ORIGINAL ARTICLE

Detection of H-*ras* Gene Point Mutations in Transitional Cell Carcinoma of Human Urinary Bladder Using Polymerase Chain Reaction

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(Received for publication on January 9, 1992)

Abstract. It has been reported that the H-*ras* gene is activated as oncogene in human bladder cancer cases, and that codon 12 and codon 61 are the major "hot spots" of its activation. A simple method to detect point mutations in these codons of H-*ras* gene was established for the use of clinical diagnosis. In this method, the DNA segments including codon 12 or codon 61 were amplified by polymerase chain reaction (PCR), and the reaction products were examined for their susceptibility to the restriction enzyme *NaeI* or *BstNI*, and by dot blot hybridization assay with oligonucleotide probes. Point mutations were detectable in small amounts of DNAs isolated from fresh or frozen tumor tissues, urine cells and paraffin sections. The method was applied for a clinical sample and a case that had a point mutation at codon 12 of H-*ras* gene was detected. The point mutation was existed in DNAs of primary tumor tissue, all recurrent tumor tissues and cells isolated from urine of this case. (Keio J Med 41 (2): 80–86, June 1992)

Key words: oncogene, clinical diagnosis, bladder neoplasm

Introduction

Activated transforming genes have been found in a number of human tumors by use of transforming assay. In most cases the detected genes are members of the *ras* gene family, Harvey (H)-*ras*, Kirsten (K)-*ras* and N-*ras*, all of which encode closely related proteins of 189 amino acid residues designated p21.^{1,2} The mechanism by which *ras* genes have been activated in tumor cells involves a single point mutation, usually resulting in the alteration of codon 12 or codon 61 of these genes.^{1,3–7} Mutations in *ras* genes have been implicated in approximately 15% of all human tumors.^{8,9} The incidence of mutation of *ras* oncogene has been reported to be as high as 95% in pancreatic carcinoma¹⁰ and 40% in colon adenocarcinoma.¹¹ In comparison, the frequency of *ras* mutation in bladder cancer has been estimated at approximately 12%.¹² In urinary tract tumors H-*ras* oncogenes are more preferentially activated than N-*ras* or K-*ras* genes¹³ and estimation of the incidence is 5 to 17%^{13–15} using the NIH/3T3 transfection assay. As these studies utilized DNA transfection assay in which

some of the *ras* oncogenes may escape detection, the incidence of point mutation of H-*ras* gene in bladder cancer must be investigated by the other methods.

The examination of urine cytology is the most common way of screening the bladder cancer patients. This examination is simple and non-invasive for patients, though, false negative rate is high especially in the cases of low grade and low stage tumors. It is therefore important to develop a method to analyze a point mutation in tumor cells released in urine.

Recently, polymerase chain reaction (PCR)¹⁶ and hybridization assays with allele-specific oligonucleotide (ASO) probes have allowed precise analysis of point mutation in a wide variety of human cancer tissues and cell lines. A simple method to detect the point mutations of codon 12 and codon 61 of H-*ras* gene that might be acceptable for clinical use is developed. This method is useful to detect the point mutations in cells released in urine or paraffin sections of the bladder tumor tissues.

