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Lack of Evidence of Association of *p21*$^{WAF1/CIP1}$ Polymorphism with Lung Cancer Susceptibility and Prognosis in Taiwan

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An association between the Arg allele of the *p21*$^{WAF1/CIP1}$ codon 31 polymorphism and lung cancer has been reported. However, the genotype distribution of the *p21* codon 31 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 the *p21* codon 31 polymorphism in 155 lung cancer patients and 189 non-cancer controls. The genotype frequencies in the Taiwanese non-cancer controls were 0.51 (Ser) and 0.49 (Arg). χ^2 analysis indicated significant differences in Taiwanese genotype distribution of *p21* from those reported for Swedes (*P*=0.001), Caucasians (*P*=0.001), Indians (*P*=0.001), and African-Americans (*P*=0.001). However, our data did not demonstrate an association of the Arg allele of the *p21* polymorphism with lung cancer risk in Taiwan. Lung cancer patients with Ser/Arg and Arg/Arg genotypes were at a non-significant 1.15-fold increased risk of lung cancer when compared to individuals with the Ser/Ser genotype (95%CI, 0.70–1.86). In addition, although *p21* is a downstream target of *p53*, we found no significant correlation of the *p21* polymorphism with the *p53* polymorphism and *p53* gene mutation in lung cancer patients. We further investigated the association of the *p21* polymorphism with prognosis in 154 lung cancer patients. Patients with the Ser/Ser genotype tended to have a poorer prognosis than those with the Ser/Arg and Arg/Arg genotypes (*P*=0.097, by the log rank test). Our data suggest that the *p21* codon 31 polymorphism may not play a significant role in cancer susceptibility and the prognosis of lung cancer patients in Taiwan.

Key words: Lung cancer — *p21* codon 31 polymorphism — Susceptibility — Prognosis — *p53* tumor suppressor gene

Cell cycle checkpoints maintain genetic integrity by arresting the cell cycle to allow for genetic errors to be repaired. An example of this is the *p53*-mediated arrest of the cell cycle at the G1/S checkpoint in response to DNA damage.[1)] p21 is one of the p53 effector proteins (also named CIP-1 and WAF-1) which has been isolated and characterized.[2,3)] p21 is transcriptionally induced by wild-type p53, and has the ability to act as a tumor suppressor.[2)] The p21 protein functions as a universal inhibitor of cyclin-dependent kinases (CDKs),[3)] and interacts with proliferation cell nuclear antigen, thereby preventing DNA replication and blocking the cell cycle in G1.[4)]

Somatic mutations in the *p21* gene appear to be very rare in human malignancies.[5–7)] However, a polymorphism in the *p21* gene, a C-to-A transversion at the third base of codon 31, resulting in the exchange of a Ser for an Arg amino acid, has been reported.[8)] This can be detected by polymerase chain reaction (PCR) and subsequent restriction enzyme digestion. The substitution leads to a loss of the *Blp*I restriction site. This codon 31 polymorphism resides in an area of greater than 90% homology at the protein level with the murine homologue, which is thought to encode a DNA-binding zinc-finger domain.[2,9)] This observation raises the possibility that this polymorphism encodes functionally distinct proteins, but transfection studies have shown no difference in the tumor suppressor abilities of the Ser and Arg alleles in a lung cancer cell line.[8)] In addition, *in vitro* CDK-cyclin kinase assays have shown that wild-type Ser p21 and the variant Arg p21 both have similar growth-inhibitory abilities.[10,11)]

Reports have demonstrated an association of the *p21*^{waf1} polymorphism with breast carcinomas, gastric carcinoma, and endometrial cancer.[12–14)] Recently, a study analyzing 144 Swedish lung cancer patients and 95 patients with chronic obstructive pulmonary disease showed an increased frequency of the *p21* codon 31 Arg allele in the lung cancer patients (7.3%), but not in the patients with chronic obstructive pulmonary disease (1.6%).[15)] In addition, Facher *et al*.[16)] found that 9 of 54 prostate adenocarcinoma samples (16.7%), and 9 of 42 squamous carcinoma of the head and neck samples (21.4%), had a significantly higher frequency of Arg allele than that in the 110 normal controls examined (9.1%). Heinzel *et al*.[17)] also reported

