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Retinoblastoma gene mutations in primary human bladder cancer

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Summary Inactivation of the retinoblastoma (RB) gene is known to be implicated in the pathogenesis of several types of human cancers. Since structural alterations of the RB gene have not been well examined in human bladder cancer, we looked for mutations in the entire coding region of this gene using polymerase chain reaction (PCR) and single-strand conformational polymorphism analysis of RNA. We also examined allelic loss of the RB gene using PCR-based restriction fragment length polymorphism analysis. Of 30 samples obtained from patients with bladder cancer, eight (27%) were found to have RB gene mutations. DNA sequencing of the PCR products revealed five cases with single point mutations and three cases with small deletions. These mutations included one (10%) of ten low-grade (grade 1) tumours, four (50%) of eight intermediate-grade (grade 2) tumours and three (25%) of 12 high-grade (grade 3) tumours. Likewise, mutations were found in four (21%) of 19 superficial (pTa and pT1) tumours and four (36%) of 11 invasive (pT2 or greater) tumours. In 15 informative cases, loss of heterozygosity at the RB locus was shown in five cases (33%), three cases with RB mutations and two without them. These results suggest that RB gene mutations are involved in low-grade and superficial bladder cancers as well as in high-grade and invasive cancers.

Keywords: retinoblastoma gene; bladder cancer; mutation; loss of heterozygosity

The retinoblastoma (RB) susceptibility gene was the first tumour-suppressor gene isolated, is located on human chromosome 13q14 (Sparkes *et al.*, 1980) and consists of 27 exons (McGee *et al.*, 1989). This gene encodes a nuclear phosphoprotein that regulates the cell cycle and forms protein complexes with the adenovirus E1A and SV40 large T oncoproteins (Goodrich *et al.*, 1991). Functional loss of the RB gene is thought to be involved in the initiation and/or progression of many human cancers.

Various studies have revealed that the inactivation of the RB gene has important roles in the development of several types of human tumours, including retinoblastoma (Friend *et al.*, 1986; Fung *et al.*, 1987; Lee *et al.*, 1987), osteosarcoma (Friend *et al.*, 1986; Fung *et al.*, 1987; Toguchida *et al.*, 1988), lung cancer (Harbour *et al.*, 1988; Yandell *et al.*, 1989; Horowitz *et al.*, 1990; Mori *et al.*, 1990), breast cancer (T'Ang *et al.*, 1988; Varley *et al.*, 1989) and prostate cancer (Bookstein *et al.*, 1990). Similarly, several studies have revealed that human bladder cancers have altered or absent RB protein, or loss of heterozygosity (LOH) at the RB locus (Horowitz *et al.*, 1990; Cairns *et al.*, 1991; Ishikawa *et al.*, 1991: Presti *et al.*, 1991; Xu *et al.*, 1993). Takahashi *et al.* (1991) and Goodrich *et al.* (1992) showed that the RB gene transfected into bladder cancer cells suppressed tumorigenicity, suggesting this gene is critical in bladder cancer development. Xu *et al.* (1993) showed alteration of RB protein expression in human bladder cancers by immunohistochemical staining. They also demonstrated that loss of RB protein expression is correlated with LOH at the RB locus. However, structural alterations of the RB gene have not been well examined. In this study, we used the recently developed method of single-strand conformational polymorphism analysis of RNA (RNA-SSCP) (Danenberg *et al.*, 1992) and examined alterations in the entire coding region of the RB gene in 30 primary human bladder cancers of different grades and stages. In addition, we examined RB-LOH using restriction fragment length polymorphism (RFLP) analysis based on the polymerase chain reaction (PCR). RB gene mutations were found in low-grade and non-invasive bladder cancers as well as in high-grade and invasive ones.