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Material and Methods

DNAs

Plasmids pP1, pKY-1 and pHs-49 were used for preliminary experiments. pP1 contains an 8.3-kbp (kilobasepair) DNA fragment of human placenta DNA including both normal codon 12 and codon 61 of *H-ras* gene.⁴ pKY-1 contains a 0.4-kbp DNA fragment of T24 cell line including mutated codon 12 of *H-ras* gene.⁷ pHs-49 contains a 6-kbp DNA fragment of Hs242 cell line including mutated codon 61 of *H-ras* gene.⁷ T24 is a cell line of human bladder cancer and Hs242 is a cell line of human lung cancer.⁷ Human bladder cancer cell line T24, human mammary carcinosarcoma cell line Hs578T and human malignant melanoma cell line SK2 have a point mutation at codon 12 (T24 and Hs578T) or codon 61 (SK2) of *H-ras* gene.^{17–19} T24 cells and Hs578T cells were obtained from Japanese Cancer Research Resources Bank (JCRB) and American Type Culture Collection (ATCC), respectively. SK2 cells were a generous gift from Dr Sekiya (National Cancer Center). All plasmids were also obtained from JCRB. Codon 12 of T24 cells and DNA fragment of pKY-1 are mutated to GTC (Val) from GGC (Gly) and codon 12 of Hs578T cells are mutated to GAC (Asp).^{17,18} while codon 61 of these DNAs are normal. Codon 61 of SK2 cells and DNA fragment of pHs-49 are mutated to CTG (Leu) from CAG (Gln), while codon 12 of these DNAs are normal.¹⁷ Frozen specimens of bladder cancer tissues, specimen of adrenal gland tissue and urine cells were obtained from Department of Urology, Tachikawa Kyosai Hospital. Histological classification and grading of malignancies were done according to the General Rules for Clinical and Pathological Studies on Bladder Cancer by the Japanese Urological Association. Frozen specimens of bladder cancer and urine cells were used for the clinical trial of the established technique. DNA isolated from normal adrenal gland tissue was used as a normal control, whose codon 12 and codon 61 are GGC (Gly) and CAG (Gln), respectively. Paraffin sections of bladder cancer tissues were also used to review the previous DNA in patients who had recurrence of tumor.

DNA isolation from cell lines, frozen or fresh specimens and urine cells were performed as follows. About 100 to 200 mg of tissue or an aliquot of cells were homogenized on ice. One milliliter of STE (10 mM Tris-HCl pH8.0, 100 mM NaCl, 1 mM EDTA pH8.0), 50 μ l of 20% SDS and 20 μ l of proteinase K (20 mg/ml) were added to homogenized tissue and incubated at 37°C overnight. This solution was extracted with phenol and chloroform (with 1/24 of isoamylalcohol) before precipitating DNA with ethanol. DNA was dissolved in TE (10 mM Tris-HCl pH7.5, 1 mM EDTA) and kept at 4°C. DNA isolation from paraffin sections was performed as follows.

The paraffin blocks were sliced in 150 to 200 μ m thick and fixed on slide glasses. Paraffin was washed out by soaking the slide glasses into xylene twice for 10 min, 100% ethanol twice for 5 min, 90% ethanol for 5 min, 80% ethanol for 5 min and into 70% ethanol for 5 min. One milliliter of STE, 50 μ l of 20% SDS and 20 μ l of proteinase K (20 mg/ml) were added to the deparaffinized tissue which was shaved off from slide glasses. After placing at 37°C overnight, they were extracted with phenol and chloroform (with 1/24 of isoamylalcohol) before precipitating DNA with ethanol.

Polymerase chain reaction

Twenty-bases oligonucleotide primers 5'GACG GAATATAAGCTGGTGG3' and 5'AATACGAC CCCACTATAGAG3' were used to amplify a 109-bp segment of exon 1 containing codon 12 and primers 5'AGAGGCTGGCTGTGTGAACT3' and 5'AAG TCTTTTGAGGACATCCA3' were used to amplify a 239-bp segment of exon 2 containing codon 61. All four primers were synthesized by a DNA synthesizer (CYCLONE, Biosearch) and purified by ion-exchange chromatography (Mono Q, Pharmacia), lyophilized and dissolved in TE.

The reaction mixture containing 500 ng of genomic DNA or 0.1 ng of plasmid DNA, 50 pmol of each primer, 0.2 mM of each dNTP, 10 mM Tris-HCl (pH8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% gelatin and 1 unit of Taq polymerase (Ampli Taq; Perkin Elmer Cetus, California USA) in a total volume of 50 μ l. The mixture was incubated at 96°C for 1 min to denature, 55°C for 1 min to anneal, 72°C for 2 min for extension, and 40 cycles of denaturation, annealing and extension of DNA were repeated by Thermal Cycler (Perkin Elmer Cetus) to amplify the segment including codon 12. The same mixture was incubated at 94°C for 30 sec to denature, 58°C for 1 min to anneal, 72°C for 2 min for extension, and 35 cycles were repeated to amplify the segment including codon 61.

