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# p53 Gene Mutations in Human Prostate Cancers in Japan: Different Mutation Spectra between Japan and Western Countries

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**The involvement of p53 mutations in prostate cancers in Japan was investigated. To evaluate any possible clinicopathological significance, p53 mutations in 40 samples from 36 Japanese prostate cancers of different stages (five cases of latent tumors, three of stage A cancers, 10 of stage B, five of stage C and 13 of stage D), including four lymph node metastases of stage D cases, were examined by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis and sequencing. Mutations were detected in five of 40 samples (12.5%); four were in primary cancers and the other in a lymph node metastasis from one of them. All mutation-positive cases were in stage D, and the mutation frequency in stage D cases was 31%. This result indicates that p53 mutations may play a role in the progression of a subgroup of prostate cancers in Japanese, as observed for Americans and Europeans. However, a difference was noted between Japanese and Americans in the p53 mutational spectrum (at CpG site), presumably arising from variation in the underlying etiologic factors.**

Key words: p53 mutation — Prostate cancer — PCR-SSCP

Prostate cancer is very common in aged men and is the second most frequent cause of cancer death among American males.[1,2] There is, however, wide international variation in the incidence and mortality rates of patients with prostate cancer. Mortality is highest in Sweden, Norway and the Caribbean, while it is very low in oriental countries.[3] African-Americans in Atlanta constitute a high risk group with an incidence of 91.3/100,000 in marked contrast to the Japanese low risk group in Osaka, with an incidence of 1.8/100,000.[4] In Japan, however, clinical diagnosis of prostate cancer has recently been increasing.[5] The available findings suggest that both genetic traits and environmental factors play important roles in carcinogenesis involving the prostate glands.[6,7]

Mutational profiles of oncogenes or tumor suppressor genes can give us clues to elucidating etiologic factors. For instance, a specific type of p53 mutation was found in skin exposed to UV[8] as well as in hepatocellular carcinomas from southern Africa and the Quindon province of China, where aflatoxin contamination has been implicated as the causative agent.[9] However, only limited information concerning genetic alterations relevant to prostate carcinogenesis has been generated so far. For the *ras* gene, low incidence (0–12.5%) of mutations were found in prostate cancers of Americans.[10–13] In contrast, a relatively high mutation rate (24%) was found in Japanese in Japan.[14]

With regard to p53 mutations, Isaacs *et al.* first found a positive prostate cancer cell line,[15] but there was no clear evidence to allow distinction of whether the mutation occurred *in vivo* or after transfer to *in vitro* culture. Immunohistochemical studies of American prostate cancers, however, have demonstrated the possible presence of p53 mutations in 6–17% of primary lesions.[16,17] Combination studies using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis and immunohistochemical analyses confirmed the frequency of p53 gene mutations in prostate cancer to be relatively low and suggested roles for such genetic changes in tumor progression in Caucasians and African-Americans.[18–21] Uchida *et al.* recently reported a low frequency of p53 mutations in prostate cancers of Japanese in Japan, examining mutations in exons 5–8 (9.5% of 21 primary prostate cancers) by PCR-SSCP analysis.[22] However, no informations has been available on the relationship between mutations and tumor progression in Japanese patients. Further, their mutation characteristics have also not been previously targeted.

The present examination of a total of 40 samples of primary and metastatic lesions, including latent tumors

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and clinical cancers at stages A–D, was therefore conducted with extensive PCR-SSCP analysis of all coding regions containing exons 1–11 for p53 mutations.

## MATERIALS AND METHODS

**Tissue samples** Samples of 40 prostate tumors were collected from 36 patients who visited Mie University Hospital, Chiba University Hospital and The Osaka Center for Adult Diseases between 1991 and 1993. The age of the patients ranged from 42 to 83 years old, with an average age of 69. Among the 40 samples, 36 samples were primary prostate tumors and four were lymph node metastases from four primary cancers included in the 36 samples. The primary prostate tumors were obtained by radical prostectomy (16 cases), radical cystoprostectomy of bladder tumors (2 cases), and autopsy (18 cases). The bladder tumors were invasive prostate cancers. The four lymph node lesions were from regional lymph node dissections.

