p53 Codon 72 Polymorphism in Taiwanese Lung Cancer Patients: Association with Lung Cancer Susceptibility and Prognosis

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ABSTRACT

An association between the BstUI (Pro/Pro) genotype of the p53 codon 72 polymorphism and lung cancer has been reported previously (X. Jin et al., Carcinogenesis (Lond.), 16: 2205–2208, 1995). However, the genotype distribution of p53 codon 72 polymorphism as well as the association of this polymorphism with lung cancer risk and prognosis remain undefined in the Taiwanese population. Therefore, we investigated the genotype distribution of p53 codon 72 polymorphism in 194 lung cancer patients and 152 noncancer controls. The genotype frequencies in Taiwanese noncancer controls were 0.56 (Arg) and 0.44 (Pro). χ² analysis indicated significant differences in genotype distribution of p53 from other reports in Swedish (P < 0.001), Spanish (P < 0.001), Caucasians in the United States (P = 0.002), and African-Americans (P = 0.027). In addition, our data suggest that the Pro allele of the p53 codon 72 polymorphism increased the risk of lung cancer among female Taiwanese. The female patients with genotype Pro/Pro showed a significantly increased odds ratio (3.14; confidence interval, 1.48–6.64; P = 0.003) of having lung adenocarcinoma, compared with normal controls with the other genotypes. Patients with the Pro/Pro genotype had an odds ratio of 2.63 (confidence interval, 1.22–5.68; P = 0.01) higher than those with the other genotypes to be diagnosed with lung cancer at the early ages. We further investigated the association of p53 codon 72 polymorphism with prognosis in 133 lung cancer patients. Patients with the Pro/Pro genotype tended to have poorer prognosis than those with the Arg/Pro genotype (P = 0.05, by the log-rank test). Our data suggested that p53 codon 72 polymorphism may play a role in cancer susceptibility and prognosis in specific classes of lung cancer patients in Taiwan.

INTRODUCTION

Lung cancer is the leading cause of cancer deaths in Taiwan (1). A low male:female ratio of 2:1 for lung cancer mortality is notably observed (2). In addition, the distribution of cigarette smokers (59.4% for males and 3.8% for females) in Taiwan differs from that in other countries (roughly 98% of males worldwide are smokers, and 70–90% of European and American females are smokers; Ref. 3). Studies conducted in mainland China and Taiwan have shown that cigarette smoking is the principal risk factor of lung cancer. However, LAD3 shows less correlation with smoking (2, 4). Most cases of lung cancer in nonsmoking women are LAD, which has been increasing in incidence and is currently the most prevalent type of lung cancer in Taiwan (1). Therefore, it is of great importance to identify factors that increase the risk of LAD in females so that effective primary preventive measures can be established.

Genetic polymorphisms at the genes involved in tumorigenesis may determine individual susceptibility of cancer. Germline p53 mutations have been reported to be associated with inherited cancer risk (5), and codon 72 polymorphic variants have also been studied as potential susceptible genotypes for lung cancer (6–9). The gene products of the two polymorphic variants differ by the presence of either Arg (CGC), a large polar amino acid residue, or Pro (CCC), a small nonpolar amino acid residue (10), and can be identified by PCR and restriction enzyme analysis (BstUI or AccII).

The genotype distribution of p53 codon 72 polymorphism is significantly different among ethnic groups. Beckman et al. (11) reported that there was 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. Weston et al. (12) also reported that the frequency of the Pro allele varied by ethnicity. 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 Pro ranging from 0.35 to 0.40 (7, 8). However, the genotype distribution of p53 codon 72 polymorphism remains undefined in the Taiwanese population.

The association of p53 codon 72 polymorphism with lung cancer risk has been studied by several groups, although with inconsistent results. The Pro allele was found to be in excess in patients with LAD in a United States study (6), but this could not be confirmed in its follow-up study (12). A study done in...
Japan (7) showed a significant association of the Pro allele with LSC but not with LAD. However, a reanalysis of this report by another group indicated that there was no significant difference between lung cancer patients and healthy controls concerning genotype frequencies (13). As to the correlation of p53 codon 72 polymorphism with smoking, there are conflicting results. For example, Murata et al. (8) found that lung cancer patients who did not smoke included a significantly larger proportion of Arg/Arg homozygotes and smaller proportion of Pro/Pro heterozygotes compared with the healthy controls. However, Jin et al. (9) reported that increased risks associated with the Pro/Pro genotype were noted in lighter smokers. The discrepancies found in various studies of p53 codon 72 polymorphism may be due to substantial interethnic and interindividual risk differences in the study populations.

