Functional mononucleotide repeat polymorphism in the promoter region of *HGF* is associated with risk and malignant aggressiveness of bladder cancer

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Abstract. Increased expression of hepatocyte growth factor (HGF) has been shown to be associated with aggressiveness in several types of cancer. Shorter variants of deoxyadenosine tract element (DATE) located in the *HGF* promoter region have been reported to enhance the expression of *HGF*. In this study, we investigated the role of *HGF* DATE variants in bladder cancer risk, *HGF* expression and clinicopathological features. The frequency of individuals with a short DATE (<28 repeats) in peripheral blood lymphocytes (PBLs) was significantly higher in bladder cancer patients compared to controls (p<0.001). Somatic mutations were observed in 37 of 70 bladder tumor (BT) tissues and the frequency of mutation to long DATE was significantly higher than that to short DATE (p=0.047). The presence of the short DATE in BT tissue was significantly associated with higher tumor grade (p=0.015). *HGF* mRNA levels were significantly higher in pT2 tumors than pTa or pT1 tumors (p=0.019), and in grade 3 tumors than grade 1 or 2 tumors (p=0.020). Furthermore, BT tissues with the short DATE showed significantly higher levels of *HGF* mRNA (p<0.001). In patients who underwent radical cystectomy, those with higher HGF expression had a significantly shorter overall survival than those with lower HGF expression (p=0.012). In conclusion, HGF may be associated with the prognosis of patients who undergo radical cystectomy, and the *HGF* DATE may affect the risk and aggressiveness of bladder cancer by altering HGF expression.

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Introduction

Hepatocyte growth factor (HGF) is a multifunctional molecule that acts as mitogen or morphogen in variety of cells (1). It is known that HGF acts as paracrine mediator that promotes proliferation, survival, migration and morphogenesis of epithelial cells (1,2). HGF also induces angiogenesis and inhibits apoptosis through c-MET (1,2). A number of reports have demonstrated that the overexpression of HGF and c-MET is associated with cancer invasion, metastasis and poor prognosis in various types of cancer (3-8). Increased serum levels of HGF were reported in breast and lung cancer patients (9,10), and an association between increased serum HGF level and poor prognosis was observed in ovarian, colorectal, hepatocellular, renal cell and bladder cancer (1,2,11-13).

Ma *et al* have reported a possible molecular mechanism for aberrant HGF expression in human breast cancer (14). They showed that the *HGF* promoter element harbors a mononucleotide repeat of approximately 30 deoxyadenosines, which has been termed the deoxyadenosine tract element (DATE), and the repeat length mutation within the DATE had a significant effect on *HGF* promoter activity in breast cancer cell lines (14). Several studies have shown the mechanism by which the poly-deoxyadenosine repeats affect gene expression levels (14-19). Namely, long deoxyadenosine repeats have been shown to prevent accessibility to promoter regions and to increase the equilibrium accessibility of other DNA target sites buried inside nucleosomes, while short deoxyadenosine tracts were suggested to stimulate transcription by improving accessibility to promoters and decreasing the stability of the DNA wrapping (18-20). While the *HGF* DATE may influence the malignant phenotype and/or the progression of breast and gastric cancers (14,21), there has been no report evaluating the significance of the *HGF* DATE in bladder cancer.

In the present study, we investigated the influence of germ line variants of the *HGF* DATE on bladder cancer risk. Furthermore, we assessed the association of clinicopathological factors and *HGF* mRNA expression with somatic variants

of the *HGF* DATE in bladder cancer, which may represent a mutational effect of DATE alteration.

Materials and methods

Subjects. A total of 140 patients with bladder cancer treated at Akita University Medical Center were enrolled in this study. All the patients were histologically diagnosed with urothelial carcinoma of the bladder with specimens obtained from transurethral biopsy or surgical resection. Clinical and histopathological information was obtained from patients' medical charts, imaging studies and pathological reports. A total of 95 healthy native Japanese men and women over the age of 60 years from Akita Prefecture, who underwent routine community health checkups, were recruited as controls. They were checked by routine urinalysis to rule out urinary tract diseases. The tumor grading system conformed to the World Health Organization grading system (22) and the tumor staging system was based on the American Joint Committee on Cancer TNM classification system (23). This study was approved by the Institutional Review Board of Akita University School of Medicine, and all the subjects provided written informed consent and were asked to provide clinical information, blood and tissue samples for genetic analysis.

