SPANDIDOS PUBLICATIONS

The allelic distribution of a single nucleotide polymorphism in the *PDCD5* gene locus of Japanese non-small cell lung cancer patients

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Abstract. It has been reported that the rs1862214 single nucleotide polymorphism (SNP) in the programmed cell death 5 gene (*PDCD5*) is associated with smoking-related lung cancer risk and prognosis in a European population with a history of smoking. The aim of this study was to investigate the status and impact of SNPs in the *PDCD5* locus of a Japanese population. We developed an assay based on real-time PCR with melting curve analysis for determining the rs1862214 SNP, and examined this SNP in 165 lung cancer patients and 180 healthy volunteers. Of the 165 lung cancer patients (107 smokers), 25 (17), 72 (47) and 68 (43) had the CC, CG and GG genotypes of rs1862214, respectively. Of the 180 volunteers (117 smokers), 31 (24), 81 (52) and 68 (41) had the CC, CG and GG genotypes of rs1862214, respectively. No significant difference in allelic distribution was found between Japanese patients and healthy controls, even among smokers. Based on the published data, the distribution of this SNP appears to be significantly different in Japanese and European populations. No significant difference in prognosis according to the SNP was observed, either in patients with a history of smoking or in the total number of patients. This too differs from the results from a European population. In conclusion, we developed a convenient real-time PCR-based assay for the genotyping of rs1862214 in the *PDCD5* locus. The distribution of the rs1862214 SNP in our Japanese population differs from its distribution in a European population, and is not related to the risk of cancer or to poor prognosis in lung cancer. This suggests the presence of an ethnicity-related difference in the role of *PDCD5* in the pathogenesis of lung cancer.

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Abbreviations: SNP, single nucleotide polymorphisms; NSCLC, non-small cell lung cancer

Key words: single nucleotide polymorphisms, *PDCD5*, Japanese, non-small cell lung cancer, cancer risk

Introduction

Lung cancer is the leading cause of cancer death in Japan and many other countries worldwide (1,2). Survival among patients with non-small cell lung cancer remains unsatisfactory because many locally advanced or metastatic cases are unresectable. Even patients with the early stages of the disease who undergo complete resections often experience recurrence, resulting in an unfavorable prognosis. In short, until now the effectiveness of therapeutic strategies has been limited.

In addition to aiding in the development of therapeutic strategies, the prevention or early detection of cancer improves its rate of mortality. Several genetic polymorphisms are considered to be molecular markers that predict the risk, progression and prognosis of various cancers (3-7). Indeed, single nucleotide polymorphisms (SNPs) in several genes are reported to be associated with the risk of lung cancer (8-10), for example SNPs in the caspase-3 and vascular endothelial growth factor genes (11,12).

Recently, Spinola *et al* reported that the rs1862214 SNP in the programmed cell death 5 gene (*PDCD5*) locus, which is known to be involved in apoptosis (13,14), was associated with lung cancer risk and prognosis in a European population with a history of smoking (15). They performed a case-control study using German and Italian lung cancer patients along with controls enrolled at the same sites, and found that patients with genotypes GG or CG for *PDCD5* showed an increased risk of lung cancer, a higher incidence of poor clinical stage disease and short-term survival compared to those with the common CC genotype. These findings indicate that *PDCD5* may be a potential molecular marker, predicting the risk of lung cancer and prognosis. However, the impact of the *PDCD5* SNP must be assessed before it is used to evaluate the risk and prognosis of lung cancer in other ethnicities, such as in a Japanese

population, since the function of polymorphisms, including SNPs, is often affected by ethnicity (16,17).

In the current study, we analyzed the allelic distribution of an SNP at rs1862214 in the *PDCD5* locus of Japanese lung cancer patients and healthy controls, and studied its impact on the risk of lung cancer and on prognosis in a Japanese population.

Materials and methods

Patients and control groups. We analyzed 165 Japanese patients who were histologically diagnosed as having primary lung cancer and who had undergone surgery at Okayama University Hospital (Okayama, Japan) between 2000 and 2006. We recruited 180 healthy volunteers in Okayama prefecture, Japan. Institutional Review Board approval and informed consent from all lung cancer patients and controls were obtained. The characteristics of the 165 lung cancer patients and 180 controls are shown in Table I. There were no significant differences in sex, age or smoking status at the time of recruitment between the lung cancer patients and the controls.

DNA extraction and genotyping of rs1862214 in the PDCD5 locus. The genomic DNA of the 165 patients was isolated from freshly-frozen non-malignant peripheral lung tissue using SDS/proteinase K treatment, phenolchloroform extraction and ethanol precipitation. The genomic DNA of the 180 controls was extracted from peripheral lymphocytes.

