ORIGINAL ARTICLE

Association of *p53* Codon 72 Polymorphism with Risk of Hypopharyngeal Squamous Cell Carcinoma in Taiwan

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Background: *p53* polymorphism at codon 72 is a known risk marker for various malignancies, but it has not been studied in hypopharyngeal cancer. This study investigated the genotype distribution of *p53* codon 72 polymorphism in hypopharyngeal cancer patients and non-cancer controls matched for age, gender, alcohol consumption and smoking habit.

Methods: Genomic DNA was extracted from peripheral blood cells of 53 patients with hypopharyngeal cancer and 53 non-cancer controls. Codon 72 polymorphism of *p53* was identified by polymerase chain reaction–restriction fragment length polymorphism.

Results: Patients with hypopharyngeal cancer had higher frequencies of Pro/Pro (26.4% vs. 13.2%) and Pro/Arg (51.0% vs. 45.3%) but lower frequencies of Arg/Arg (22.6% vs. 45.1%) compared to controls. Compared to Arg/Arg genotypes, Pro/Pro genotypes had a relative risk of hypopharyngeal cancer of 3.667 (95% confidence interval, 1.16–11.56; $p = 0.03$). As a group, patients with Pro/Pro or Arg/Pro who were carriers of the Pro allele had a higher relative risk of hypopharyngeal cancer compared to Arg homozygous carriers (odds ratio, 2.415; 95% confidence interval, 1.04–5.64; $p = 0.04$).

Conclusion: This study demonstrated that *p53* codon 72 Pro homozygosity is associated with a higher risk of developing hypopharyngeal cancer. [*J Formos Med Assoc* 2006;105(2):99–104]

Key Words: hypopharyngeal cancer, *p53*, polymorphism

Squamous cell carcinoma of the hypopharynx is one of the most malignant of all head and neck cancers because of its high incidence of locoregional recurrence and distant metastasis. Despite recent advances in surgery, radiotherapy and chemotherapy, the reported 5-year disease-specific survival rates from different centers worldwide ranged from 25% to 33.4%.[1] Because of difficulty in early detection of hypopharyngeal cancer and the high frequency of occult metastasis, most patients present with advance-stage disease at diagnosis. Thus, identification of new biomarkers is urgently needed to determine cancer susceptibility, predict prognosis and guide appropriate treatment.

In head and neck squamous cell carcinoma (HNSCC), the *p53* gene is frequently either mutated or shows altered expression of the gene product.[2,3] Polymorphism in the wild-type *p53* gene at codon 72 of exon 4 has been described, resulting in either a proline (CCC) or an arginine (CGC) residue.[4] The association of polymorphism at codon 72 in the *p53* gene with susceptibility to cancers of the cervix,[5–11] lung,[12–18] bladder,[19–21] prostate,[22] skin,[23,24] esophagus,[25,26] stomach,[27] and breast[28–30] has been studied. However, studies of the role of *p53* codon 72 polymorphism in HNSCC have been limited,[31–33] and specific studies on hypopharyngeal cancer are lacking. This

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study investigated the genotype distribution of *p53* codon 72 polymorphism and its association with risk of hypopharyngeal cancer.

Methods

Study population

Between July 2000 and February 2003, 53 patients with pathologically-confirmed squamous cell carcinoma of the hypopharynx were enrolled in this study after informed consent was obtained. A control group was selected by screening 53 subjects from health examination clinics, matched for age, gender, alcohol consumption, smoking habit, and no past history of malignancy. A 10 mL peripheral blood sample was collected from all patients and controls.

Blood processing and DNA extraction

Whole blood samples were centrifuged at 1000*g* for 15 minutes. The supernatant was discarded. The erythrocytes were lysed by incubating with 40–45 mL lysis buffer (155 mmol/L NH_4Cl, 7.5 mmol/L $KHCO_3$, 2.5 mmol/L K_2CO_3, 0.1 mmol/L EDTA; pH 7.8) on ice for 15 minutes, and vortexed briefly twice during incubation. Immediately after centrifugation, the cell pellet was washed twice in phosphate-buffered saline (pH 7.3). The cell pellet was treated by adding 2–4 mL TRIzole (GIBCO/BRL, Bethesda, MD, USA) at room temperature for 5 minutes. The organic phase was collected and mixed with 0.3 mL 100% ethanol/1 mL TRIzole at room temperature for 3 minutes. The resulting DNA pellet was transferred to a new 1.5 mL Eppendorf tube. The DNA pellet was washed twice in 1 mL 10% ethanol/0.1 M sodium citrate, and then subjected to 1 mL 75% ethanol at room temperature for 15 minutes. Pelleted DNA was vacuum-dried and resuspended in 8 mM NaOH. After quantification by spectrophotometry, the DNA was divided into different vials and stored at –30° C until use.

