**RUNX3 Inactivation by Point Mutations and Aberrant DNA Methylation in Bladder Tumors**

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**Abstract**

RUNX3 is inactivated at high frequency in many tumors. However, in most cases, inactivation is caused by silencing of the gene due to promoter hypermethylation. Because epigenetic silencing is known to affect many major tumor suppressor genes in cancer cells, it is not clear whether RUNX3 is primarily responsible for the induction of carcinogenesis in these cases, except for the gastric cancer cases that we reported previously. We investigated genetic and epigenetic alterations of RUNX3 in 124 bladder tumor cases and seven bladder tumor–derived cell lines. Here we show that RUNX3 is inactivated by aberrant DNA methylation in 73% (90 of 124) of primary bladder tumor specimens and 86% (six of seven) of bladder tumor cell lines. In contrast, the promoter regions of 20 normal bladder mucosae were unmethylated. Importantly, one patient bore missense mutations, each of which resulted in amino acid substitutions in the highly conserved Runt domain. The mutations abolished the DNA-binding ability of RUNX3. A second patient had a single nucleotide deletion within the Runt domain coding region that resulted in truncation of the protein. RUNX3 methylation was a significant risk factor for bladder tumor development, superficial bladder tumor recurrence, and subsequent tumor progression. These results strongly suggest that inactivation of RUNX3 may contribute to bladder tumor development and that promoter methylation and silencing of RUNX3 could be useful prognostic markers for both bladder tumor recurrence and progression. (Cancer Res 2005; 65(20): 9347-54)

**Introduction**

Over 70% of human bladder tumors are superficial transitional cell carcinomas that can be treated by transurethral resection. However, the majority of these tumors recur. More importantly, ~15% will progress to invasive disease with poor prognosis (1). However, conventional histopathologic evaluation, encompassing tumor grade and stage, is inadequate to accurately predict the behavior of most bladder tumors. The need to establish which superficial tumors will recur or progress and which invasive tumors will metastasize has led to the identification of a variety of potential prognostic markers for bladder tumor patients. Important determinants of population risk to bladder tumor may be mutation of proto-oncogenes and tumor suppressor genes, loss of heterozygosity for specific alleles, and methylation patterns of promoter regions in specific genes (2–4). Genetic alterations associated with bladder tumor have been described (5, 6), such as mutations in c-erbB-2, EGFR, c-myc, cyclin D1, H-ras, p53, p16\(^{INK4A}\), and Rb; frequent deletions involving the 2q, 3p, 4q, 5q, 6q, 8p, 9p, 9q, 11p, 11q, 13q, and 17p loci; and frequent gains of the 1q, 3q, 5p, 6p, 8q, 17q, and 20q loci and of chromosome 7. However, a practically useful marker gene that is able to accurately predict the clinical course of bladder tumors has not yet been identified.

Alteration of DNA methylation in CpG islands is emerging as a key event in the inheritance of transcriptionally repressed regions of the genome. The direct relationship between the density of methylated cytosine residues in CpG islands and local transcriptional inactivation has been widely documented (7). Transcriptional repression by DNA methylation is mediated by changes in chromatin structure. Specific proteins bound to methylated DNA recruit a complex containing transcriptional corepressors and histone deacetylases (8). Histone deacetylation results in chromatin compaction and hence transcriptional inhibition.

Tumor cells exhibit genome-wide hypomethylation accompanied by region-specific hypermethylation (7, 9). Inactivation of gene expression by abnormal methylation of CpG islands can act as a “hit” for tumor generation (7, 9). Many tumor suppressor genes contain CpG islands and show evidence of methylation-specific silencing. The Rb gene was the first tumor suppressor gene for which hypermethylation was linked to tumorigenesis, and about 9% of retinoblastomas exhibit hypermethylation of the Rb 5’ region (10). Thus far, several genes, including p16, RAR-β, H-cadherin, DAPK, and RASSF1A, have been reported to undergo methylation in bladder tumor (11–17). In some of these tumors, hypermethylation is associated with loss of heterozygosity, and in others, it affects both alleles. It has recently been recognized that aberrant hypermethylation events can occur early in tumorigenesis, predisposing cells to malignant transformation.