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Materials and methods

Patients and tissues

Tissue samples of human bladder cancers were obtained from 30 patients [18 males and 12 females, 67.1 ± 14.3 years of age (mean ± s.d.)] by transurethral cold-knife resection, and matching normal tissue samples from heparinised venous blood were collected at Yokohama City University and Yokohama Municipal Citizen's Hospital. Two or three pieces obtained from one tumour in each patient were used for RNA and DNA analyses. The remaining tumours were fixed and haematoxylin and eosin staining was performed for histological diagnosis. All the tumours were histologically diagnosed as transitional cell carcinoma. Twenty-five cases were fresh tumours and five cases were recurrent ones after initial transurethral surgery. None of them had been treated with radiation or anti-cancer drugs before surgery. Detailed clinical and histopathological data on each patient were evaluated at the same institutions according to the General Rule for Clinical and Pathological Studies on Bladder Cancer (Japanese Urological Association and the Japanese Pathological Society, 1980), which adopts the TNM classification system of malignant tumours (Table I). All the samples selected for this study were rapidly frozen in liquid nitrogen and stored at −80°C until RNA or DNA was extracted. Written informed consent was obtained from each patient according to the guidelines of the Human Subjects Reviews Committee.

RNA extraction, PCR and RNA-SSCP

Total cellular RNAs from these tissues were isolated by the acid guanidinium–phenol–chloroform method (Chomczynski and Sacchi, 1987). Isolated RNAs were reverse transcribed to cDNA using random hexamers (Pharmacia, Uppsala, Sweden). The 16 sets of PCR primers shown in Table II and Figure 1 were synthesised according to the RB cDNA sequence described by McGee *et al.* (1989) using an Applied Biosystems model of 392 DNA synthesiser. These primers were designed to cover the coding region from exons 1 to 27 of the RB gene. The T7 RNA polymerase promoter sequence, TAATACGACTCACTATAGGG, was attached to the 5′ end of each upstream PCR primer to produce a single-strand RNA with the same sequence as the sense strand of the cDNA. PCR with each primer set provided PCR products of 150–300 base pairs, covering overlapping

Table I Clinical profile of bladder cancer and mutation of the RB gene

Case no.	Grade	Stage	RB gene mutation Exon	Codon	Amino acid change	LOH[a]
1	3	pT4N2M0	26	896-897	3 bp deletion[b]	NE
2[c]	3	pT1N0M0		ND[d]		NE
3	1	pTaN0M0		ND		–
4	1	pT1N0M0		ND		NE
5	1	pT1N0M0	20	693	1 bp deletion[e]	–
6	3	pT4N0M1		ND		NE
7	1	pT1N0M0		ND		NE
8	2	pT1N0M0		ND		H
9	2	pT1N0M0		ND		–
10	3	pT3N0M0		ND		–
11	1	pT1N0M0		ND		–
12	2	pT2N0M0	7	208	Met(ATG)→Val(GTG)	NE
13	2	pTaN0M0	4	158	Leu(TTG)→Ser(TCG)	–
14	3	pT1N0M0		ND		–
15[c]	2	pT1N0M0		ND		H
16	3	pT1N0M0		ND		H
17	1	pT1N0M0		ND		H
18	2	pTaN0M0	20	661	Arg(CGG)→Trp(TGG)	H
19	3	pT2N0M0	4	138	Ile(ATT)→Ser(AGT)	+
20[c]	1	pTaN0M0		ND		H
21	3	pT3N4M1		ND		+
22	3	pT4N1M0		ND		H
23[c]	2	pT1N0M0	20	677	1 bp deletion[e]	+
24	3	pT2N0M0		ND		–
25	1	pTaN0M0		ND		–
26	3	pT2N0M0		ND		H
27	2	pT2N2M0		ND		+
28	3	pT2N2M0	23	794	Ser(AGT)→Ile(ATT)	+
29	1	pTaN0M0		ND		–
30	1	pTaN0M0		ND		H

[a]LOH: H, homozygous (uninformative); +, LOH positive; –, heterozygous; NE, not examined. Primer set used in PCR-LOH assays was as follows: upstream, 5′ TTCC-AATGAAGAACAAATGG-3′; downstream, 5′GCAATTGCACAATCCAAGTT-3′, originally reported by McGee *et al.* (1990). [b]AAATTT (Lys-Phe)→ATT(Ile). [c]Recurrent case. [d]ND, not detected. [e]Novel stop codon (TGA) at codon 695 caused by these deletions.

