

## **P53 gene codon 72 polymorphism and risk of esophageal squamous cell carcinoma: a case/control study in a Chinese population**

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**SUMMARY.** The aims of this study were to investigate whether p53 gene codon 72 polymorphism was a biomarker associated with esophageal squamous cell carcinoma (ESCC) and its relationship with smoking status in China. The p53 genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism among 673 patients with ESCC and 694 healthy controls. The association between p53 genotypes and risk of developing ESCC was estimated by odds ratios (OR) and their 95% confidence intervals (CIs) computed by logistic regression. Compared with Arg/Arg homozygotes, Pro/Pro homozygotes had a nearly twofold increased risk (adjusted OR, 1.83; 95% CI, 1.35–2.48). For the Pro/Arg heterozygotes, there was no evident increased risk (adjusted OR, 1.01, 95% CI, 0.78–1.30). Furthermore, the risk associated with the Pro/Pro variant genotype was more pronounced in younger patients at diagnosis ( $\leq 45$  years) (OR, 7.4; 95% CI, 1.44–37.89,  $P = 0.02$ ), in women (OR, 3.15; 95% CI, 1.52–4.53,  $P = 0.02$ ) and in non-smokers (OR, 2.49; 95% CI, 1.58–3.94) and light smokers (OR, 2.13; 95% CI, 1.15–3.93). But tests for homogeneity between smoking-related OR showed no significant differences ( $P = 0.4$ ). The p53 gene codon 72 Pro/Pro genotype was significantly associated with the increased risk of ESCC in a Chinese mainland population and may be an independent factor in susceptibility to ESCC. The association was especially noteworthy in women and in younger patients.

**KEY WORDS:** esophageal squamous cell carcinoma, p53, polymorphism.

### **INTRODUCTION**

Esophageal cancer is the 6th most common cancer globally<sup>1</sup> and also is one of the most interesting tumors because of the great diversity in its incidence worldwide and in its pathological type. In Europe and the USA the overall incidence does not exceed 10/100 000/year in men and 2/100 000/year in women and the pathological type is mainly the adenocarcinoma. In the central part of China (Henan and Shanxi provinces) and northern Iran, however, incidence rates of over 100/100 000/year have been reported in both men and women<sup>2</sup> and about 150 000 patients died of esophageal squamous cell carcinoma (ESCC) per year in China, accounting for 75% of the world deaths from this disease.<sup>3</sup> Higher incidence rates of ESCC in certain geographic

areas have suggested that environmental factors contribute to the carcinogenesis of ESCC. However, even in the high-risk areas, only a portion of exposed individuals develop ESCC, indicating that there may be important genetic basis inducing susceptibility to the disease. Therefore it is of great importance to investigate genetic variation in susceptibility to ESCC.

The tumor suppressor gene p53 is altered in almost all kinds of the human malignancies. The possible mechanism is that p53 can induce cell cycle arrest for DNA repair and/or apoptosis in response to cellular stress such as DNA damage or hypoxia.<sup>4</sup> Germline mutations of the p53 gene not only result in its losing its normal function but also in increasing the promoting tumor's function. P53 gene codon 72 polymorphism (Arg or Pro), which results from a single base change (from CGC to CCC), has been known since 1987;<sup>5</sup> however, its significance as a genetic susceptibility factor for cancer is still a matter of controversy. Association studies between this polymorphism and cervix,<sup>6</sup> lung,<sup>7</sup> breast,<sup>8</sup> colorectal,<sup>9</sup> skin<sup>10</sup>

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and gastric<sup>11</sup> cancer have revealed inconsistent results. With respect to ESCC, the relationship between the p53 gene codon 72 polymorphism and risk of ESCC is also controversial. Some studies have shown that patients carrying the p53 gene codon 72 Pro/Pro genotype had an approximately twofold increased risk of developing ESCC,<sup>12-14</sup> while others have reported that the p53 gene codon 72 Arg/Arg genotype was an at risk genotype for the human papillomavirus-associated ECSS.<sup>15-17</sup> In the present study, we determined the genotype frequencies of the p53 gene codon 72 polymorphism in ESCC patients and healthy controls matched by age, sex and ethnicity. Our aims were to understand whether this polymorphism was a biomarker associated with susceptibility to ESCC and to study its relationship to smoking status in a Chinese population.