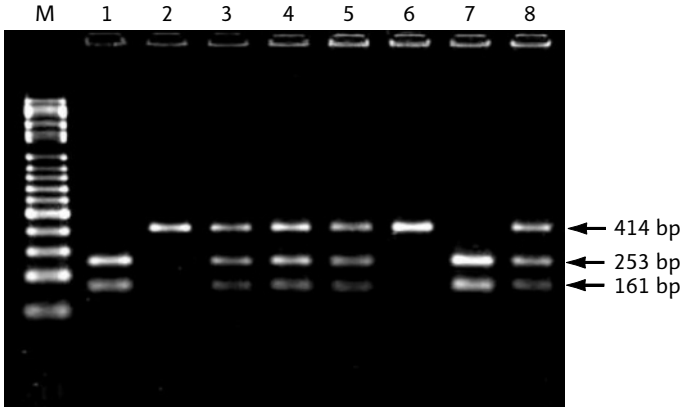

Figure 1. Polymerase chain reaction–restriction fragment length polymorphism analysis of the *p53* gene. The Pro allele is not cleaved by *BstU* I at codon 72 and has a single band with a fragment length of 414 bp. The Arg allele is cleaved by *BstU* I and yields two small fragments (253 bp and 161 bp). The heterozygote has three bands (414 bp, 253 bp, 161 bp). Lane M = 100 bp ladder DNA marker; Lanes 1 and 7 = Arg/Arg; Lanes 2 and 6 = Pro/Pro; Lanes 3, 4, 5 and 8 = Arg/Pro.

Genotyping

Purified genomic DNA was amplified by polymerase chain reaction (PCR) using standard buffer, nucleotides, *Taq* DNA polymerase and the primer pairs (forward, 5′-TGAGGACCTGGTCCTCTGACT-3′; reverse, 5′-AAGAGGAATCCCAAAGTTCCA-3′). The PCR protocol consisted of an initial melting step of 94° C for 5 minutes, 40 cycles of 94° C for 30 seconds, 60° C for 30 seconds, and 72° C for 30 seconds, and a final extension step of 72° C for 10 minutes. The PCR products were purified and digested with 5 U of *Bst*U I (New England Biolabs, Beverly, MA, USA) in a buffer containing 25 mM Tris/acetate (pH 7.8), 100 mM potassium acetate, 10 mM magnesium acetate, and 1 mM dithiothreitol, and incubated at 37° C for 15 hours. An aliquot was subjected to electrophoresis on 2% agarose gel and bands were visualized with ethidium bromide. The arginine form of codon 72 contains a *Bst*U I site that is not present in the proline allele. The Arg allele, when cleaved by the *Bst*U I enzyme, yields two fragments of 253 bp and 161 bp, while the Pro allele remained as a 414-bp single band on the gel. Patients with the heterozygous Arg/Pro will produce three bands (Figure 1). To verify the accuracy of the genotyping obtained by this PCR and restriction fragment length polymorphism (RFLP) technique, we performed direct sequencing of PCR products from 30 randomly selected patients. All tested samples were confirmed by accurate sequencing data (Figure 2).

Statistical analysis

The chi-square test was used to compare the genotype frequencies of the *p53* gene polymorphism between hypopharyngeal cancer patients and controls. A *p* value of less than 0.05 was considered to be statistically significant. The association between *p53* codon 72 polymorphism and hypopharyngeal cancer was estimated by computing the odds ratio (OR) and 95% confidence interval (CI) by logistic regression analysis. All statistical analyses were performed using SPSS version 10.0 software (SPSS Inc, Chicago, IL, USA).