Thirty-five samples were immediately frozen after resection and kept at −80°C until use, and five were formalin-fixed. Portions of the frozen samples were subjected to light microscopic review after hematoxylin-eosin staining by a pathologist experienced in the diagnosis of prostate cancer, and normal tissues were removed to obtain tissues containing more than 75% tumor cells for DNA extraction. The five formalin-fixed samples were all of latent cancers, taken from subjects with no clinical features of prostate cancer.

All samples were staged and graded according to the General Rules for Clinical and Pathological Studies on Prostatic Cancer using the Gleason system of classification.[23,24] Table I summarizes the clinical information for all prostate tumor samples.

**DNA extraction** From frozen tissues, genomic DNA was extracted by the standard method, with proteinase K digestion, serial phenol and chloroform extractions, and ethanol precipitation.[25]

From paraffin-embedded tissues, partially purified DNA was extracted as follows. First 5-$\mu$m thick sections on slide glasses were trimmed to remove non-tumorous portions as far as possible. Then they were stripped off, deparaffinized with xylene, and the DNA extracted with PCR buffer containing Nonidet P-40 and Tween 20 in a total volume of 90 $\mu$l.[26]

**PCR-SSCP analysis** Twelve sets of primers were prepared to amplify DNA fragments covering exons 1 through 11 of the p53 gene, mostly based on the reported sequences (Table II).[27–30] The primers for exons 4-1 and 4-2 were newly designed to obtain shorter PCR products. Primers were end-labeled with [$\gamma$-$^{32}$P]ATP using T4 polynucleotide kinase (Takara, Kyoto). Samples of 50 ng of genomic DNA were amplified by PCR in a 5 $\mu$l reaction mixture, which consisted of 80 n*M* end-labeled 5′ and 3′ primers, 100 $\mu$*M* each of dNTP, 10 m*M* Tris-Cl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.001% gelatin, and 0.1 U/$\mu$l of *Taq* polymerase (Perkin Elmer Cetus, Norwalk, CT). The reaction conditions were 94°C (0.5 min), 55°C (0.5 min), and 72°C (1 min) for 35 cycles. The reaction was initiated with one 3-min incubation at

Table I. Clinicopathological Features of the Examined Prostate Cancer Patients

| Sample | Age | Site | Clinical diagnosis | Stage | Histological grade | Gleason score |
|---|---|---|---|---|---|---|
| 1 | 53 | Primary | None | L | W | 2 |
| 2 | 84 | Primary | None | L | M | 6 |
| 3 | 71 | Primary | None | L | M | 6 |
| 4 | 75 | Primary | None | L | P | 8 |
| 5 | 66 | Primary | None | L | P | 8 |
| 6 | 76 | Primary | BPH | A | W | 4 |
| 7 | 78 | Primary | BPH | A | P | 8 |
| 8 | 45 | Primary | PC | A | P | 9 |
| 9 | 58 | Primary | PC | B | M | 6 |
| 10 | 70 | Primary | PC | B | M | 5 |
| 11 | 64 | Primary | PC | B | M | 5 |
| 12 | 65 | Primary | PC | B | M | 6 |
| 13 | 57 | Primary | PC | B | M | 7 |
| 14 | 73 | Primary | PC | B | M | 6 |
| 15 | 66 | Primary | PC | B | M | 5 |
| 16 | 82 | Primary | BPH | B | M | 7 |
| 17 | 57 | Primary | PC | B | P | 8 |
| 18 | 72 | Primary | PC | B | P | 7 |
| 19 | 64 | Primary | PC | C | M | 5 |
| 20 | 73 | Primary | PCB | C | M | 6 |
| 21 | 73 | Primary | BPH | C | M | 6 |
| 22 | 61 | Primary | PC | C | M | 6 |
| 23 | 67 | Primary | PCB | C | P | 8 |
| 24 | 67 | Primary | PC | D | W | 5 |
| 25 | 77 | Primary | PC | D | M | 7 |
| 26 | 77 | LN (#25) | PC | D | M | 7 |
| 27 | 73 | Primary | PC | D | M | 7 |
| 28 | 65 | Primary | PC | D | M | 8 |
| 29 | 65 | LN (#28) | PC | D | P | 7 |
| 30 | 71 | Primary | PC | D | P | 7 |
| 31 | 75 | Primary | PC | D | P | 9 |
| 32 | 75 | Primary | PC | D | P | 9 |
| 33 | 42 | Primary | PC | D | P | 9 |
| 34 | 66 | Primary | PC | D | P | 9 |
| 35 | 80 | Primary | PC | D | P | 9 |
| 36 | 83 | Primary | PC | D | P | 9 |
| 37 | 83 | LN (#36) | PC | D | P | 9 |
| 38 | 83 | Primary | PC | D | P | 9 |
| 39 | 83 | LN (#38) | PC | D | P | 9 |
| 40 | 78 | Primary | PC | D | P | 10 |
| Average | 69 | | | | | 7.0 |