## MATERIALS AND METHODS

### Study participants

This study included 673 patients with ESCC and 694 healthy controls. All participants were unrelated ethnic Chinese and residents in Beijing and the surrounding regions. Patients with ESCC were consecutively recruited from January 1997 to July 2002 at the Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China. All cases were diagnosed as ESCC by histopathology and had been previously untreated by radiotherapy or chemotherapy. Most patients ( $n = 656$ ) were enrolled in our previous study, which was reported elsewhere.<sup>18</sup> In the present study we added 17 cases, which were further consecutively recruited after the previous study was terminated. There were no sex, age or stage restrictions. The control group matched by age, sex and ethnicity comprised non-cancer patients randomly selected from a nutritional survey database consisting of 2500 individuals. At recruitment, informed consent was obtained from each subject, and each subject was interviewed to collect detailed information on their characteristics and lifetime history of tobacco use. The patients who smoked up to 1 years before the date of diagnosis and the controls who smoked up to the date of the interview were considered current smokers. Light or heavy smokers were categorized by the approximate 50th percentile pack-year value among controls, i.e.  $< 27$  or  $\geq 27$  pack-year [(cigarettes per day/20)  $\times$  (years smoked)]. This study was approved by the Institutional Review Board of the Chinese Academy of Medical Sciences Cancer Institute.

### P53 gene codon 72 polymorphism analysis

Genomic DNA was isolated from the peripheral blood of the controls and the cases. A polymerase

chain reaction (PCR)-restriction fragment length polymorphism (PCR-RFLP) assay was used to identify the p53 gene codon 72 genotypes with the primers of 5'-TTGCCGTCCCAAGCAATGGATGA-3' and 5'-TCTGGGAAGGGACGAAAGATGAC-3'. The 199-bp target DNA fragment contains the CGC/CCC site of the p53 gene codon 72 located in 4exon. The 25- $\mu$ L PCR mixture contained 1  $\mu$ L (approximately 100 ng) DNA, 0.6  $\mu$ L (12.5 mmol/L) each of primer, 0.2  $\mu$ L (10 mmol/L) dNTP, 1.5  $\mu$ L (25 mmol/L) MgCl<sub>2</sub>, 2.5  $\mu$ L 10  $\times$  PCR buffer, 0.2  $\mu$ L (5 U/ $\mu$ L) Taq DNA polymerase and 18.4  $\mu$ L dH<sub>2</sub>O. The reaction was carried out in the following conditions: an initial melting step of 94°C for 2 min, followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, 45 s at 72°C, and a final elongation of 7 min at 72°C. The 199-bp fragment was then digested with 4  $\mu$  BstI (New England Biolabs, Inc. Ipswich, MA, US) for 4 h at 37°C. The digested product was separated on a 3% agarose gel with ethidium bromide and photographed with an Ultra Violet Product Image Store system. The Pro/Pro genotype produced a single 199-bp band due to loss of BstI restriction site; the wild type Arg/Arg genotype produced two bands (86 bp and 113 bp) and the Pro/Arg genotype produced three bands (86 bp, 113 bp and 199 bp). The results were evaluated by one of us being blinded to the participants' case-control status. More than 10% of the samples were randomly selected for repeated assay, and the results were 100% in agreement.

### Statistical analysis

Differences in age, sex and smoking status between ESCC patients and healthy controls were evaluated by using the  $\chi^2$  test. The association between the p53 gene codon 72 polymorphism and risk of ESCC was estimated by odds ratios (OR) and 95% confidence intervals (CI), which were calculated by unconditional logistic regression models. The OR were all adjusted for age, sex or smoking status. A probability level of  $< 0.05$  was used as the criterion of significance and all tests were two-sided. All analyses were carried out using Statistical Analysis System software (version 6.12, SAS Institute Cary, NC).

## RESULTS

The selected characteristics of the 673 ESCC patients and 694 controls are summarized in Table 1. The ESCC patients and the controls were adequately matched on age, sex and ethnicity. The mean age was 58.1 years ( $\pm 9.8$  years; range, 25–89 years) for the cases and 57.6 years ( $\pm 7.6$  years; range, 39–87 years) for the controls ( $P = 0.38$ ). There was no significant difference between patients and controls in sex distribution (79.1% men in the cases vs 83%

**Table 1** Distribution of age, sex and smoking status between cases and controls

	Cases ( <i>n</i> = 673)		Controls ( <i>n</i> = 694)		<i>P</i> *
	No.	%	No.	%	
Sex					0.06
Male	532	79.0	576	83.0	
Female	141	21.0	118	17.0	
Age (year)					0.38
≤ 45	63	9.4	34	4.9	
> 45	610	90.6	660	95.1	
Smoking status					0.59
Never	290	43.1	309	44.5	
Ever	383	56.9	385	55.5	
< 27 pack-years	176	46.0	191	49.6	0.31
≥ 27 pack-years	207	54.0	194	50.4	

\*Two-sided  $\chi^2$  test.**Table 2** P53 genotype and allele frequencies of cases and controls and their association with esophageal squamous cell carcinoma

