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PRLTS Gene Alterations in Human Prostate Cancer

Akira Komiya,[1] Hiroyoshi Suzuki,[1] Takeshi Ueda,[1] Sara Aida,[1] Naomi Ito,[1] Taizo Shiraishi,[2] Ryuichi Yatani,[2] Mitsuru Emi,[3] Kosaku Yasuda,[1] Jun Shimazaki[1] and Haruo Ito[1,4]

[1]*Department of Urology, School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260,* [2]*Department of Pathology, Faculty of Medicine, Mie University, 2-714 Edobashi, Tsu 514 and* [3]*Department of Molecular Biology, Institute of Gerontology, Nippon Medical School, Nakahara-ku, Kawasaki 211*

Since loss of heterozygosity on 8p22-p21.3 has been found frequently in prostate cancer, the status of a candidate tumor suppressor gene named *PRLTS* gene, recently cloned from the same region in some human malignancies, was examined in the present study. DNAs were isolated from 69 Japanese prostate cancer patients (37 localized and 32 cancer-death cases). Loss of heterozygosity at this gene locus was observed in 15 of 36 (42%) localized prostate cancer patients and 22 of 32 (69%) cancer-death patients. One cancer-death patient had a missense mutation, ACG→ATG (Thr→Met) at codon 64 in metastatic tumor tissues of pelvic lymph node and liver, and these tissues showed loss of the homologous allele, indicating that "two-hit" mutation of the *PRLTS* gene had occurred in this case. The others did not show any mutation, regardless of the presence or absence of loss of heterozygosity. Although loss of heterozygosity at the *PRLTS* gene locus is a relatively common abnormality, mutation of this gene is rare in prostate cancer.

Key words: *PRLTS* gene — Chromosome 8 — Tumor suppressor gene — Prostate cancer — PCR-SSCP

There have been many reports on the abnormality of chromosome arm 8p in a variety of human tumor types, including hepatocellular carcinoma, colorectal carcinoma, non-small cell lung carcinoma,[1] bladder carcinoma[2] and prostate cancer.[3,4] We recently narrowed the commonly deleted region of 8p22-p21.3 to 1.2 Mb in human prostate cancer,[5] and therefore this region may contain one or more putative tumor suppressor genes for this tumor. Fujiwara *et al.*[6] isolated a candidate tumor suppressor gene named *PRLTS* gene within this region. Somatic mutations of this gene in colorectal carcinoma and hepatocellular carcinoma, which showed LOH at the loci on 8p22-p21.3, suggested a two-hit mutation inactivation pattern in the *PRLTS* gene. These findings led us to examine whether allelic losses at this gene locus and mutations in the six exons (exons 2–7) of this gene are present in prostate cancer. To this end, the present study was undertaken to examine the status of the *PRLTS* gene using LOH analysis, PCR-SSCP analysis, and direct sequencing.

MATERIALS AND METHODS

DNA preparation Tissue samples from 69 Japanese patients with adenocarcinoma of the prostate who were treated at Chiba University Hospital and affiliated hospitals were obtained from surgery or autopsy between April, 1992 and November, 1995. Each tumor was classified according to the TNM classification.[7] Tumor specimens from 37 cases of localized prostate cancers (T2-T3 N0-N2 M0) which had been surgically removed, and from 32 cancer-death cases at autopsy who had initially responded to endocrine therapy and thereafter relapsed, were used in the present study. A total of 166 samples consisting of 69 cancerous prostates, 69 normal tissues and 27 metastatic cancer foci obtained at autopsy from liver, lung and pelvic lymph nodes were examined. Tumor characteristics of these cases are summarized in Table I. Specimens from surgery or autopsy were frozen immediately after removal and stored at −80°C. In cancer-death cases, tissues were obtained 2–12 h postmortem. Surgically resected prostates were cut into 5 mm serial sections, each section was examined with hematoxylin/eosin staining, and cancer foci were enucleated. For autopsy cases, a piece of cancerous tissue was removed and confirmed histologically. Genomic DNAs were prepared by proteinase K digestion and phenol/chloroform extraction. Concentrations of DNA stocks were estimated by spectrophotometry.

