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State of Adenomatous Polyposis Coli Gene and *ras* Oncogenes in Japanese Prostate Cancer

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Genetic alterations of *ras* oncogenes (K-, H- and N-*ras*) and adenomatous polyposis coli (APC) gene in tissues of prostate cancer from Japanese patients were examined using PCR-SSCP (polymerase chain reaction-single strand conformation polymorphism) analysis and direct sequencing. Tissues from 8 cases of untreated stage B prostate cancer surgically removed and from 10 cases of endocrine therapy-resistant metastatic disease obtained at autopsy were used in the present study. In four out of 18 cases (22%), *ras* point mutations were found, two in either codon 12 or 61 of K-*ras* and two in either 13 or 61 of H-*ras*. These point mutations were detected in one of the stage B cases (13%) and in three of the autopsy cases (30%). All these cases were poorly differentiated adenocarcinoma. In autopsy cases showing *ras* mutation in cancerous prostate, the same alteration was observed in metastatic tissues. No APC gene mutation was detected in any sample, although polymorphism was found in some cases. These results indicate that *ras* oncogene mutations are related to the progression of prostate cancer, whereas APC gene alteration is not involved in tumorigenesis and development of this cancer.

Key words: APC gene — *ras* oncogene — Prostate cancer — Genetic alteration — PCR-SSCP

Prostate cancer is one of the common cancers in human males.[1] The growth of prostate cancer is sensitive to androgen, and hormonal manipulation has been used for treatment.[2] About 75% of prostate cancers initially respond to endocrine therapy, but more than half of the responders gradually become resistant to the endocrine therapy.[3] Changes in tumors from an androgen-responsive to an androgen-unresponsive state have been widely discussed, and the mechanism is accepted to be a multistep process in which the accumulation of genetic changes results in uncontrolled cellular proliferation.[4] Our previous report using histochemical examination revealed increased expression of *ras* p21 protein in advanced prostate cancer, correlating with metastatic potency.[5] Other reports have suggested that genetic alterations of *ras* oncognens occur in some prostate cancer cases.[6,7]

The APC[5] gene, which is responsible for familial adenomatous polyposis and sporadic tumors of colon, was recently isolated.[8–11] Somatic mutations of this gene were also detected in cancers of the stomach[12] and pancreas.[13] To shed light on genetic alterations in prostate cancer, the present study was undertaken to examine structural abnormalities of *ras* (K-, H- and N-*ras*) and APC genes.

MATERIALS AND METHODS

Specimens Samples from 18 patients with adenocarcinoma of the prostate who were treated in Chiba University and affiliated hospitals were used in the study. Tissues were removed from eight stage B cancer cases by total prostatectomy (5 moderately differentiated and 3 poorly differentiated adenocarcinomas). The others were obtained at autopsy from 10 cases of endocrine therapy-resistant metastatic disease including bone lesion, who had initially responded to the therapy, and thereafter relapsed (all were poorly differentiated adenocarcinoma). All cases were unrelated Japanese individuals. A total of 51 samples, 18 from cancerous prostate, 18 from normal tissues including normal prostate and 15 from metastatic cancer tissues (liver, kidney and pelvic lymph node), were examined.

Genomic DNA preparation Specimens from surgery or autopsy were frozen immediately after removal and stored at −80°C until DNA extraction. In cases of autopsy samples, tissues were obtained 2–12 h after death. Resected prostate from surgery was cut in 5 mm serial sections, each section was stained with hematoxylin and eosin, then cancer foci were enucleated. For autopsy

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[5] Abbreviations: APC, adenomatous polyposis coli; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism.

cases, a piece of cancer tissue was removed and confirmed histologically. Genomic DNA was extracted as described.[14, 15] The concentration of DNA was estimated spectrophotometrically. Fifty ng/μl of genomic DNA was employed as the template for PCR.

PCR To examine *ras* oncogenes, amplified fragments surrounding codons 12, 13 and 61 of K-, H- and N-*ras* oncogenes were studied. Primers for PCR of these oncogenes are shown in Table I. For screening of APC gene mutations, the 5′-half of exon 15 (codons 742–1702), where more than two-thirds of the somatic mutations in colorectal tumors were observed,[16, 17] was examined. Primers for PCR of APC gene were prepared according to Groden *et al.*[8]

The reaction mixture was made up of the following components: 200 ng of DNA, 0.5 μM of each primer, 70 μM of each deoxynucleotide triphosphate, 10 mM Tris-HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.25 U of *Taq* polymerase (Perkin-Elmer Cetus Corp., Norwalk, CT), 0.25 mM spermidine, and 0.1 μl of [α-^{32}P]dCTP (3000 Ci/mmol) in a final volume of 10 μl. PCR for *ras* oncogenes was performed with 32 cycles of denaturation (94°C, 60 s) annealing (K and N-*ras*: 55°C, 120 s; H-*ras*: 55°C, 60 s) and extension (72°C, 60 s) by a thermal sequencer (Iwaki No. TSR-300, Tokyo). PCR for APC gene was done according to Groden *et al.*[8]

