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Original Paper

Prognostic Significance of *p53* Codon 72 Polymorphism in Lung Carcinomas

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Lung cancer is the leading cause of cancer death in Taiwan. Potential molecular markers associated with cancer susceptibility and prognosis are the genes involved in tumorigenesis. Therefore, we investigated the association of *p53* codon 72 polymorphism with prognosis in 114 lung cancer patients. The estimated median survival times for patients with proline (Pro)/Pro, arginine (Arg)/Arg, and Arg/Pro genotypes were 25, 26 and 36 months, respectively. We also found that patients with the Pro/Pro genotype had a worse prognosis compared with those with Arg/Pro genotypes, especially for patients with squamous cell lung cancer ($P = 0.013$), male patients ($P = 0.028$) and those aged 60–69 years ($P = 0.052$). In patients with early stage lung cancer, patients with Pro/Pro and Arg/Arg genotypes had a tendency for a worse prognosis than those with the Arg/Pro genotype ($P = 0.057$). Our data suggest that *p53* codon 72 polymorphism may be a potential prognostic factor in certain sub groups of lung cancer patients in Taiwan.

Key words: lung cancer, *p53* tumour suppressor gene, polymorphism, prognosis
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INTRODUCTION

CANCER OF the lung is the leading cause of cancer death in Taiwan [1]. Lung cancer patients who undergo potentially curative resection often die due to recurrence within 5 years of resection. Patients with the same stage show varied prognoses. It is often difficult to distinguish the patients with a poor prognosis from others with a better prognosis based on the existing diagnostic tools. Therefore, identifying unfavourable prognostic factors, other than the TNM system, may stimulate patients to receive adjuvant treatment.

Potential molecular markers associated with cancer susceptibility, as well as metastases and adverse survival, are the genes involved in tumorigenesis and/or drug metabolism. Several studies have suggested a clear genetic influence on the incidence of metastases, and consequently poor prognosis, in lung cancer patients. For example, Okada and colleagues demonstrated that lymph node or distant metastasis was more frequently observed among squamous cell carcinoma (SCC) lung cancer patients with susceptible homozygous genotype C in the *MspI* polymorphism of the cytochrome P450 *1A1* gene (*CYP1A1*) [2]. Goto and associates reported that lung cancer patients with a combination of susceptible *CYP1A1* genotypes and a deficient null genotype in the glutathione S-transferase M1 gene had remarkably shortened survivals [3]. In addition, Kawashima and co-workers found a close correlation between long band products with *Eco*RI restriction fragment length polymorphism of L-*myc* and the extent of metastasis, particularly to lymph nodes at the time of surgery [4].

Polymorphism at codon 72 in the *p53* gene has been studied as potential susceptible genotypes for lung cancer [5–8]. The gene products of the two polymorphic variants differ by the presence of either arginine (Arg, CGC), a large polar amino acid residue, or proline (Pro, CCC), a small, nonpolar amino acid residue [9] and can be identified by polymerase chain reaction (PCR) and restriction enzyme analysis (*BstUI* or *AccII*). 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 adenocarcinoma (AD) in an American study [5]. A study carried out in Japan [6] showed

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a significant association of the Pro allele with the SCC lung cancer group, but not in AD. As to the correlation of *p53* codon 72 polymorphism with smoking, Murata and colleagues found that lung cancer patients who did not smoke included a significantly larger proportion of Arg/Arg homozygotes and a smaller proportion of Arg/Pro heterozygotes compared with healthy controls [7]. They also found that Pro allele carriers were significantly more frequent in patients who smoked [10]. However, Jin and associates reported that high risks associated with the Pro/Pro genotype were noted in lighter smokers [8]. We also found that genotype distribution of *p53* in the Taiwanese population differs significantly from other reports in Europe and the U.S.A. (data not shown). Therefore, these discrepancies may be due to substantial interethnic and interindividual risk differences. Nevertheless, the effects of *p53* codon 72 polymorphism on the prognosis in lung cancer remain poorly defined.