To determine the genotype of rs1862214 in the *PDCD5* locus with rapidity, a convenient genotyping assay using real-time PCR was developed. We designed the primers and hybridization probes for the rs1862214 SNP in the *PDCD5* locus to be used in real-time PCR, then detected the genotype of each sample by melting curve analysis. Two different fluorescent-labeled hybridization probes, which hybridize to an internal sequence of the amplified fragment, were added during PCR. One probe was labeled at the 3'-end with fluorescein (anchor). The other was labeled at the 5'-end with a LightCycler-Red fluorophore (LC-Red 705) and, to avoid extension, modified at the 3'-end by phosphorylation (sensor).

Using the LightCycler 1.5 System (Roche Applied Science, Indianapolis, IN), real-time PCR was performed in capillaries with a reaction volume of 20 μl containing 200 ng of DNA, 0.5 μM sense (5'-GCC AGA AGC AAG ACT GAT AC-3') and antisense (5'-CCT GAA AGG AAA CCC ACA TTT A-3') primers, 0.2 μM anchor (5'-GGC CTC CTG GCC TCC TGT TTT CCT GAT CAC ACC AC-3') and sensor (5'-CAC TGC ACG AGC ATT TTC-3') hybridization probes, 1X reaction buffer (LightCycler FastStart DNA Master HybProbe, Roche Applied Science) and 2 mM $MgCl_2$. The sensor hybridization probe was specific for the G allele. PCR conditions were as follows: one cycle at 95˚C for 10 min, 45 cycles at 95˚C for 10 sec, 61˚C for 10 sec and 72˚C for 10 sec. After PCR, derivative melting curves [(-dF/dT) vs T] were generated by slowly heating the amplicon/probe heteroduplex and measuring the dramatic changes in fluorescence.

In order to genotype rs1862214, we also performed direct sequencing using 93 out of the 165 samples to confirm the reliability of the PCR-based convenient assay.

Table I. Characteristics of lung cancer patients and healthy controls.

Characteristic	Lung cancer patients (n=165)	Healthy controls (n=180)	P-value
Mean age (years ± SD)	66.6±10.8	68.1±11.4	0.212
Sex			
Male	108	115	0.761
Female	57	65	
Smoking status			
Non-smoker	58	63	0.977
Smoker	107	117	

SD, standard deviation.

Statistical analysis. All statistical analyses were performed using SPSS 12.0 (SPSS Inc., Chicago, IL). The odds ratio (OR) and 95% confidence interval (CI) were adjusted for age, sex and smoking status using an unconditional logistic regression model. Accordance with Hardy-Weinberg equilibrium was determined for lung cancer patients and healthy controls using the χ^2 test. P-values <0.05 were considered to be significant.

Results

Detection of rs1862214 SNPs by real-time PCR with melting curve analysis. We investigated the distribution of the genotype of rs1862214 in the *PDCD5* locus of Japanese lung cancer patients and healthy controls. Initially, 93 cases were tested by both PCR-based convenient assay and direct sequencing. The results of the assay were consistent with those of the direct sequencing analysis (Fig. 1). Consequently, we used the assay alone for the remaining cases, as it enabled us to rapidly determine the SNP.

Impact of the rs1862214 SNP in the PDCD5 locus on lung cancer risk. In 165 primary Japanese lung cancers, there were 25, 72 and 68 cases of CC, CG and GG genotypes, respectively (Table II). When limited to patients with a history of smoking, 17 (15.9%) had a CC, 47 (43.9%) had a CG and 43 (40.2%) had a GG genotype. In 180 healthy volunteers, the incidence of the CC, CG and GG genotypes was 31, 81 and 68, respectively. When limited to volunteers with a history of smoking, 24 (20.5%) had a CC, 52 (44.4%) had a CG and 41 (35.0%) had a GG genotype. None of these numbers deviated from those expected according to Hardy-Weinberg equilibrium (P=0.870 and 0.832 in total patients and total controls; P=0.878 and 0.764 in smoking patients and smoking controls, respectively). There was no significant difference between the frequency of the G genotype in lung cancer patients and in healthy controls. In addition, in the Japanese population, there was no significant difference between the frequency of the G genotype in lung cancer patients and in controls with a history of smoking. This is inconsistent with the frequencies previously