Table II PCR primer sets for the RB gene

Set	Exon		Sequence of primers		Nucleotide number
a	1	5′	T7[a]-CTCTCGTCAGGCTTGAGTTT	3′	266-285
	4	5′	ATGGACACTGATTTCTATGT	3′	525-506
b	3	5′	T7-TTGACCTAGATGAGATGTCG	3′	461-480
	6	5′	GAAACTTTTAGCACCAATGC	3′	719-700
c	5	5′	T7-ACACAACCCAGCAGTTCGAT	3′	661-680
	8	5′	TGCTATCCGTGCACTCCTGT	3′	930-911
d	8	5′	T7-CCCATTAATGGTTCACCTCG	3′	871-890
	10	5′	GAAAGATTTTCAACCTCTGG	3′	1091-1072
e	9	5′	T7-GGACTTGTAACATCTAATGG	3′	1048-1067
	12	5′	CATAACAGTCCTAACTGGAG	3′	1275-1256
f	11	5′	T7-CCACACACTCCAGTTAGGAC	3′	1249-1268
	13	5′	CTGTCCCACAGCTTTAGCAA	3′	1446-1427
g	13	5′	T7-TAAAGCTGTGGGACAGGGTT	3′	1431-1450
	16	5′	TTACAACCTCAAGAGCGCAC	3′	1621-1602
h	16	5′	T7-TCTTTATTGGCGTGCGCTCT	3′	1591-1610
	17	5′	CTTGTCAAGTTGCCTTCTGC	3′	1769-1750
i	17	5′	T7-GGAACAGATTTGTCTTTCCC	3′	1663-1682
	18	5′	AATCTGAGAGCCATGCAAGG	3′	1837-1818
j	17	5′	T7-TCATGGAATCCCTTGCATGG	3′	1808-1827
	19	5′	TTTACACGCGTAGTTGAACC	3′	2006-1987
k	18	5′	T7-TCACACTGCAGCAGAT	3′	1935-1950
	20	5′	GAGATAGGCTAGCCGATACA	3′	2118-2099
l	19	5′	T7-CGCGTGTAAATTCTACTGCA	3′	1997-2016
	21	5′	ATTTGGTCCAAATGCCTGTC	3′	2246-2227
m	20	5′	T7-CACACCCTGCAGAATGAGTA	3′	2194-2213
	22	5′	GAATGTCTCCTGAACAGC	3′	2355-2338
n	22	5′	T7-ACAAGGATCTTCCTCATG	3′	2321-2338
	23	5′	ACTTGTAAGGGCTTCGAGGA	3′	2512-2493
o	23	5′	T7-CCTCACATTCCTCGAAGCCCTTACA	3′	2485-2509
	25	5′	CACTTCTTTTGAGCACACGGTCGC	3′	2725-2702
p	24	5′	T7-GGTGAATCATTCGGGACTTCTGAGA	3′	2644-2668
	27	5′	GCTTTTGCATTCGTGTTCGAGTAGA	3′	2878-2854

[a]Denotes that the fixed T7 promoter sequence of TAATACGACTCACTATAGGG was attached to the 5′ end of each upstream PCR primer.

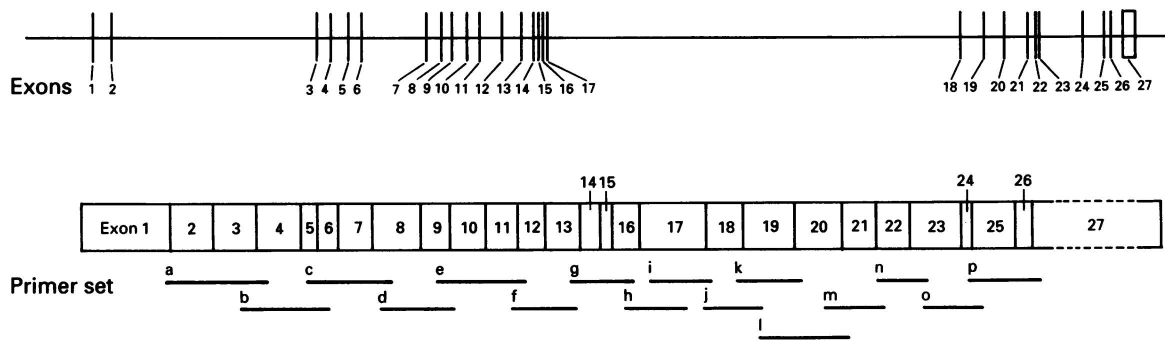

Figure 1 Exon map of the RB gene and locations of PCR primers in RB cDNA.