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that 6 of 11 oral cancers in Indians were Arg genotypes in the *p21* gene. It is notable that, in the latter two studies, the Arg polymorphism only exists in patients without a *p53* mutation. This suggests that the *p21* polymorphism may, in some cases, be incompatible with *p53* mutations. Nevertheless, no association between the *p21* genotype and cancer risk was observed in nasopharyngeal carcinomas,[11] brain tumors,[7] and other studies of breast, ovary, and endometrium carcinomas.[18, 19]

The codon 31 polymorphism of *p21* shows distinct differences among major ethnic groups.[20] The frequency of Arg allele ranges from 4% in Caucasians,[11] to 16% in Indians,[17] 29% in African Blacks in the USA[20] and 50% in Chinese (Guizhou and Singapore).[20] A case-control study, including 76 nasopharyngeal carcinoma patients in Taiwan and 66 normal controls, showed that the frequencies of Arg allele in cases and controls were 56% and 55%, respectively.[11] However, the observation that nearly 83% were heterozygous at the *p21* gene is puzzling because the genotype distribution was not in Hardy-Weinberg equilibrium. Therefore, an investigation of the genotype distribution of the *p21* gene in more samples is important, in order to understand the possible mechanism of the involvement of the *p21* tumor suppressor gene in tumorigenesis in Taiwan. The purpose of this study therefore is to investigate the genotypic frequency of the *p21* codon 31 polymorphism in lung cancer patients in Taiwan, and to examine the association of this polymorphism with lung cancer risk and prognosis.

MATERIALS AND METHODS

Study population The cases included in this study were 155 lung cancer patients who were admitted to Veterans General Hospital-Taichung, Taichung, Taiwan, between 1993 and 1998. Of these, 138 patients had non-small-cell lung cancers [73 adenocarcinomas (AD), 58 squamous carcinomas (SQ), 2 adenosquamous carcinomas, 2 large-cell carcinomas, 2 mixed-type large-cell carcinoma and small-cell lung cancer, and 1 mixed-type AD and large-cell carcinoma], and 17 patients had small-cell lung cancers. The histologies of the tumor types and stages were determined according to the WHO classification method[21] and the TNM system,[22] 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 154 patients was performed at 2-month intervals in the first year after surgery, and at 3-month intervals thereafter at out-patient clinics or by routine phone calls. The end of the follow-up period was defined as Apr. 15, 1999, for all 154 patients. The mean follow-up period for all patients was 15.9 months (range 0.5–67 months). For the 68 patients who survived the follow-up period (censored patients), the mean follow-up time was 20.3 months. For the 86 patients who died during the follow-up period, the mean follow-up period was 12.4 months. For controls, 152 non-cancer and unrelated controls were recruited from Chung Shan Hospital and Veterans General Hospital-Taichung, Taichung, Taiwan. They were randomly selected individuals from the physical check-up center, with the only restriction being a matching of age distribution to that of the patient group. The mean ages of patients and controls were 66 years (range, 33–86) and 62 years (range, 24–92), respectively.

Polymorphism analysis Blood samples (5–10 ml) were obtained and genomic DNA was extracted from the peripheral lymphocytes using standard methods. Purified genomic DNA was amplified by PCR for exon 2 of the *p21* tumor suppressor gene. Oligodeoxynucleotide primers and thermocycle PCR conditions were as indicated in ref. 11. The Ser-coded allele, but not the Arg-coded allele, has a single *Blp*I site in the amplified fragment (recognition site GCTNAGC, New England Biolabs, Beverly, MA). Thus, after electrophoresis in 3.0% agarose gel, and staining with ethidium bromide, the genotype of the codon 31 polymorphism was determined (Fig. 1).