Genotype	Controls ( <i>n</i> = 694)		Cases ( <i>n</i> = 673)		Adjusted OR† (95% CI)
	No.	%	No.	%	
Arg/Arg	195	28.1	163	24.2	1.00 (ref.)
Pro/Arg	366	52.7	306	45.5	1.01 (0.78–1.30)
Pro/Pro	133	19.2	204	30.3	1.83 (1.35–2.48)
Pro allele	0.46		0.54		$\chi^2 = 7.72$ , <i>P</i> = 0.005 (allele) $\chi^2 = 22.9$ , <i>P</i> = 0.000002 (genotype)

†OR were adjusted for age, sex and pack-years by unconditional logistic regression. CI, confidence interval

**Table 3** Risk of esophageal squamous cell carcinoma associated with P53 genotypes by age, sex and smoking status

	Cases/controls, (nos) OR† and 95% CI					
	Arg/Arg		Arg/Pro		Pro/Pro	
Total	163/195	1.00 (ref.)	306/366	1.01 (0.78–1.30)	204/133	1.83 (1.35–2.48)
Age (years)						
≤ 45	16/13	1.00 (ref.)	30/18	1.47 (0.55–3.95)	17/3	7.40 (1.44–37.89)*
> 45	147/182	1.00 (ref.)	276/348	0.99 (0.75–1.30)	187/130	1.78 (1.30–2.44)
Sex						
Female	30/33	1.00 (ref.)	59/65	1.04 (0.57–1.93)	52/20	3.15 (1.52–4.35)**
Male	133/162	1.00 (ref.)	247/301	1.00 (0.75–1.36)	152/113	1.64 (1.18–2.31)
Smoking status (pack-years)						
0	61/90	1.00 (ref.)	128/159	1.19 (0.80–1.77)	101/60	2.49 (1.58–3.94)***
< 27	41/53	1.00 (ref.)	84/108	0.98 (0.59–1.62)	51/30	2.13 (1.15–3.93)
≥ 27	61/52	1.00 (ref.)	94/99	0.83 (0.52–1.32)	52/43	1.05 (0.60–1.83)

\**P* < 0.05, test for homogeneity between age-related OR. \*\**P* < 0.05, test for homogeneity between sex-related OR. \*\*\**P* > 0.05, test for homogeneity between smoking-related OR. †Adjusted for age, sex or pack-years smoked of ESCC within the strata. CI, confidence interval.

in the controls; *P* = 0.06). In the smoking status of the cases and controls, there was also no significant difference (56.9% vs 55.5%, *P* = 0.59).

The p53 gene codon 72Pro allele frequencies and genotype distributions in the cases and controls are summarized in Table 2. The observed genotype frequencies of p53 polymorphism in the controls were in agreement with Hardy–Weinberg equilibrium ( $\chi^2 = 2.84$ , *P* = 0.092). We observed genotype frequencies of Arg/Arg 24.2% and 28.1%, Arg/Pro 45.5% and 52.7%, Pro/Pro 30.3% and 19.2%, Pro/Arg or Arg/Arg 69.7% and 80.8% in the cases and the controls, respectively, and the differences between

the cases and the controls were statistically significant ( $\chi^2 = 22.9$ , *P* = 0.000002). By using logistic regression analysis, we evaluated the association between the Pro variant and risk of ESCC. Compared with Arg/Arg homozygotes, Pro/Pro homozygotes carried a nearly twofold increased risk (adjusted OR, 1.83; 95% CI, 1.35–2.48). For the Pro/Arg heterozygotes, there was no evident increased risk (adjusted OR, 1.01; 95% CI, 0.78–1.30).

Associations between the p53 gene codon 72 genotype and ESCC stratified on age, sex and smoking status are shown in Table 3. Because the common age for ESCC to occur in China is around 50 years

old, we classified the age group of  $\geq 45$  years as the younger patients. The risk associated with the Pro/Pro variant genotype was more pronounced in younger patients at diagnosis (OR, 7.40; 95% CI, 1.44–37.89) and in women (OR, 3.15; 95% CI, 1.52–4.53). In non-smokers and light smokers, the risk associated with the Pro/Pro variant genotype was more pronounced, too, with OR being 2.49 (95% CI, 1.58–3.94) and 2.13 (95% CI, 1.15–3.93), respectively. But the test for homogeneity showed no significant difference ( $P = 0.40$ ).