HGF genotyping analysis. Germ line DNA was extracted from peripheral blood lymphocyte (PBL) samples collected from individuals using a QIAamp Blood Kit (Qiagen, Hilden, Germany). Tumor DNA was extracted from bladder tumor tissues obtained at transurethral resection (TURBT). The length of the DATE in the *HGF* promoter region was determined by a polymerase chain reaction (PCR). The PCR primers (forward, 5'-GGGACAGGTATTGTGGGGCCAA AATAAG-3'; and reverse, 5'-GGGTGTGGTATTGTGGGG CCAAAATAAG-3') generated a 247-bp product when the length of DATE was 30 deoxyadenosines. PCR reactions were performed in a 25 μl volume containing 20 ng of genomic DNA, 1X PCR buffer (PE Applied Biosystems, Branchburg, NJ, USA), 0.2 mM of each dNTP, 2.5 mM $MgCl_2$, 50 pM of each primer, and one unit of Ampli-Taq Gold DNA polymerase (PE Applied Biosystems). The PCR amplification conditions were as follows: 10 min at 94˚C, followed by 35 cycles of 30 sec at 94˚C, 30 sec at 62˚C and 60 sec at 72˚C, with a final extension of 10 min at 72˚C in a thermal cycler (GeneAmp PCR System 9700, Perkin-Elmer, Branchburg, NJ). The samples were purified using the Illustra AutoSeq G-50 Kit (GE Healthcare Life Sciences, Little Chalfont, UK). All purified fragments were subjected to DNA sequencing with forward and reverse primers using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA), and analyzed with a Genetic Analyzer (ABI PRISM 310 Genetic Analyzer, Applied Biosystems). Because we could clearly define only the length of the shorter DATE owing to the direct sequencing method, the data of the shorter DATE length were used in further statistical analyses (Fig. 1A). The short DATE was defined as 27 deoxyadenosine repeats or fewer, while the long DATE was defined as more than 27 repeats, as described in the Results.

Expression analysis of HGF mRNA using RT-PCR. Total RNA was extracted from approximately 30 mg of tumor tissues obtained by TURBT using TRIzol RNA Isolation Reagent (Life Technologies Inc., Rockville, MD, USA) and was reverse-transcribed to cDNA using a SuperScript VILO cDNA Synthesis Kit (Life Technologies Inc.).

Real-time RT-PCR amplication mixtures (20 μl) contained 2 μl template cDNA, 2X SYBR-Green Master mix buffer (10 μl) (Takara Bio Inc., Otsu, Japan) and 100 μM forward and reverse primers (0.8 μl). Reactions were run on a Thermal Cycler Dice Real-Time System (Takara Bio Inc.) with cycling conditions of 30 sec at 95˚C, 40 cycles at 95˚C for 5 sec, and 60˚C for 60 sec. The primers used were as follows: 5'-ATGAT GATGCTCATGGACCCT-3' (forward) and 5'-CTGGCAAGC TTCATTAAAACC-3' (reverse) for *HGF* and 5'-TGATGACA TCAAGAAGGTGGTGAAG-3' (forward) and 5'-TCCTTGG AGGCCATGTGGGCCAT-3' (reverse) for *GAPDH*. PCR reactions were simultaneously performed for *HGF* and the reference *GAPDH*. The *HGF* expression levels normalized by the *GAPDH* were used for further analyses.

