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Microsatellite Instability and Other Molecular Abnormalities in Human Prostate Cancer

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Microsatellites are highly polymorphic, short-tandem repeat sequences dispersed throughout the genome. Instability of these repeat sequences at multiple genetic loci may result from mismatch repair errors, and occurs in hereditary nonpolyposis colorectal carcinoma and certain sporadic cancers. To examine microsatellite instability during the pathogenesis of human prostate cancer, we screened 48 prostate cancer cases (20 stage B, 10 stage C and 18 endocrine therapy-resistant cancer-death cases) for replication error at 17 microsatellite marker loci on 9 chromosomes. Microsatellite instabilities were found in 7 of 48 cases (14.6%), and all 7 cases showing the instability were poorly differentiated adenocarcinomas. Moreover, microsatellite instabilities were more frequently observed in cancer-death cases (6/18, 33%) than in stage B+C cases (1/30, 3.3%). These data suggest that microsatellite instability is an important genetic change related to the progression of a subset of human prostate cancer cases. It is suggested to be associated with extensive, concurrent molecular changes including androgen receptor gene mutations, as well as frequent loss of heterozygosity at chromosomal regions 8p, 10q, and 16q.

Key words: Microsatellite instability — Prostate cancer — Loss of heterozygosity — Genetic alteration

Prostate cancer is a major cause of cancer death among elderly men. In spite of its high prevalence, little is known regarding the molecular pathogenesis of this neoplasm. Numerous studies have disclosed some of the genetic events associated with carcinogenesis of solid tumors, and it is now generally accepted that transformation of normal to malignant tissue follows an accumulation of genetic changes in oncogenes and tumor suppressor genes within a cell lineage.[1)] We and others have shown that genetic alterations of the androgen receptor gene,[2,3)] of the *ras* oncogenes[4,5)] and of the p53 tumor suppressor gene[6–8)] are related to the development of prostate cancer. Allelic loss of chromosome 8p[9,10)] has been frequently observed in this cancer (50–65%), and approximately 20–30% of the cancers show loss of heterozygosity (LOH) for chromosome 10q, 16p and 17p.[11–13)]

Microsatellite instability (MSI), representing mutations of the short-tandem repeat sequences distributed within the genome, were initially reported in sporadic colorectal cancers and hereditary nonpolyposis colorectal cancer.[14,15)] MSI appears to be a novel molecular mechanism in carcinogenesis and is thought to reflect multiple replication errors arising from abnormalities of the mismatch repair genes.[16,17)] Besides colorectal cancer, MSI has also been described in a variety of other human tumors. However, the incidence of MSI is strikingly different depending on the organ of origin; MSI is more frequently observed in gastric carcinomas and pancreatic carcinomas than in tumors of the breast, liver, proximal colon, uterine cervix, and ovary.[18)] Recent studies have found that MSI was related to the progression of cancers of the stomach,[18)], pancreas[18)] and kidney,[19)] although MSI occurred as an early event in tumorigenesis of the breast,[20)] urinary bladder,[21)] and lung.[22)] To clarify whether MSI is an initial event or a later one in the development of prostate cancer, we examined specimens from various stages of prostate cancer. We also evaluated the relationship between MSI and other genetic changes in prostate cancer.

MATERIALS AND METHODS

Tissue samples Samples from 48 Japanese patients with adenocarcinoma of the prostate who were treated in Chiba University and affiliated hospitals were obtained from surgery or autopsy between April 1992 and August 1994. There was no family history of hereditary nonpolyposis colorectal cancer in any case studied. Tumor tissues were removed by total prostatectomy from 20 patients with stage B adenocarcinomas (6 well differentiated, Gleason pattern 1-2; 8 moderately differentiated, Gleason pattern 3; and 6 poorly differentiated, Gleason pattern 4-5) and 10 patients with stage C adenocarcinomas (4 moderately differentiated and 6 poorly differen-

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tiated). The other 18 tumors were obtained at autopsy from endocrine therapy-resistant cases who had initially responded to therapy and relapsed thereafter (all tumors were poorly differentiated adenocarcinomas). Paired DNA samples from primary cancers and normal tissues including normal prostate were obtained in all 48 cases. Twelve metastatic cancer samples obtained at autopsy from liver, kidney, lung and pelvic lymph nodes were also examined.

DNA preparation Specimens from surgery or autopsy were frozen immediately after removal and stored at −80°C until extraction of DNA. In cancer-death cases, tissues were obtained 2–12 h postmortem. Surgically resected prostate tissues were cut into 5 mm serial sections. Each section was examined with hematoxylin/eosin staining, then cancer foci were enucleated. For cancer-death cases, a piece of cancerous tissue was removed and confirmed histologically. Genomic DNA was extracted as described previously.[23] Concentrations of the DNA stocks were estimated by spectrophotometry.