Runt domain transcription factors (RUNXs) are homologous to products encoded by the *Drosophila* segmentation genes *runt* and *lozenge*. They contain a conserved region termed the Runt domain, which is required for dimerization with a β subunit and for the recognition of cognate DNA-binding sequences (18). The RUNX gene family consists of three members, RUNX1/AML1, RUNX2, and RUNX3 (19). A single gene encodes the β subunit, *CEBβ/PEBP2β* (20, 21). All three RUNX family members play important roles in normal developmental processes and in carcinogenesis (19, 22, 23). The RUNX1 locus, required for definitive hematopoiesis, is the most frequent target of chromosome translocation in leukemia and is responsible for about 30% of the cases of human acute leukemia (24). RUNX2, essential for osteogenesis, is involved in the human disease cleidocranial
dysplasia, an autosomal-dominant bone disorder (25–29). The \textit{RUNX3} gene is located on human chromosome 1p36, a region that has long been suspected to harbor one or more suppressors of various tumors (30). Recent studies suggested that \textit{RUNX3} is one of the tumor suppressors located in this region (31).

Here, we show that inactivation of \textit{RUNX3} which is caused mainly by epigenetic alteration is closely associated with bladder tumor development, recurrence, and progression. Furthermore, we identify two bladder tumor patients bearing point mutations within the \textit{RUNX3}-coding region; these mutations abolish the sequence-specific DNA-binding ability of the protein. Our results suggest that inactivation of \textit{RUNX3} is associated with bladder tumor development and that this feature could be used as a diagnostic and prognostic marker.

**Materials and Methods**

**Cell lines and tissue samples.** The human bladder tumor cell lines EJ, 5637, T24, J82, UMUC3, TCCSUP, and HT1197 were obtained from the Korea Research Institute of Bioscience and Biotechnology (Daejon, South Korea). All media (Invitrogen, Carlsbad, CA) were supplemented with 10% fetal bovine serum (Invitrogen) and cells were grown at 37°C in a 5% CO$_2$ atmosphere.

Fresh bladder tissues were obtained from the Department of Urology, Chungbuk National University Hospital, Cheongju, South Korea. The samples comprised 124 bladder tumor specimens and 20 normal bladder mucosae. The samples comprised 124 bladder tumor specimens and 20 normal bladder mucosae. The patients (113 males and 11 females) had a mean age of 64.7 ± 11.5 years and were recruited in the same department. Normal subjects (17 males and three females) had a mean age of 61.9 ± 14.1 years and were also recruited in the same department for benign diseases, mainly benign prostatic hyperplasia and stress urinary incontinence. The normal controls were determined to be tumor free as judged by urine cytology.

**Figure 1. \textit{RUNX3} methylation in bladder tumor cell lines.** A, nucleotide sequence of the \textit{RUNX3} exon 1 region, from 211 to 61 from the translation initiation codon. CpG dinucleotides are indicated in bold. B, methylation-specific PCR (top) and RT-PCR (bottom) analyses of \textit{RUNX3} in bladder tumor cell lines. Normal bladder tissue was used for a control. Abbreviations: M, methylation-specific PCR product; U, methylation-nonspecific PCR product. C, methylation of \textit{RUNX3} in bladder tumor cell lines. DNAs from seven bladder tumor cell lines were analyzed by methylation-specific PCR followed by direct DNA sequencing. Methylated cytosines remain cytosines, whereas unmethylated cytosines are converted to thymidine. Absence of \textit{RUNX3} expression (−) and presence of \textit{RUNX3} expression (+). D, reactivation of \textit{RUNX3} expression. Cells were cultured for 3 days in the presence or absence of 5-aza-2’-deoxycytidine (5-AZA, 2 μmol/L). RT-PCR was done using the RX3-A5 and RX3-A3 primers.
(absence of malignant cells) and by cystoscopic examination of the bladder during surgery. Informed consent was obtained from each subject, and the study was approved by the Institutional Review Board of Chungbuk National University College of Medicine. In this study, superficial recurrence is defined as a recurrence of primary superficial bladder tumor without progression, and progression is defined as the progression of superficial bladder tumors to invasive or metastatic disease, or of invasive tumors to metastatic disease after adequate treatment. All specimens were rapidly frozen in liquid nitrogen and stored at −80°C until DNA and RNA extraction.