The purpose of this study was to investigate the association of *p53* codon 72 polymorphism with prognosis of lung cancer. The effect of *p53* codon 72 polymorphism on survival was analysed in patients stratified into more precise categories. Our data are the first to show the interactive nature and prognostic capabilities of *p53* codon 72 polymorphism in lung tumorigenesis.

PATIENTS AND METHODS

Study population

The cases were 114 surgical lung cancer patients who were consecutively admitted to Veteran General Hospital-Taichung, Taichung, Taiwan between 1993 and 1996. 110 patients had non-small cell lung cancers (53 SCC, 49 AD, 3 adenosquamous carcinomas, 4 large cell carcinomas and 1 mixed type of AD and large cell carcinomas), and 4 patients had small cell lung cancer. The histologies of tumour type and stage were determined according to the World Health Organization classification method. Patient follow-up was performed at 2 month intervals in the first year after surgery and at 3 month intervals thereafter. The end of the follow-up period was 15 January 1998. The mean follow-up period for all patients was 16.4 months (range 0.5–45 months). For the 63 patients who survived the follow-up period (censored patients), the mean follow-up time was 20.3 months. For the 51 patients who died during the follow-up period, the mean follow-up time was 11.7 months.

Polymorphism analysis

Representative proportions of well-separated normal lung tissues were taken after surgical resection and 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. Purified genomic DNA was amplified by PCR for the *p53* tumour suppressor gene. Oligodeoxynucleotide primers and thermocycle PCR conditions were as described previously [11]. The polymorphic site of codon 72 was detected by *BstUI* restriction enzyme digestion (recognition site CGCG, New England Biolabs, Beverly, Massachusetts, U.S.A.) 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 2.0% agarose gels and staining with ethidium bromide, the genotype of codon 72 polymorphism was determined. We performed the dideoxy-chain termination DNA sequencing of 12 PCR products to confirm the authenticity of the genotype analysis. We found an identical genotype for all patients analysed using both PCR-based genotype analysis and DNA sequencing analysis.

Statistical analysis

Type III censoring was performed on subjects who were still alive at the end of the study [12]. The Kaplan–Meier method was used to estimate the probability of survival as a function of time and the median survival [13]. The log rank test was used to assess the significance of the difference between pairs of survival probabilities [14]. We treated only one potential prognostic factor each time using a multivariate technique, i.e. the Cox proportional hazard model, to obtain the results of the univariate analysis. The estimated relative risk (hazard) ratio was calculated by the exponential of coefficient obtained from the Cox proportional hazard model.

RESULTS

Overall survival rate in patients with lung cancer in relation to the p53 *codon 72 polymorphism*

The relationship between *p53* codon 72 polymorphism and postoperative survival was analysed for 114 surgically resected lung cancer patients. Survival times in patients correlating with the three genotypes are shown in Figure 1. The estimated median survival times for patients with Pro/Pro, Arg/Arg and Arg/Pro genotypes were 25, 26 and 36 months, respectively. Patients with the Arg/Pro genotype showed a 10 month increase in median survival time over those with the other genotypes. Survival rates in patients with Pro/Pro and Arg/Arg genotypes were lower than those in patients with the Arg/Pro genotype. These differences were examined by the log rank test, but were not statistically significant ($P = 0.063$ and $P = 0.088$, respectively, Figure 1).