DNA analysis The DNA samples were examined for genetic alterations at 17 different microsatellites by the polymerase chain reaction (PCR) method. The microsatellite loci examined are shown in Table I. 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 (Takara, Kyoto). Prior to the PCR reaction, each sense primer was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. PCR conditions were initial denaturation at 94°C for 5 min, 27 cycles of 94°C for 60 s, 55°C for 60 s, and 72°C for 90 s, and final extension at 72°C for 7 min. 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. As defined in Refs. 14 and 15, MSI is characterized by a shift and/or gain of electrophoretic bands. In contrast, LOH is characterized by a loss of bands. If two or more markers showed alterations in the allele sizes of the tumor DNA, the tumor was categorized as MSI-positive.

Southern blotting A 10 μg aliquot of each DNA sample was digested overnight with a more than 10-fold excess of restriction enzyme, *Msp* I (Takara) or *Taq* I (Boehringer Mannheim GmbH, Germany), then fractionated by electrophoresis using 0.8–1% Tris-borate buffer agarose gels. 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.

Probes and hybridization The 22 RFLP markers used in this study are shown in Table II. All RFLP markers were physically localized on chromosome 8p, 10q and 16q by fluorescent *in situ* hybridization and linkage analysis as reported by others.[24–26] Probes were labeled with [α-^{32}P]dCTP by random primer extension. Prehybridization, hybridization and autoradiography were carried out as described previously.[10] The membranes were stripped in 0.4 *N* NaOH and hybridized repeatedly. Densitometry was performed with a GS300 (Hoefer Scientific Instruments, San Francisco, CA) or BAS2000 (Fujix, Tokyo) scanning densitometer. After correction for differences in the amounts of DNA loaded in each lane, the intensities

Table I. The Microsatellite Repeats Examined

Marker	Chromosome	Repeat	Frequency of instability (%)	Cases showing MSI
D2S119	2	(CA)n	3/48 (6.3)	9, 16, 52
D2S123	2	(CA)n	3/48 (6.3)	9, 27, 52
D2S147	2	(CA)n	3/48 (6.3)	9, 16, 52
D7S461	7	(CA)n	2/48 (4.2)	9, 52
D8S206	8	(GT)n	4/48 (8.3)	5, 9, 14, 52
LPL	8	(Gt)n	3/48 (6.3)	5, 9, 52
D10S197	10	(CA)n	3/48 (6.3)	5, 9, 52
D10S172	10	(GT)n	3/48 (6.3)	9, 45, 52
D11S904	11	(CA)n	3/48 (6.3)	9, 27, 52
D11S1344	11	(CA)n	2/48 (4.2)	9, 52
D13S115	13	(CA)n	3/48 (6.3)	9, 14, 52
D16S186	16	(CA)n	3/48 (6.3)	5, 9, 52
D16S305	16	(CA)n	4/48 (8.3)	9, 14, 27, 52
D16S310	16	(ATAG)n	4/48 (8.3)	9, 14, 27, 52
D16S402	16	(CA)n	4/48 (8.3)	5, 9, 45, 52
TP53	17	(CA)n	2/48 (4.2)	9, 52
DCC	18	(CA)n	3/48 (6.3)	9, 14, 52

Table II. Chromosomal Locations of RFLP Markers Used

Probe name	Enzyme	Chromosomal localization
CI8-388	*Msp*I	8p23.1-p23.2
pKSR2	*Taq*I	8p22-pter
CI8-2014	*Taq*I	8p21.3-p22
cMSR-32	*Msp*I	8p22
CI8-2644	*Msp*I	8p21.3-p22
	*Taq*I	
CI8-1051	*Msp*I	8p22-p21.3
CI8-1312	*Taq*I	8p21
CI8-190	*Taq*I	8p21.3
CI8-319	*Taq*I	8p21.2-p21.3
pHNF-L	*Taq*I	8p21
CI8-494	*Msp*I	8p11.22-p11.23
p9-12A	*Taq*I	10q21.2
Dry5-1	*Taq*I	10q22-q23
p1-101	*Taq*I	10q22-q23
OS-2	*Taq*I	10q22.3-q24.1
pGB2862	*Taq*I	10q21.2-24.3
pEFD75	*Taq*I	10q26
pCETP11	*Taq*I	16q13
CJ52.209M1	*Msp*I	16q21
CJ52.1	*Taq*I	16q22.1
CJ52.96	*Taq*I	16q24.2-q24.3
p79-2-23	*Taq*I	16q24.3

Table III. Correlation of Microsatellite Instability with Clinical Stage and Histological Grade

	Incidence (%)	
Stage		
B	1/20 (5)	*
C	0/10 (0)	
Cancer-death	6/18 (33)	
Grade		
Well differentiated	0/6 (0)	**
Moderately differentiated	0/12 (0)	
Poorly differentiated	7/30 (23)	
Total	7/48 (14.6)	

* $P=0.00800$, stage B+C vs. cancer-death cases.
** $P=0.0276$, well+moderately vs. poorly differentiated (Fisher's exact test).