Restriction enzyme digestion

*Nae*I cleavage site (GCCGGC) overlaps the nucleotides of codon 12 of *H-ras* gene. This *Nae*I site is lost when a mutation in codon 12 occurs. Two *Bst*NI cleavage sites (CCAGG and CCTGG) are present in the DNA segment including codon 61 of *H-ras* gene, one overlaps the nucleotides of codon 61 and the other overlaps the nucleotides unrelated to codon 61. One of these is lost when the mutation of codon 61 occurs except in the cases whose mutation is CTG. *Hpa*II cleavage site (CCGG) overlaps first two nucleotides of codon 12. This *Hpa*II site is lost when mutation occurs in the first two nucleotides of codon 12.

The reaction mixture for the digestion with these restriction enzymes contained about 40 ng of amplified DNA, 2 units of restriction enzyme and 1 μ l of 0.1% bovine serum albumin in a total volume of 9 μ l. This mixture was incubated at 37°C for 1 hr.

Electrophoresis

The DNA was resolved by electrophoresis in an 8% or 10% polyacrylamide gel with 120V for 45 min and stained with ethidium bromide.

Dot blot hybridization

Ten and 5 ng of amplified DNA in 3 μ l was applied to nylon filter and denaturation was done with alkali. The filters were prehybridized overnight at 37°C in 5 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl, 0.015 M sodium citrate pH7.0), 0.1% SDS, salmon sperm DNA (100 μ g/ml) and 5 $\times$ Denhardt's solution (1 $\times$ Denhardt's solution = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin). Allele-specific oligonucleotide probes were purchased and radiolabeled by [γ - 32 P] ATP using T4 polynucleotide kinase according to the manufacture's instruction (Takara). The filters were hybridized overnight at 37°C in the same solution plus the end-labeled oligomer probes. Filters were washed twice in 2 $\times$ SSPE (1 $\times$ SSPE = 10 mM sodiumphosphate pH7.2, 0.18 M NaCl, 1 mM EDTA) containing 0.1% SDS for 15 min at room temperature. Subsequently, the filters were rinsed in TCMA buffer (3 M tetramethylammonium chloride, 50 mM Tris-HCl pH8.3, 2 mM EDTA, 0.1% SDS) for 30 min at 63°C. The filters were soaked in 2 $\times$ SSPE and 0.1% SDS for 10 min at room temperature and autoradiographed at -80°C overnight.

Detection of mutated DNA mingled with normal DNA

This is model experiment for the detection of small quantity of mutated DNA which may be contained in DNA isolated from cells in urine of the bladder cancer patient. DNAs isolated from T24 cells and normal adrenal tissue were mixed at each consistency as shown in Figure 4. PCR was performed in adequate condition and DNA fragments including codon 12 were amplified. Amplified DNA was digested by *NaeI* to clarify the DNA with point mutation at codon 12, which may remain undigested.

Results

DNA fragments corresponding to codon 12 or codon 61 of H-ras gene were generated by PCR amplification of various recombinant plasmids. These DNA fragments were separated by polyacrylamide gel electrophoresis (8%) and visualized with ethidium bromide staining.

Plasmids pP1 and pKY-1 were amplified with PCR and the DNA fragments of 109 bp including codon 12 were produced, while plasmids pP1 and pHs-49 were amplified and the DNA fragments of 239 bp including codon 61 were produced (Fig 1).

Upon incubating the amplified DNA fragments including codon 12 with *NaeI*, DNA fragments of pP1 were split into two DNA bands of 78 bp and 31 bp, while DNA fragments of pKY-1 remained undigested (Fig 2A). Upon incubating the amplified DNA fragments including codon 61 with *BstNI*, both DNA fragments of pP1 and pHs-49 were digested to three DNA bands of 124 bp and 100 bp and 15 bp, although the smallest DNA band was not apparent in the gel (Fig 2B). The cloned H-ras gene in the plasmid pKY-1 has a point

Fig 1 Analysis of amplified DNA including codon 12 (109bp) and codon 61 (239bp). DNA segments including codon 12 of pKY-1 (lane a and b) and pP1 (lane c and d), and DNA segments including codon 61 of pHs-49 (lane e and f) and pP1 (lane g and h) were amplified with PCR. *HaeIII* digested ϕ X174 was used as size marker. The DNA bands were analyzed by polyacrylamide gel electrophoresis (8%), stained with ethidium bromide, and photographed.