A study done in Hong Kong analyzing p53 intron 2 polymorphism indicated that there was no significant difference in genotype distribution among 34 non-small cell lung cancers and 27 normal controls (14). However, the distribution of p53 codon 72 polymorphism remains poorly defined in the Chinese population. The purposes of this study were to investigate the genotypic frequency of p53 codon 72 polymorphism in lung cancer patients in Taiwan and to examine the association of this polymorphism with lung cancer risk and prognosis, especially in female LAD patients.

MATERIALS AND METHODS

Study Population. The cases were 194 surgically resected lung cancer patients who were admitted to Veteran General Hospital-Taichung (Taichung, Taiwan) between 1993 and 1998. One hundred eighty-five patients had non-small cell lung cancers (102 LADs, 69 LSCs, 5 adenosquamous carcinomas, 7 large-cell carcinomas, 1 mixed type of large-cell carcinoma and small cell lung cancer, and 1 mixed type of LAD and large-cell carcinoma), and 9 patients had small cell lung cancers. The histologies of tumor types and stages were determined according to the WHO classification method (15) and Tumor-Node-Metasis system (16), respectively. Information on the smoking history of the lung cancer patients was obtained from hospital records. The patients were classified into smoking and non-smoking groups, the former included both current smokers and ex-smokers. Follow-up of 133 patients was performed at 2-month intervals in the 1st year after surgery and at 3-month intervals thereafter at outpatient clinics or by routine phone calls. The end of the follow-up period was defined as June 15, 1998, for all of the 133 patients. The mean follow-up period for all of the patients was 18.9 months (range, 0.5–51 months). For the 73 patients who survived the follow-up period (censored patients), the mean follow-up time was 24.7 months. For the 60 patients who died during the follow-up period, the mean follow-up time was 11.9 months. For controls, 152 noncancer and unrelated controls were from Chung Shan Hospital (Taichung, Taiwan). They were randomly selected individuals from physical check-up center, with the only restriction being the matching of age distribution with that of the patient group. The mean ages of patients and controls were 64 years (range, 37–85) and 62 years (range, 35–87), respectively.

Polymorphism Analysis. Representative proportions of well-separated normal lung tissues of lung cancer patients were taken after surgical resection, immediately snap-frozen, and subsequently stored in liquid nitrogen. Genomic DNA was prepared using proteinase K digestion and phenol/chloroform extraction followed by ethanol precipitation. For controls, blood samples (5–10 ml) were obtained and genomic DNA was extracted from peripheral lymphocytes using the standard methods. Purified genomic DNA was amplified by PCR for exon 4 of p53 tumor suppressor gene. Oligodeoxynucleotide primers and thermocycle PCR conditions were as indicated in Ref. 17. The polymorphic site of codon 72 was detected by BstUI restriction enzyme digestion (recognition site CGCG, New England Biolabs, Beverly, MA) for 4–8 h at 60°C. The Arg-coded allele, but not the Pro-coded allele, has a single BstUI site in the amplified fragment. Thus, after electrophoresis in 2% agarose gel and staining with ethidium bromide, the genotype of codon 72 polymorphism was determined. To examine the accuracy of our polymorphism analysis, we performed a genotyping assay of 24 cancer patients using the DNA from well-separated normal lung tissues and tumor lung tissues as well as the DNA from blood samples of the same patients. We found an identical genotype for all of the patients analyzed.