HGF immunohistochemical analysis. Ninety-one bladder cancer specimens obtained at radical cystectomy were subjected to immunohistochemical analysis. The specimens were fixed in 10% buffered formalin and embedded in paraffin. Serial sections (5-μm thick) were deparaffinized in xylene, rehydrated in a graded series of decreasing ethanol concentration, and then rinsed in Tris-buffered saline. For HGF immnostaining, HGF was detected with a goat polyclonal anti-human HGF antibody (1:50; R&D Systems, Minneapolis, MN, USA) as primary antibody. Antigen retrieval treatment was performed at 121˚C for 10 min in 10 mM sodium citric acid (pH 6.0), and endogenous peroxidases were blocked using 0.3% hydrogen peroxidase/methanol for 30 min. After washing in phosphate-buffered saline (PBS) for 5 min and 10% bovine serum albumin/PBS for 30 min, the sections were exposed to a primary antibody diluted 1:50 overnight at 4˚C. After washing in PBS, a secondary antibody conjugated with anti-goat IgG was applied, followed by incubation at room temperature for 30 min. Immunoreactions were visualized using 3,3'-diaminobenzidine as chromogen. Positive control was represented by a normal hepatic cell showing strong cytoplasmic expression for HGF.

To assess HGF immunoreactivity, we used the following scoring systems. Cytoplasmic HGF staining intensity was scored on a semi-quantitative scale as: 1, weak; 2, moderate; 3, strong; and 4, very strong. The percent of cytoplasmic HGF-positive cells was divided into four groups: 1 (<25%), 2 (25-50%), 3 (50-75%) and 4 (>75%). Total immunoreactivity was finally calculated by multiplying the two scores and was classified into two groups: low expression (≤9) and high expression (>9) (Fig. 1B).

Statistical analysis. The data was analyzed using SPSS software (version 19.0J, SPSS Inc., Chicago, IL, USA). Two group comparisons were performed with the Mann-Whitney U test for continuous variables and χ^2 test for categorical variables. A probability of <0.05 was required for statistical significance. Kaplan-Meier survival curves with the log-rank test were used to compare overall survival and disease-specific survival between patients with high and low HGF expression.

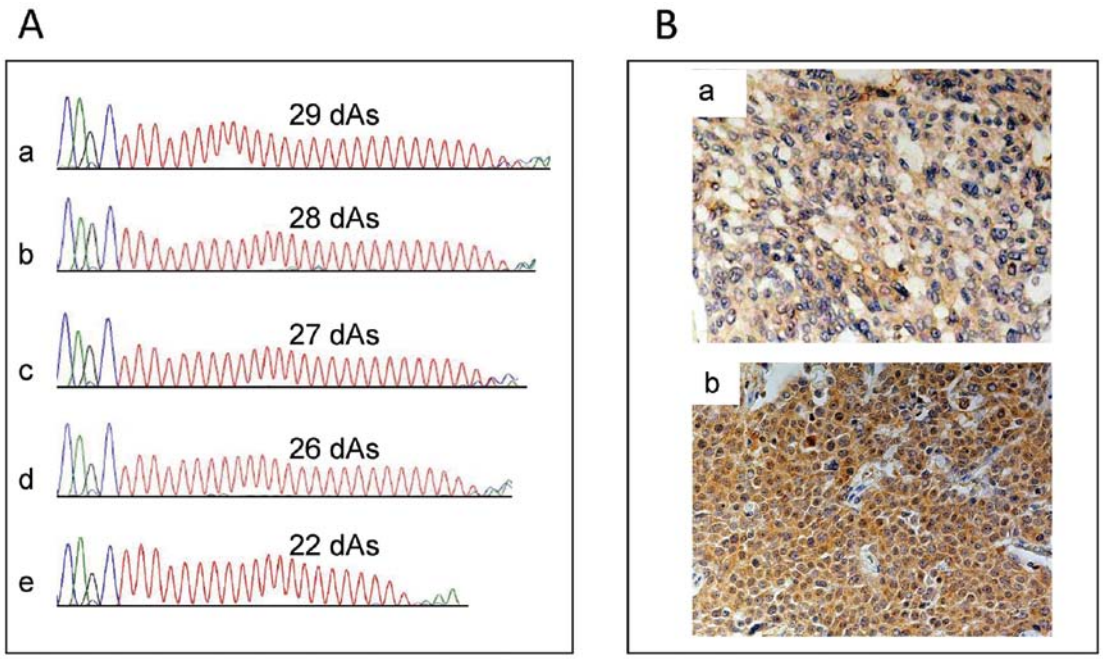

Figure 1. (A) Representative results for direct sequencing of the *HGF* DATE polymorphic region. Letters a, b, c, d and e show 29, 28, 27, 26 and 22 deoxyadenosine-repeats (dAs), respectively. (B) Representative immunohistochemistry demonstrating (a) low and (b) high HGF expression scores.

