KCOT (adjusted OR (95% CI) =2.17(1.23-3.84),  $p=0.0062$ ). **Conclusions:** The C/C genotype of *P53* gene codon 72 increases the risk of developing sporadic KCOT and may be a useful tool for screening and diagnostic purposes.

**Keywords:** Keratocystic odontogenic tumor - *P53* - polymorphism - risk

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### Introduction

Keratocystic odontogenic tumor (KCOT) represents a common benign lesion of primarily odontogenic origin that arise from the dental lamina and its remnants. It is a rare and benign but develops into a locally aggressive cystic neoplasia. Histopathologically, KCOT displays typical feature of corrugated and thin parakeratinized stratified squamous epithelium lining the cystic wall (Philipsen and Reichart, 2006; Bhargava et al., 2012). Furthermore, KCOT lesions are usually classified as sporadic or those associated with nevoid basal cell carcinoma syndrome (NBCCS) that mainly comprises multiple basal cell carcinomas of skin, calcification of falx cerebri, rib and vertebrate anomalies and KCOT (Philipsen and Reichart, 2006). Nonetheless, what remains crucial is that KCOT can manifest either as asymptomatic or as aggressive infiltrating lesions with a high recurrent rate. A challenge though, is that underlying molecular basis of this variable behavior remains unclear and an area of interest for further investigation. Research activities from several groups have now reported the involvement of key important molecules that may be playing a role in the pathogenesis of KCOT. For example, a study by Barreto, et al found a 5

bp deletion in exon 3 in 1 of 3 sporadic cyst and nonsense (C2760A) and missense (G3499A) mutation in 2 of 3 cyst associated with NBCCS (Barreto et al., 2000). A study by Guo, et al showed 11 germline and 4 somatic cell mutation of *PTCH* in 11 of 14 patients with NBCCS associated with KCOT (Guo et al., 2013). In addition, Moreira, et al, detected *p21* promoter methylation in 3 of 10 KCOT samples whereas no methylation could be detected in 10 samples of dental follicle and 6 samples of normal mucosa (Moreira et al., 2009). Moreover, work from our group has demonstrated that levels of methylation are essentially different in KCOT and ameloblastoma (Kitkumthorn and Mutirangura, 2010). Notably, these observed differences between the two sample groups were irrespective of clinical symptom and radiographic features which alone are largely inseparable. In this regard, a definitive diagnosis is essentially determined by a pathologist, after reviewing histopathological H&E stained tissue sections, however subtle differences between KCOT samples may be missed.

The *p53* tumor suppressor gene plays a crucial role in modulating key cellular processes such as cell cycle checkpoint, cell cycle arrest and apoptosis (Hanahan and Weinberg, 2011). Invariably, the *p53* gene is a frequent

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target for alterations, for example, deletions, frame shift, missense, nonsense and promoter methylation resulting in loss of function and tumorigenesis (Chen et al., 1994; Rivlin et al., 2011; Chmelarova et al., 2013). Moreover, *p53* single nucleotide polymorphism (SNP) at codon 72, has also been associated as a risk factor in many precancers and cancer including those of oral lichen planus (Yanatatsaneejit et al., 2010), cancer of cervical (Zhou et al., 2012), colorectal (Aizat et al., 2011), lung (Ren et al., 2013), breast (Vijayaraman et al., 2012) and bladder (Xu et al., 2012). In this regard, two forms of this *p53* SNP, Arg and Pro are now recognized as playing a role in tumorigenesis and notably in patients with differing demographics. For example, recent studies have found that Arg is likely to be associated with colorectal and breast cancers in Greek patient population (Kalemi et al., 2005; Dakouras et al., 2008), while largely prevalent in cervical cancer patients of Sudanese origin (Eltahir et al., 2012). The Pro SNP on the other hand, was noted to be more common in colorectal cancer patients of Malaysian (Aizat et al., 2011) and Chinese origin (Zhu et al., 2007), and lung cancer patients of Korean origin (Piao et al., 2011). In this light, follow up studies from our group observed that *p53* codon 72 polymorphism was closely aligned with ameloblastoma and suggested that this alteration could be the risk factor of this disease (Kitkumthorn et al., 2010).