Statistical Analysis. The Pearson χ² test was used to compare genotype distributions between different ethnic groups as well as between lung cancer cases and controls. The statistical modeling using logistic regression was used to calculate the relative risk (OR) of Pro/Pro genotype to Arg/Arg or Arg/Pro genotype for case-control study. ORs were expressed together with the 95% CI. Multivariate logistic regression analysis was adjusted for age and sex. Type III censoring was performed on subjects who were still alive at the end of study (18). The Kaplan-Meier method was used to estimate the probability of survival as a function of time and the median survival (19). The log-rank test was used to assess the significance of the difference between pairs of survival probabilities (20).

RESULTS

Distribution of p53 Polymorphism in Taiwanese Compared with Other Ethnic Groups Worldwide. We studied a total of 346 individuals: 194 lung cancer patients and 152 noncancer controls. The frequencies of the three p53 genotypes Arg/Arg, Arg/Pro, and Pro/Pro found in the noncancer controls in Taiwan were 30.9, 49.3, and 19.7%, respectively, and fitted the Hardy-Weinberg equilibrium (χ² = 0.02; df = 2; P = 0.99) with the allele frequencies of 0.56 (Arg) and 0.44 (Pro); (Table 1). The genotype distribution was similar between male and female controls. The Pro/Pro genotype was strongly associated with ethnicity as compared with the distribution of the p53 genotype in our controls with the data reported previously for other study populations. χ² analysis indicated significant differences in genotype distributions of p53 from other reports in Swedish (P < 0.001; Ref. 12), Spanish (P < 0.001; Ref. 21), and Caucasians in the United States (P = 0.002; Ref. 12), in which a lower frequency of the Pro allele was found. The genotype distribution also differed significantly between the Taiwanese population and African-Americans (P = 0.027; Ref. 9), in which a higher frequency of Pro allele was found. How-
Table 1 Distribution of p53 polymorphism by case/control status and clinico-pathological parameters of lung cancer patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Genotypes</th>
<th>Crude OR (95% CI) Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noncancer control</td>
<td>Arg/Arg, n (%)</td>
<td>Arg/Pro, n (%)</td>
</tr>
<tr>
<td>Male</td>
<td>27 (33.3)</td>
<td>37 (45.7)</td>
</tr>
<tr>
<td>Female</td>
<td>20 (28.2)</td>
<td>38 (53.5)</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>68 (35.1)</td>
<td>74 (38.1)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>53 (37.6)</td>
<td>56 (39.7)</td>
</tr>
<tr>
<td>Female</td>
<td>15 (28.3)</td>
<td>18 (34.0)</td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD</td>
<td>33 (32.4)</td>
<td>40 (39.2)</td>
</tr>
<tr>
<td>LSC</td>
<td>28 (40.1)</td>
<td>28 (40.1)</td>
</tr>
<tr>
<td>LAD (female)</td>
<td>11 (28.2)</td>
<td>11 (28.2)</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I + II</td>
<td>27 (29.7)</td>
<td>35 (38.5)</td>
</tr>
<tr>
<td>III + IV</td>
<td>37 (43.0)</td>
<td>30 (34.9)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>41 (36.9)</td>
<td>46 (41.4)</td>
</tr>
<tr>
<td>No</td>
<td>25 (35.7)</td>
<td>23 (32.9)</td>
</tr>
</tbody>
</table>

* ORs were calculated to measure the association of the Pro/Pro genotype and lung cancer risk.
* Adjusted for age and sex.
* Adjusted for age.
* P < 0.01.
* P < 0.05.

Table 2 Characteristics of cases and controls by p53 genotype and age

<table>
<thead>
<tr>
<th>Ages (years)</th>
<th>Cases</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arg/Arg, n (%)</td>
<td>Arg/Pro, n (%)</td>
</tr>
<tr>
<td>≥60</td>
<td>33 (29.5)</td>
<td>57 (50.8)</td>
</tr>
<tr>
<td>&lt;60</td>
<td>14 (35.0)</td>
<td>18 (45.0)</td>
</tr>
</tbody>
</table>

* Patients with the Pro/Pro genotype had an OR of 2.63 (CI, 1.22–5.68; P = 0.01) of being diagnosed as having lung cancer at earlier ages (<60 years old) than patients with the other genotypes.

ever, there was no difference among Japanese (8), Chinese (11), and Taiwanese.