BPH, benign prostatic hyperplasia; PC, prostate cancer; PCB, prostate cancer invaded to bladder; L, latent cancer; LN, lymph node; W, well-differentiated; M, moderately differentiated; P, poorly differentiated.

94°C and ended with 7 min at 72°C. Five $\mu$l of PCR products was added to 45 $\mu$l of loading buffer (95% formamide, 20 m*M* EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol), heat-denatured, and a 1-$\mu$l portion was loaded per lane of 4.9% polyacrylamide gel with or without 5% glycerol. Electrophoresis was carried out at 40 W at 18°C with a water jacket. Gels were dried and exposed to XAR-5 (Kodak, Rochester, NY) at −80°C for 0.5–1 h.

**DNA sequencing** DNA was extracted from shifted bands obtained by PCR-SSCP analysis. Fragments were directly subcloned into the pCR vector using a TA cloning system kit (Invitrogen, San Diego, CA) and sequenced by the Sanger dideoxynucleotide method with a

Table II. Sequences of the Oligonucleotides Used as PCR and Sequencing Primers

| Exon | Direction | Sequence |
|---|---|---|
| 1 | F | 5′-GGAATTCGGATTCCTCCAAAATGATTT-3′ |
| | R | 5′-GGAATTCCAGTCAGGAGCTTACCCA-3′ |
| 2 | F | 5′-TGGATCCTCTTGCAGCAG-3′ |
| | R | 5′-TGGGTCTTCAGTGAACCA-3′ |
| 3 | F | 5′-TGGATCCTCTTGCAGCAG-3′ |
| | R | 5′-TGGGGTCTTCAGTGAACA-3′ |
| 4-1 | F | 5′-CCATCTACAGTCCCCCTTGC-3′ |
| | R | 5′-CTGCCCTGGTAGGTTTTCTG-3′ |
| 4-2 | F | 5′-CCCAGGTCCAGATGAAGCTC-3′ |
| | R | 5′-GGGCAACTGACCGTGCAAGT-3′ |
| 5 | F | 5′-TGTTCACTTGTGCCCTGACT-3′ |
| | R | 5′-AGCAATCAGTGAGGAATCAG-3′ |
| 6 | F | 5′-TGGTTGCCCAGGGTCCCCAG-3′ |
| | R | 5′-GGAGGGCCACTGACAACCA-3′ |
| 7 | F | 5′-CTTGCCACAGGTCTCCCCAA-3′ |
| | R | 5′-AGGGGTCAGCGGCAAGCAGA-3′ |
| 8 | F | 5′-TTGGGAGTAGATGGAGCCT-3′ |
| | R | 5′-AGTGTTAGACTGGAAACTTT-3′ |
| 9 | F | 5′-GTTATGCCTCAGATTGAT-3′ |
| | R | 5′-TGGAAACTTTCCACTTGAT-3′ |
| 10 | F | 5′-TGTTGCTGCAGATCCGTGGG-3′ |
| | R | 5′-GAGGTCACTCACCTGGAGTG-3′ |
| 11 | F | 5′-TCTCCTACAGCCACCTGAAG-3′ |
| | R | 5′-CTGACGCACACCTATTGCAA-3′ |

F, forward; R, reverse.

