TABLE III – COX PROPORTIONAL HAZARD REGRESSION ANALYSIS OF PREDICTING FACTORS FOR PSA RECURRENCE-FREE SURVIVAL IN PROSTATE CANCER PATIENTS WHO UNDERWENT RADICAL PROSTATECTOMY (A) AND IN PATIENTS WITH BONE METASTASIS (D2) AT DIAGNOSIS (B)

Variable	Category for statistical analysis	PSA recurrence-free survival		
		HR[1]	95% CI[2]	*p*
(A)				
Univariate analysis				
Preoperative PSA	≥9.6 *vs.*<9.6 (ng/ml)	2.643	1.377–5.074	0.003
Pathological T status	T3-4 *vs.* T2	3.934	2.065–7.493	<0.001
Surgical margin status	Positive *vs.* Negative	3.637	1.876–7.050	<0.001
Gleason Score	≥8 *vs.*<8	1.348	0.731–2.485	0.399
Mel-18 polymorphism	*GG vs. AA/GA*	3.222	1.458–7.120	0.004
Immuohistochemical staining of Mel-18	<4 *vs.*≥4	2.013	1.025–3.955	0.042
Multivariate analysis				
Preoperative PSA	≥9.6 *vs.*<9.6 (ng/ml)	3.095	1.352–7.083	0.007
Pathological T status	T3-4 *vs.* T2	1.311	0.5528–3.188	0.540
Surgical margin status	Positive *vs.* Negative	4.759	1.857–12.191	0.001
Mel-18 polymorphism	*GG vs. AA/GA*	2.757	1.154–6.588	0.022
Immuohistochemical staining of Mel-18	<4 *vs.* ≥4	2.271	1.018–5.066	0.045
Variable	Category for statistical analysis	Cancer-specific survial		
		HR[1]	95% CI[2]	*p*
(B)				
Univariate analysis				
Age	≥71 *vs.* <71 (yrs)	1.899	1.018–3.542	0.044
Tumor grade	High *vs.* Low/Intermediate	1.097	0.598–2.012	0.764
PSA	≥176 *vs.*<176 (ng/ml)	2.823	1.484–5.370	0.002
Hemoglobin	<11.5 *vs.* ≥11.5 (g/dl)	15.790	5.304–47.006	<0.001
Alkaline phosphatase	Increased *vs.* Normal	3.623	1.832–7.165	<0.001
Lactate dehydrogenase	Increased *vs.* Normal	2.319	1.086–4.956	0.030
Mel-18 polymorphism	*GG/GA vs. AA*	3.165	1.304–7.685	0.011
Multivariate analysis				
Age	≥71 *vs.* <71 (yrs)	1.212	0.442–3.321	0.709
PSA	≥176 *vs.* <176 (ng/ml)	2.825	1.122–7.110	0.027
Hemoglobin	<11.5 *vs.* ≥11.5 (g/dl)	1.973	0.317–12.268	0.466
Alkaline phosphatase	>ULN *vs.* Normal	27.093	4.676–156.970	<0.001
Lactate dehydrogenase	>ULN *vs.* Normal	1.427	0.391–5.199	0.590
Mel-18 polymorphism	*GG/GA vs. AA*	4.658	1.287–16.858	0.019

[1]HR: hazard ratio.–[2]CI: confidence interval.–[3] ULN, upper limits of the normal range.

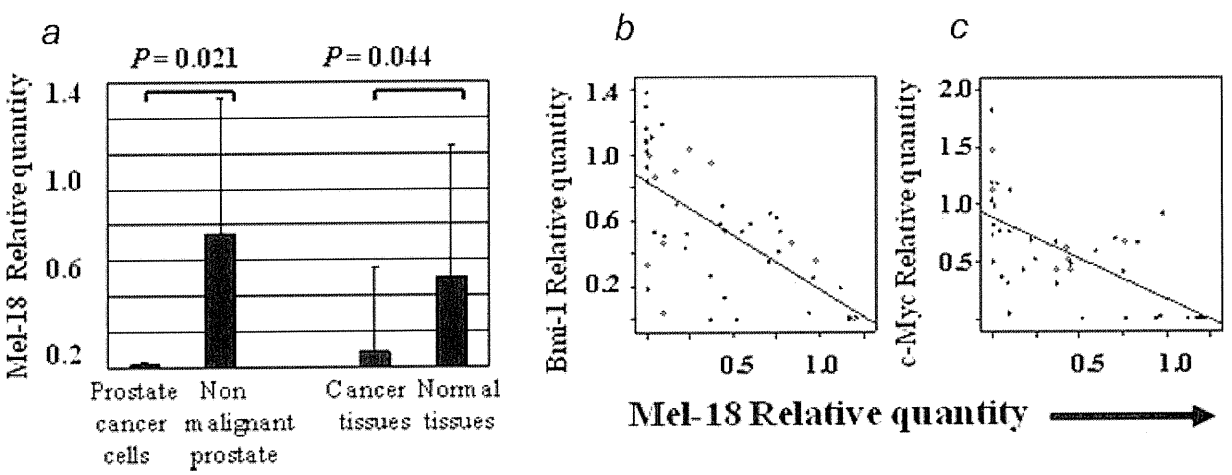

FIGURE 4 – Comparison of relative Mel-18 expression. Comparison of Mel-18 expression between prostate cancer cell line and non-malignant prostate tissues (left side), and between the renal and bladder cancer tissues and surrounding normal tissues (*a*). Correlation between *Mel-18* and *Bmi-1* expression in the malignant and non-malignant tissues (*b*). Correlation between *Mel-18* and c-*Myc* expression in the malignant and non-malignant tissues (*c*).