PCR-LOH assay Tumor and normal DNAs were amplified by PCR at the following 3 microsatellite loci: D8S254 (8p22), LPL (8p22), and D8S258 (8p22). Approximately 0.1 μg of genomic DNA was amplified in each 20-μl PCR reaction mixture consisting of 10 m*M* Tris-HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 m*M* of each deoxynucleotide triphosphate, 1 μM concentration of each primer, and 2.5 units of Taq polymerase

[4] To whom correspondence should be addressed.

[5] The abbreviations used are: *PRLTS*, platelet-derived growth factor receptor-like tumor suppressor; PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism; LOH, loss of heterozygosity; RFLP, restriction fragment length polymorphism; PDGFR, platelet-derived growth factor receptor.

Table I. Characteristics of 69 Prostate Cancers

TNM	Differentiation			Total
	Well differentiated	Moderately differentiated	Poorly differentiated	
T2 N0-N1 M0	10 (2)	3 (1)	7 (2)	20 (5)[a]
T3 N0-N2 M0	3 (3)	9 (4)	5 (3)	17 (10)[a]
Cancer-death	0 (0)	0 (0)	32 (22)[b]	32 (22)[c]
Total	13 (5)	12 (5)	44 (27)	69 (37)

Numbers in parentheses are the numbers of LOH at the *PRLTS* gene locus (8p22-p21.3).
a) Removed by total prostatectomy.
b) One case showed a *PRLTS* gene mutation.
c) Obtained at autopsy from cancer-death patients who had initially responded to endocrine therapy and thereafter relapsed.

(Takara, Kyoto). Prior to the PCR reaction, each sense primer was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The PCR protocol consisted of 27 cycles of denaturation (94°C, 60 s), annealing (55°C, 60 s) and extension (72°C, 90 s) in a Thermal Sequencer (Iwaki, Chiba). PCR products were denatured by 95% formamide-dye and electrophoresed on polyacrylamide gels containing 7 *M* urea for 2–4 h at 40 W, followed by autoradiography. Densitometry was performed with a Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA). A reduction in signal intensity of more than 50% was judged as LOH.

Southern blotting A 10 μg aliquot of each DNA sample was digested overnight with a more than 10-fold excess of restriction enzymes, *Msp* I (Takara) or *Taq* I (Boehringer Mannheim GmbH, Germany), and then fractionated by electrophoresis in agarose gels containing 1% Tris-borate buffer. The DNAs were transferred to nylon membranes (Hybond-N+, Amersham, Buckinghamshire, UK) in 0.1 *N* NaOH-0.1 *M* NaCl and fixed by UV cross-linking. The 3 RFLP markers used in this study were cMSR-32 (8p22), CI8-2644 (8p21.3-p22), and CI8-1051 (8p22-p21.3). All RFLP markers were physically localized on chromosome arm 8p by fluorescent *in situ* hybridization and linkage analysis as reported by others.[8,9] Probes were labeled with [α-^{32}P]dCTP by random primer extension. Prehybridization, hybridization and autoradiography were carried out as described previously.[5] The membranes were stripped in 0.4 *N* NaOH and hybridized repeatedly. In this study, LOH was determined according to the same criteria as those used in the PCR-LOH assay described above for microsatellite markers.

Search for *PRLTS* gene mutations by PCR-SSCP analysis SSCP analysis of the *PRLTS* gene was performed by PCR amplification of the six exons (exons 2–7) corresponding to the coding region. PCR primers were prepared in introns according to Fujiwara *et al.*[6] to amplify each exon:

