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Allelic Frequency of p53 Gene Codon 72 Polymorphism in Urologic Cancers

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Alterations in the p53 tumor suppressor gene appear to be important in the development of many human tumors. The wild-type p53 gene has a polymorphism at codon 72 that presents the arginine (CGC) or proline (CCC) genotype, which recently has been reported to be associated with genetically determined susceptibility to smoking-related lung cancers. To determine whether this p53 genotype influences individual risk of urologic cancer and/or its progression, we used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis to assay the allelic frequencies of this polymorphism in 85 renal cell carcinoma patients, 151 urothelial cancer patients, 33 testicular cancer patients, 28 prostatic cancer patients and 56 patients without neoplastic disease. The allelic distributions of the three genotypes (Arg/Arg, Arg/Pro, Pro/Pro) in patients with renal cell carcinoma (29.4%, 55.3%, 15.3%), urothelial cancers (45.7%, 39.7%, 14.6%), testicular cancer (45.4%, 48.5%, 6.1%) or prostate cancer (42.9%, 50.0%, 7.1%) did not differ significantly from those in the normal controls. However, Pro/Pro genotype in renal cell carcinoma and urothelial cancer (smoking-related cancers) was more frequent than that in prostate cancer and testicular cancer (smoking-unrelated cancers) with borderline significance ($P=0.0881$). There was no particular correlation between frequency of the three genotypes and grade or stage of each type of tumor. The association of genetic predisposition to urologic cancers with p53 gene codon 72 polymorphism is not so clear as the previous study of Japanese lung cancer patients, but this polymorphism may play some role in urothelial cancers and renal cell carcinoma, in which smoking is an epidemiological risk factor.

Key words: p53 gene — Codon 72 polymorphism — Allelic frequency — Urologic cancer

The p53 gene is a tumor suppressor gene located on chromosome 17p13.[1,2] Recent studies of the function of the wild-type p53 gene showed that its antiproliferative effect is caused by stimulation of a 21 kilodalton protein ($p21^{CIP1/WAF1}$) that inhibits cyclin-dependent kinase activities and thereby cell division.[3,4] This negative cell cycle controller effect may explain why the wild-type p53 gene can suppress the transformation of cells by activated oncogenes, thereby inhibiting the growth of malignant cells *in vitro* and suppressing the tumorigenic phenotype *in vivo*.[5,6] The accumulated clinical evidence is also consistent with this important function because in the development of many human cancers, the wild-type p53 allele is frequently lost, whereas the mutant allele is retained, providing a growth advantage for malignant cells.[7–9] Indeed, the alterations in the p53 gene are among the most common changes yet seen in human malignancies.[3,6,9]

p53 gene alteration in the evolution of cancers can be detected by using intragenic polymorphic probes.[10] Only a few polymorphisms at the wild-type p53 gene locus have been described so far.[1,11–18] Of these, codon 72 polymorphism on the 4th exon of the p53 gene, which results in two variant proteins with a single base-pair change, arginine (CGC) or proline (CCC), has been frequently studied for the detection of allele loss in tumors.[19–26]

Because p53 is an extremely important cellular regulatory molecule, it is necessary to understand the functional and epidemiological significance of its polymorphism. The half-lives of the two polymorphic variants of wild-type p53 are equivalent in normal phytohemagglutinin-stimulated lymphocytes, but the proline variant is twice as stable as the arginine variant in Daudi cells.[23] Biological function studies, however, have shown that there is no difference in the biological activities of the variants, on the basis of results of post-transcriptional selection against accumulation in 3T3-A31 cells, stabilization by SV-40 large T, and the variants' abilities to reduce the SV-40-mediated anchorage-independent growth of 3T3-A31 cells.[27] Although no obvious biological differences between the two variants has been found in *in vitro* assays, the association of this polymorphism with genetically determined susceptibility to smoking-induced lung cancer has been reported by Kawajiri *et al.*[25] They found a 1.7-fold excess of the Pro/Pro genotype in Japanese patients with this type of lung cancer. This suggests that these p53 genotypes may be linked to a predisposition to certain subsets of malignancies.