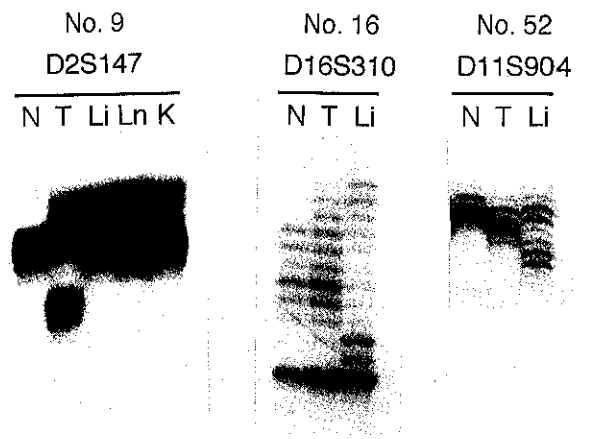

Fig. 1. Examples of microsatellite instability at the D2S147, D16S310, and D11S904 loci. In tumor DNAs, abnormal patterns indicating expansion or deletion are shown at each microsatellite locus. Normal alleles appear as major bands with their ladders. All patterns were reproducible in repeated assays. The case number and microsatellite loci are shown above each column. N, T, Li, Ln, K indicate normal tissue, primary tumor, and metastatic cancer tissue of liver, lymph node and kidney, respectively.

of signals in tumor-tissue DNAs were compared with those of the corresponding normal DNAs. A reduction in signal intensity of more than 50% was judged as LOH.

PCR-single strand conformation polymorphism (PCR-SSCP) analysis and direct sequencing of p53 and androgen receptor genes PCR-SSCP analysis was performed as described previously.[2, 8] We examined exons 4–9 of p53 gene and exons B-H of androgen receptor gene. Oligonucleotide primers for amplification of p53 and androgen receptor genes were the same as in the previous reports.[2, 8, 27] The 5′-terminal of each primer was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Takara). PCR mixture was prepared as in the previous reports[2, 8, 27] and the PCR protocol used was as described previously.[2, 8, 27] After the reaction, the PCR products were diluted with formamide-dye mixture. One μl of diluted PCR products was heat-denatured (80°C, 3 min), chilled on ice and immediately loaded on 5% polyacrylamide gel containing 45 m*M* Tris-borate buffer (pH 8.3), 4 m*M* EDTA, 0.05% ammonium peroxydisulfate and 5% glycerol, then electrophoresed at 30 W for 2–3 h at room temperature. After electrophoresis, the gel was vacuum-dried and autoradiographed.

Abnormal bands detected by SSCP analyses were eluted from the gels and amplified in a scale of 100 μl by PCR according to the manufacturer's instructions. 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 by means of a cycle sequencing method as described previously.[28, 29] 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

Table IV. Other Genetic Changes in Microsatellite Instability-positive Cases

Case No.	Stage	Grade	Mutations		Loss of heterozygosity		
			p53[a]	AR[b]	8p	10q	16q
5	Cancer-death	poorly	−	−	+	−	+
9	Cancer-death	poorly	+	+	+	−	−
14	Cancer-death	poorly	−	−	+	+	+
16	Cancer-death	poorly	−	−	+	+	+
27	Cancer-death	poorly	−	−	−	+	−
45	B	poorly	−	−	−	+	−
52	Cancer-death	poorly	+	+	+	−	+

a) p53 gene mutation: No. 9: CGG (Arg)-GGG (Gly) at codon 248 (exon 7). No. 52: GGC (Gly)-GTC (Val) at codon 154 (exon 5).
b) AR (androgen receptor) gene mutation: No. 9: ACT (Thr)-GCT (Ala) at codon 877 (exon H). No. 52: ACT (Thr)-GCT (Ala) at codon 877 (exon H).

Table V. Correlation of Microsatellite Instability with Other Genetic Changes in Cancer-death Patients

Genetic change	MSI (+)	MSI (−)
p53	2/6 (33%)	4/12 (33%)
Androgen receptor	2/6 (33%)	0/12 (0%)
8pLOH	5/6 (83%)	8/12 (66%)
10qLOH	4/6 (66%)	7/12 (58%)
16qLOH	4/6 (66%)	4/12 (33%)

purified PCR products were further amplified and denatured by using a ds cycle sequencing system (BRL, Gaithersburg, MD). After electrophoresis, gels were transferred to Whatman 3MM paper, dried on vacuum slab dryers and autoradiographed.