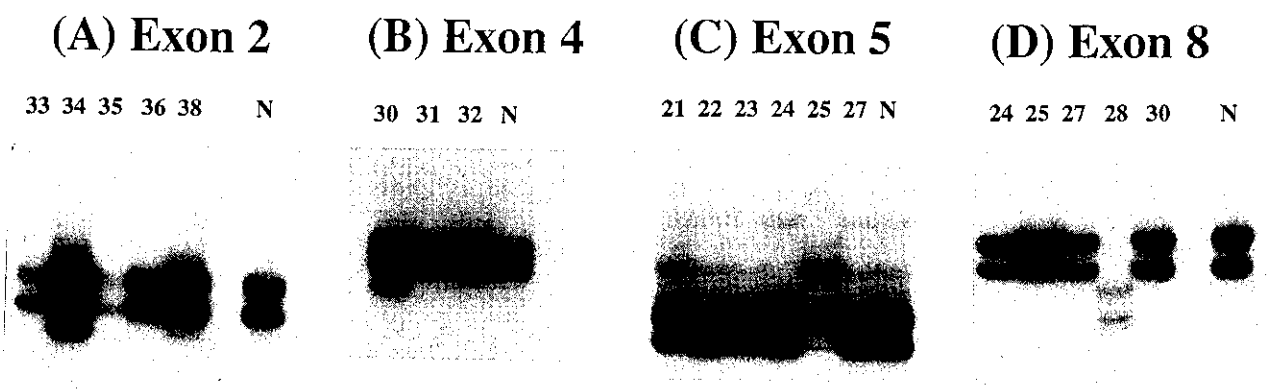


Fig. 1. PCR-SSCP analyses of p53 in prostate tumors. Sample numbers are shown above each panel. The samples were analyzed on 5% acrylamide gels with 5% glycerol. (A) Exon 2, Sample No. 34 has an abnormal band. (B) Exon 4, No. 30 has an abnormal band. (C) Exon 5, No. 25 has abnormal bands. (D) Exon 8, No. 28 has abnormal bands.

Sequenase Ver 2.0 kit (United States Biochemical, Cleveland, OH).

**Statistical analysis** Associations between p53 mutations and other factors were evaluated using the $\chi^2$ test. The criterion of significance was $P<0.05$.

## RESULTS

**PCR-SSCP analysis** The PCR-SSCP analysis of exons 1–11, which include the entire p53 gene coding region, was performed for all 40 samples. Mobility shifts were detected with four primary cancers and one lymph node metastasis of one of the four mobility-shift-positive primary cancers. Sample No. 34 showed a band shift in exon 2 (Fig. 1A), No. 30 in exon 4-1 (Fig. 1B), No. 25 and its lymph node metastasis, No. 26, in exon 5 (Fig. 1C), and No. 28 in exon 8 (Fig. 1D). Nos. 25 and 26 were demonstrated to have no bands corresponding to the wild-type allele at least for exon 5, and No. 28 lacked such bands for exon 8 (Fig. 1C and 1D), suggesting deletion of the wild-type allele.

**Sequencing** PCR-products of all of the five samples which showed band shifts in SSCP-analysis were subcloned and sequenced (Fig. 2). For each sample, sequences of 10 subclones were determined. Point mutations were detected in more than four clones of all samples analyzed, all mutations resulting in amino acid substitutions. No. 34 had a GAG-to-CAG transversion at codon 11 in exon 2. Nos. 25 and 26 both had a TGC-to-TTC transversion at codon 176 in exon 5. No. 30 had a CCA-to-TCA transition at codon 67 in exon 4. No. 28 had an AAT-to-AGT transition at codon 288 in exon 8.