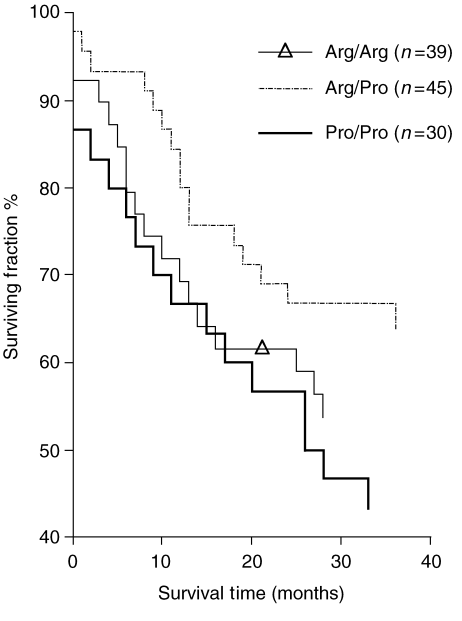

Figure 1. Kaplan–Meier survival curves with respect to *p53* codon 72 genotypes in 114 lung cancer patients. *P* values were calculated using the log rank test. The estimated median survival times for patients with Pro/Pro, Arg/Arg and Arg/Pro genotypes were 25, 26 and 36 months, respectively. The patients with Pro/Pro and Arg/Arg genotypes had a worse prognosis than those with the Arg/Pro genotype ($P = 0.063$ and $P = 0.088$, respectively).

Survival rate in patients stratified to various clinicopathological parameters in relation to the p53 *codon 72 polymorphism*

We also analysed the prognosis significance of *p53* codon 72 polymorphism by stratifying the various clinicopathological parameters of patients. Patients with the Pro/Pro genotype had the shortest median survival time among patient groups of SCC lung cancer, men, and those aged 60–69 years (Figure 2). The survival rates in SCC patients with the Pro/Pro genotype were significantly lower than those in SCC patients with the Arg/Pro genotype ($P = 0.013$; Figure 2a). When all three genotypes were considered for SCC, a borderline significance was apparent (Table 1; $P = 0.046$). In male patients, the median survival time of 20 months for the Pro/Pro genotype was shorter than that of 36 months for the