**Plasmids and preparation of bacterially expressed proteins.** pET-28a(+)-RX3 and pET-28a(+)-RX3-mt4 were constructed by subcloning wild-type and mutant RUNX3 cDNA (amino acids 44-200; SWISSPROT: Q13761-1) into pET-28a(+) (Novagen, Madison, WI). pET-28a(+)-CBFh2, which expresses His-tagged full-length human CBFh2, was similarly constructed. Proteins were expressed in the bacterial strain BL21 and purified with Ni-NTA columns (Qiagen, Hilden, Germany) as suggested by the manufacturer. The concentration of each protein was determined by SDS-PAGE followed by Coomassie blue staining.

**Methylation-specific PCR.** Genomic DNA was extracted by standard methods using the Wizard Genomic DNA Purification System (Promega, Madison, WI). Methylation-specific PCR of the RUNX3 exon 1 region (between 267 and 56 from the translation initiation site of the P2 isoform) was done as previously reported (32). The Rx3-5W (5′-GAGG-GGGTGTCGTACGCGGG-3′) and Rx3-3W (5′-CGGCCGGCCGCGTCG-3′) primer set was used for untreated DNA. The Rx3-5M (5′-GAGGGGTGGTTGTATGTGGG-3′) and Rx3-3M (5′-AAAACGACCGACGCGAACGCCTCC-3′) primer set was used for detecting methylated DNA. The Rx3-5U (5′-GAGGGGTGGTTGTATGTGGG-3′) and Rx3-3U (5′-AAAACGACCGACGCGAACGCCTCC-3′) primer set was used for detecting unmethylated DNA.

### Table 1. Relationship between RUNX3 methylation status and stage, likelihood of recurrence, and progression of bladder tumor

<table>
<thead>
<tr>
<th>Variable</th>
<th>Methylation status</th>
<th>Unmethylated</th>
<th>Methylated</th>
<th>P</th>
<th>OR (95% CI)</th>
</tr>
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<tr>
<td>Normal versus tumor (n = 124)</td>
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<tr>
<td>Normal bladder mucosa</td>
<td></td>
<td>20/20 (100%)</td>
<td>0/20 (0%)</td>
<td>&lt;0.01</td>
<td>107.55 (6.33-1827.39)</td>
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<tr>
<td>Bladder tumor</td>
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<td>34/124 (27.42%)</td>
<td>90/124 (72.58%)</td>
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<td></td>
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<tr>
<td>Stage (n = 124)</td>
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<tr>
<td>Superficial</td>
<td></td>
<td>27/78 (34.62%)</td>
<td>51/78 (65.38%)</td>
<td>0.01</td>
<td>2.95 (1.16-7.47)</td>
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<tr>
<td>Invasive</td>
<td></td>
<td>7/46 (15.22%)</td>
<td>39/46 (84.78%)</td>
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<td>Recurrence (n = 73)</td>
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<tr>
<td>No</td>
<td></td>
<td>21/46 (35.94%)</td>
<td>25/46 (64.06%)</td>
<td>0.02</td>
<td>3.70 (1.19-11.46)</td>
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<td>5/27 (15.22%)</td>
<td>22/27 (84.78%)</td>
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<td>Progression (n = 101)</td>
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<td></td>
<td>23/74 (31.08%)</td>
<td>51/74 (68.92%)</td>
<td>0.01</td>
<td>5.63 (1.23-25.82)</td>
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<tr>
<td>Yes</td>
<td></td>
<td>2/27 (7.41%)</td>
<td>25/27 (92.59%)</td>
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</table>