segments (Figure 1, Table II). The PCR was run in a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA) for 40 cycles using the following parameters; denaturation 95°C, 1 min; annealing 63°C, 1 min; elongation 72°C, 1 min. Aliquots of 3 µl of the 30 PCR products derived from their sets of PCR primers were analysed by agarose gel electrophoresis. Each PCR product was then transcribed to RNA with T7 RNA polymerase (Pharmacia) as described by Danenberg *et al.* (1992). Aliquots of 3 µl of the RNA transcripts produced by T7 RNA polymerase were electrophoresed on a 6% polyacrylamide gel (acrylamide:bis-acrylamide 19:1) in a Hoefer 650 dual-cooled PAGE unit (Hoefer Scientific Instruments, San Francisco, CA, USA). The running conditions of gel electrophoresis were 25 W per plate at 6–7°C with circulating ice-cooled water, as described previously (Danenberg *et al.*, 1992). The resultant gels were then stained with ethidium bromide and abnormal electrophoretic patterns were analysed under UV light.

Ligation of PCR products and DNA sequencing

PCR products were directly ligated to the plasmid vector, pCR 1000, in the TA cloning system (InVitrogen, San Diego, CA, USA), according to the manufacturer's protocol. At least ten colonies of the ligated PCR products were sequenced with Sequenase version 2.0 (United States Biochemical, Cleveland, OH, USA). The mutations were confirmed by the same protocols of ligation of PCR products and DNA sequencing.

RFLP analysis

DNA was extracted using a model 341 nucleic acid purification system (Applied Biosystems) from tissue samples. When genomic DNAs from both tumours and normal cells from venous blood from the same patients were available, DNA was amplified using a primer set flanking an *Xba*I RFLP site within intron 17 of the RB gene originally reported by McGee *et al.* (1990) (Table I). The PCR conditions were the same as for amplification of cDNA for exons of the RB gene. These PCR products were digested with *Xba*I (Takara Shuzo, Kyoto, Japan) into the two fragments of 630 and 315 bp if its recognition site (TCTAGA) was present. LOH appears as loss of the cleaved (630 + 315) or the uncleaved (945) allele in the tumour.

Results

SSCP and sequence analysis

We electrophoresed T7 transcription products from PCR samples derived from primer set (a) to (p) for RNA-SSCP analysis. The results of 30 bladder cancer samples are summarised in Table I. SSCP analysis revealed ten tumour samples suggestive of a mutation (Figure 2). Sequence studies of these ten PCR products revealed single missense mutations in five samples, small deletions in three samples (Figure 3, Table I) and no mutations in two samples. The types of point mutations were a T to G transversion at the second position of codon 138 (exon 4), a T to C transition at the second position of codon 158 (exon 4), an A to G transition at the

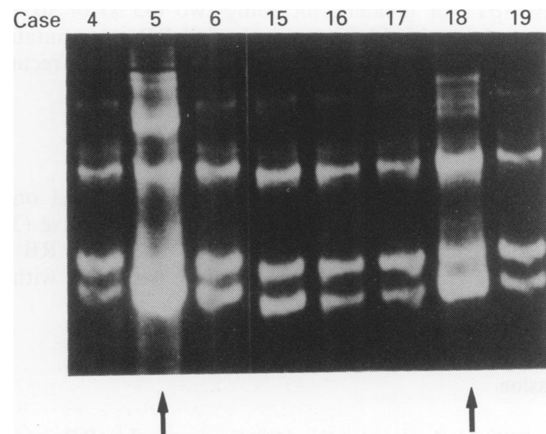

Figure 2 RNA-SSCP analysis of human bladder cancers with primer set (e). Abnormal electrophoretic patterns (arrowheads) are observed in cases 5 and 18.