Results

Comparison of the length of the HGF DATE in germ-line DNA. We evaluated the length of the DATEs in PBLs of 140 bladder cancer patients and 95 healthy controls. Distributions of the DATE lengths in bladder cancer patients and controls were as follows: in bladder cancer patients, the frequency of 30, 29, 28, 27, 26, 25, 24 and 22 doxyadenosine repeats in the DATE were 3 (2.1%), 15 (10.7%), 52 (37.1%), 48 (34.3%), 13 (9.3%), 5 (3.6%), 2 (1.4%) and 2 (1.4%), respectively. In the controls, the frequency of 30, 29, 28, 27, 26 and 25 doxyadenosine repeats in the DATE were 4 (4.2%), 32 (33.7%), 36 (37.9%), 18 (18.9%), 4 (4.2%) and 1 (1.1%), respectively. Because the median length of DATE was 28 in both groups, we classified the length of DATE into two categories as short (≤27) and long (>27). When we applied our classification, the frequency of individuals with a short DATE was significantly higher in bladder cancer patients than controls (49.3 vs. 24.2%, P<0.001) (Table I).

Comparison of the frequency of short DATE between bladder tumor tissue and PBLs. We assessed the difference in the frequency of short DATEs between bladder tumor tissue and PBLs. In addition, we evaluated the association between the clinicopathological factors of bladder cancer and the presence of a short DATE in bladder tumor tissue. The patients comprised 55 males and 15 females with a mean age of 73.2 years. There were 51 non-muscle invasive tumors (NMIT) (pTa-1) and 19 muscle invasive tumors (MIT) (pT2), and 42 low grade tumors (grade 1 or 2) and 28 high grade tumors (grade 3). The frequency of patients with a short DATE in their PBLs was 41.4% (29/70), while that in their bladder tumor tissue was 52.9% (37/70) (Table II). The frequency of patients with a short DATE in their bladder tumor tissue was significantly higher than that in their PBLs (p=0.008) (Table II). In 25 (35.7%) patients, the length of the DATE in their bladder tumor tissues was shorter than that in their PBLs. In 12 (17.1%) patients, the length of the DATE in their bladder tumor tissues was longer than that in their PBLs. The frequency of the mutation to long DATE was significantly higher than that to short DATE (p=0.047 by binominal test). The results suggested that the DATE was a frequent target of

Table I. Bladder cancer patient and control demographics and status of short deoxyadenosine repeats in the *HGF* DATE.

	Bladder cancer	Control	P-value
Total	140	95	
Age	68.4±7.8	68.1±4.5	0.694
(Range)	(16-94)	(60-75)	
Gender			0.399
Male	107	77	
Female	33	18	
Grade			
G1-2	48 (34.3)		
G3	92 (65.7)		
pT			
pTa-1	63 (45)		
>pT1	77 (55)		
HGF DATE			<0.001
Short DATE (-)	71 (50.7)	72 (75.8)	
Short DATE (+)	69 (49.3)	23 (24.2)	

DATE, deoxyadenosine tract element. Short DATE was defined as 27 or fewer deoxyadenosine repeats in the *HGF* DATE.

Table II. Comparison of clinicopathological factors with the status of the *HGF* DATE in bladder tumor tissues and peripheral blood lymphocytes.

		BT tissue			PBL		
	Total	Short DATE (-)	Short DATE (+)	P-value	Short DATE (-)	Short DATE (+)	P-value
	70	33	37		41	29	0.008
Age	73.2±10.5	73.2±10.6	73.1±10.5	0.949	71.6±11.0	75.3±9.5	0.144
(Range)		(43-88)	(53-94)		(53-89)	(56-94)	
Gender				0.784			0.780
Male	55	26	29		31	23	
Female	15	7	8		10	6	
pT				0.178			0.786
pTa-1	51	27 (82)	24 (66)		29 (71)	22 (76)	
pT2	19	6 (18)	13 (34)		12 (29)	7 (24)	
Grade				0.015			0.621
G1-2	42	25 (76)	17 (46)		26 (65)	16 (55)	
G3	28	8 (24)	20 (54)		15 (35)	13 (45)	