PCR-SSCP analysis SSCP analysis was performed according to the method described previously.[14, 15] After the reaction, the PCR products were diluted with an adequate volume of formamide-dye mixture. One μl of diluted PCR products was heat-denatured, chilled on ice and immediately loaded on polyacrylamide gels according to the method described previously.[14, 15] Two types of gel conditions were employed (glycerol concentration: 5% and 10%). After electrophoresis at 30 W for 3–6 h at 10°C, the gel was vacuum dried and autoradiographed. Duplicate examinations were performed to confirm conformation abnormality.

Direct sequencing Another aliquot of genomic DNA showing a variant pattern on SSCP analyses was amplified by PCR according to the manufacturer's instructions. Amplified DNA fragments were electrophoresed on low-melting-point agarose gels (Sea Plague GTG or Nusieve GTG, FMC Bio Products, Rockland, ME). After electrophoresis, the fragments were separated from gels and purified by phenol-chloroform extraction, followed by ethanol precipitation. The purified PCR products were sequenced by means of the cycle sequencing method.[18, 19] The sequencing primer was the same as used for the preceding PCR and the 5′-terminal of each primer was labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Using the labeled sequencing primer, the purified PCR products were further amplified and denatured with a ds cycle sequencing system (BRL, Gaithersburg, MD). After electrophoresis performed according to the reported method,[14] gels were transferred to Whatman 3MM paper, dried on vacuum slab dryers and autoradiographed.

RESULTS

Detection of *ras* oncogene mutations Four out of 18 prostate cancer cases contained *ras* point mutations in cancer foci of the prostate (22%). Moreover, the same mutations were detected in metastatic tissues of the respective cancers. Two mutations of K-*ras* were detected in one stage B case and one autopsy case (Figs. 1 and 2 and Table II). Two mutations of H-*ras* were found in two autopsy cases (Table II). All cases showing *ras* point mutations were poorly differentiated adenocarcinoma. None of the samples showed mutation in N-*ras*. To sum up, *ras* point mutations were detected in one out of eight stage B cases (13%) and three out of 10 autopsy cases

Table I. Primers for PCR of *ras* Oncogenes

K-*ras* codons 12,13	U: 5′-GACTGAATATAAACTTGT-3′ (exon)
	D: 5′-CTATTGTTGGATCATATTCG-3′ (exon)
K-*ras* codon 61	U: 5′-TTCCTACAGGAAGCAAGTAG-3′ (exon)
	D: 5′-CACAAAGAAAGCCCTCCCCA-3′ (exon)
H-*ras* codons 12,13	U: 5′-GGCAGGAGACCCTGTAGGAG-3′ (intron)
	D: 5′-GTATTCGTCCACAAAATGGTTCT-3′ (exon)
H-*ras* codon 61	U: 5′-TCCTGCAGGATTCCTACCGG-3′ (intron-exon)
	D: 5′-GGTTCACCTGTACTGGTGGA-3′ (exon-intron)
N-*ras* codons 12,13	U: 5′-GACTGAGTACAAACTGGTGG-3′ (exon)
	D: 5′-CTCTATGGTGGGATCATATT-3′ (exon)
N-*ras* codon 61	U: 5′-GGTGAAACCTGTTTGTTGGA-3′ (exon)
	D: 5′-ATACACAGAGGAAGCCTTC-3′ (exon)

U and D indicate up and down primer, respectively.

(30%). These *ras* gene alterations seem to be late events in the development of prostate cancer.

Detection of APC gene mutations Polymorphism within the coding region was observed in both normal and cancerous tissues from five out of 18 cases (28%). Polymorphisms were detected in codon 1494 (one case from autopsy) and codon 1679 (five cases from autopsy). One case contained both polymorphisms. No somatic mutation, however, was detected in any of the cancer foci, including metastatic tissues. One polymorphism at codon 1679 (from GGA to GGG), resulting in no amino acid alteration, is shown in Figs. 3 and 4. Substitution of ACG for ACA at codon 1494 did not alter the amino acid sequence, either.

DISCUSSION

Carcinogenesis consists of multiple steps of genetic alteration.[20] The successive acquisition of activating mutations in cellular proto-oncogenes and inactivating mutations in, or deletion of, tumor suppressor genes has been documented during the evolution of human colorectal tumors from benign polyps to metastasizing carcinoma. Activation of *ras* oncogenes occurs during the middle period of tumorigenesis and APC gene alteration is an early event in colorectal tumorigenesis.[20] The sequence may provide a model for other kinds of neoplasms.