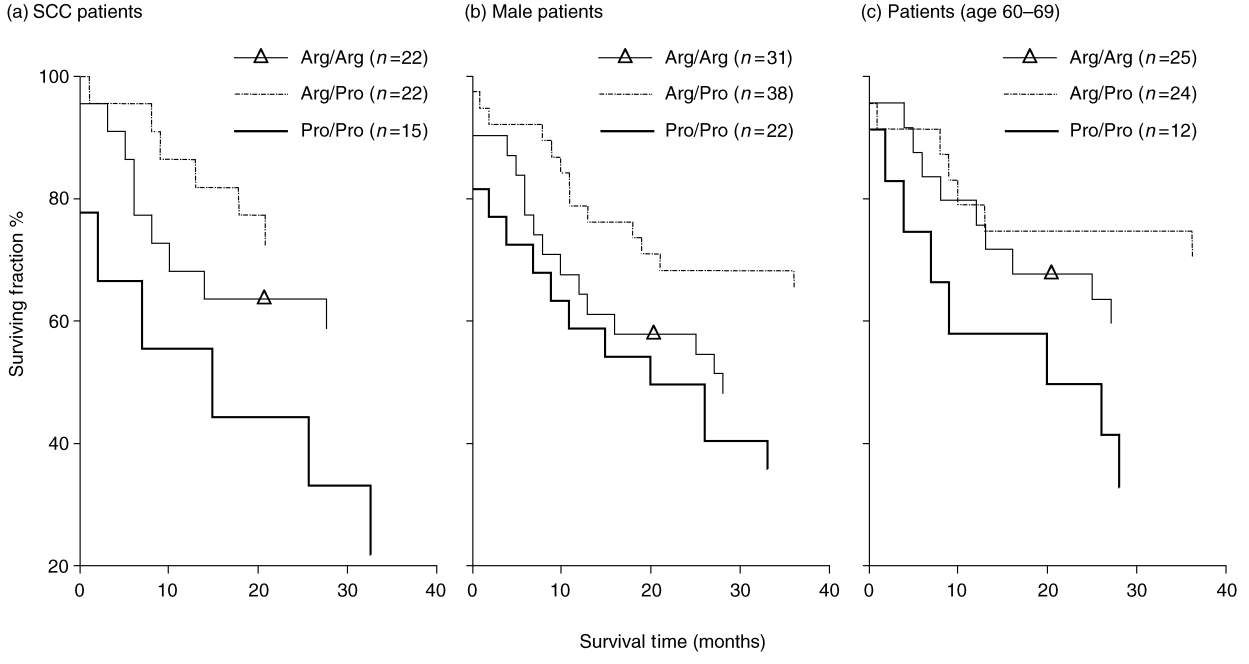

Figure 2. Kaplan–Meier survival curves with respect to *p53* codon 72 genotypes in relation to clinicopathological parameters of lung cancer patients. (a) Patients with squamous cell carcinoma. The survival rate in patients with the Pro/Pro genotype was significantly lower than in those with the Arg/Pro genotype (P=0.013, using the log rank test). (b) Male patients. The survival rate in patients with the Pro/Pro genotype was lower than in those with the Arg/Pro genotype (P=0.028). (c) Patients aged 60–69 years. The survival rate in patients with the Pro/Pro genotype was markedly lower than in those with the Arg/Pro genotype (P=0.052).

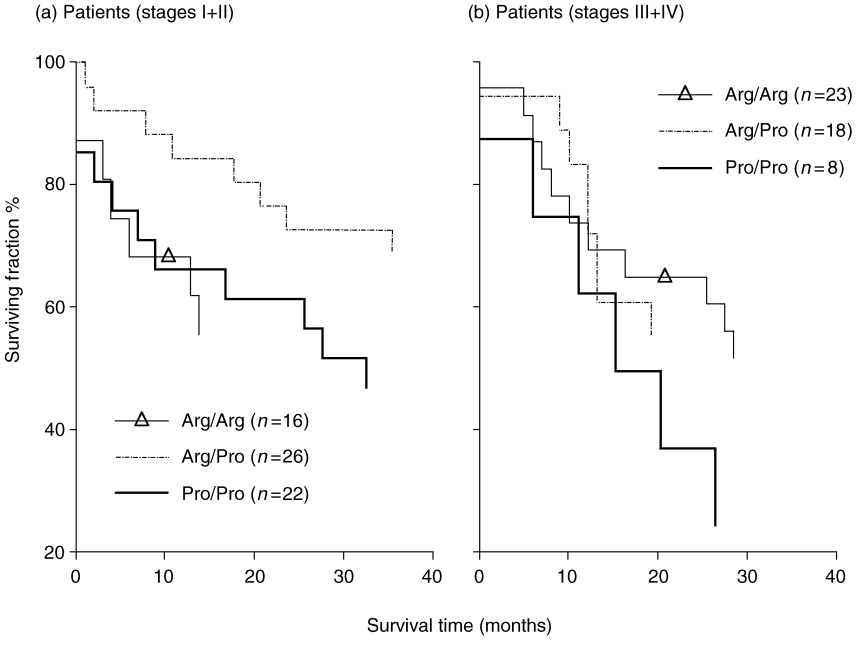

Figure 3. Kaplan–Meier survival curves with respect to *p53* codon 72 genotypes in relation to tumour stage of lung cancer patients. (a) Early stage patients (stages I and II). The survival rates in patients with Pro/Pro and Arg/Arg genotypes were lower than in those with the Arg/Pro genotype (P=0.057, using the log rank test). (b) Late stage patients (stages III and IV). The survival rate in patients with the Pro/Pro genotype was lower than in those with the Arg/Arg genotype (P=0.052).