BT, bladder tumor; PBL, peripheral blood lymphocyte; DATE, deoxyadenosine tract element. Short DATE was defined as 27 or fewer deoxyadenosine repeats in the *HGF* DATE.

somatic mutation (37/70, 52.9%) in bladder cancers and the mutation from long DATE to short DATE might be dominant and play a role in the progression of bladder cancer. There was no significant difference between NMIT and MIT in the frequency of patients with a short DATE either in PBLs or bladder tumor tissue (Table II). No significant association was observed between the presence of a short DATE in PBLs and tumor grade, while tumor grade was significantly higher in bladder tumor tissue with a short DATE than those without a short DATE (p=0.015, Table II).

Association of HGF mRNA expression levels with DATE length in bladder tumor tissue. We compared *HGF* mRNA levels between bladder tumor tissue with and without a short DATE. MIT showed significantly higher *HGF* mRNA expression levels (p=0.019) than NMIT (Fig. 2A). High grade tumors showed significantly higher *HGF* mRNA expression levels than low grade tumors (p=0.020) (Fig. 2B). *HGF* mRNA expression was significantly higher in bladder tumor tissue with a short DATE than that without a short DATE (p<0.001) (Fig. 2C).

HGF immunohistochemical analysis. We evaluated the relationship between HGF immunohistochemical expression in bladder cancer and clinicopathological factors. Overall and disease-free survival rates were compared according to the HGF expression scores in 91 patients who underwent radical cystectomy. The patients comprised 70 males and 21 females with a mean age of 69.8 years. The mean follow-up duration was 19 months (range 0-98). Low and high HGF expression scores were observed in 61 (67.0%) and 30 (33.0%) patients, respectively (Table III). The high HGF expression group had a significantly worse overall survival than the low HGF expression group (p=0.012). Furthermore, although not statistically significant, the high HGF expression group had a tendency towards worse disease-free survival than the low HGF expression group (p=0.056) (Fig. 3).

Table III. Comparison of bladder tumor HGF expression score with the clinical characteristics of patients who had undergone radical cystectomy.

	Total	Low	High	P-value
Number	91	61	30	
Age	69.8±11.0	67.5±11.6	74.5±7.7	0.004
(Range)	(25-91)	(25-85)	(56-91)	
Gender				0.298
Male	70	49	21	
Female	21	12	9	
pT				0.502
≤pT2	51	36	15	
>pT2	40	25	15	
pN				0.395
pN0	67	47	20	
pN1-3	17	10	7	
pNx	7	4	3	

Low and high were defined as HGF expression score 9 or lower and greater than 9, respectively.

Discussion

In this study, we showed that the frequency of the short *HGF* DATE in germ line DNA was significantly higher in bladder cancer patients than that of healthy controls, suggesting that the *HGF* DATE plays a role in the carcinogenesis of bladder cancer. Ma *et al* reported that the frequency of healthy