***p53* mutation and polymorphism analyses** PCR/single strand conformation polymorphism (PCR/SSCP) was used to detect the presence of mutations in the *p53* tumor suppressor gene of 63 patients. Oligodeoxynucleotide primers and thermocycle PCR conditions designed to produce DNA fragments of *p53* gene exons 4–11 are described in ref. 23. PCR products were subjected to electrophoresis at 30W for 4–5 h in a 6% nondenaturing polyacrylamide gel with 5% glycerol, and fan-cooled at room temperature. Abnormal DNA fragments detected during PCR/SSCP

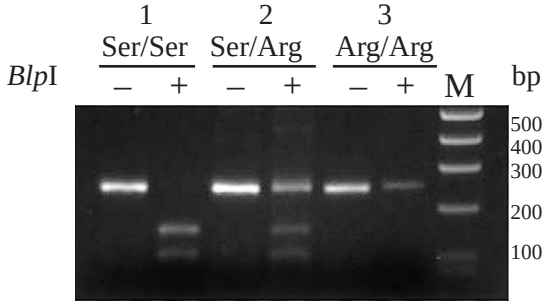

Fig. 1. Detection of *p21* codon 31 polymorphism by PCR and *Blp*I digestion. PCR product digested (+) or undigested (–) with *Blp*I for 4–8 h at 37°C. The Ser encoding and Arg encoding fragments are determined as the 245 plus 145 bp and 100 bp bands, respectively. 1 is an example of a Ser/Ser homozygote, 2 is an example of a Ser/Arg heterozygote, and 3 is an example of an Arg/Arg homozygote.

analysis were sequenced using the dideoxy chain termination method, with α-^{35}S-dATP, PCR amplified primers, and a Sequenase II kit (United States Biochemical Corporation, Cleveland, Ohio). The polymorphic site of codon 72 was detected in 126 patients by *BstU*I restriction enzyme digestion of the PCR product of exon 4 for 4–8 h at 60°C. The Arg-coded allele, but not the Pro-coded allele, has a single *BstU*I site in the amplified fragment. Thus, after electrophoresis in 2.0% agarose gel and staining with ethidium bromide, the genotype of codon 72 polymorphism was determined.

Statistical analysis The Pearson χ^2 test was used to compare genotype distributions among different ethnic groups, as well as between the lung cancer cases and controls. Statistical modeling, using logistic regression, was used to calculate the relative risk (odds ratio, OR) of Ser/Arg and Arg/Arg genotypes to the Ser/Ser genotype for the case-control study. ORs were expressed together with the 95% confidence interval (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 the study.[24] The Kaplan-Meier method was used to estimate the probability of survival as a function of time and median survival.[25] The log rank test was used to assess the significance of the difference between pairs of survival probabilities.[26]

RESULTS

Distribution of the *p21* polymorphism in Taiwanese compared to other ethnic groups worldwide We studied a total of 344 individuals: 155 lung cancer patients and 189 non-cancer controls. The frequencies of the three *p21* genotypes Ser/Ser, Ser/Arg, and Arg/Arg found in the non-cancer controls in Taiwan were 27.0%, 47.1%, and 25.9%, respectively, and fitted the Hardy-Weinberg equilibrium with allele frequencies of 0.51 (Ser) and 0.49 (Arg) (Table I). Comparing the distribution of the *p21* genotype in our controls with the data reported previously for other study populations (Table I), it is clear that the Arg variant genotype was strongly associated with ethnicity. χ^2 analysis indicated significant differences in the genotype distributions of *p21* between the Taiwanese and reported data for Swedish (P=0.001),[15] U.S. Caucasian (P=0.001),[11] Indian (P=0.001),[20] and African-American (P=0.001)[20] populations, in which lower frequencies of the Arg allele were found. However, there was no difference among Japanese,[14] Chinese,[20] and Taiwanese.

Distribution of the *p21* polymorphism among healthy controls and lung cancer patients, as well as the correlation with clinicopathological parameters of patients Genomic DNA from lung cancer patients and non-cancer controls was analyzed to determine the distribution of the *p21* codon 31 polymorphism. The mean age of the cancer patients was 66 years, compared with 62 for the controls. Women were over-represented in the control group (53.4%, 101/189 versus 20.6%, 32/155 in the control versus patient groups, respectively). Table II shows the distribution of the *p21* polymorphism by case/control status, and the clinicopathological parameters of lung cancer patients. Overall, there was no difference in genotype distributions between non-cancer controls and lung cancer patients (P>0.05, using the logistic regression model), no matter whether we adjusted for age and sex or not. As the patients' group was stratified by sex, tumor type, tumor stage, and smoking habit, there was also no significant difference between cases and controls.