Fig 2 Analysis of DNA digested with restriction enzymes. (A) Bands of amplified DNA fragments including codon 12 of pKY-1 (lane a) and pP1 (lane b) are shown. *NaeI* digested the DNA fragments including codon 12 into two bands of 78 bp and 31 bp in pP1 (lane d), while pKY-1 remained undigested (lane c). (B) Bands of amplified DNA fragments including codon 61 of pHs-49 (lane e) and pP1 (lane f) are shown. *BstNI* digested the DNA fragments including codon 61 into two large bands of 124 bp and 100 bp, and one small band of 15 bp which was not visible in this gel, in both pHs-49 (lane g) and pP1 (lane h). *HaeIII* digested ϕ X174 was used as size marker. The DNA bands were analyzed by polyacrylamide gel electrophoresis (10%), stained with ethidium bromide, and photographed.

mutation of GTC at codon 12 and the cloned *H-ras* gene in the plasmid pP1 has a normal nucleotide sequence containing the *NaeI* restriction cleavage site (GCCGGC). The cloned *H-ras* gene in the plasmid pHs-49 has a point mutation of CTG at codon 61 and the cloned *H-ras* gene in the plasmid pP1 has a normal codon of CAG, both contains two *BstNI* restriction cleavage sites (CCTGG and CCAGG).

When DNA fragments were not digested with *NaeI*, dot blot hybridization assays were performed to determine the base substitution of codon 12 utilizing the allele-specific oligonucleotide probes GGC and GTC. The amplified DNAs of plasmids pP1 and pKY-1 were used for confirming the codon 12 sequence in each of the cloned *H-ras* gene. Restriction enzyme *BstNI* digestion cannot detect the point mutation of codon 61 if it is mutated to CTG. In these cases, dot blot hybridization assay utilizing the synthetic oligonucleotide probes CAG and CTG were performed. It was confirmed that codon 12 of pP1 was wild type GGC (Gly), while codon 12 of pKY-1 was mutated to GTC (Val) (Fig 3). Similarly, codon 61 of pHs-49 was mutated to CTG (Leu) and codon 61 of pP1 was normal CAG (Gln). Thus, this method using PCR, restriction enzyme and dot blot hybridization is simple for the determination of point mutations in the codon 12 or codon 61 of *H-ras* gene.

Detection of the *H-ras* gene point mutation mingled in substantial amounts of normal DNA was attempted using various samples of DNA mixtures (Fig 4). DNA mixtures were amplified with PCR and digested by *NaeI*

(Fig 4). DNA component with the point mutation in codon 12, which was derived from human bladder cancer T24 cells, remained undigested. While normal DNA component, which was derived from normal adrenal gland tissue, was digested to bands of 78 bp and 31 bp. This model experiment indicates that the method is sensitive enough to identify 1% of mutated DNA mingled in normal DNA. This suggests that as little as 1% of cancer cells mingled in normal cells are detectable by this method.

The method was applied for clinical samples such as frozen or paraffin blocked specimens and urine cells from a bladder cancer patient. A 44-year-old male patient first had transurethral resection of bladder tumor (TUR-BT) in November 1988 (Primary). The intravesical tumor recurrence was noted three times: June 1989 (Recurrence-1), August 1989 (Recurrence-2) and January 1990 (Recurrence-3). All tumors were resected by TUR and they were all pathologically diagnosed as transitional cell carcinoma, papillary non-invasive type. The primary and recurrence-1 tumors were grade 1 and recurrence-2 and recurrence-3 tumors were grade 2. Their clinical stages were all pT₁. DNAs were isolated from frozen tumor specimens resected by three times of TUR-BT.

Fig 3 Characterizaion of point mutations at codon 12 and codon 61 of *H-ras* gene. Ten (a, c, e and g) and 5 ng (b, d, f and h) of amplified DNA in 3 μ l was dotted onto 4 filters. Line 1 and 3 are pP1, line 2 is pKY-1 and line 4 is pHs-49. Dot blot hybridization was done using codon 12 allele-specific oligonucleotide probe GGC (a and b) and GTC (c and d), or codon 61 probe CAG (e and f) and CTG (g and h).

Fig 4 Detection of mutant *H-ras* allele mingled with normal allele. DNA from T24 cell and adrenal cell (Adr) were mixed at each consistency as shown in table and amplified with PCR (A). Each amplified DNA was digested by restriction enzyme *NaeI* (B). *HaeIII* digested ϕ X174 was used as size marker. The DNA bands were analyzed by polyacrylamide gel electrophoresis (A: 8%, B: 10%) stained with ethidium bromide, and photographed.