exon 2: 5′-ACC TTG TTC CTC CTG AAG AAAC-3′ (upstream),
5′-ACC GAG GGC CGA ACG TTA GA-3′ (downstream)
exon 3: 5′-ATT TCT CTC TCC TTA CGT TTT GC-3′ (upstream),
5′-TTA CAA ATC CTC AAC CCT ACA CA-3′ (downstream)
exon 4: 5′-CCA AGC CAA GGT CTG ATT TGC-3′ (upstream),
5′-GTT CCA TTA ATG CAC CAA GTA TTT-3′ (downstream)
exon 5: 5′-AGC CTG TGC TCT TCC TTC CC-3′ (upstream),
5′-CTG ACC AGG GGA CTA ACT AGA-3′ (downstream)
exon 6: 5′-ACT CGG GGT ACT CAC TCT GC-3′ (upstream),
5′-GGC TGA GAT GCA GGT ACA ACA-3′ (downstream)
exon 7: 5′-CTT CAG TCT TTG TGG GTG TCG-3′ (upstream),
5′-GTA CAC AAA TGG GCT TTC CAT AA-3′ (downstream)

The 5′-terminal of each primer was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Takara). The PCR mixture was prepared as follows: 50 ng of genomic DNA, 0.125 pmol of each primer, 0.3125 nmol of each deoxynucleotide triphosphate, 10 m*M* Tris-HCl buffer (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 5% dimethyl sulfoxide and 0.0625 U Taq polymerase (Takara) in a final volume of 5 μl. The PCR protocol consisted of 35 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min) and extension (72°C, 1 min) in a Thermal Sequencer (Iwaki). The PCR products were diluted with a suitable volume of formamide-dye mixture and electrophoresed on 5% polyacrylamide gel containing 45 m*M* Tris-borate buffer, 4 m*M* EDTA, 0.05% ammonium peroxydisulfate and 5% glycerol at 4°C. After electrophoresis, the gels were dried under vacuum and autoradiographed at −80°C. Duplicate examinations were performed to confirm conformation abnormality.

Direct sequencing Abnormal bands detected by SSCP analysis were eluted from the gels and amplified on a scale of 100 μl by PCR according to the manufacturer's instructions (Gene Amp PCR Reagent Kit, Perkin Elmer Cetus, Norwalk, CT). Amplified DNA fragments were electrophoresed on low-melting-point agarose gels (Sea Plaque GTG, FMC Bio Products, Rockland, ME). After electrophoresis, the fragments were separated from the gels and purified by phenol/chloroform extraction followed by ethanol precipitation. The purified PCR products were sequenced according to a cycle sequencing method described previously.[10,11] The sequencing primers were the same as those used for PCR-SSCP.

Statistical analysis Significant differences were calculated using Fisher's exact test. One-tailed P values of less than 0.05 were considered statistically significant.

RESULTS

Allelic loss at the *PRLTS* gene locus Since the *PRLTS* gene was isolated from 8p22-p21.3,[6] we used RFLP and microsatellite markers overlapping with this region. Among 69 cases, LOH was detected in 37 (54% of 68 informative cases) with at least one locus on 8p22-p21.3. A significant association was found between allelic losses and clinicopathological findings (Table I). Allelic loss was observed more frequently in cancer-death patients (22/32, 69%) than in localized cancers: T2 (5/20, 25%; $P=0.0394058$) or T2+T3 (15/36, 42%; $P=0.0225802$). Moreover, LOH was observed more frequently in poorly differentiated adenocarcinomas (28/43, 65%) than in well+moderately differentiated ones (9/25, 36%; $P=0.0189377$). Representative autoradiograms are presented in Fig. 1a.

Mutational analysis of the *PRLTS* gene DNA samples from 69 cases were examined with PCR-SSCP and abnormal bands were further analyzed by direct sequencing. Metastatic foci from pelvic lymph node and liver of one cancer-death case (P-4) showed a variant SSCP pattern in exon 3 (Fig. 1b). Direct DNA sequencing confirmed a point mutation, C-to-T transition, at the second