Association of the *p21* polymorphism with *p53* gene mutation and genotype Because *p21* is a downstream target of *p53*, we analyzed the correlation of the *p21* polymorphism with the *p53* polymorphism and gene mutation. The 126 cancer patients analyzed for the *p21* polymorphism in this study had been tested for *p53* genotype.[27] Table III shows the distribution of the *p21* polymorphism by *p53* genotype in the lung cancer patients. In the cancer patients with the Pro/Pro variant type of *p53*, 29% con-

Table I. Frequency of Codon 31 Alleles of *p21* in Different Populations

Population	Genotypes			Arg allele frequency	*P* value[a]	Reference
	Ser/Ser (%)	Ser/Arg (%)	Arg/Arg (%)			
Taiwanese (n=189)	51 (27.0)	89 (47.1)	48 (25.4)	0.49		This study
Swedes (n=761)	692 (90.9)	67 (8.8)	2 (0.3)	0.05	0.001	Själander *et al.*
Caucasians (n=65)	58 (89.2)	7 (10.8)	0 (0.0)	0.05	0.001	Sun *et al.*
Indians (n=92)	66 (71.7)	23 (25.0)	3 (3.3)	0.15	0.001	Birgander *et al.*
African-American (n=122)	56 (45.9)	61 (50.0)	5 (4.1)	0.29	0.001	Birgander *et al.*
Japanese (n=55)	23 (41.2)	19 (34.5)	13 (23.6)	0.46	0.098	Hachiya *et al.*
Chinese, Singapore (n=121)	27 (22.3)	69 (57.0)	25 (20.7)	0.49	0.251	Birgander *et al.*
Chinese, Guizhou (n=105)	25 (23.8)	55 (52.4)	25 (23.8)	0.50	0.700	Birgander *et al.*

a) *P* values were calculated using the χ^2 test.

Table II. Distribution of *p21* Polymorphism by Case/Control Status and Clinicopathological Parameters of Lung Cancer Patients

Characteristics		Genotypes			Total	Crude OR[a)] (95%CI)	Adjusted OR[b)] (95%CI)
		Ser/Ser (%)	Ser/Arg (%)	Arg/Arg (%)			
Non-cancer control		51 (27.0)	89 (47.1)	49 (25.9)	189	1.00	1.00
Sex	Male	23 (26.1)	45 (51.1)	20 (22.7)	88	1.00	1.00
	Female	28 (27.7)	44 (43.6)	29 (28.7)	101		
Age	≥65	30 (31.9)	40 (42.6)	24 (25.5)	94	1.00	1.00
	<65	21 (22.1)	49 (51.6)	25 (26.3)	95		
Lung cancer		38 (24.5)	85 (54.8)	32 (20.6)	155	1.15 (0.70–1.86)	1.19 (0.71–2.01)
Sex	Male	31 (25.2)	68 (55.3)	24 (19.5)	123	1.05 (0.56–1.96)[c)]	1.15 (0.60–2.22)[d)]
	Female	7 (21.9)	17 (53.1)	8 (25.0)	32	1.39 (0.54–3.57)[c)]	1.41 (0.54–3.70)[d)]
Age	≥65	28 (25.7)	59 (54.1)	22 (20.2)	109	1.36 (0.74–2.50)[e)]	1.36 (0.74–2.50)[f)]
	<65	10 (21.7)	26 (56.3)	10 (21.7)	46	1.02 (0.44–2.39)[e)]	1.36 (0.74–2.50)[f)]
Tumor type							
	AD	18 (25.0)	41 (56.9)	13 (18.1)	72	1.12 (0.60–2.08)	1.17 (0.62–2.19)
	SQ	15 (25.9)	30 (51.7)	13 (22.4)	58	1.07 (0.55–2.09)	1.17 (0.55–2.48)
Tumor stage							
	I+II	13 (25.5)	26 (51.0)	12 (23.5)	51	1.09 (0.54–2.21)	1.30 (0.61–2.78)
	III+IV	21 (24.7)	49 (57.6)	15 (17.6)	85	1.14 (0.63–2.04)	1.12 (0.62–2.04)
Smoking							
	Yes	26 (24.8)	55 (52.4)	24 (22.9)	105	1.13 (0.65–1.96)	1.38 (0.68–2.79)
	No	11 (22.4)	30 (61.2)	8 (16.3)	49	1.29 (0.61–2.71)	1.30 (0.61–2.76)

a) Odds ratios were calculated to measure the association of the mutant genotypes (Ser/Arg and Arg/Arg) with lung cancer risk.
b) Adjusted for age and sex.
c) Odds ratios were calculated by using male controls and female controls as references.
d) Adjusted for age.
e) Odds ratios were calculated by using controls aged ≥65 and controls aged <65 as references.
f) Adjusted for sex.