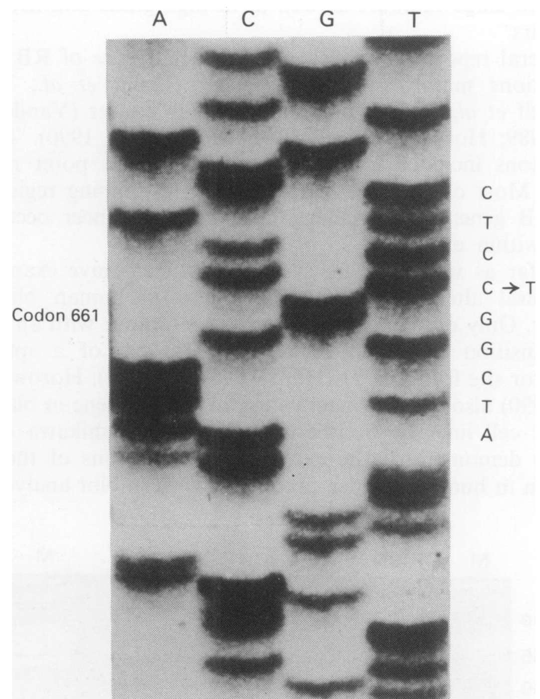

Figure 3 The DNA sequences of PCR products of case 18. The point mutation was identified, resulting in a change from arginine (CGG) to tryptophan (TGG) at codon 661 in the region of exon 20.

first position of codon 208 (exon 7), a C to T transition at the first position of codon 661 (exon 20) and an A to G transition at the first position of codon 794 (exon 23). Two cases with single base pair deletions were identified at the second or third position of codon 677 (exon 20) and at the first position of codon 693 (exon 20), and both these deletions resulted in generation of a novel stop codon at codon 695 in exon 20. The remaining one case with mutation was found to have a deletion of 3 bp (AAT) (codons 896 to 897) in exon 26. Comparisons of these sequences with DNA from matching normal tissues confirmed that the alterations were somatic events.

RB gene mutations were found in one (10%) of ten low-grade (grade 1) tumours, four (50%) of eight intermediate-grade (grade 2) tumours and three (25%) of 12 high-grade (grade 3) tumours. In relation to pathological stages, four (21%) of 19 superficial (pTa and PT1) and four (36%) of all invasive (pT2 or greater), including two (33%) of six cases with metastases, bladder cancers showed RB gene mutations. There were no mutations in all the five cases with recurrent bladder cancer.

Allelic loss of the RB gene

To detect RB–LOH, we used RFLP assays based on the PCR. Allelic loss of the RB gene was detected in five (33%) of 15 informative cases, including three cases with RB gene mutations (Figure 4, Table I). However, two cases with RB mutation did not have LOH.

Discussion

We investigated structural alterations of the RB gene in tissue specimens of 30 human bladder cancers by use of rapid and sensitive RNA–SSCP analysis. We found eight mutated cases, including five of single missense mutations and three of small deletions, and these mutations were found in low-grade and low-stage tumours as well as in high-grade and invasive tumours.

Several reports have identified the occurrence of RB gene alterations mainly in retinoblastoma (Dunn *et al.*, 1989; Yandell *et al.*, 1989) and small-cell lung cancer (Yandell *et al.*, 1989; Horowitz *et al.*, 1990; Mori *et al.*, 1990). These mutations included small deletions and single point mutations. Most of them occurred in the E1A-binding region of the RB gene; in particular, those in lung cancer occurred only within exons 20–23 of this gene.

So far as we know, no systematic studies have examined structural alterations of the RB gene in human bladder cancer. Only one report has shown one tumour with an A to G transition at intron 21 resulting in loss of a splicing acceptor site for exon 21 (Horowitz *et al.*, 1989). Horowitz *et al.* (1990) also showed inactivation of the RB gene in bladder cancer cell lines by Southern blot analysis. Ishikawa *et al.* (1991) demonstrated the existence of alterations of the RB protein in human bladder cancer by Western blot analysis or immunohistochemical staining. Since these studies showed that RB abnormalities were mainly observed in high-grade or high-stage bladder cancers, RB gene mutations were suggested to occur as late events. Several reports confirmed these findings and further suggested that altered RB expression might be useful clinical indicators for bladder cancers (Cordon-Cardo *et al.*, 1992; Logothetis *et al.*, 1992). However, a recent report by Xu *et al.* (1993) showed that a portion of low-grade or superficial bladder cancers had loss of RB protein expression, although the loss was more frequently seen in high-grade or invasive tumours. The present study systematically analysing the abnormalities in the entire coding region of the RB gene revealed the presence of RB gene mutations irrespective of tumour grade and stage. Therefore, RB gene alterations themselves were not specific to high-grade or invasive bladder cancers.