Thus the focus of this study was aimed to evaluate if there was any indication of an association between *p53* codon 72 polymorphism and the risk of developing sporadic KCOT in a Thai patient population. Consequently, any new emerging knowledge can likely help in our understanding of the molecular basis of KCOT as well as identifying new strategies to help different forms of KCOT and help to distinguish between KCOT and ameloblastoma lesions, with the overall view of facilitating the overall diagnosis to aide in the choice of correct treatment options.

## Materials and Methods

### Sample collection and DNA extraction

One hundred (100) formalin fixed and paraffin-embedded (FFPE) and histopathologically confirmed KCOT biopsies collected and archived between January 2006 and December 2010 at the Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Mahidol University, were used in this study.

All specimens further underwent histopathological assessment by an oral pathologist, for reconfirmation of the presence of KCOT. In parallel, clinical data for each case was collected from archived patients' charts. Healthy controls undergoing routine health examination and found to have no past history of malignancies were enrolled into this study. Six milliliter of peripheral blood was collected from each of the 160 healthy controls after obtaining written informed consent. Both the KCOT and healthy control patient cohorts were of Thai demographics. This arm of the experiment design was approved by the Human Research Ethics Committee of Chulalongkorn University (IRB no 94/54). This study was done using a double-blind technique. In addition to obtaining sections for H&E staining, FFPE KCOT tissues were used to

obtain ~5  $\mu$ M sections in eppendorf tubes (3-5 sections) which subsequently underwent lysis followed by DNA isolation and purification using the MagneSil® Genomic fixed tissue system kit (Promega, Madison WI, USA). DNA from peripheral blood of each of the healthy subject was extracted by first digesting with proteinase K and an overnight incubation at 50°C followed by phenol/chloroform extraction and ethanol precipitation. After washing in 70% ethanol and suspending in ddH<sub>2</sub>O the genomic DNA was quantified and 100 nanogram used as a template in genotyping analysis.

### *p53* genotyping analysis

Polymorphisms *p53* at codon 72, were determined using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method using primers (5' CCCGGACGATATTGAACA3' and 5' AGAAGCCCAGACGGAAAC 3'). PCR conditions include denaturation 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 with a final extension for 7 min at 72°C. A 10  $\mu$ L aliquot of the PCR product was digested overnight at 37°C in a 15  $\mu$ L reaction volume containing 10 units of *Bst*UI (New England BioLabs, Ipswich MA). After, DNA fragments were separated by electrophoresis using a 2.5% agarose gel and after staining with ethidium bromide the bands are visualized. The premise of the band size is based on the fact that Arg/Arg homozygote cleaved by *Bst*UI and essentially yielding two size bands of 125 and 79 bp. On the other hand, the Pro/Pro homozygote is resistant to cleavage by *Bst*UI and represented by a single band of 204 bp, whereas the Arg/Pro heterozygote contained all three bands (204, 125, and 79 bp) following digestion. Twenty percent of samples were confirmed by direct sequencing of PCR products to verify the accuracy of the genotyping.

### Statistical analysis

Genotype frequencies were checked for consistency among normal controls with those expected from the Hardy-Weinberg equilibrium (HWE). All statistical analysis was calculated by using Statcalc Program (AcaStat Software, Leesburg, VA, USA). The correlation between genotype and KCOT was analyzed by chi-square test. A value of <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 a risk factor in KCOT. If OR is >1, this indicates a positive association or increase risk between the SNP allele and KCOT and by contrast, <1 is referred to a decrease susceptibility to disease outcome at the 95% confidence interval.

## Results

### Demographics

All 160 healthy controls and 100 KCOT patients were Thai population. Average age of healthy control was 32.51 years (male 31.92 years and female 33.05 years). Average age of the KCOT cohort was 32.23 years (male 35.46 and female 29 years) as shown in Table 1. Patients with KCOT in this study were all sporadic cases.