Table III. Distribution of *p21* polymorphism by *p53* Genotypes and Mutation of Lung Cancer Patients

Characteristics	Genotypes			Total	$P^{a)}$ value
	Ser/Ser (%)	Ser/Arg (%)	Arg/Arg (%)		
p53 genotypes (n=126)					
Arg/Arg	10 (23.2)	26 (60.5)	7 (16.3)	43	0.69
Arg/Pro	11 (21.1)	27 (51.9)	14 (26.9)	52	
Pro/Pro	9 (29.0)	15 (48.4)	7 (22.6)	31	
p53 mutation (n=63)					
Yes	4 (36.4)	4 (36.4)	3 (27.3)	11	0.82
No	15 (28.8)	24 (46.2)	13 (25.0)	52	

a) P values were calculated using the χ^2 test.

tained the Ser/Ser wild type of *p21*. The frequency of the Ser/Ser genotype was slightly increased compared to that in those with the Arg/Arg and Arg/Pro genotypes of the *p53* gene (P>0.05). The 63 cancer patients analyzed for the *p21* polymorphism in this study had been tested for *p53* gene mutation.[28)] Table III shows the distribution of the *p21* polymorphism by *p53* mutation in lung cancer patients. In the cancer patients with *p53* mutation, 36.4% contained the Ser/Ser wild-type of the *p21*. The frequency of the Ser/Ser genotype was slightly increased compared to that in those without the *p53* gene mutation (P>0.05).

***p21* polymorphism and prognosis** The relationship between the *p21* codon 31 polymorphism and postoperative survival was analyzed for 154 patients. There was a

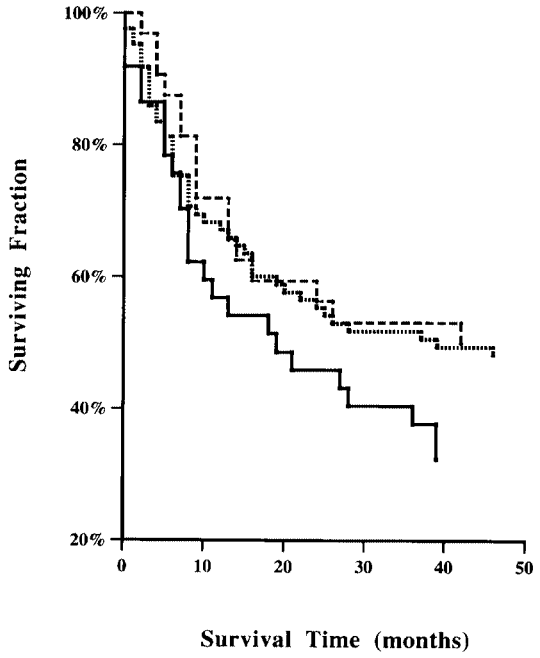

Fig. 2. The Kaplan-Meier survival curves with respect to *p21* codon 31 genotypes in 154 lung cancer patients. *P* values were calculated using the log rank test. The estimated median survival times for patients with Ser/Ser, Ser/Arg, and Arg/Arg were 18, 20, and 24 months, respectively. The patients with Ser/Ser genotype had poorer prognoses than those with Ser/Arg and Arg/Arg genotypes ($P=0.097$). —— Ser/Ser ($n=37$), ····· Ser/Arg ($n=85$), ------ Arg/Arg ($n=32$).

near-significant trend for shorter survival in the patients with the Ser/Ser genotype ($P=0.097$, by the log rank test) compared to those with Ser/Arg and Arg/Arg genotypes. The estimated median survival times for patients with Ser/Ser, Ser/Arg, and Arg/Arg were 18, 20, and 24 months, respectively (Fig. 2).