RESULTS

Microsatellite instability in prostate cancers Specimens from 7 of the 48 (14.6%) showed MSI at one or more chromosomal loci (Tables I and III). In 2 out of 7 cases with MSI, the genetic alteration was detected in all loci examined in this study (Case Nos. 9 and 52, Fig. 1). MSI was exclusively found in poorly differentiated adenocarcinomas (7/30, 23%, Table III). MSI was observed more frequently in cancer-death cases (6/18, 33%) than in stage B+C cases (1/30, 3.3%), suggesting that MSI is involved in the progression of prostate cancer. DNAs from metastatic cancer tissues were also analyzed in cancer-death cases. All six cancer-death cases with MSI showed instabilities in both primary tumors and metastatic tissues (Fig. 1).

Correlation between MSI and mutations of the p53 and androgen receptor genes The relationship between MSI and p53 or androgen receptor gene mutations is summarized in Tables IV and V. In cancer-death cases, p53 gene mutations were observed in both MSI-positive (2/6, 33%) and -negative cases (4/12, 33%). Two out of the 6 cancer-death cases with MSI showed androgen receptor gene mutations, but other cancer-death cases revealed no androgen receptor abnormality, irrespective of MSI. Thus, androgen receptor gene mutations exclusively occurred in MSI-positive cases.

Correlation between MSI and LOH at 8p, 10q, and 16q The relationship between MSI and LOHs at 8p, 10q and 16q is also summarized in Tables IV and V. There was a trend toward higher frequencies of LOHs in the cases with MSI, although this trend was not statistically significant.

DISCUSSION

It has been accepted that growth of prostate cancer is initially controlled by androgen, but gradually lose sensitivity to androgen. Change from an androgen-responsive to an androgen-unresponsive state appears to be related to acquisition in the tumor of a phenotype of uncontrolled cellular proliferation brought about by accumulation of genetic changes.[25] MSI was recently found to be associated with hereditary nonpolyposis-type colon cancer and other sporadic and familial kinds of neoplasms.[14, 15, 18–22] Some hereditary nonpolyposis-type colon cancer patients carry mutations in *hMSH2, hMLH1, hPMS1,* and *hPMS2,* which are homologues of bacterial mismatch repair genes and are likely to be the cause of disease. MSI is thus defined as hypermutability of microsatellite sequences due to a defect in DNA repair caused by a mutation(s) of the gene(s) responsible for this enzymatic activity. Gao *et al.* reported a high frequency (37 out of 57, 65%) of MSI in prostate cancer.[30] Another report[31] found MSI in 37.5% of Japanese prostate cancer cases. In the present study only 7 of 48 Japanese patients (14.6%) showed MSI. The reason for

these differences are unclear, but perhaps there are geographical and/or ethnic factors. Geographical differences have been shown in the frequency of *ras* oncogene mutations[4, 5] and in p53 gene mutation spectra[7, 8] between Japan and Western countries.

Alternatively, differences in the frequency of MSI may be attributable to tumor grade and stage examined. The present study found a significant association between frequency of MSI and histological grade. Similar to our observations, the previous reports showed that MSI is more likely to occur in poorly differentiated carcinomas of the prostate.[30, 31] However, Gao *et al.* found no association between frequency of MSI and tumor stage.[30] In the present study, the frequency of MSI in cancer-death cases was significantly higher than that in stage B+C cases. Thus, we suggest that MSI is an important genetic change related to the progression of a subset of prostate cancer. In this context, it is worth noting that MSI is also associated with tumor progression in pancreatic cancer,[18] gastric cancer[18] and renal cell carcinomas.[19] However, MSI occurs as an early event in other tumors.[20–22] Thus, the significance of MSI is strikingly different depending on the organ of origin.

Allelotyping studies of prostate cancer have revealed frequent allelic losses on chromosomes 8p, 10q and 16q,[9–13] suggesting the presence of tumor suppressor genes on these chromosomes. The present study also revealed frequent allelic losses on these chromosomes. Moreover, a very high frequency of LOHs on chromosomal arms 8p, 10q and 16q was found in MSI-positive cases. In addition to LOH, androgen receptor gene mutations exclusively appear in MSI-positive cases. Other reports have described a similar association of frequent LOH with MSI in non-small and small cell lung cancers.[32, 33] In marked contrast, however, MSI correlated inversely with allelic loss in sporadic colon cancers.[14, 15] These data taken together suggest that MSI has different roles in different organs of origin.

The present data clearly imply that genetic instability is one of the important genetic changes related to the progression of a subset of prostate cancer. Therefore, screening of MSI may be a potentially useful way to identify patients at high risk. However, further studies are required on the course of this cancer.

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