*Genotyping of SNP: P53 codon 72*

Paraffin-embedded KCOT samples and healthy control blood samples were used as specimen for genotyping. The genotype of *p53* codon 72 in KCOT and control samples was analyzed after performing PCR-RFLP. From the analysis, three genotype groups of *p53* codon 72 in patients with KCOT and control blood samples were detected. As shown in Table 1, genotypes Pro/Pro, Arg/Pro and Arg/Arg were 23.75%, 49.37% and 26.88%, respectively in controls. While in KCOT samples, this genotype was 43%, 39% and 18%, respectively. The distribution of the genotype of this SNP among the control was in Hardy-Weinberg equilibrium ( $>0.05$ ). An additional 39 heterozygous samples KCOT samples were tested for % density of 3 PCR to ensure that there was no mosaicism from epithelium cells and the % CV of all bands were less than 10%.

*SNP analysis of the P53 gene at codon 72 in KCOT*

The emerging data and allele frequency between KCOT and healthy controls is shown in Table 1. These observations were used to perform detailed statistical analysis. From the analysis, we observed that there was a significant risk associated with the *p53* codon 72 SNP. The OR (95% CI) was 1.77 (1.22-2.59) among KCOT cases with C as the susceptibility alleles. The OR (95% CI) in male was 1.39 (0.82-1.38) and in female this was 2.13 (1.22-3.71) and sex adjusted, this value came out as 1.71 (1.17-2.5).

*Model of inheritance*

Next, we sought to determine the model of inheritance. From this analysis, we noted that when the model of inheritance of this SNP was recessive, the OR (95% CI)

of CC was 2.42 (1.37-4.30), sex adjusted was 2.17 (1.23-3.84). When the mode of inheritance was dominant, the OR (95% CI) of CC or CG was 1.67 (0.87-3.26), sex adjusted was 1.68 (0.87-3.28). The data is shown in Table 2. Collectively, the data from this analysis suggest that the model of inheritance of *p53* codon 72 in KCOT, is recessive.

**Discussion**

Single nucleotide polymorphism identified at codon 72 of the *p53* gene results in an aberrant amino acid sequence and the variants, Arg and Pro translate into a protein that exhibit very different biochemical properties. For example, Arg is an amino acid with a positive charge and basic-R group whereas Pro is non-polar and cyclic amino acid (Thomas et al., 1999). Notably, the protein resulting from the Arg variant is more potent than the Pro form in its tumor suppressive properties (Dumont et al., 2003). In addition, the Arg form has now been reported to be more efficient in inducing apoptosis than Pro counterpart. Notwithstanding, cancer is a multistep process with an aberrant *p53* playing a pivotal role but whether the SNP at codon 72 of this gene, is involved in carcinogenesis still remains to be determined. In some studies, Pro/Pro and Arg/Arg variants have been found be risk factors for same type of cancers such as lung and breast cancer (Papadakis et al., 2000; Buyru et al., 2008; Piao et al., 2011; Proestling et al., 2012). Perhaps pertinent, frequencies of *p53* codon 72 genotypes have been postulated to vary according to ethnicity (Xu et al., 2012). Thus, this emerging data suggests that the codon 72 alleles of *p53* might not to be functionally involved in cancer but, rather, may be a marker in linkage disequilibrium. However, more studies