**Figure 2.** RUNX3 methylation in primary bladder tumor tissues. A, the RUNX3 methylation status of 124 primary bladder tumors was examined by MS-PCR. Ninety cases (73%) were positive for RUNX3 methylation. Typical results. B, bisulfite-modified DNA was amplified and sequenced. An example of a direct DNA sequencing chromatogram. Methylated (red arrow), unmethylated (blue arrow), and partially methylated cytosines (green arrow). C, frequency of methylation of each CpG dinucleotide within the sequenced region.
RNA isolation, reverse transcription-PCR, and DNA sequencing. Total RNAs were obtained from various bladder tumor cell lines and tissues including bladder tumors and normal bladder mucosa by the method of Chomczynski and Sacchi (33). cDNAs were synthesized from 1 μg total RNA by random priming using a First-Strand cDNA Synthesis Kit (Amersham Biosciences, Buckinghamshire, United Kingdom). RUNX3 cDNA was amplified by PCR with the sense primer RX3-A5 (5′-ATGGCTATCCCGCCAAGAGGAA-3′) and the antisense primer RX3-A3 (5′-AGGCGTTCCAGGTTCCAGAA-3′). cDNA was extracted from the gel, purified with a QIAquick Gel Extraction Kit (Qiagen) and sequenced by the dye terminator DNA sequencing method (BIONEX, Hanyang University, South Korea). As a control, GAPDH cDNA was amplified using the sense primer h-GAPDH-F (5′-ACCACAGTCATGCC ATCAC-3′) and the antisense primer h-GAPDH-B (5′-TCCACACCTGTGTTGTA-3′). Semiquantitative PCR amplification was done in a Perkin-Elmer-Cetus 9700 Gene-Amp PCR system under the following conditions: preheating at 95°C for 5 minutes followed by 30 cycles (for RUNX3) or 25 cycles (for GAPDH) of denaturation for 20 seconds at 95°C, annealing for 1 minute at 55°C, and extension for 1 minute 30 seconds at 72°C, with a final extension for 10 minutes at 72°C.

Electrophoretic mobility shift assay. The double-stranded DNA probe RXE (sense strand, 5′-gatccaccacagccaGACCACAggcagacatgagga-3′) was used for electrophoretic mobility shift assay (EMSA), and the oligonucleotide M1 (sense strand, 5′-gatccaccacagccaGACCACAggcagacatgagga-3′) was used as a specific competitor. A perfect match to the RUNX binding site is indicated by capital letters. Binding assays were done at 25°C for 10 minutes in 20 μL binding buffer [20 mmol/L HEPES (pH 7.6), 4% (w/v) Ficoll type 400, 50 mmol/L KCl, 2 mmol/L EDTA, and 2 μg of poly(doxyinosinic-deoxyctydilic acid) containing 1 nmol/L 32P end-labeled probe (20,000 cpm) and bacterially expressed proteins. Samples were electrophoresed on a 4% polyacrylamide gel in 0.25% Tris-borate EDTA buffer at 250 V for 1 hour and subjected to autoradiography at −70°C.

Statistical analysis. Results were analyzed with the SAS statistical analysis program, version 8.1 (SAS Institutes, Cary, NC), using the χ² test, and P < 0.05 was considered as the criterion of statistical significance.