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We have analyzed the frequency of each polymorphic allele of p53 at codon 72 in 85 patients with renal cell carcinoma, 151 with urothelial cancer, 33 with testicular cancer, 28 with prostate cancer, and 56 non-cancerous control patients to determine whether there is a genetic influence for urologic cancers. We tested for a correlation between each polymorphic allele and the grade and stage of the disease in the study population to investigate the role of these genotypes in urologic cancer progression. Moreover, as cigarette smoking is an important etiologic factor in the development of renal cell carcinoma[28] and urothelial cancers,[29] we compared the allelic frequency in these smoking-related urologic cancers with those in smoking-unrelated urologic cancers (testicular cancer and prostate cancer). The habitual smoking status in urothelial cancer patients was further analyzed to check the relationship between smoking and p53 allelic distribution frequency in this group of patients.

MATERIALS AND METHODS

Case selection and samples All the samples were collected at Kyoto University Hospital or associated community hospitals. All the patients were Japanese except for one prostate cancer patient who was South American.

The source of the high-molecular-weight DNA used to determine the p53 genotypes was peripheral blood or normal tissue. Ten milliliters of peripheral blood was collected from each patient with urologic cancer: 22 renal cell carcinomas, 81 urothelial cancers, 33 testicular cancers and 28 prostate cancers, as well as from each of the 56 non-cancerous controls. In addition, paired samples of tumor tissue and normal counterpart tissue were obtained surgically from 63 renal cell carcinoma patients and 70 urothelial cancer patients. These tissues were immediately snap-frozen in liquid nitrogen and kept at $-80^{\circ}C$ until DNA extraction.

Consequently, 151 patients with urothelial tumor (115 males and 36 females, mean age of 70.0 ± 10.0 years), 85 patients with renal cell carcinoma (62 males and 23 females, mean age of 59.5 ± 11.8 years), 28 patients with prostatic cancer (mean age of 66.9 ± 7.5 years), 33 patients with testicular cancer (mean age of 47.3 ± 19.5 years), and 56 non-cancerous controls (mean age of 35.1 ± 16.0 years), were analyzed for polymorphic pattern of p53 codon 72.

Histopathology, tumor grades and stages The standard histopathological criteria were used to classify the tumor grades and stages for each type of cancer. Renal cell carcinoma was classified according to the nuclear grading system of Fuhrman *et al.*[30] and Robson's modified staging system.[31] Urothelial cancer classification was based on the WHO[32] and TNM[33] systems. Gleason's grading system[34] and the modified stages of the Whitmore/Jewett system[35, 36] were used for prostate cancer classification. For easier separation of testis cancers, pure seminoma and nonseminoma were classified histologically. The stages of the testis cancers followed the TNM classification advocated by the UICC.[33]

PCR-RFLP analysis After proteinase K digestion of tissue samples and peripheral blood leukocytes, high-molecular-weight genomic DNA was extracted by the phenol/chloroform method.[37] Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of the codon 72 polymorphism of the p53 gene was done as described elsewhere.[1] Briefly, a 199

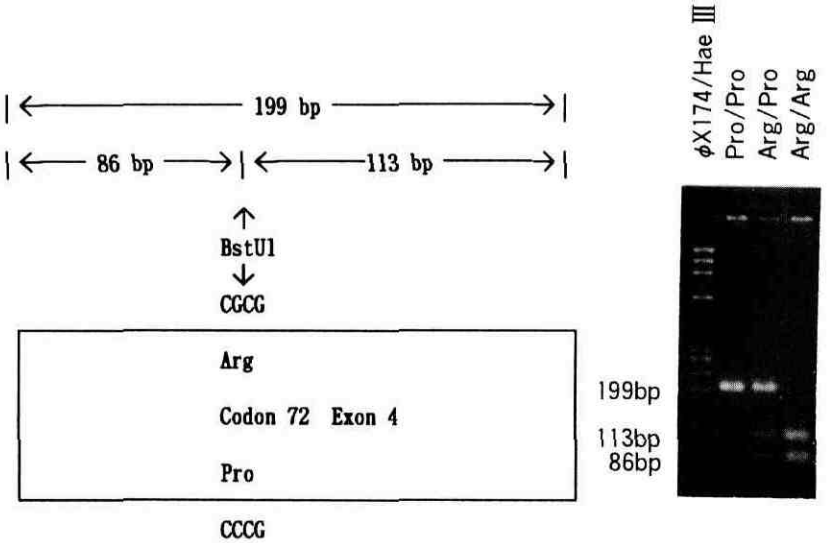

Fig. 1. PCR-RFLP analysis of the p53 gene. The PCR product from the proline allele, which is not cleaved by *Bst*UI at codon 72, has a single band with a fragment length of 199 bp. The arginine allele, which has the CG ↓ CG site and is cleaved by *Bst*UI, yields two small fragments (113 bp and 86 bp). The three representative analysis patterns (Arg/Arg, Arg/Pro and Pro/Pro) are shown.