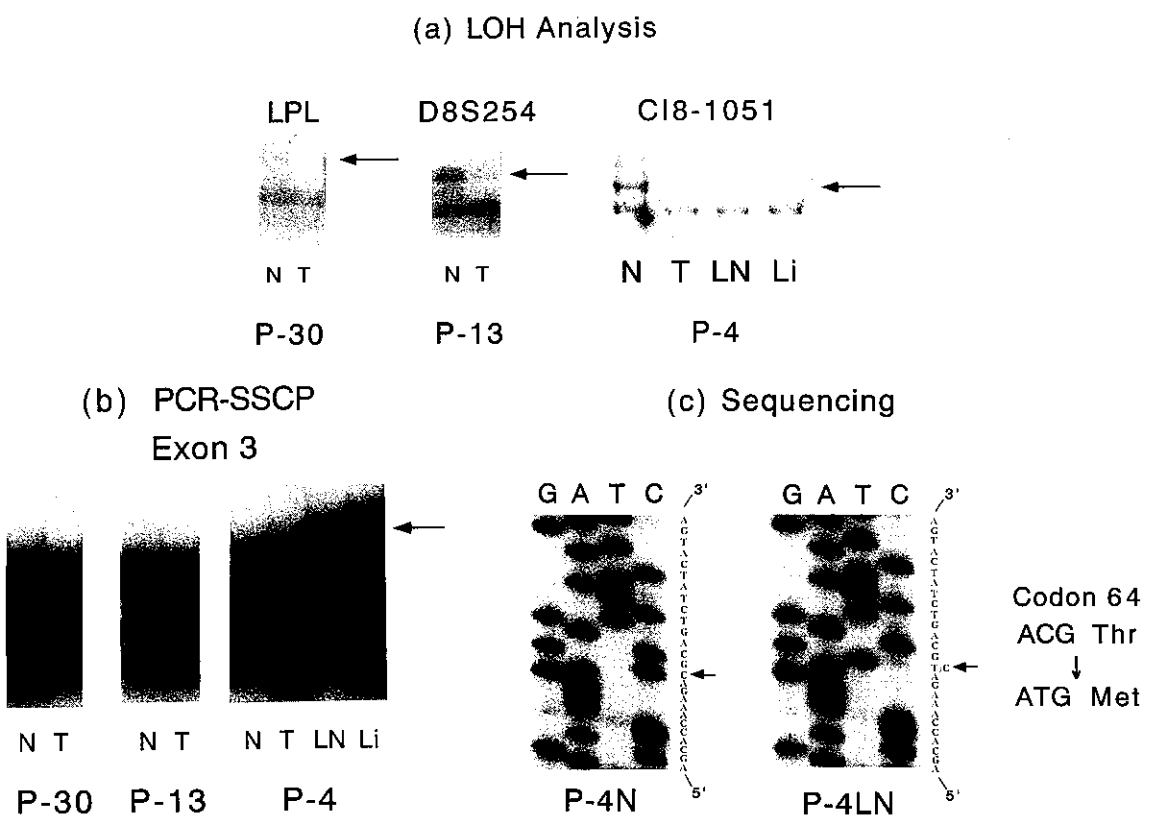

Fig. 1. LOH analysis at the *PRLTS* gene locus (a), and PCR-SSCP analysis (b) and direct sequencing (c) of the *PRLTS* gene. N, normal tissue; T, primary tumor; LN, metastatic tissue of lymph node; Li, metastatic tissue of liver. a, Arrows indicate allelic loss. LOH was observed at *LPL* in case P-30 (T2 N0 M0, well differentiated adenocarcinoma), at D8S254 in case P-13 (T3 N0 M0, poorly differentiated adenocarcinoma) and at CI8-1051 in both primary and metastatic tumors in case P-4 (cancer-death, poorly differentiated adenocarcinoma). b, Abnormally shifted bands (arrow) were observed in the metastatic tissues of pelvic lymph node and liver of case P-4. c, Codon number is used according to Fujiwara *et al.*[6] In case P-4, C-to-T transition at codon 64 resulted in a substitution of Met (ATG) for Thr (ACG) in metastatic tumors of both lymph node and liver. Case P-4 is 80 years old, staged as T3 NX M0, with poorly differentiated adenocarcinoma (Gleason score 5-4) in a biopsy specimen of the prostate. After 2 years and 2 months of endocrine therapy (castration and antiandrogen), this patient had a relapse, and 1 year later he died of widespread metastases of prostate cancer. The tissues examined were obtained at autopsy.

position of codon 64, resulting in a substitution of Met for Thr (Fig. 1c) in the lymph node metastasis. In this case, the other metastatic site (liver) was also examined and the same mutation at codon 64 of the *PRLTS* gene was found. However, the cancerous tissue in the prostate of the same patient showed LOH at this gene locus without any point mutation of the *PRLTS* gene. Except for case P-4, no *PRLTS* gene mutation was found in the cancerous tissues of primary and metastatic foci from any of the other patients, regardless of the presence or absence of LOH.