**Clinicopathological correlations** The clinical significance of the observed p53 mutations was investigated by ex-

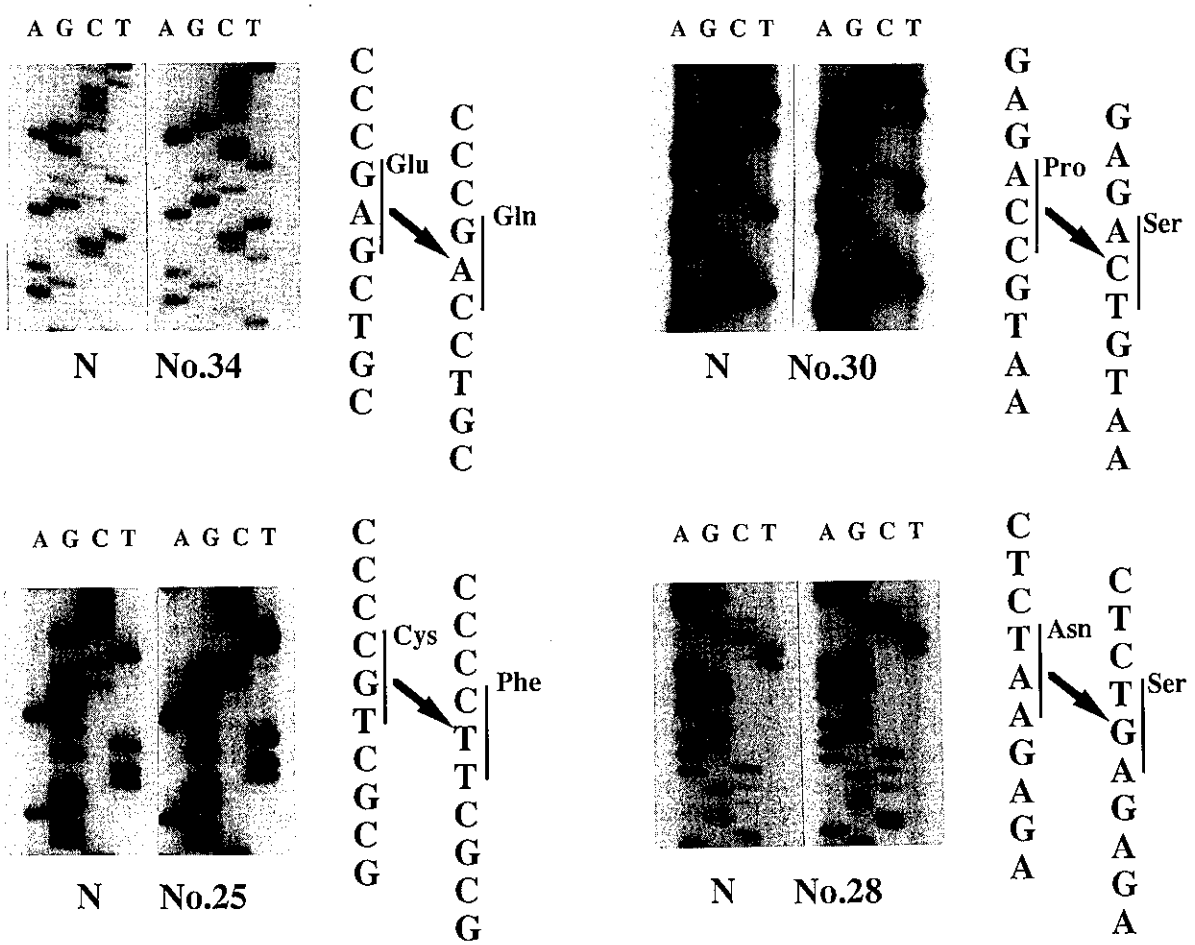


Fig. 2. Sequencing analyses. Sample numbers are indicated below each panel. N indicates findings for normal portions of tissue from the same patient. Nucleotide changes were detected in No. 34 at codon 11, in No. 30 at codon 67, in No. 25 at codon 176 and in No. 28 at codon 288.

Table III. Summary of p53 Mutations Found in Human Prostate Cancer Patients

| Sample | Age | Site | Stage | Gleason score | p53 gene mutation | | |
|---|---|---|---|---|---|---|---|
| | | | | | Exon-codon | Nucleotide change | Amino acid change |
| 25 | 77 | Primary | D | 7 | 5-176 | TGC to TTC | Cys to Phe |
| 26 | | LN (#25) | | | 5-176 | TGC to TTC | Cys to Phe |
| 28 | 65 | Primary | D | 8 | 8-288 | AAT to AGT | Asn to Ser |
| 30 | 71 | Primary | D | 7 | 4-67 | CCA to TCA | Pro to Ser |
| 34 | 66 | Primary | D | 9 | 2-11 | GAG to CAG | Glu to Gln |
| Average | 69.8 | | | 7.8 | | | |

amining correlations with disease stage or histological grade. Of 36 primary tumors examined, five were latent tumors, three were stage A cancers, 10 were stage B, five were stage C and 13 were stage D. All the mutations were in stage D primary tumors, and the incidence of p53 mutations in stage D lesions was 31%. Application of the chi-square test ($P<0.05$, $\chi^2=7.96$) gave statistical confirmation of a positive association between p53 gene mutations and stage D. The involved cancers were all moderately or poorly differentiated carcinomas. However, examination of three well differentiated and 17 moderately differentiated tumors revealed no significant association between histological grade and p53 mutation. Although p53 mutations were detected in samples with Gleason scores $\geq 7$, there was again no statistically significant association between Gleason score and p53 mutation, partly because of the small number of tumors with Gleason scores $<7$ (15 against 25).