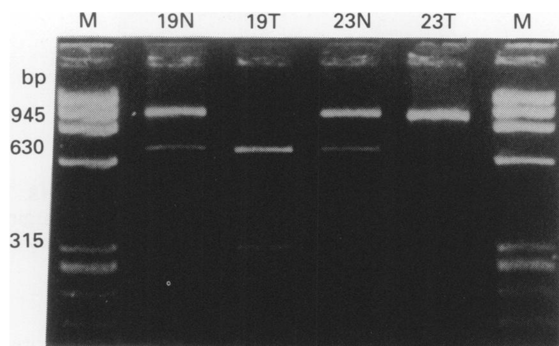

Figure 4 LOH demonstrated by PCR in cases 19 and 23. These products were run on an agarose gel, which was stained with ethidium bromide. N, normal tissue; T, tumour; M, marker.

Three mutations in this study occurred in the region of exon 20, which encodes the parts of E1A-binding region of the RB gene and is regarded as a 'hot-spot' in several malignancies. In the five other cases RB gene mutations were found in exons 4, 7, 23 and 26 which are outside this region. The biological significance of these regions has not been determined to date. Thus, the importance of the mutations in these regions needs to be clarified.

We also examined allelic loss of the RB gene in this study. Cairns *et al.* (1991) reported frequent loss of the RB gene in 162 bladder tumours using Southern blot analysis. That report showed that 3% of low-grade, 32% of intermediate-grade, 56% of high-grade, 4% of superficial and 57% of invasive bladder cancers had LOH at the RB locus, and concluded that RB-LOH strongly correlated with both tumour grade and muscle invasion. The incidence of RB loss in our results is similar to Cairns' results, but two mutated cases without LOH were identified. This discrepancy might be attributable to the possibility that normal tissues contaminated the tumour samples so that LOH could not be detected. However, separate RB mutations could have occurred in each RB allele.

We reported previously that structural alterations in the p53 tumour-suppressor gene occurred in the same 25 bladder cancer samples (cases 1–25) as detected by the RNA–SSCP analysis used here (Miyamoto *et al.*, 1993). Although p53 gene mutations were detected in six cases (cases 1, 6, 14, 21, 22 and 25), only one case has mutations of both the p53 and the RB genes (case 1). In addition, p53 alterations were mostly detected in high grade and high stage bladder cancers. In contrast, RB alterations were detected in low-grade and non-invasive tumours as well as in high-grade and invasive tumours in the present study. It is suggested that mutations of the RB gene are involved in earlier steps of bladder carcinogenesis than those of the p53 gene.

RB gene mutations detected by RNA–SSCP analysis in the region of exons 1–27 in 30 bladder cancer tissues were found in eight (27%) cases. This frequency might be underestimated for several reasons. One is amplification of cDNA from the normal stromal component of the tumour or contamination by normal tissue. Another is the fact that the SSCP method may not identify all mutations. It has recently been suggested that immunohistochemical staining is more sensitive and specific than examining RB gene mutations by molecular techniques (Zhang *et al.*, 1994). Therefore, immunohistochemical analysis should be performed both to clarify the relative value of these approaches and to strengthen the significance of the observation of RB mutations.

Finally, coexistence of abnormalities of multiple tumour-suppressor genes are commonly found in cancers (Murakami *et al.*, 1991), and cooperative roles for the RB and the p53 genes have been suggested (Shay *et al.*, 1991). Further studies are required to clarify the multistep process of bladder cancer development in relation to the alterations of tumour-suppressor genes, and it will be interesting to see whether mutations of the RB gene as well as other tumour-suppressor genes can be used as clinical predictors for determining prognosis and guiding treatment in patients with bladder cancer.

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