The *ras* oncogene family, which codes for membrane-associated protein products of molecular weight 21,000 (p21), is the most notable of the oncogenes activated in human solid tumors.[21,22] Increased expression of *ras* p21 has been observed in various human cancers, including prostate cancer.[5,23-25] The previous report from our laboratory[5] showed that *ras* p21 expression was dependent on grade and stage. Moreover, this report also indicated that cancer cells exhibiting positive *ras* p21

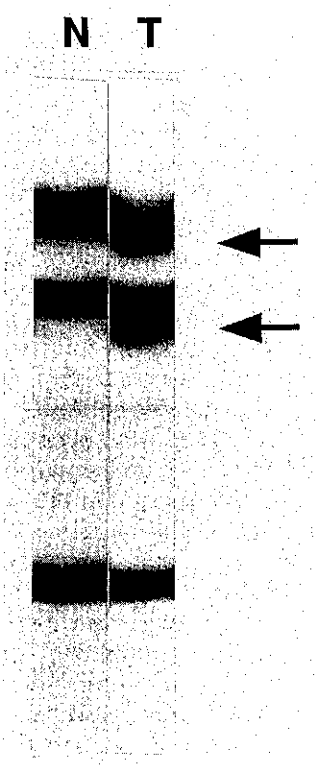

Fig. 1. SSCP analysis of K-*ras* codon 61 in patient No. 8 (stage B prostate cancer). N: normal prostate tissue, T: cancerous prostate. Arrows indicate abnormally shifted bands in cancerous prostate.

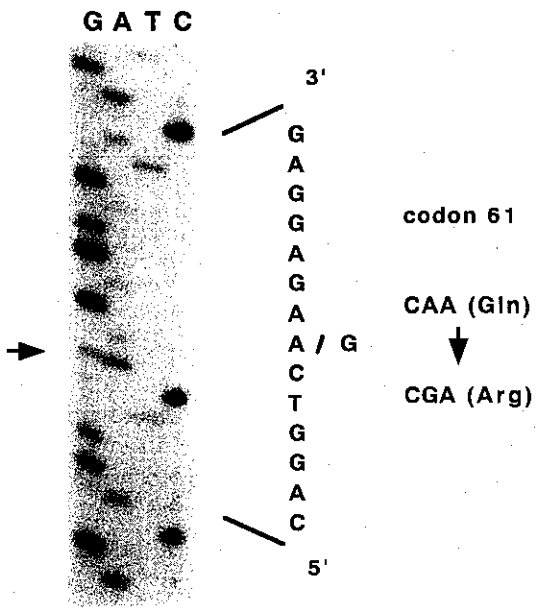

Fig. 2. Partial sequence of K-*ras* codon 61 in patient No. 8 (shown in Fig. 1). An A-to-G substitution at codon 61 resulting in Gln (CAA) to Arg (CGA) mutation.

Table II. Somatic Mutations of *ras* Oncogenes

Age (years)	Origin	*ras* mutation		
		Type	Codon	Nucleotide change
77	autopsy[a]	H	13.1	GGT(Gly)→CGT(Arg)
72	autopsy[a]	H	61.2	CAG(Gln)→CTG(Leu)
75	autopsy[a]	K	12.2	GGT(Gly)→CGT(Arg)
68	surgery[b]	K	61.2	CAA(Gln)→CGA(Arg)

a) The tissues were obtained from cases of endocrine therapy-resistant metastatic disease at autopsy.
b) The tissue was obtained from surgical specimens of a stage B patient at total prostatectomy.

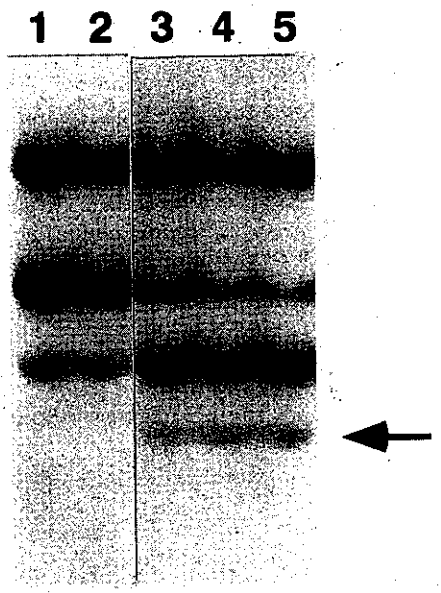

Fig. 3. SSCP analysis of APC gene exon 15 codons. Lane 1: normal prostate in patient No. 17 (stage B), lane 2: cancerous prostate in patient No. 17, lane 3: normal kidney in patient No. 18 (autopsy cancer case), lane 4: cancerous prostate in patient No. 18, lane 5: metastatic lesion of liver in patient No. 18. The arrow indicates a shifted band.