Also DNAs were isolated from pathologically non-cancer tissue resected at the same time with the operation of recurrence-2 (Normal-2), cells taken from urine just before the operation of recurrence-2 (Cell-2) and recurrence-3 (Cell-3), and paraffin sections of primary cancer tissue. All these DNAs were amplified with PCR before digesting by restriction enzyme *NaeI* and *HpaII* (Fig 5A). *NaeI* digested the DNA fragments of normal-2 into two bands of 78 bp and 31 bp, and *HpaII* digested them into bands of 79 bp and 30 bp. These restriction enzymes digested some DNA fragments of recurrence-2 and recurrence-3, while DNAs of recurrence-1, cell-2, cell-3 and paraffin sections of the primary tumor tissue appeared to be undigested (Fig 5B). Tumor tissues contain non-cancer components as interstitial cells or blood cells. DNAs from those normal cells included in tumor tissues were digested by *NaeI* or *HpaII*, while DNAs from tumors remained undigested. These results showed that primary and all recurrent bladder cancer tissues had the point mutation at codon 12 of H-ras gene, while pathologically non-cancer tissue exhibited no point mutation. The point mutation was also detected in DNAs from cells released in urine. To determine the base substitution of this case, dot blot hybridization assays were performed for DNAs of the tumor tissue of this case, T24 cells, Hs578T cells and normal adrenal gland tissue utilizing the allele-specific oligonucleotide probe sets. Figure 6 shows that nucleotide sequence in the codon 12's of T24 cells, Hs578T cells and normal adrenal gland tissue are GTC, GAC and wild type GGC, respectively, while the codon 12 in the tumor case is mutated to AGC. This mutation leads to the substitution of serine for glycine at codon 12 of H-ras gene.

Discussion

Activated transforming genes have been found in a number of human tumors and molecular characterization has revealed many of them are the member of the *ras* gene family, which are H-ras, K-ras and N-ras. H-ras gene which locates on the short arm of chromosome 11²⁰ is reported to be most commonly activated in cases of human urinary tract tumors.¹³ The mechanism by which *ras* genes are activated in tumor cells involves a single point mutation.^{3,7}

The incidence of point mutation of H-ras gene in bladder cancer is estimated to be 5 to 17%¹³⁻¹⁵ using the NIH/3T3 transfection assay. This rate is rather low compared with other cancers such as pancreatic cancer and colorectal cancer.^{10,11} The DNA transfection assay is likely to miss the detection of point mutation because greater than 1,000-fold differences are found in transforming efficiency among members of the mutated *ras* genes.²¹ The *ras* gene mutations in bladder cancer may be found in different rate if investigated

Fig 5 Restriction enzyme analysis of amplified DNA of a clinical case. Genomic DNA prepared from three recurrent tumor tissues; Recurrence-1 (lane f, h and i), Recurrence-2 (lane a, j and k), Recurrence-3 (lane d, l and m), pathologically non-tumor tissue resected simultaneously with Recurrence-2; Normal-2 (lane b, n and o), cells taken from urine just before the operation of TUR-BT resecting Recurrence-2 and Recurrence-3; Cell-2 (lane c, p and q) and Cell-3 (lane e, r and s), and paraffin sections of primary tumor tissue (lane g, t and u). A: analysis of amplified DNA including codon 12 and B: restriction enzyme analysis of amplified DNA. Lane h, j, l, n, p, r and t are digestion of *NaeI* and lane j, k, m, o, q, s and u are digestion of *HpaII*. *HaeIII* digested ϕ X174 was used as size marker. The DNA bands were analyzed by polyacrylamide gel electrophoresis (A: 8%, B: 10%), stained with ethidium bromide, and photographed.

Fig 6 Characterization of point mutations at codon 12 of H-ras gene. Ten and 5 ng of amplified DNA in 3 μ l was dotted onto seven filters. Number 1 is a clinical case, 2 is T24 cell line, 3 is Hs578T cell line and 4 is normal adrenal gland tissue. Dot blot hybridization was done using seven types of allele-specific oligonucleotide probes as noted; GGC(a), GTC(b), GAC(c), GCC(d), AGC(e), TGC(f) and CGC(g).

with other methods.