Results

DNA methylation of the RUNX3 exon 1 in bladder tumor cell lines. Because RUNX3 transcription is frequently suppressed in various tumors by DNA methylation, we analyzed the expression and methylation status of RUNX3 in bladder tumor cell lines by methylation-specific PCR using the bisulfite method (32). The CpG island in the RUNX3 exon 1 region, the first exon transcribed from the RUNX3 p2 promoter (31) between −211 and −61 relative to the translation start site (Fig. 1A), was methylated in six of seven cell lines; the HT1197 line was the exception (Fig. 1B, top). Reverse transcription-PCR (RT-PCR) analysis revealed that RUNX3 expression was undetectable in the RUNX3-methylated cell lines (Fig. 1B, bottom). DNA sequence analysis of the methylation-specific PCR products revealed that the amplified region was fully unmethylated in the HT1197 line but was completely methylated at C residues followed by G residues in the other lines (Fig. 1C). This result suggests that the absence of RUNX3 expression is due to methylation of the CpG island in the exon 1 region of the RUNX3 promoter.

To confirm this interpretation, the EJ, 5637, and UMUC3 cell lines were cultured in the presence of 5′-aza-2′-deoxycytidine, an inhibitor of DNA methylase that reactivates gene expression that is suppressed by CpG island hypermethylation (34). As shown in Fig. 1D, RUNX3 expression was restored in all three cases. This result strongly supports the notion that hypermethylation of the CpG island of RUNX3 exon 1 region results in inactivation of RUNX3 expression.

DNA methylation of RUNX3 in bladder tumor tissues. To determine whether RUNX3 inactivation by DNA methylation is characteristic of human bladder tumors, we collected 124 bladder tumor specimens and 20 normal bladder mucosa and analyzed the methylation status of the RUNX3 exon 1 region by methylation-specific PCR. RUNX3 in all of the normal bladder mucosa was completely methylation free, but on the other hand, for 73% (90 of 124) of bladder tumor tissues, a methylation-positive pattern was observed (Table 1). Typical results are shown in Fig. 2A. Only methylation-specific products were seen for most of these RUNX3 sequences. However, both methylation-specific and methylation-nonspecific products were observed for others, which may be due to contamination with normal surrounding tissue. DNA sequence analysis of the methylation-specific PCR products revealed that most were partially methylated (Fig. 2B). All methylation-specific PCR products were sequenced and the frequency of methylation of each CpG site is shown in Fig. 2C.

To examine whether RUNX3 exon 1 methylation results in the suppression of RUNX3 expression, we measured RUNX3 mRNA levels by semiquantitative RT-PCR in seven normal bladder mucosa and 99 bladder tumor specimens. RUNX3 expression was detected in all the normal bladder mucosa (seven of seven; Fig. 3A) and in most of the unmethylated bladder tumor tissues (30 of 34). Typical results are shown in Fig. 3B. In contrast, RUNX3 expression was not detected in the RUNX3-methylated bladder tumor tissues (62 of 65), except for a few tissues (Fig. 3C). This result suggests that methylation of the RUNX3 exon 1 region is closely correlated with the inactivation of RUNX3 expression in bladder tumor tissues.
Statistical analysis of these results showed that RUNX3 methylation is a significant risk factor for bladder tumor development \[P < 0.01; \text{odds ratio (OR)} 107.55; 95\% \text{confidence interval (95\% CI), 6.33-1827.39; Table 1}]. Furthermore, RUNX3 methylation was also associated with tumor stage, recurrence, and progression, and it was more frequently observed in invasive tumors (39 of 46, 85\%) than in superficial bladder tumors (51 of 78, 65\%; \(P = 0.01\); OR, 2.95; 95\% CI, 1.16-7.47). The RUNX3