DISCUSSION

This study evaluated the association between the risk of developing lung cancer and its prognosis, and the genotype at codon 31 of the *p21* gene. The results show that, (a) the genotype distribution of the Arg allele *p21* polymorphism in the Taiwanese population differs significantly from those reported for Swedish, Indians, Caucasians, and African-Americans; (b) the Arg allele of the *p21* polymorphism was not associated with increased risk of lung cancer in Taiwan; (c) the *p21* polymorphism was not associated with *p53* gene mutation and polymorphism; and (d) patients with the Ser/Ser genotype tended to have a shorter postoperative survival compared to those with the Ser/Arg or Arg/Arg genotype.

We identified ethnicity as an important confounding factor in epidemiological studies involving hereditary factors (Table I). This agrees with the findings of Birgander *et al.*[20] who reported significant differences in the frequency of the Arg allele in major ethnic groups. Beckmen *et al.* also found a significant correlation between the frequency of the Pro allele of the *p53* gene and latitude.[29] However, there seems to be no correlation between the geographical patterns of the *p53* and *p21* alleles.[20]

The distribution of the *p21* genotypes was similar in both cases and controls, and no significant association between the *p21* polymorphism and lung cancer (Table II) was observed. Lung cancer patients with Ser/Arg and Arg/Arg genotypes were at a non-significant 1.15-fold increased risk of lung cancer when compared to individuals with the Ser/Ser genotype (95%CI, 0.70−1.86). In contrast, Själander *et al.* found an increased frequency of the *p21* codon 31 Arg allele in lung cancer patients in Sweden, especially in comparison with chronic obstructive pulmonary disease patients.[15] The discrepancies between the present study and the last-mentioned study may result from differences in the control populations chosen. Själander *et al.* found that the association of the Arg allele with lung cancer is not significant, based on a comparison between lung cancer patients and healthy population controls. Alternatively, the discrepancies may be due to substantial inter-ethnic and inter-individual risk differences in the study populations of Sweden and Taiwan. In fact, the association of the *p21* codon 31 polymorphism with cancer risk has been studied in various tumors with inconsistent results (see the opening section). The association may depend on the tumor type analyzed. Note that no functional difference, with regard to the inhibition of CDKs or to the inhibition of tumor cell growth, has so far been demonstrated for the Arg-containing p21 protein,[8, 10, 11] though the possible occurrence of post-transcriptional and/or post-translational modifications of the Arg-protein has not been ruled out.

An interesting question is whether *p53* alleles may interact synergistically with the alleles of its effector protein p21 in cancer. We found, however, no association between the *p53* codon 72 and *p21* codon 31 polymorphisms in lung cancer (Table III). This is in agreement with the results of previous studies.[15, 20] In addition, we found no significant difference in the distribution of the variant allele in lung cancer patients whose tumors had (7/11) or did not have (37/52) *p53* gene mutation (Table III). The lack of association between the *p21* polymorphism and *p53* mutations was also found in studies of sporadic ovarian tumors[19] and brain tumors.[7] Nevertheless, this should be confirmed in larger patient subsets.

The observation of a tendency toward a worse prognosis with the Ser/Ser wild-type allele of the *p21* polymorphism in lung cancer is intriguing. This suggests a possible asso-

ciation of the Ser/Ser allele with a poor survival rate for lung cancer patients. A further possibility is that the Ser/Ser allele of the *p21* gene may be a genetic marker of other genes that affect the prognosis of lung cancer patients. Interestingly, we have previously shown that patients with the Pro/Pro genotype of the *p53* gene have a poor prognosis.[27] In the present study, we observed that patients with the Ser/Ser genotype tended to have an increase frequency of the Pro/Pro variant type of *p53* (Table III).

In summary, our data suggest that the *p21* codon 31 polymorphism may not be significantly associated with cancer risk or the prognosis of lung cancer patients in Taiwan. It may also have no significant effect on the *p53* gene mutation and polymorphism of lung cancer patients in Taiwan. However, the analysis of larger case populations for correlation with *p53* gene mutation would be desirable. In addition, we are currently examining the association of the *p21* genotype with the p21 protein expression in lung cancer specimens.

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