## DISCUSSION

The tumor suppressor gene p53 is located on human 17q 3.1 and is composed of 11 exons. It encodes for a 53 KD protein with 393 amino acids, which can be phosphorylated and activated by signals of DNA damage and arrest the cell cycle in G1 phase to allow DNA repair or apoptosis.<sup>19</sup> The polymorphism of codon 72 located in a proline-rich domain of p53, which is required for the growth suppression activity of p53,<sup>20</sup> plays an important role in p53-mediated apoptosis<sup>21</sup> and has been known for almost 20 years. But the association between the polymorphism and the function is still unclear. Although both variants are morphologically wild-type and do not differ in their ability to bind to DNA in a sequence-specific manner,<sup>22</sup> the Pro/Pro genotype seems to be a stronger inducer of downstream transcription and less effective at the suppression of cellular transformation, and the Arg/Arg genotype is at least five times more efficient in apoptosis induction than the Pro/Pro genotype.<sup>23</sup> It has been found that this region comprises five PxxPSH3 (SRC-homology-3) binding motifs, one of which will be lost when the proline at codon 72 is replaced by arginine.<sup>21</sup> This may be the biological base of the change of function. The difference of function between the two genotypes accords with the result of molecular epidemiology studies: the Pro/Pro homozygous genotype increases individual susceptibility to lung,<sup>7</sup> breast<sup>8</sup> and colorectal<sup>9</sup> cancer and ESCC.<sup>12–14</sup>

In the present study, we verified that the Pro/Pro genotype of p53 gene codon 72 carried a nearly twofold increased risk of developing ESCC when compared with the Arg/Arg genotype, which was consistent with previous studies.<sup>12–14</sup> We also found that the association between the p53 variant and ESCC risk was more pronounced in younger patients and women, suggesting the younger patients and women with the Pro/Pro genotype of p53 gene codon 72 were more susceptible to ESCC. An interesting finding in our study was that women with the Pro/Pro genotype were conferred with an OR of  $> 7$  for developing ESCC. Why are women with the Pro/Pro genotype more susceptible to ESCC

than men with Pro/Pro genotype? The reason is not clear. It has been shown that there are joint effects between the p53 gene and estrogen.<sup>24</sup> The associations between the p53 gene and estrogen and the risk of ESCC should be further investigated. Although we found that the risk of ESCC associated with Pro/Pro genotype was more pronounced in non-smokers and light smokers than heavy smokers, there was no significant difference by test for homogeneity. In baseline data, there was also no significant difference in the smoking status of the cases and controls. In our study, we found no association between smoking and the occurrence of ESCC, and P53 genotype and smoking status had no joint effect on the carcinogenesis of esophagus. This result was in agreement with the findings of Lee and Wang,<sup>13,25</sup> but it was not in agreement with other previous studies.<sup>26–28</sup> These inconsistent results may be due to the relative small size of the sample. The associations between the p53 genotype and smoking status and other environmental factors and the risk of ESCC need further investigating.

Human papilloma virus (HPV) infection has been considered a potential oncogenic factor for the development of several carcinomas, including ESCC. The relationship between HPV and p53 mutation or polymorphism has always been noticed, because the E6 protein, which is encoded by HPV-16/18, can bind to p53 and direct degradation through the ubiquitin pathway.<sup>29–31</sup> It is widely assumed that p53 is functionally inactivated by the viral E6 protein in HPV-associated cancer cell. An *in vitro* assay demonstrated that the Arg/Arg genotype of p53 gene codon 72 was more susceptible to degradation by the HPV-E6 protein than the p53 Pro variant.<sup>32</sup> Kawaguchi *et al.* found the frequency of the Arg allele in HPV-positive cases was significantly higher than in HPV-negative cases of ESCC.<sup>16</sup> Our result is not in agreement with these studies, because the Arg allele was a protective factor of the carcinogenesis of the esophagus in the present study. A possible explanation is that HPV infection is independent of the carcinogenesis of the esophagus. However, in a recent study including 99 ESCC patients and 381 controls showed that there was no significant association between HPV infection and ESCC.<sup>33</sup> The associations between the p53 gene codon 72 polymorphism and HPV infection and ESCC need to be further studied.

It is reported that there was a significant decrease in the frequency of the Pro allele with increasing latitude, ranging from 0.63 in black Africans to 0.17 in Swedish Saamis.<sup>34</sup> In this study, we calculated the frequency of the Pro allele of the north China population (0.46), which is lower than that of African-Americans (0.50)<sup>34</sup> and slightly higher than that reported from Japan (0.35–0.40),<sup>35,36</sup> where the latitude is slightly higher than Beijing. This is a noteworthy phenomenon and implies that the p53 polymorphism

is maintained by natural selection. This polymorphism may be an important role in an ecological adaptation to ultra-violet (UV) radiation, probably through UV-induced p53-mediated DNA repair.<sup>34</sup>

In conclusion, the p53 gene codon 72 Pro/Pro genotype is significantly associated with increased risk of ESCC in a Chinese mainland population and may be an independent factor in susceptibility to ESCC. This association is especially noteworthy in women and in younger patients.

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