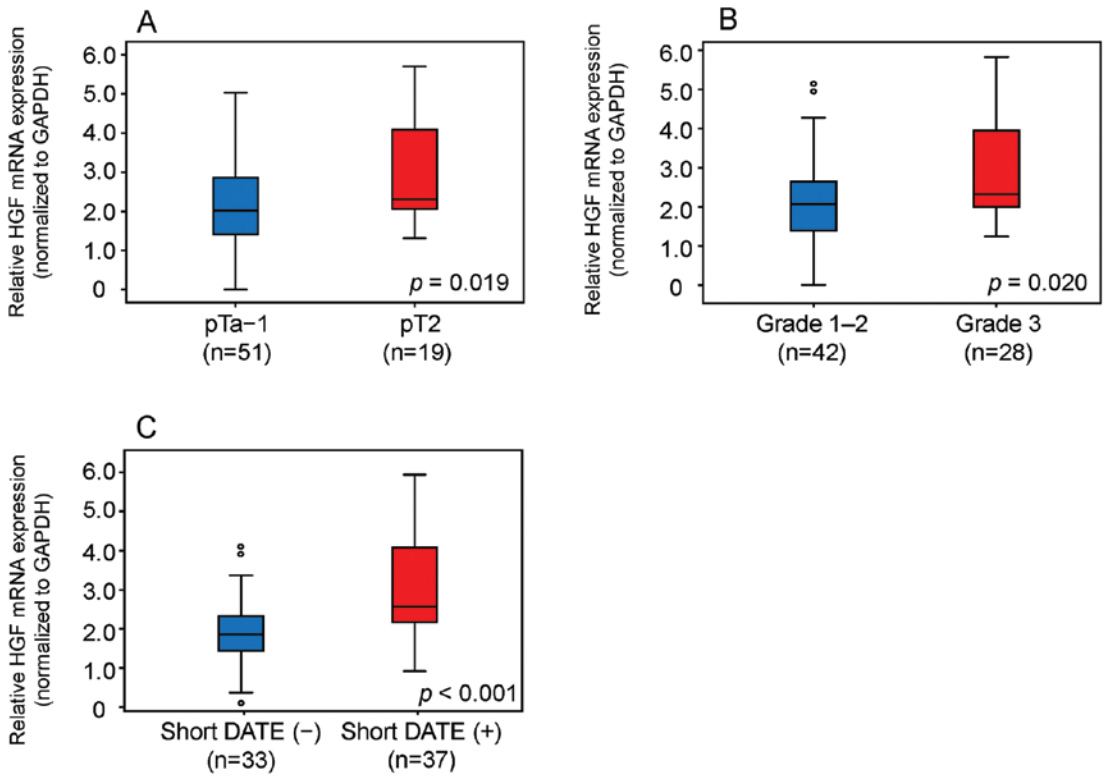

Figure 2. Relationship between *HGF* mRNA expression levels and DATE length in bladder tumor tissue. The expression levels are shown as box plots. Significantly higher *HGF* mRNA expression levels were observed in (A) invasive tumors (p=0.019); (B) high grade tumors (p=0.020); and (C) tumors with short DATEs (p<0.001).

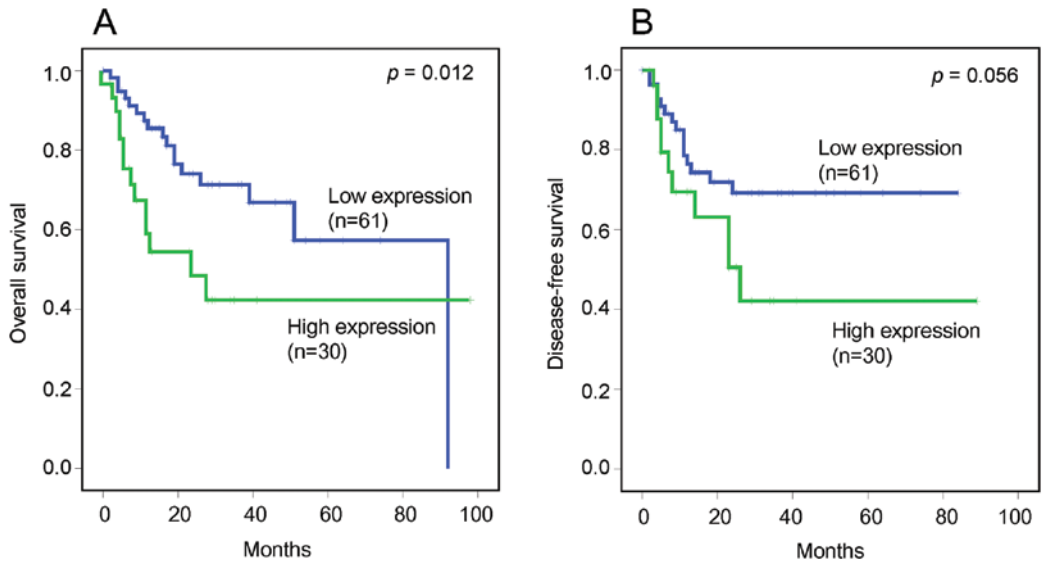

Figure 3. Relationship between HGF expression in bladder tumors and clinical outcome of patients after radical cystectomy. (A) Patients with high tumor HGF expression had significantly worse overall survival than those with low tumor HGF expression (p=0.012). (B) Although not significant, patients with high tumor HGF expression had worse disease-free survival than those with low tumor HGF expression (p=0.056).