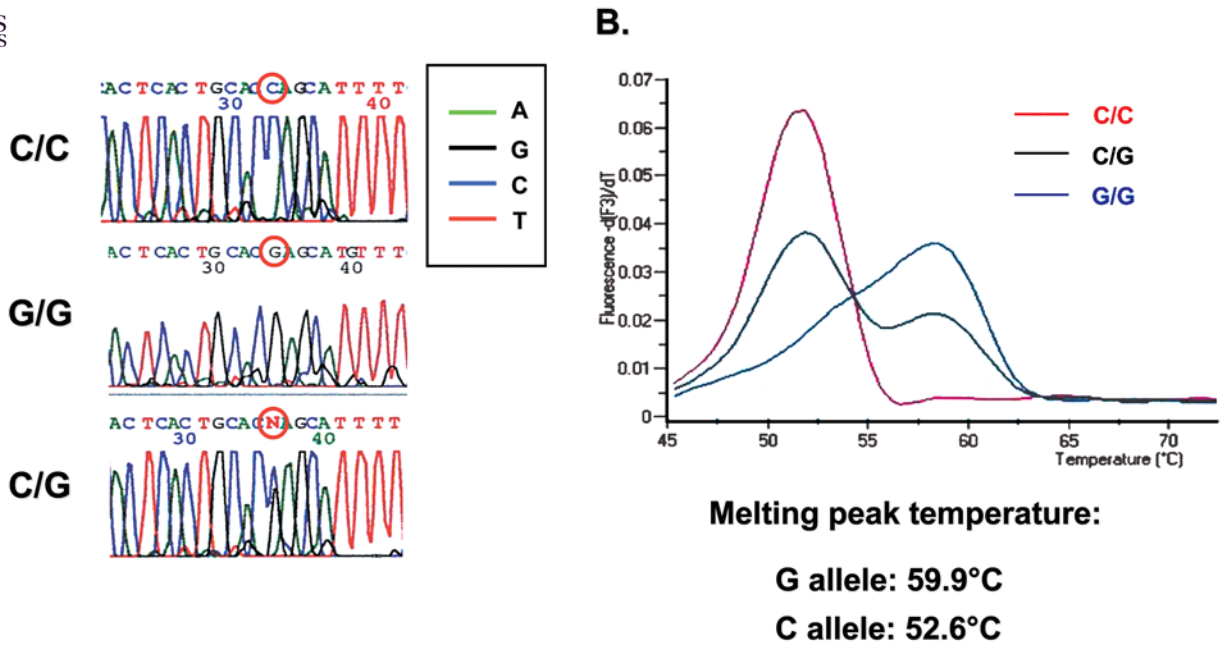

Figure 1. Representative figures of direct sequencing and melting curve analysis for genotyping at rs1862214 in the *PDCD5* locus. (A) Data from direct sequencing show three different genotypes of rs1862214 in the *PDCD5* locus. The top row shows the homozygous genotype of C (C/C), the middle row the homozygous genotype of G (G/G), and the bottom row the heterozygous genotype of rs1862214 (C/G). (B) Melting curve analysis distinguishes three different genotypes of rs1862214. The homozygous genotype of C generates a melting curve with its peak at 52.6˚C. The homozygous genotype of G generates its peak at 59.9˚C. The heterozygous genotype generates double peaks at 52.6 and 59.9˚C.

Table II. Genotypes of rs1862214 in the *PDCD5* locus of Japanese lung cancer patients.

	Controls[a]	Patients[a]	Odds ratio[b]	95% CI	P-value
Total cases					
C/C	31	25	1.00		
C/G	81	72	1.10	0.58-2.0	0.797
G/G	68	68	1.20	0.66-2.3	0.509
C/G or G/G	149	140	1.20	0.65-2.1	0.627
Smokers					
C/C	24	17	1.00		
C/G	52	47	1.30	0.62-2.7	0.502
G/G	41	43	1.50	0.70-3.1	0.310
C/G or G/G	93	90	1.40	0.69-2.7	0.366
Non-smokers					
C/C	7	8	1.00		
C/G	29	25	0.67	0.21-2.2	0.497
G/G	27	25	0.76	0.24-2.4	0.644
C/G or G/G	56	50	0.71	0.24-2.1	0.547

[a]The observed genotype distributions of patients and controls were in agreement with the Hardy-Weinberg equilibrium. [b]Odds ratios were adjusted for age, sex and smoking status.

reported by Spinola *et al* (15) in a European population. Regarding ethnic differences in the genotype, in the Japanese population the G haplotype was dominant in both patients and in healthy volunteers. According to the report by Spinola *et al*, (15) this is rare in European patients and points to an ethnic difference between the Japanese and European populations.

Prognosis for lung cancer patients stratified by the rs1862214 SNP in the PDCD5 locus. We examined the impact of the rs1862214 SNP in the *PDCD5* locus on the prognosis for Japanese lung cancer patients. There was no significant difference between the prognosis for the CC and G-contained variants (GG or CG). Since Spinola *et al* had found that the