Distribution of p53 Polymorphism among Healthy Controls and Lung Cancer Patients as well as the Correlation with Clinico-pathological Parameters of Patients. Genomic DNA from both lung cancer patients and noncancer controls was analyzed to determine the distribution of p53 codon 72 polymorphism. The mean age of the patients was 64 years, compared with 62 years for the controls. Women were over-represented in the control group (46.7%, 71 of 152 versus 27.3%, 53 of 194 in control versus patient groups, respectively). Table 1 shows the distribution of p53 polymorphism by case/control status and clinico-pathological parameters of lung cancer patients. Overall, there was no difference in genotype distributions between noncancer controls and lung cancer patients (P > 0.05, using the χ² test). However, when the patients’ group was stratified by sex, tumor type, tumor stage, smoking habit, and age, some interesting points were revealed (Tables 1 and 2).

An increased frequency of the Pro/Pro genotype was observed in female patients compared to noncancer controls after adjustment for age (Table 1). The female patients with the Pro/Pro genotype showed an increased OR (2.17; CI, 1.04–4.50; P = 0.04) of having lung cancer compared with the controls with other genotypes. As the LAD patients were stratified according to sex, the Pro allele was over-represented in the female LAD patients compared with the noncancer controls (χ² = 10.33, df = 2, P = 0.006). The female patients with the Pro/Pro genotype showed an increased OR (3.14; CI, 1.48–6.64; P = 0.003) of having LAD, compared with the controls with other genotypes (Table 1). This difference was also statistically significant when the analysis was adjusted for age. These data suggest that the Pro allele of the p53 codon 72 polymorphism potentially increases the risk of LAD among female Taiwanese.

When the patients’ group was stratified according to smoking status, we found a slight increase of the Pro/Pro genotype frequency in lung cancer patients who did not smoke, compared with the noncancer controls, although not statistically significant (P = 0.06; Table 1). When the patients’ group was stratified according to age, we found an increase in the Pro/Pro genotype frequency was observed as patients’ age decreased (Table 2). Patients with the Pro/Pro genotype had an OR of 2.63 (CI, 1.22–5.68; P = 0.01) to be diagnosed as lung cancer at earlier ages than those with the other genotypes.
**p53 Polymorphism and Prognosis.** The relationship between p53 codon 72 polymorphism and postoperative survival was analyzed for 133 patients. The patients with the Arg/Pro genotype had the longest survival times among the three genotypes, up to 51 months postoperation (Fig. 1). The estimated median survival times for patients with Pro/Pro and Arg/Arg genotypes were 26 and 27 months, respectively. Patients with the Arg/Pro genotype did not reach the estimated median survival time during the observation period. The patients with the Pro/Pro genotype had poorer prognoses than those with the Arg/Pro genotype (P = 0.05).

**DISCUSSION**

This study evaluates the association between the risk of developing lung cancer, the prognosis, and the genotype at codon 72 of the p53 gene. The results show that (a) the genotype distribution of the Pro allele p53 polymorphism in the Taiwanese population differs significantly from other reports in Swedish, Spanish, Caucasians in the United States, and African-Americans; (b) the Pro allele of p53 polymorphism was associated with an increased risk of LAD among female Taiwanese; (c) the genotype distribution differed significantly among patients with different ages; and (d) the patients with the Pro/Pro genotype tended to have a shorter postoperative survival compared with those with the Arg/Pro genotype.

We demonstrated ethnicity as an important confounding factor in epidemiological studies involving hereditary factors. This agrees with the finding of Beckmen et al. (11) who reported a significant correlation between the frequency of the Pro allele and latitude. It has been suggested that the frequency of the Pro/Pro allele may partly explain the incidence of lung cancer among different ethnic groups, that is, the higher the Pro/Pro genotype frequency, the higher the lung cancer incidence (9). However, the substantially high Pro genotype frequency (0.44) still cannot explain the lower lung cancer incidence rate of Taiwanese (25.4 of 100,000) compared to that of other populations worldwide.