bp fragment carrying codon 72 of the p53 gene was amplified by 35 PCR cycles with the primers (sense oligo: 5′-TTGCCGTCCCAAGCAATGGATGA-3′; antisense oligo: 5′-TCTGGGAAGGGACAGAAGATGAC-3′). Each PCR reaction mixture (50 μl) contained 50 pmol of each primer, 1.0 m*M* $MgCl_2$, 200 m*M* each dNTP, 2 U of *Taq* polymerase and 150–300 ng of genomic DNA. After confirmation of an amplified fragment of the expected size on an agarose gel, the PCR product was digested with 10 U of restriction enzyme *Bst*UI (Biolabs, New England, ME) at 60°C for 16 h. DNA fragments that had been resolved by electrophoresis through a 4% NuSieve agarose gel (FMC Bioproduct, Rockland, ME) were stained with ethidium bromide. Representative gel electrophoresis of the three genotypes, Arg/Arg, Arg/Pro and Pro/Pro, is shown in Fig. 1.

The chi-square test was used for the statistical analysis. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Distributions of the three genotypes (Arg/Arg, Arg/Pro, Pro/Pro) of the p53 gene in patients with various urologic cancers and in non-cancerous control patients are shown in Table I. The genotypic frequency (46.4% for Arg/Arg, 42.9% for Arg/Pro, 10.7% for Pro/Pro) of the randomly selected, non-cancerous, hospitalized controls fits the Hardy-Weinberg equilibrium and the reported frequencies in Caucasian and Japanese populations.[1, 15, 22, 25] We therefore formed a pooled control group using our control population and the control group reported previously for the normal Japanese population (Kawajiri *et al.*[25]) after statistical confirmation of no significant difference ($\chi^2=0.507$, df=2, $P=0.776$). This step increased the number of control cases (both normal and non-cancerous in the hospitalized Japanese population) for better comparison with the numbers of cancer patient groups. Using this pooled control as the basis for comparison, we analyzed the statistical significance of the genotypic distribution frequency in each cancer group. No significant difference in the distributions of the three genotypes was found for any urologic cancer group ($P>0.05$, in each cancer group) (Table I). However, the frequency of Pro/Pro genotype in urothelial cancers and renal cell carcinoma, in which smoking is considered as an epidemiological risk factor, was higher than that in either normal control or testicular cancer and prostate cancer groups. We compared the frequency difference in each genotype between urothelial cancers plus renal cell carcinoma (smoking-related group) and testicular cancer plus prostate cancer (smoking-unrelated group) (Table II). Pro/Pro genotype versus Arg/Arg plus Arg/Pro genotype in the smoking-related group was higher than that in the smoking unrelated group with borderline significance ($\chi^2=2.908$, df=1, $P=0.0881$).

We next examined the genotypic frequency difference in each cancer group with respect to histologic grading and clinical pathologic staging to test the possible influence of genotype on a particular cancer progression risk. The relationships of grade and stage to the p53 allelotype in patients with renal cell carcinoma and urothelial cancer are shown in Tables III and IV. We found no cancer progression risk susceptibility for the Pro/Pro genotype in either of our types of cancer patient with respect to grade and stage ($P>0.05$, in both groups). Results were similar (data not shown) for the testicular and prostatic cancer patients. Table V shows the relationship between habitual smoking status and the p53 alleles in 131 urothelial cancer patients. There was no statistically significant difference in allelic distribution frequencies among smokers, but a trend of correlation

Table I. Distributions of Allelic Frequency in 3 Genotypes

Study group	Arg/Arg	Arg/Pro	Pro/Pro	Total
Normal controls				
Present series	26 (46.4)	24 (42.9)	6 (10.7)	56 (100)
Kawajiri's series	144 (41.5)	165 (47.6)	38 (10.9)	347 (100)
Pooled control	170 (42.2)	189 (46.9)	44 (10.9)	403 (100)
Renal cell carcinoma[a)]	25 (29.4)	47 (55.3)	13 (15.3)	85 (100)
Urothelial cancer[b)]	69 (45.7)	60 (39.7)	22 (14.6)	151 (100)
Testicular cancer[c)]	15 (45.4)	16 (48.5)	2 (6.1)	33 (100)
Prostate cancer[d)]	12 (42.9)	14 (50.0)	2 (7.1)	28 (100)

The pooled control, the sum of the present and Kawajiri's series, is used for comparison with each cancer group. Numbers in parentheses are the percents of distribution in each group.
a) $\chi^2=5.039$, df=2, $P=0.080$.
b) $\chi^2=2.797$, df=2, $P=0.247$.
c) $\chi^2=0.775$, df=2, $P=0.679$.
d) $\chi^2=0.448$, df=2, $P=0.799$.