Table IV. Comparison of the p53 Gene Mutation Spectrum of Japanese with That of Americans and Europeans

| Type of p53 gene mutation | Japanese[a] (%) | Americans and Europeans (%)[b] |
|---|---|---|
| Transition | | |
| GC to AT | 1 (16%) | 12 (60%) |
| (CpG) | 0 | 8 (40%) |
| AT to GC | 1 (16%) | 2 (10%) |
| Transversion | | |
| AT to CG | 1 (16%) | 2 (10%) |
| AT to TA | 0 | 1 (5%) |
| GC to CG | 1 (16%) | 0 |
| GC to TA | 2 (32%) | 2 (10%) |
| Others | 0 | 1[c] (5%) |
| Total | 6 | 20 |

*a*) Combined data from our study and Ref. 22.
*b*) Combined data from Refs. 18, 19, 20, 21 and 33.
*c*) One base pair deletion.

## DISCUSSION

The present examination of 36 primary prostate tumors at various stages and four lymph node metastases revealed p53 mutations in four primary cancers. The one of these four primary cancers which had metastasized also demonstrated the same mutation in its lymph node metastasis (Table III). Since PCR-SSCP analysis is highly sensitive,[31] false negative can be considered very few and all of the coding region of the p53 was covered. One of the four mutations detected in the primary cancers was in exon 2, one in exon 4 and one each in exons 5 and 8, where most of the mutations documented for various tumors have been found.[32] Since all the other reported studies of p53 mutations in human prostate cancers only examined exons 5–8,[18–22] it is possible that the actual p53 mutation frequencies were higher than those reported.

Correlations between p53 mutations and clinicopathological features, including histologic grade, stage and androgen sensitivity have been examined for prostate cancers in Americans and Europeans.[18–22, 33–35] p53 mutations have been found to be quite rare in early prostate cancers (4% or 74 stage 0–II tumors) versus advanced cancers (23% of 69 stage III–IV cancers).[20] Immunohistological analysis of p53, also demonstrated a higher positive rate (24% of 97 tumors) in high grade or stage D lesions than in the lower grade or stage A–C prostate cancers in Americans.[21] In the present study, we found that all primary tumors which were proven to have p53 mutations belonged to stage D, with an incidence of 31% (4 or 13 cases). The lack of involvement of p53 mutations in localized tumor (latent tumors, stage A and B cancers) was statistically significant. Though the situation for p53 mutations in stage C cancers could not be clarified because of the small sample number, these results suggest that p53 mutations might play a role in prostate tumor progression.

There is no essential difference in the p53 mutation frequency in advanced primary prostate cancers between Japanese (this study) and Americans,[20, 21] the values in both cases being relatively low when compared with data for colon, lung, bladder and breast cancers.[36–39]

However, analysis of mutation spectra, which often provides us with information about etiologic factors,

revealed clear differences for the p53 gene between our Japanese patients and earlier reported American/European prostate cancers (Table IV).[18-22, 33] In Japanese, four of six (67%) mutations were transversions and the remaining two (23%) were transitions. No mutations occurred at CpG dinucleotides. In contrast, in Americans and Europeans, 14 of the 20 (70%) were transitions and eight of these occurred at CpG dinucleotides, which might be caused by spontaneous deamination of 5-methylcytosine, due to endogenous factors, such as nitric oxide.[40] Further, transversions at G·C pairs were relatively low in Americans and Europeans as compared with Japanese (10% versus 48%). Though the size of the available data base for Japanese is small, the difference in p53 mutational spectra does suggest variation in etiologic factors between the two populations. The incidence of *ras* mutations has been reported to be higher in prostate cancers in Japanese than in Americans.[14, 41] This difference might also indicate different etiologic factors. The possibility of identifying environmental agents which might be playing a role in causing progression of prostate cancer in Japanese by more extensive studies of p53 gene mutation spectra deserves consideration.

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