Increasing frequency of the Pro/Pro genotype was noted in female and female LAD patients (Table 1). Registry data indicate a low male:female ratio of 2:1 for lung cancer mortality in Taiwan (2). However, few Taiwanese females smoke cigarettes. The distribution of cigarette smokers in Taiwan is 59.4% for males and 3.8% for females (3). If Pro/Pro is a susceptible genotype, its high prevalence in female lung cancer patients may partly explain their high rate of LAD. Moreover, patients with the susceptible Pro/Pro genotype had a tendency to be diagnosed with lung cancer at earlier ages than individuals with the other genotypes (Table 2). This finding is consistent with the data reported by Jin et al. (9), as well as that for the CYP1A1 gene and cancer susceptibility (22). Smoking habits would also be a confounding factor. We found that Pro/Pro allele was slightly overrepresented in lung cancer patients who were non-smokers (Table 1). The association between smoking habits and p53 genotype distribution is controversial (8, 9). Our data agree with Jin et al. (9) that high risks are associated with the Pro/Pro genotype in lighter smokers. It is conceivable that genetic differences in risk tend to be more important at exposure to low doses of a carcinogen such as a low level of cigarette smoking.

Our data also suggest that p53 codon 72 polymorphism may be a potential factor indicative of prognosis of lung cancer in Taiwan. To the best of our knowledge, this is the first report of an association of p53 codon 72 polymorphism with prognosis in lung cancer patients. Buller et al. (23) reported that the Arg/Arg and Pro/Pro genotypes were more frequently observed than the Arg/Pro genotype among the invasive ovarian cancer patients, suggesting an association of Arg/Arg and Pro/Pro genotypes with tumor progression. We found that patients with the Pro/Pro genotype were more than five times as likely to die at early postoperation stages than those with the Arg/Pro genotype. It is possible that patients with the Pro/Pro genotype of p53 tumor suppressor gene were susceptible to additional genetic changes that may have resulted in worse postoperative survival. However, there is no direct evidence clarifying how the Pro/Pro genotype of the p53 gene causes worse prognosis in lung cancer patients at the present time.

It would be interesting to know why the Pro/Pro genotype of p53 gene is associated with the lung cancer risk and adverse
prognosis. The codon 72 is located in a Pro-rich linking region between amino acids 60 and 92, which begins the hydrophobic midsection of the p53 protein. The hydrophobic region between amino acids 100 and 295 determines p53 conformation, specific binding to DNA consensus sequences, and sequence-specific transcriptional activity, which may be essential for growth suppression (24). The effect of p53 codon 72 polymorphism on the function of p53 protein remains unknown. However, functional differences of these variants of p53 protein encoded by different genotypes have been studied in several cell lines. P53 protein with Pro is structurally different from p53 with Arg, which is reflected by its altered electrophoretic mobility. However, no foci of transformed cells are observed with either type of p53 protein when they are cotransfected with H-ras in rat cells (10). The half-lives of the two variants of p53 protein are the same in most cells with the exception of Daudi cells, in which the Pro variant is twice as stable as the Arg form (25). However, it is possible that p53 codon 72 polymorphism may influence expression of p53 gene, which encodes a nucleoprotein functioning as a transcription factor that regulates cell cycle-related genes (26). Two studies analyzed the p53 status and cell cycle checkpoint pathway in bladder cancer cell lines with different polymorphism at p53 codon 72. The results showed that cells with the Arg/Arg genotype express a higher level of RNA but a lower level of protein compared with cells with the Pro/Pro genotype (27). In addition, these cell lines all demonstrated defects in radiation response regardless of p53 status. However, other defects in the cell-cycle checkpoint pathway may be involved in these cell lines (28). A further possibility is that the codon 72 genotype at the p53 gene may be a genetic marker of other genes that affect the susceptibility and prognosis of lung cancer patients. These genes may be cosegregated with the p53 gene.

p53 codon 72 polymorphism may play a role in cancer susceptibility and prognosis in specific classes of lung cancer patients in Taiwan. We recently examined the mutation spectrum of the p53 gene in lung cancer patients in Taiwan (29), and we also found that patients with or without the p53 mutation had similar genotypic distribution of the p53 gene (30), which suggests that p53 codon 72 polymorphism may be unassociated with p53 gene mutation. The association of p53 codon 72 polymorphism with alterations in other genes involved in tumorogenesis is currently under investigation.

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