DISCUSSION

Previously, we reported frequent loss of heterozygosity within a 1.2 Mb region of 8p22-p21.3[5] flanked by the markers cMSR-32 and CI8-1051 in prostate cancers. In the present study, LOH in this region was examined in a larger number of cases and a similar incidence to the previous result was obtained: 22 out of 32 (69%) cancer-death cases vs. 15 out of 36 (42%) localized tumors (T2-T3, $P=0.0225802$). Therefore, this region may be correlated to the progression of prostate cancer.

From the same chromosomal region of 8p22-p21.3, a candidate tumor suppressor gene, the *PRLTS* gene, was isolated in hepatocellular carcinoma, colorectal carcinoma, and non-small cell lung carcinoma.[6] The predicted product of the *PRLTS* gene bears a significant homology (27% identity) with the ligand-binding domain of a receptor-type tyrosine kinase, PDGFR-β, and this suggests an antagonistic interaction between PDGF and its receptor. Somatic mutations of the *PRLTS* gene were observed in one colorectal cancer and two hepatocellular carcinomas with LOH on 8p22-p21.3. The *PRLTS* gene in the three tumors had sustained two-hit mutations, indicating that development or progression of these tumors may be explained by inactivation of the *PRLTS* gene. In the present study, the metastatic sites of one cancer-death case of prostate cancer had also undergone a two-hit mutation inactivation of the *PRLTS* gene by loss of the second allele and a missense mutation, which may be related to acquisition of metastatic ability, because the primary prostate foci revealed only LOH with no mutation of the *PRLTS* gene. Since the missense mutation in the present study (codon 64, Thr→Met) was different from those reported in colorectal cancer (codon 23, His→Tyr) and hepatocellular carcinoma (codon 302, Val→Ala; codon 175, a two-nucleotide deletion),[6] this can not be regarded as a mutational "hot spot" in the *PRLTS* gene. As the frequency of *PRLTS* gene mutation was low, the inactivation of this gene during development or progression may actually occur only in a few cases in a variety of solid tumors including prostate cancer. However 36 of 69 (52%) prostate cancer patients had lost one of the alleles of the *PRLTS* gene in the present study. Of 95 informative cases consisting of hepatocellular carcinoma, colorectal carcinoma, and non-small cell lung carcinoma, LOH at the *PRLTS* gene locus was found in 39 (41%).[6] Therefore, it is also possible that the dosage effect due to alteration of the copy number of the gene affects development or progression of these cancers. In fact, the dosage change of some genes has been implicated as a cause of human neurological disorders.[12, 13] A change of copy number of retinoblastoma gene was shown to affect the susceptibility to spontaneous pituitary tumors of adult mice.[14] A reduction of *p53* gene dosage enhanced the malignant progression of chemically induced skin tumors in mice.[15] A similar situation in the *CDKN2* gene was found in human prostate cancer.[11]

If dosage effects are considered, the *PRLTS* gene may play a significant role in the progression of human prostate cancer. This may be one of the reasons for the infrequency of somatic mutations, in spite of the frequent LOH at the *PRLTS* gene locus in tumors. However, another putative tumor suppressor gene may be present within the chromosomal region of 8p21.3-p22, or aberrant hypermethylation may contribute to inactivation of the *PRLTS* gene. Since development or progression of prostate cancer is based on a complex pattern of gene alterations, clarification of the *PRLTS* gene status will represent only one step in the process of shedding light on the carcinogenesis of prostate cancer.

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