Table II. Distribution of p53 Alleles in Smoking-related and Smoking-unrelated Urologic Cancers

Study group	Arg/Arg	Arg/Pro	Pro/Pro	Total
Smoking-related[a] urologic cancers	94 (39.8)	107 (45.3)	35 (14.8)	236 (100)
Smoking-unrelated[b] urologic cancers	27 (44.3)	30 (49.2)	4 (6.6)	61 (100)

[c]

Numbers in parentheses are the percents of distribution in each group.
a) Renal cell carcinoma plus urothelial cancer.
b) Testicular cancer plus prostate cancer.
c) A 2×2 table (Pro/Pro vs. Arg/Arg plus Arg/Pro) was used to calculate the *P* value. $\chi^2=2.908$, df=1, $P=0.0881$.

Table III. Relationship of Cancer Grade and Stage to p53 Allelotypes in Renal Cell Carcinoma Patients

	Arg/Arg	Arg/Pro	Pro/Pro
Grade I[a]	10 (29.4)	21 (61.8)	3 (8.8)
Grade II+III[b]	13 (28.9)	24 (53.3)	8 (17.8)
Stage A+B[c]	14 (29.2)	27 (56.3)	7 (14.6)
Stage C+D[d]	8 (28.6)	15 (53.6)	5 (17.8)

The pooled control is used for comparison with each group. Numbers in parentheses are the percents of distribution in each group.
a) $\chi^2=2.812$, df=2, $P=0.245$.
b) $\chi^2=3.745$, df=2, $P=0.154$.
c) $\chi^2=3.074$, df=2, $P=0.215$.
d) $\chi^2=2.530$, df=2, $P=0.282$.

Table IV. Relationship of the Cancer Grade and Stage to p53 Allelotypes in Urothelial Cancer Patients

	Arg/Arg	Arg/Pro	Pro/Pro
Grade I+II[a]	38 (45.2)	33 (39.3)	13 (15.5)
Grade III+IV[b]	26 (49.1)	21 (39.6)	6 (11.3)
Stage (≤pT1)[c]	36 (48.0)	29 (38.7)	10 (13.3)
Stage (≥pT2)[d]	28 (45.9)	25 (41.0)	8 (13.1)

The pooled control is used for comparison with each group. Numbers in parentheses are the percents of distribution in each group.
a) $\chi^2=2.269$, df=2, $P=0.322$.
b) $\chi^2=1.060$, df=2, $P=0.589$.
c) $\chi^2=1.762$, df=2, $P=0.414$.
d) $\chi^2=0.802$, df=2, $P=0.670$.

was found among non-smokers ($P=0.079$). Further analysis of non-smokers with urothelial cancers disclosed that the correlation resulted from the low frequency of Arg/Pro genotype (95% confidence interval (CI)= 19.9–44.3) and high frequency of Pro/Pro genotype (95% CI=7.9–27.9) in this group.

Table V. Relationship between Habitual Smoking Status and p53 Allelotypes in Urothelial Cancer Patients

Group	Arg/Arg	Arg/Pro	Pro/Pro
Smokers[a]	31 (41.3)	36 (48.0)	8 (10.7)
Non-smokers[b]	28 (50.0)	18 (32.1)	10 (17.9)

The pooled control is used for comparison with each group. Numbers in parentheses are the percents of distribution in each group.
a) $\chi^2=0.031$, df=2, $P=0.985$.
b) $\chi^2=5.082$, df=2, $P=0.079$.

A simultaneous study of polymorphism in both normal and tumor tissues was done for 30 renal cell carcinoma and 70 urothelial cancer patients. Genomic DNAs from 16 patients with renal cell carcinoma and 26 patients with urothelial cancer showed heterozygosity at codon 72 of the p53 gene. Of the heterozygous patients, 4 with renal cell carcinoma (25%) and 10 with urothelial cancer (38.5%) showed loss of heterozygosity (LOH) of the p53 gene. These two polymorphic alleles of the p53 gene were evenly involved in allelic loss in urothelial cancer patients, whereas in renal cell carcinoma patients only the arginine allele was lost.