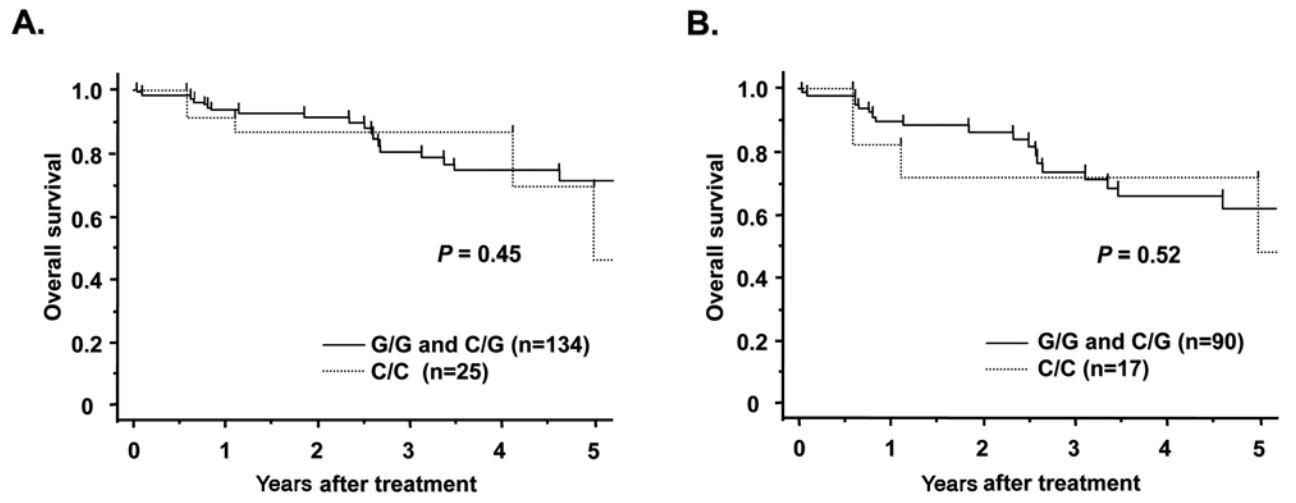

Figure 2. Kaplan-Meier curves showing overall survival according to the genotype of *PDCD5*. (A) Kaplan-Meier curves for all cases. (B) Kaplan-Meier curves for the smokers. No significant differences in overall survival were observed in the total number of patients (A) or in patients with a history of smoking (B) based on the genotype of rs1862214.

G-contained genotype had a significant prognostic impact on lung cancer patients with a history of smoking, we performed a similar analysis for our subgroups classified as having a history of smoking. However, even when restricted to smokers, no significant difference was observed between the prognosis for the two genotypes (Fig. 2).

Discussion

We developed a hybridization probe-based real-time PCR assay with melting curve analysis for genotyping rs1862214 in the *PDCD5* locus, the reliability of which was confirmed by direct sequencing. This convenient assay is less time-consuming and more cost-effective than direct sequencing, and is useful for the analysis of SNPs. These benefits would be meaningful were SNP genotyping to be applied for screening in the clinical field. The genotype of other significant genes can be determined using this real-time PCR-based assay, and a large scale of samples can be dealt with.

The biological function of *PDCD5* is the regulation of apoptosis. PDCD5 expression levels are significantly increased in cells undergoing apoptosis, and its protein translocation from the cytoplasm to the nucleus of apoptotic cells precedes the externalization of phosphatidylserine and the fragmentation of chromosome DNA (13). Spinola *et al* investigated whether the rs1862214 SNP had a functional role in *PDCD5* mRNA expression in normal and tumor lung tissue (15). However, in healthy lung parenchyma, PDCD5 expression was similar in CC genotype and G-allele carriers. The functional difference of PDCD5 in both genotypes is therefore unclear.

In the present study, we investigated an SNP at rs1862214 in the *PDCD5* locus of a Japanese population. As polymorphisms, including SNPs, generally display ethnic variations, our aim was to ascertain whether *PDCD5* can be used as a molecular marker for the prediction of lung cancer risk and prognosis in a Japanese as well as in a European population (16). We found a different allelic distribution at rs1862214 compared to that found by Spinola *et al*. In the Japanese population, haplotype G was more frequent than C, while Spinola *et al* had reported that the frequency of the G allele was 22% in both a German and an Italian population. These results suggest that there is a significant difference between SNP distribution at rs1862214 in European and Japanese controls and cancer patients (P<0.0001). Additionally, there was no significant association between cancer risk and a specific genotype of rs1862214 among the total population or among the smokers alone. Moreoever, there was no difference in prognosis according to genotype among the cases with a history of smoking, which again differed from the results taken from the European population with a history of smoking. Taken together, these findings suggest that the impact of an SNP in the *PDCD5* locus on lung cancer may vary in Japanese and European populations, and that this SNP may not be useful for predicting the risk of lung cancer in Japanese patients.

One limitation of the present study is that the study population may not have been large enough to be conclusive. The degree of smoking can also be an important factor and should ideally be considered when estimating risk and survival. Thus, further study is necessary to elucidate the impact of the SNP at rs1862214 in a Japanese population on the risk of lung cancer and on clinical outcome.

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