![Figure 4. RUNX3 point mutations found in bladder tumors. Direct sequencing results of sense and antisense RUNX3 cDNA amplified by PCR. (A) A → C, T → C, G → T, and C → A transitions in the same allele (clone 23); the other allele is normal (clone 19). All four transitions result in amino acid changes. (B) A G → A transition and a G deletion are in the same allele (clone 10) and the other allele is normal (clone 11). The G → A transition is a silent mutation and the G deletion results in a frameshift. Direct sequencing of the PCR product, with the appearance of doublet peaks after deletion of the G residue (arrow).]
methylation status in primary bladder tumor tissues was determined for patients with recurrent and nonrecurring bladder tumors. Methylation was found to be more frequent in the former (22 of 27, 85%) than in the latter group (21 of 46, 46%). Recurrence of superficial bladder tumor significantly correlated with *RUNX3* methylation (P = 0.02; OR, 3.70; 95% CI, 1.19-11.46). Of primary bladder tumors in patients with disease progression, 93% (25 of 27) showed *RUNX3* methylation, whereas 69% (51 of 74) of those with nonprogressive tumors did so. Subsequent tumor progression after curative treatment in patients with bladder tumor was significantly associated with *RUNX3* methylation (P = 0.01; OR, 5.63; 95% CI, 1.23-25.82). Statistical analysis of *RUNX3* expression and *RUNX3* methylation pattern showed that they were essentially the same.

**RUNX3 point mutations.** To further establish that inactivation of *RUNX3* is associated with bladder tumorigenesis, we determined whether primary bladder tumors bear mutations within the *RUNX3* coding region. In the present study, the *RUNX3* isoform with the NH$_2$-terminal sequence MRIPV was used throughout (SWISSPROT: Q13761-1). Thirty-four cases of bladder tumor with unmethylated *RUNX3* were analyzed. Full-length *RUNX3* cDNAs were obtained by RT-PCR and directly sequenced. One of the patients (BT158) was found to have four point mutations, T to C, C to A, C to A, and A to G (Fig. 4A), resulting in substitutions of Leu$^{89}$ with proline, Pro$^{102}$ with threonine, Ala$^{199}$ with aspartic acid, and Met$^{129}$ with valine, respectively. Subcloning and sequence analysis revealed that all four mutations occurred on the same allele (RX3-mt4) and that the other allele was normal. Another patient (BT91) had a silent mutation and a single nucleotide deletion within the same allele (Fig. 4B). The nucleotide deletion resulted in a frame shift from amino acid number 100 (RX3-fs100) and an early termination seven amino acids downstream from the deletion, thereby removing about two thirds of the Runt domain and the COOH-terminal region. The genomic DNA sequences of all of the mutant alleles were determined and these bore the same mutations as the cDNA sequences. On the other hand, the nucleotide sequences of the *RUNX3* coding region in patient blood samples were all normal.

**Impaired DNA-binding ability of *RUNX3* mutants.** Because all of the mutations occurred in the Runt domain of the *RUNX3* protein, they were predicted to impair DNA binding. To determine whether this was the case, histidine-tagged wild-type and mutant Runt domains (RX3-mt4) were expressed in bacteria (Fig. 5A). EMSA using the consensus RUNX binding site revealed that none of the mutated Runt domains bound to this site, whereas the wild-type Runt domain did bind (Fig. 5B). The inability of the mutated gene product to bind DNA suggests that the mutations abolish the ability of *RUNX3* to function as a transcription factor.

**Discussion**

In this study, we analyzed the nucleotide sequence and methylation status of the *RUNX3* gene in bladder tumor and showed that *RUNX3* was silenced in 86% of bladder cancer cell lines and in 73% of primary bladder tumor specimens by hypermethylation of its promoter region. Most importantly, loss-of-function mutations of *RUNX3* were found in two cases.