DISCUSSION

The wild-type p53 gene is generally accepted to act as a tumor suppressor gene, negatively regulating the cell cycle, whereas mutant p53 contributes to tumorigenesis or tumor progression.[2]

The genetic predisposition to malignancy in the germline p53 mutation that predominates in cancer-prone individuals with the Li-Fraumeni syndrome, in which affected relatives develop a diverse set of malignancies, is well documented.[9] With regard to urologic cancer development, alterations in the p53 gene have been found mainly in patients with urothelial cancer,[38,39] followed by renal cell carcinoma[40] and prostate cancer,[41] where

involvement is relatively infrequent. Alterations in the p53 gene are rare in testicular cancer.[42]

Although the presence of the p53 gene mutation has been shown in urologic cancers, whether there is genetically determined susceptibility to any urologic cancer with respect to codon 72 genotypic polymorphism of the p53 gene is unclear. In lung cancer, the Pro/Pro genotype has been shown to be related to genetically determined susceptibility to adenocarcinoma in Weston's series[22] and smoking-related lung cancer in Kawajiri's series.[25] Ours is the first study to investigate this point in connection with urologic cancers. The data presented here show that with regard to the allelic distribution frequency of the p53 gene, as compared with the control data, there is no significant association of this polymorphism with the development of renal cell carcinomas, urothelial cancers, testicular cancers or prostate cancers. However, if we take the smoking-related urologic cancers (renal cell carcinoma and urothelial cancers) as one group and compare it with normal controls or the smoking-unrelated urologic cancers (testicular cancer and prostate cancer), a relative higher proportion of Pro/Pro genotype in this group of patients is noted. Our results suggest that the codon 72 germ-line polymorphism of the p53 gene, especially the Pro/Pro genotype, may be worthy of further study to determine the role of this polymorphism in the genetically determined susceptibility to renal cell carcinoma and to urothelial cancers.

Further analysis of the relationship between the allelic distribution frequencies and the grade or stage of each type of cancer did not show a statistically significant difference. Based upon our data, although the case numbers are limited, the association of this germ-line polymorphism with the disease progression status of these urologic cancers seems weak. Thus, to use this polymorphism in the estimation of disease progression may be of little value.

Tobacco smoke is an important, causally related risk factor for urothelial cancers.[29] Previous studies showed that it did not significantly influence the incidence of p53 gene mutation but that there was a specific mutation pattern.[39, 43] The data presented here show that in patients with urothelial cancers the actual smoking status does not have a significant impact on genetically determined susceptibility to this type of cancer. In contrast, the non-smokers who develop urothelial cancers possibly have a relatively lower frequency of Arg/Pro genotype and higher frequency of Pro/Pro genotype. These observations suggest that other factors may be important in the determination of urothelial cancer risk in the non-smokers with these genotypes. Thus, on the basis of these findings it seems that although the smoking effect contributes to the risk of urothelial cancers, its role in relation to the genetically determined susceptibility to these cancers needs further study.

The involvement of germ-line polymorphism of p53 gene in human cancers has only been reported by Weston *et al.*[22] and Kawajiri *et al.*[25] in Caucasians, African-Americans and Japanese populations. Both reports showed a variable degree of correlation between Pro/Pro genotype and lung cancer susceptibility, although the related histological types were different. A racial difference was also found to be significant between Caucasians and African-Americans.[21] Taking these results and our data together, it seems that involvement of the Pro/Pro genotype in cancers is tissue- or organ-specific and possibly also race-specific. Why there is organ-specific involvement of this polymorphism and linkage of a specific genotype (Pro/Pro) with the risk of particular cancers is not known. The hypothesis suggested by Kawajiri *et al.*[25] is that different carcinogenesis processes are involved in the genesis of various tumor types because of the presence of functionally different p53 alleles (Pro- or Arg-type). The limited information obtained by Zhang *et al.*[23] and Moreau and Matlashewski[27] regarding the function of the polymorphism variants indicates that there is no remarkable difference between them. The only difference in these variants reported so far is the two-fold greater half-life of the Pro variant in Daudi cells.[23] This phenomenon, however, was not seen in the other three types of cells analyzed in their study. To date, including the results of the study reported here, only limited data have been obtained concerning this germ-line polymorphism and clinical cancer sample analysis. To clarify the complex mechanisms involved, more clinical evidence is needed to confirm that there is genetically determined susceptibility to cancer development and progression. More knowledge about the wild-type p53 gene functions as well as the actual role of polymorphism in this important gene is also needed.

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