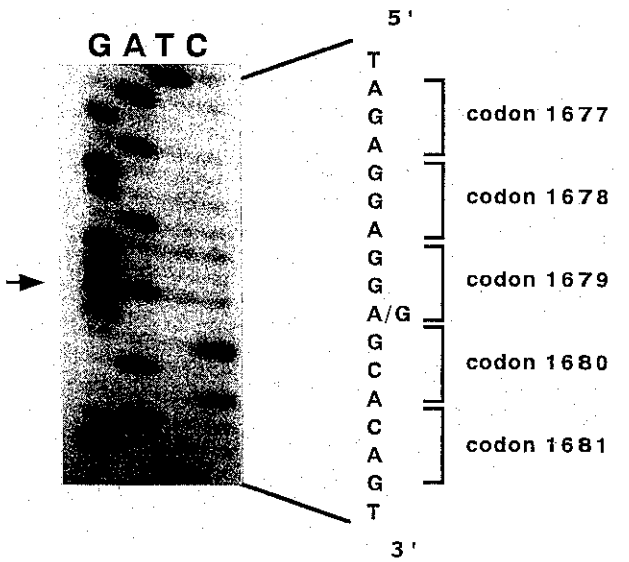

Fig. 4. Partial sequence of APC gene exon 15. An A-to-G substitution at codon 1679, resulting in no amino acid change, was recognized in patient No. 17 (see Fig. 3).

expression tended to have a higher frequency of nodal metastasis. It was claimed that histochemically detectable expression of p21 was not necessarily associated with K-*ras* mutation in latent prostate cancer.[6] *Ras* point mutations were observed in 22% of cases in the present study, and this frequency is quite low when compared with the increased expression of *ras* p21 detected histochemically by ourselves[5] and other.[26] The reason for the difference in frequencies found by histochemical examination and by biochemical analysis is unknown.

When incidences of *ras* mutation in prostate cancer are compared, there are differences among countries. It was reported that in Japanese men at autopsy, point mutations in codon 12 of K-*ras* gene were detected in six cases out of 23 clinically silent prostate cancers (26%).[6] Another report demonstrated that *ras* point mutations were present in 16 out of 68 Japanese prostate cancer cases (24%).[7] Thus, the frequency of *ras* gene mutation is 22–26% in Japan. On the other hand, Carter *et al.* reported that only one *ras* gene mutation was detected in 24 human prostate tissues (Ha-*ras* codon 61).[27] Gumerlock *et al.* detected only one *ras* gene mutation in 19 human prostate tissues (Ha-*ras* codon 61).[28] Thus, the prevalence of *ras* gene mutations in the USA seems to be rather low. It remains to be clarified why the frequency of *ras* gene mutations in Japan is higher than that in the USA.

The present study showed that mutations in all four *ras* gene were present in 13 poorly differentiated carcinoma (31%), but no mutation was detected in five moderately differentiated carcinoma. Moreover, the frequency of *ras* point mutations in autopsy cases was higher than that in stage B cases, suggesting that *ras* gene alteration is a rather late event in the development of prostate cancer. On the contrary, a study using dot blot hybridization detected *ras* mutations in rather early stage cases, and they increased in frequency with progression. Further studies are required to permit a final conclusion.

The APC gene, located on the long arm of chromosome 5,[29] is mutated in patients with familial adenomatous polyposis and sporadic colorectal cancers.[8–11, 16] Recently, we reported that APC gene mutations were detected in three out of 24 oral squamous cell cancer cases by the same method as used in the present study.[30] To our knowledge, there is no report on the structure of the APC gene in human prostate cancer, so we examined this point. No somatic mutation was detected in 18 prostate cancer cases ranging from early to advanced stage. Therefore, APC gene alteration is not associated with pathogenesis of prostate cancer. Polymorphism was detected in 28% of all prostate cases. Since this change was shared in normal and cancerous tissues, the polymorphism was independent of tumorigenesis of this cancer. The polymorphism at codon 1679 was identical to that found previously.[8, 31] Thus, polymorphism seems to be rather common in the APC gene.

The p53 gene is one of the tumor suppressor genes and its deletions or point mutations are observed in many kinds of tumors.[15, 32, 33] In the literature, p53 genetic alterations in prostate cancer are relatively rare.[34, 35] It is possible that the frequencies of genetic abnormalities of notable oncogenes and tumor suppressor genes, such as *ras*, APC, and p53, are rather low in prostate cancer, when compared with cancers of colon and lung.[32, 33, 36] In an allelotyping study of prostate cancer, however, high frequencies of allelic deletions on chromosomes 8 (65%), 10 (55%) and 16 (56%) were found.[37, 38] Genetic alterations on these chromosomes might be involved in tumorigenesis and development of prostate cancer. Further studies including searches for these genetic alterations are required to elucidate the course of prostate cancer.

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