The development of techniques for the detection of molecular lesions made possible to diagnose many germline and somatic disease. PCR is one of such techniques that provide simple, accurate and rapid assay. There are great merits for the diagnosis of bladder cancer if its occurrence can be detected by the methods related to molecular biology. It is desirable to establish a simple method for reinspection of the incidence of point mutations in *ras* gene. And a simple assay to detect and characterize *ras* gene mutation should be useful for further understanding their roles in carcinogenesis.

H-ras oncogenes are more preferentially activated in urinary tract tumor as compared to *N-ras* or *K-ras* genes,¹³ and codon 12 and codon 61 of the *H-ras* gene are the "hot spots" for the incidence of point mutation.^{1,3–7} In this study, detection of point mutations at codon 12 or codon 61 of *H-ras* gene was attempted by the simple method involving PCR amplification. Adequate conditions for PCR, restriction enzyme digestion and dot blot hybridization were established using cloned *H-ras* gene DNAs as a model system. Then, DNAs isolated from fresh or frozen tumor specimens of bladder tumor were used. An *H-ras* gene point mutation in frozen specimen, urine cells and paraffin block specimens was readily detected by our method. In one clinical case, the *H-ras* gene point mutation existed in recurrent tumors which were noted only a few months after the previous operation. This point mutation might have existed in DNA of visually or pathologically non-cancer bladder tissue at the time of first operation. If the biopsy specimen was taken from that place, the mutation might have been detected. It is widely believed that *ras* gene mutation is an early initiating event in tumorigenesis.^{11,22–26} The data that the point mutation was detected in urine cells and that 1% of mutated DNA mingled in normal DNA was detectable, suggest the possibility of molecular diagnosis for the occurrence of bladder cancer in an early stage.

In the cases of *K-ras* gene in colorectal cancer, same mutation was found in tumors diagnosed as adenoma and carcinoma.^{11,26,27} It was suggested that the mutation preceded the development of malignancy from a pre-existing adenoma in most cases and therefore the *K-ras* gene mutation might be involved in the early stages of human colorectal tumorigenesis.²⁷ It also suggested that DNA from pathologically diagnosed dysplasia of bladder mucosa, which was considered to be precancer, might contain some kinds of gene abnormalities like point mutations of *ras* genes. The incidence of mutations in *ras* genes in the bladder cancer is significantly lower than that of colorectal cancer,^{11,27} and therefore bladder cancer and colorectal cancer may arise through different pathogenic mechanisms, that would not be detected by the usual assay. Attempts to discover new oncogenes or

tumor suppressor genes that might be involved in the bladder cancer formation are indispensable for further investigation.

Epidemiological studies have indicated that bladder cancer can be associated with exposure of carcinogen. It is well known that N-methyl N-nitrosourea (MNU) leads to G·C to A·T base transition in codon 12.²³ 7,12-dimethyl-benzanthracene (DMBA) results in mutations of adenine residues in codon 61.²⁸ Zarbl *et al*²⁵ detected activated *H-ras* oncogenes in MNU-induced rat mammary carcinoma and DMBA-initiated mouse skin carcinoma.²⁹ Workers exposed to some organic compounds are thought to have a high risk for the occurrence of bladder cancer, and therefore examining the incidence of a point mutation in *H-ras* gene of the bladder cancer tissues from these workers will be of great interest.

The method described in this report provides simple, sensitive and accurate analysis of *H-ras* gene mutation. The method appears useful for the early diagnosis of occurrence or recurrence of bladder cancer.

Acknowledgements: The author is grateful for a direction of Dr Nobuyoshi Shimizu, Professor of the Department of Molecular Biology, School of Medicine, Keio University, and for a guidance of Dr Hiroshi Tazaki, Professor of the Department of Urology, School of Medicine, Keio University. He is also deeply indebted to Dr Ichiro Nagakubo, Chief of the Department of Urology, Tachikawa Kyosai Hospital, Dr Makoto Hata, Associate Professor of the Department of Urology, School of Medicine, Keio University, and to Dr Jun Kudoh, Dr Ryuichi Fukuyama and Mr Kosuke Sakai, members of the Department of Molecular Biology, School of Medicine, Keio University for their great advice. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education Science and Culture (No 02454374).

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