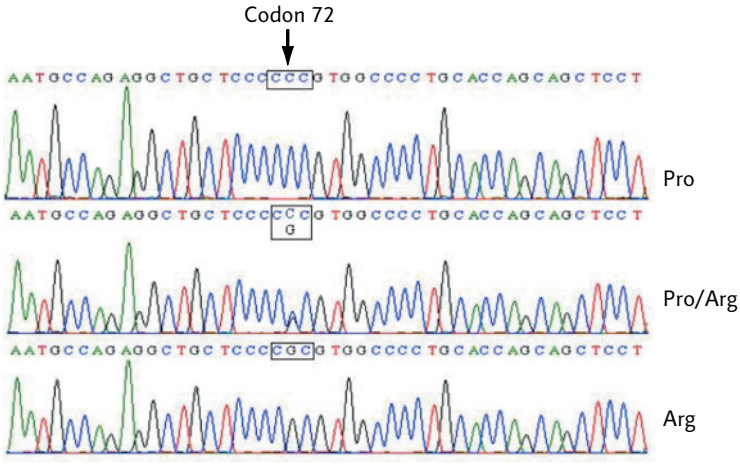

Figure 2. Direct sequencing data confirm results of the polymerase chain reaction–restriction fragment length polymorphism analysis.

Results

All patients and controls were male and had similar smoking and alcohol consumption habits. The mean age of patients was 60.5 years (range, 33–86 years) and of controls was 58.1 years (range, 33–81 years).

The distribution of *p53* genotypes among controls and patients is shown in the Table. For patients with hypopharyngeal cancer, frequencies for the Arg/Arg, Arg/Pro and Pro/Pro genotypes were 22.6%, 51.0% and 26.4%, respectively. In controls, these frequencies were 41.5%, 45.3% and 13.2%, respectively (p = 0.07). Compared to Arg/Arg genotypes, Pro/Pro genotypes had a relative risk of hypopharyngeal cancer of 3.667 (95% CI, 1.16–11.56; p = 0.03). The relative risk of hypopharyngeal cancer among Arg/Pro genotypes was 1.778 (95% CI, 0.62–5.14; p = 0.29). Patients with Arg/Pro or Pro/Pro genotype who were carriers of the Pro allele had a higher relative risk of hypopharyngeal cancer compared to Arg homozygotes (OR, 2.415; 95% CI, 1.04–5.64; p = 0.04).

According to the 1997 TNM staging system of the American Joint Committee on Cancer, 47 patients presented with stage IV, two with stage II and four with stage III disease. For patients with stage IV disease, frequencies of the Arg/Arg, Arg/Pro and Pro/Pro genotypes were 21.3%, 48.9% and 29.8%, respectively. For patients with stage II/III disease, the frequencies of the Arg/Arg, Arg/Pro and Pro/Pro genotypes were 33.3%, 66.7% and 0%, respectively. Analysis of whether or not there was an association between *p53* codon 72 polymorphism and stage was not meaningful in this study because of the small sample size of patients with stage II/III disease.

Discussion

There is increasing evidence suggesting that host factors, including genetic polymorphisms, may explain some of the individual differences in can-

Table. Distributions of codon 72 polymorphism in patients and controls

	Patients (n = 53)	Controls (n = 53)	OR (95% CI)	p
Arg/Arg	12 (22.6%)	22 (41.5%)	1	
Arg/Pro or Pro/Pro	41 (77.4%)	31 (58.5%)	2.415 (1.043–5.639)	0.040
Arg/Pro	27 (51.0%)	24 (45.3%)	1.778 (0.615–5.136)	0.288
Pro/Pro	14 (26.4%)	7 (13.2%)	3.667 (1.163–11.557)	0.027

OR = odds ratio; CI = confidence interval.

cer occurrence. The *p53* tumor suppressor gene, located on chromosome 17p13, is one of the most commonly mutated genes in all types of human cancer.[34] After DNA-damaging insult, wild-type *p53* induces either growth arrest or apoptosis.[35] Mutations within *p53* abolish these activities and allow uncontrolled proliferation of cells harboring mutations, ultimately resulting in the progression to cancer.[36]

The proline-rich domain of *p53* has been shown to be necessary for its ability to induce apoptosis and growth suppression.[37,38] Interestingly, within this domain, there is a common polymorphism at position 72 encoding either an arginine or a proline residue.[4] Both variant alleles differ in biochemical and biologic activity.[39] Several mechanisms have been proposed to explain the role of the Arg allele in cancer development. The Arg allele has weaker affinity for several transcription-activating factors *in vitro*.[39] In addition, the Arg allele has been shown to be more susceptible to degradation by the human papillomavirus (HPV) E6 protein than the Pro allele *in vivo*.[5] The Arg allele may enhance mutant *p53* binding to *p73*, thus neutralizing *p73*-induced apoptosis independently of HPV-related mechanisms in squamous cell cancers.[40,41]