Arg/Pro genotype ($P = 0.028$; Figure 2b). Similarly, the log rank test revealed that patients aged 60–69 years with the Pro/Pro genotype had a worse prognosis (of borderline significance) than did patients with the Arg/Pro genotype ($P = 0.052$; Figure 2c). When analysing the data by tumour stage, the effect of the *p53* codon 72 polymorphism was not statistically significant (Table 1). However, patients with stage III–IV disease with the Pro/Pro genotype had a tendency for a shorter median survival time than those with the Arg/Arg genotype ($P = 0.052$, Figure 3b). For stage I–II disease, patients with Arg/Arg and Pro/Pro genotypes had a tendency for a worse prognosis than those with the Arg/Pro genotype ($P = 0.057$; Figure 3a).

Table 1. *Median survival of lung cancer patients stratified by various clinicopathological parameters in relation to* p53 *polymorphism*

Characteristics	*p53* genotypes	Cases	MST (months)	*P* values*
Male	Arg/Arg	31	25	0.074
	Arg/Pro	38	36	
	Pro/Pro	22	20	
Female	Arg/Arg	8	–	0.969
	Arg/Pro	7	–	
	Pro/Pro	8	28	
Age (years)				
≤ 59	Arg/Arg	6	14	0.756
	Arg/Pro	5	21	
	Pro/Pro	10	26	
60–69	Arg/Arg	25	25	0.075
	Arg/Pro	24	–	
	Pro/Pro	12	20	
≥ 70	Arg/Arg	9	10	0.482
	Arg/Pro	15	21	
	Pro/Pro	7	33	
Tumour types				
SCC	Arg/Arg	22	28	0.046
	Arg/Pro	22	–	
	Pro/Pro	9	15	
AD	Arg/Arg	12	25	0.604
	Arg/Pro	22	36	
	Pro/Pro	15	28	
Tumour stages				
I + II	Arg/Arg	16	14	0.063
	Arg/Pro	26	–	
	Pro/Pro	21	28	
III + IV	Arg/Arg	23	27	0.187
	Arg/Pro	18	19	
	Pro/Pro	8	15	
N factors				
N –	Arg/Arg	23	14	0.117
	Arg/Pro	32	–	
	Pro/Pro	23	28	
N +	Arg/Arg	14	25	0.138
	Arg/Pro	10	19	
	Pro/Pro	7	15	
Smokers	Arg/Arg	27	25	0.085
	Arg/Pro	32	36	
	Pro/Pro	15	17	
Non-smokers	Arg/Arg	12	–	0.788
	Arg/Pro	13	24	
	Pro/Pro	14	28	

MST, median survival time; –, median survival not reached; SCC, squamous cell carcinomas; AD, adenocarcinomas; N –, no metastasis to lymph nodes; N +, metastasis to lymph nodes; Pro, proline; Arg, arginine.* *P* values were calculated by the log rank test on the difference among the three genotypes.

Table 2 shows the results of univariate analyses using *p53* genotypes as well as variables including sex, age, smoking habit, tumour type, tumour stage, and lymph node metastasis of patients. Lymph node metastasis and *p53* genotypes were not statistically significant factors. The relative risks of death for patients with Pro/Pro and Arg/Arg compared with those with the Arg/Pro genotype were 2.280 and 1.796, respectively (1.016–5.117, $P = 0.045$ and 0.908–3.553, $P = 0.092$, respectively, Table 2).

DISCUSSION

In this study, we have shown that codon 72 polymorphism of the *p53* gene is a potential prognostic factor of specific classes of lung cancer in Taiwan. Patients with the Pro/Pro genotype had a worse prognosis compared with those with the other genotypes, but this was only significant for male patients, those aged 60–69 years and for those with SCC.