individuals with a short DATE, defined as 25 or fewer deoxyadenosine repeats, was 26% in African Americans and 3% in Caucasians (14). In our study, because the frequency of healthy controls having 25 or fewer repeats of the DATE was only 1% and the median was 28 repeats among all subjects, we defined 27 or fewer deoxyadenosine repeats as the short DATE. Based on our criterion, the frequency of Japanese healthy controls having the short DATE was 24.2% (23/95). It is interesting to note that there is an ethnic difference in the distribution of the length of the *HGF* DATE. A distinct role of the *HGF* DATE in the carcinogenesis of bladder cancer requires validation in an epidemiological study designed to assess gene-environment

interaction in a large population. Additionally, our study showed that the DATE was a frequent target for somatic mutation in bladder cancer and the frequency of the short DATE in bladder tumor tissues was significantly higher than that in matched PBLs. The results suggested that the somatic mutational shortening of the DATE is possibly involved in the genesis and progression of bladder cancer. The results may further strengthen the significance of the DATE in bladder cancer carcinogenesis.

A previous study demonstrated that HGF protein expression in both breast cancer and normal epithelium was significantly increased with decreased length of the *HGF* DATE (14). In our study, the tumor grade was significantly higher in bladder tumor tissues with a short DATE than those without a short DATE (Table II). Furthermore, *HGF* mRNA expression in bladder tumor tissues with the short DATE was significantly higher than in those without the short DATE (Fig. 2C). Our results were consistent with a report by Ma *et al* in which *HGF* mRNA expression in human breast cancer tissues increased with decreased length of the DATE (14). Our study also demonstrated that MIT showed significantly higher *HGF* mRNA expression levels than NMIT, and high grade tumors showed significantly higher *HGF* mRNA expression levels than low grade tumors. In a previous study, HGF was reported to stimulate invasion of tumor cells and to induce angiogenesis *in vivo* (1). It was also reported that HGF signaling might be involved in tumor progression and invasion by directly regulating the transcription of downstream functional molecules (1,2). Taken together, the short DATE is suggested to be associated with higher malignant potential and tumor progression by regulating the transcriptional activity of the *HGF* gene in bladder cancer cells.

The association between prognosis and high serum level of HGF was previously reported in several types of cancer, and serum HGF was suggested to be a useful marker for discriminating a malignant tumor from benign disease and as a potential therapeutic target (3-5,10). In our study, patients with high HGF expression in their bladder cancer tissue had a significantly worse overall survival than those with low HGF expression, and patients with high HGF expression tended to have worse disease-free survival than those with low HGF expression. These results suggest that high HGF expression in bladder cancer tissue is a predictor of survival and recurrence after radical treatment of bladder cancer.

The results of this study should be interpreted with some caution. One limitation was that we genotyped only the length of deoxyadenosine repeat in the shorter DATE allele because the number of repeats in the longer DATE allele could not be clearly defined owing to the use of a direct sequencing method. According to a previous study, however, only the presence of shorter deoxyadenosine repeats has clinical significance and longer deoxyadenosine repeats may be ignored in the analysis (14). Second, our studies indicated a significant relationship between HGF protein expression and the clinical outcome of patients with bladder cancer in immunohistochemical analysis. However, we failed to genotype the *HGF* DATE in the same patients group using paraffin-embedded tumor tissues obtained by radical cystectomy, and could not directly analyze the relationship between the short DATE and the prognosis of bladder cancer patients. Further studies are required to clarify whether the short DATE may be a prognostic marker for invasive bladder cancer.

In conclusion, our results suggested that the DATE in the *HGF* promoter region is associated with carcinogenesis and aggressiveness of bladder cancer by aberrantly activating HGF expression. Further investigation is warranted to evaluate the clinical usefulness of the short DATE as a marker of poor prognosis in invasive bladder cancer patients.

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