In recent years, it has become increasingly obvious that genetic abnormalities are not the only mechanism by which tumor suppressor genes are repressed during tumorigenesis. Growing evidence now indicates that epigenetic alteration plays an important role in carcinogenesis and indeed, that it may be as significant as genetic abnormalities. For example, the *APC*, *p16$^{INK4a}$*, hMLH1, RASSF1A, and *E-cadherin* genes are inactivated by DNA hypermethylation in various tumors (11–17). Earlier, we reported strong evidence suggesting that *RUNX3* is a gastric cancer tumor suppressor gene and that it is frequently inactivated by hypermethylation of the promoter region. Therefore, *RUNX3* seems a new addition to the list of tumor suppressors that are often silenced by promoter hypermethylation in cancer cells. Although we observe silencing of *RUNX3* by promoter methylation in cancer cells, this does not prove that *RUNX3* is responsible for carcinogenesis, because genes other than *RUNX3* may also be silenced and we cannot specify the gene(s) responsible for the cancer phenotype. In the case of gastric cancer, however, we identified a mutation within the highly conserved Runt domain of *RUNX3*, RUNX3(R122C), that destroys tumor suppressor activity in nude mice (31). Identification of RUNX3(R122C) was therefore the key finding that identifies RUNX3 as the gene responsible for the induction of gastric cancer, or at least one of such genes.

In this study, we identified mutations in *RUNX3* within the Runt domain in two cases of bladder tumor. One of these was amino acid substitutions that abolished the DNA-binding ability of *RUNX3*, and the second truncated *RUNX3* in the middle of the domain, also destroying RUNX3 function. The existence of these
mutations strongly suggests that RUNX3 is a tumor suppressor in bladder tumor and in gastric cancer. Components of the transforming growth factor-β signaling cascade are frequently altered in many types of cancers, and this cascade is therefore referred to as a tumor suppressor pathway (35, 36). If RUNX3 is an integral component of this pathway (31), it may be involved in many different types of cancers, in addition to gastric and bladder tumors. Indeed, inactivation of RUNX3 by silencing due to promoter hypermethylation has been reported for cancers of the lung, colon, pancreas, liver, prostate, bile duct, breast, larynx, esophagus, endometrium, uterine cervix, and testicular yolk sac (37–43). It is thus of interest to obtain more definitive evidence showing that RUNX3 is also a tumor suppressor in these cancers.

Statistical analysis revealed that RUNX3 methylation confers a 100-fold increase in the risk for bladder tumor development (OR, 107.55). RUNX3 methylation also seems associated with tumor stage (OR, 2.95), recurrence (OR, 3.70), and progression (OR, 5.63), suggesting that RUNX3 is required not only to inhibit tumor initiation but also to suppress the aggressiveness of primary bladder tumors (Table 1). Although various diagnostic markers for bladder tumor development, recurrence, and progression have been reported, none adequate to predict the behavior of most tumors. Our results show that the methylation status of RUNX3 could be a better diagnostic marker for bladder tumor than previously described markers.

It is noteworthy that the two patients from whom we identified RUNX3 mutations still have a wild-type RUNX3 allele that is normally expressed. This observation suggests that haploinsufficiency of RUNX3 predisposes to bladder tumor or the mutant RUNX3 function as a dominant-negative form. In fact, haploinsufficiency of RUNX3 is closely associated with human diseases. For example, heterozygous loss-of-function mutation of RUNX3 is associated with familial platelet disorder with predisposition to acute myeloid leukemia (44). Sporadic heterozygous mutation of RUNX1 is also leukemogenic (45). Similarly, haploinsufficiency of RUNX2 caused by germ line mutation results in the congenital bone disorder cleidocranial dysplasia (25, 28). Further analysis is required to understand how decreases in RUNX3 activity and point mutations of the gene result in bladder tumor.

Frequent inactivation of RUNX3 in bladder tumor prompted us to examine for bladder abnormalities in RUNX3 knockout mice. However, no abnormal bladder phenotypes could be detected. This suggests that RUNX3 knockout mice, which die soon after birth (31), probably do not have the time to acquire the secondary mutations that aid bladder tumor progression, even if they are predisposed to acquiring bladder tumor.

Although current pathologic and clinical variables provide important prognostic information, these variables still have limitations for assessing the true malignant potential of most bladder tumors. Our results suggest that inactivation of RUNX3 is causally associated with bladder tumor and that the methylation status of RUNX3 could be useful as a diagnostic marker for bladder tumor and as an indicator for bladder tumor recurrence and progression in the clinical setting.

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