It would be interesting to know why the Pro/Pro genotype and the Arg/Pro genotype of the *p53* gene associate with an adverse and improved prognosis, respectively. Codon 72 is located in a Pro rich linking region between amino acids 60 and 92, and begins the hydrophobic mid-section 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, and which may be essential for growth suppression [15]. The effects of *p53* codon 72 polymorphism on the function of p53 protein is at present unknown. However, the 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, and this is reflected by its altered electrophoretic mobility. However, no foci of transformed cells are observed with either type of p53 protein as they are cotransfected with *H-ras* in rat cells [9]. 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 [16]. Nevertheless, it is possible that these polymorphic variants of p53 protein might differ in damage-induced cell cycle arrest and/or apoptosis [17]. We have previously found that mutation of the *p53* tumour suppressor gene may be involved in squamous cell lung cancer in Taiwan [18]. We investigated whether *p53* codon 72

Table 2. *Univariate analysis of prognostic factors in* 114 *patients with lung cancer*

Variable	Risk ratio	*P* value
Sex	1.732 (male versus female)	0.154
Age	1.189 (≤ 59 versus 60–69)	0.634
	1.052 (≥ 70 versus 60–69)	0.880
Smoking	1.373 (non-smoker versus smoker)	0.304
Tumour types	1.117 (AD versus SCC)	0.722
Tumour stage	1.427 (III + IV versus I + II)	0.210
N factors	1.723 (N + versus N –)	0.068
p53 genotypes	2.280 (Pro/Pro versus Arg/Pro)	0.045
	1.796 (Arg/Arg versus Arg/Pro)	0.092

SCC, squamous cell carcinomas; AD, adenocarcinomas; N–, no metastasis to lymph nodes; N +, metastasis to lymph nodes; Pro, proline; Arg, arginine.

genotypes were associated with *p53* gene mutation in 60 surgical specimens of lung cancer, and found that there was no significant difference with respect to the genotype distribution of the *p53* gene between patients with and without *p53* mutation (data not shown). However, it is possible that *p53* codon 72 polymorphism may influence expression of the *p53* gene, which encodes a nucleoprotein functioning as a transcription factor that regulates cell cycle-related genes [19, 20]. This hypothesis is currently under investigation. A further possibility is that the codon 72 genotype in the *p53* gene might be a genetic marker of other genes that affect the prognosis of lung cancer patients. These genes may be cosegregated with the *p53* gene. It is also possible that patients with the Pro/Pro genotype of the *p53* tumour suppressor gene were susceptible to further genetic changes and this may result in worse postoperative survival. Studies on the association of *p53* codon 72 polymorphism with alterations in other genes involved in tumorigenesis will be an interesting approach to this problem.

Several *p53* polymorphisms have been reported [21–23]. However, the association of *p53* polymorphism with postoperative survival in lung cancer was rarely reported. Buller and colleagues reported that the Arg/Arg and Pro/Pro genotypes were more frequently observed than the Arg/Pro genotype among invasive ovarian cancer patients, suggesting an association of Arg/Arg and Pro/Pro genotypes with tumour progression [24]. A study carried out in Hong Kong analysing *p53* intron 2 polymorphism indicated that there was a tendency for a lower survival rate in patients with the A1/A1 genotype than those with A1/A2 and A2/A2 genotypes ($0.25 < P < 0.5$) among 34 non-small cell lung cancers [25]. Tagawa and associates [26] recently reported that there was no significant association of the *p53* polymorphism with prognosis of Japanese lung cancer patients. The discrepancy between this study and ours may be due to the differences in patients' characteristics. There were more patients with SCC and early stages of lung cancer in our study compared with that of Tagawa and associates. Note that in the present study, we found that patients with the Arg/Pro genotype had a better prognosis compared with those with other genotypes, especially in the patients with SCC lung cancer ($P = 0.013$) and early stage of lung cancer ($P = 0.057$). In addition, there were differences in the genotype distribution between the two studies. Ethnicity may also be an important confounding factor in epidemiological studies involving hereditary factors. To our knowledge, we are the first to report that the *p53* codon 72 polymorphism could be used as a potential prognostic indicator in patients with specific classes of lung cancer. If a genetic marker of genomic DNA could be used to identify patients at either low or high risk, then such markers could facilitate the stratification of patients for clinical trials. These tests could be performed on DNA obtained from peripheral white blood cells prior to therapeutic intervention.

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