Many previous studies have investigated the association between *p53* codon 72 polymorphism and the risk of various cancers. Carcinomas of the cervix and lung were the most frequently investigated. Storey et al first suggested that *p53* codon 72 polymorphism played a role in the development of HPV-related cancers.[5] They showed that women with the Arg/Arg genotype variant were seven times more susceptible to HPV-associated cervical cancer than heterozygotes. However, subsequent studies did not consistently confirm this association.[6–11] A meta-analysis of 50 articles of cervical neoplasia showed that there was no evidence of association or heterogeneity for preinvasive lesions.[42] For invasive cervical cancer with undefined histology, the Arg/Arg genotype was not found to affect risk (OR, 1.1; 95% CI, 0.9–1.3). Only a slightly increased risk was observed for squamous cell carcinoma (OR, 1.5; 95% CI, 1.2–1.9) and adenocarcinoma (OR, 1.7; 95% CI, 1.0–2.7). Three large case-control studies found that the *p53* Pro allele was associated with an increased risk of lung cancer, especially in adenocarcinoma rather than squamous cell carcinoma.[14–16] However, a recent systematic review and meta-analysis showed little support for this hypothesis.[43] These conflicting results from epidemiologic and functional studies of *p53* codon 72 polymorphism suggest that the Arg and Pro alleles may play different roles in different types of cancers, depending on the interplay of each allele with different pathogens.

Compared with other types of cancer, there has been little study of the association of *p53* codon 72 polymorphism with HNSCC.[31–33] Indeed, tumors arising from the head and neck region contain many different anatomic sites with different behaviors. Recently, three large case-control studies of HNSCC failed to demonstrate a positive association with *p53* codon 72 polymorphism.[31–33] Another two studies also failed to identify any evidence for association between *p53* codon 72 polymorphism and risk of oral cancer in subjects with or without HPV infection.[44,45] Oral cancers occur in a heterogeneous group of patients with tumors arising from the buccal mucosa, gingiva, tongue, hard palate, and lip. However, previous studies of specific types of head and neck cancers usually found positive association with *p53* codon 72 polymorphism, such as nasopharyngeal carcinoma[46] and laryngeal tumors.[47] In the present study, we demonstrated a positive association of hypopharyngeal cancer with *p53* codon 72 polymorphism.

The genotype distribution of *p53* codon 72 polymorphism shows significant variation among ethnic groups. Beckman et al reported a significant decrease in the frequency of the Pro allele with increasing latitude, ranging from 0.63 in African Blacks to 0.17 in Swedish Saamis.[48] Weston et al also reported that the frequency of the Pro allele varied by ethnicity.[49] The *p53* Pro allele was found to be more common in African-Americans (0.50) than in Caucasians (0.29). Two Japanese studies showed genotype frequencies of the Pro allele ranging from 0.35 to 0.40.[13,17]

In order to clarify the extent of possible bias in the selection of the control group for this study, we compared the characteristics of our control group with control groups in various previous studies of *p53* codon 72 polymorphism in patients with cancer in Taiwan.[18,23,26,46,50] No significant difference in *p53* codon 72 polymorphism distribution in non-cancer controls was found between our study and those previous studies. The Pro/Pro genotype frequency in our controls was 13.2%, which was in the range of other Taiwanese series (12.0–19.7%).[18,23,26,46,50] Another interesting finding from our literature review is that Taiwanese patients with the Pro/Pro genotype have a significantly higher risk of upper aerodigestive cancers, including carcinomas of the nasopharynx (OR, 3.0; $p < 0.05$),[46] hypopharynx (the current study: OR, 2.42; $p = 0.04$), esophagus (OR, 2.2; $p < 0.05$),[26] and lung (OR, 3.14; $p = 0.003$).[18]

In conclusion, our data show that the Pro/Pro genotype may be a useful marker for identifying individuals susceptible to the development of hypopharyngeal cancer and possibly other upper aerodigestive cancers.

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