**Table 1. P53 Codon 72 Polymorphism in KCOTs and Controls**

Group	No. of samples	Ave. age (y.)	P53 genotype [n(%)]			Allele frequency [n(%)]		Allelic test OR (95% CI)	P-value
			Pro C/C	Hetero C/G	Arg G/G	C	G		
Controls	160	32.51	38(23.75%)	79(49.37%)	43(26.88%)	155(48.44%)	165(51.56%)	Ref.	
Sex									
Male	80(50.00%)	31.92	20(25.00%)	38(47.50%)	22(27.50%)	78(48.75%)	82(51.25%)	Ref.	
Female	80(50.00%)	33.05	21(26.25%)	38(47.50%)	21(26.25%)	80(50.00%)	80(50.00%)	Ref.	
KCOT	100	32.23	43(43.00%)	39(39.00%)	18(18.00%)	125(62.50%)	75(37.50%)	1.77(1.22-2.59)	0.0024
Sex									
Male	50(50.00%)	35.46	20(40.00%)	17(34.00%)	13(26.00%)	57(57.00%)	43(43.00%)	1.39(0.82-2.38)	0.2429
Female	50(50.00%)	29	23(46.00%)	22(44.00%)	5(10.00%)	68(68.00%)	32(32.00%)	2.13(1.22-3.71)	0.0065
Adjust OR								1.71(1.17-2.50)	0.0046

OR; odd ratio, CI; confidence interval

**Table 2. Risk of KCOT Associated with P53 Codon 72 Genotype according to Different Models of Inheritance**

	KCOT OR (95%CI)	P-value	Male OR (95%CI)	P-value	Female OR (95%CI)	P-value	Adjusted OR (95%CI)	P-value
C dominance, G wild type								
GG	1.00		1.00		1.00		1.00	
CC or CG	1.67(0.87-3.26)	0.1356	1.08(0.45-2.60)	0.9875	1.97(0.86-4.51)	0.1184	1.68(0.87-3.28)	0.1358
C recessive, G wild type								
CG or GG	1.00		1.00		1.00		1.00	
CC	2.42(1.37-4.30)	0.0018	3.20(1.03-10.57)	0.0425	2.39(1.06-5.41)	0.0336	2.17(1.23-3.84)	0.0062

\*CC and GG are the homozygous C and G, respectively. CG is the heterozygous. OR(95%) is odd ratios and 95% interval between allele and compared allele; \*Fisher exact test: 1 tailed test

are needed to definitively define a role of codon 72 alleles of *p53* with the multistep process of cancer including KCOT.

The molecular approach for investigating KCOT and specifically to distinguish the different forms that could be missed by histopathological evaluation is in need. In this regard, molecular evaluation could be a useful addition for clinical diagnosis, prevention and treatment. A number of genetic based studies have shown a role for *PTCH* and *p21WAF1* in the etiology of KCOT (Barreto et al., 2000; Moreira et al., 2009; Guo et al., 2013). A study by Barreto, et al suggested that mutations in *PTCH* could be an important first step in KCOT (Barreto et al., 2000). A study by Guo YY et al, 2013, reported that *PTCH* was mutated in KCOT in Chinese patients (Guo et al., 2013). While the study by Moreira, et al. reported that the *p21WAF1* promoter was methylated in 3 of 10 KCOT samples whereas no methylation could be detected in 10 dental follicles and 6 normal mucosa samples (Moreira et al., 2009).

In this study, we addressed whether an association of *p53* codon 72 alleles with KCOT was present in a Thai patient population. From the emerging data, we observed that the genotype Pro/Pro (proline) was estimated to have a 2.17 fold increased risk to developing KCOT compared to normal controls. In addition, we also noted that the mode of inheritance of this SNP was recessive. From these observations, we report that the Pro/Pro genotype is a risk factor for KCOT in Thai patient population. Notably, our previous study also found that there was a significant association between the Arg/Arg (arginine) genotype of *p53* and a prominent risk for ameloblastoma (Kitkumthorn et al., 2010). This difference, where the arginine and proline isoforms increases risk for ameloblastoma and KCOT, respectively, is likely due to different biological function of these variants.

In conclusion, we present emerging data from our current study that furthers our understanding of molecular basis of KCOT and when combined with observation from our previous study, genotyping *p53* codon 72 in both KCOT and ameloblastoma may discriminate these two lesions with have similar clinical symptoms and histopathological and radiographical features making diagnosis very challenging. Our molecular approach offers a simple and a relatively efficient approach to investigate KCOT and ameloblastoma for accurate diagnosis in the present.

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