Mel-18 expression than those with *GA* or *GG* genotypes although the difference was not statistically significant ($p = 0.188$). Other confounding factors such as promoter activity or modulation by microRNAs may modify Mel-18 expression in prostate cancer tissues.

Finally, we examined the expression levels of *Mel-18*, *Bmi-1*, and *c-Myc* mRNA in 2 prostate cancer cell lines, PC-3 and DU145, 12 non-cancerous prostate tissues, 7 renal cancers and the surrounding non-cancerous tissues, and 8 bladder cancers and the surrounding non-cancerous tissues. Using quantitative real time-

RT-PCR, *Mel-18* mRNA levels in prostate cancer cell lines were significantly lower than those in non-cancerous prostate tissues (Fig. 4*a*). We also found that levels of *Mel-18* mRNA in renal and bladder cancer tissues were significantly lower than those in surrounding non-cancerous tissues (Fig. 4*a*). In addition, statistically significant direct correlations were observed between levels of expression of *Mel-18* and *Bmi-1* and *c-Myc*, in all samples. A strong negative correlation was observed between *Mel-18* and *Bmi-1* ($r = -0.678$, $p < 0.001$) and between *Mel-18* and *c-Myc* ($r = -0.670$, $p < 0.001$) (Figs. 4*b*,*c*).

Discussion

Our immunohistochemical and clinicopathological data are consistent with the hypothesis that Mel-18 functions as a tumor suppressor protein in prostate cancer and possibly represses expression of the oncoproteins, c-Myc and Bmi-1. These observations are also consistent with previous examinations of prostate and breast cancer cells.[8,9] Because of the difficulty in comparing inter-institutional evaluations, immunohistochemical evaluation is often not clinically useful. Therefore, we sought an alternative method of investigating the association between *Mel-18* and prostate cancer. Our study demonstrated a *Mel-18* allele with low expression, the *1805 G* allele, was correlated with risk not only for poorer survival rates of patients with localized prostate cancer, but also with a poor prognosis in patients with metastatic prostate cancer. In this study, we examined Mel-18 gene expression corresponding to each allele of the *1805A/G* polymorphism in the prostate and in other cancer cell lines and found that the expression of the *1805A* allele was consistently higher than the *1805G* allele in seven cancer cell lines (Figs. 2*c*,*d*). Since we examined differences in the allelic mRNA expression in individual cancer cell lines from heterozygous subjects, the effects of environmental and/or other non-genetic factors would be exclude. In this study, the patients with the patients with the *GG* genotype had a significantly higher rate of PSA recurrence after prostatectomy compared to the *AA* or *GA* genotype ($p = 0.002$, Fig. 3*a*), whereas the *GG/GA* genotypes were associated with significantly worse cancer-specific and overall survival of the patients with metastasis compared with the *AA* genotype ($p = 0.007$ and $p = 0.002$, respectively; Fig. 3b and 3*c*). The sensitivity to hormonal and/or chemotherapeutic treatments might modify the survival of the metastatic prostate cancer patients.

Recent studies have provided evidence that SNPs in microRNA-binding sites in the 3′ untranslated region of tumor associated genes may be important factors for cancer risk.[19–21] The microRNA–RNA silencing complexes can inhibit translation when the microRNA binds to the 3′ untranslated-region (UTR)-mRNA target with an imperfect complementarity. This binding results in a reduced level of protein without reductions in the mRNA level.[22,23] In our study, the miRNA181a and 3′ UTR of the *Mel-18* showed an imperfect complementarity. However, the mRNA levels of the respective alleles demonstrated significant differences, suggesting that this altered expression may result from some as yet unidentified mechanism. Alterations in the 3' UTR can also affect the stability of an mRNA due to increased sensitivity to RNase. Partial folding structures of the *1805G* and *1805A* mRNAs, predicted by MFOLD, are shown in Figure 2*b*. These clearly different secondary structures may suggest that the different mRNAs differ in their protein-translational efficiencies.

Mel-18 is structurally highly similar to *Bmi-1*, another PcG member whose over-expression has been linked to the highly malignant behavior of various cancer cells including prostate cancer.[5,6] The N-terminal region of *Mel-18*, which contains a RING finger domain, is 93% homologous to the corresponding region of Bmi-1.[7] *Bmi-1* is negatively regulated by *Mel-18 via* repression of the *c-Myc* oncogene, which is amplified and/or overexpressed in a variety of malignancies.[8–11] Recent genome-wide analysis has identified variants in five chromosomal regions, including three independent regions, 8q24, 17q12, and 17q24.3, that are significantly associated with prostate cancer.[12,13] Although we failed to demonstrate an association between the *Mel-18 1805A/G* polymorphism and a risk of prostate cancer, prostate cancer is a slow growing cancer with a long period between initiation and formation of a clinically significant cancer, suggesting that progression rather than initiation may be the rate-limiting factor in the diagnosis of clinical cancer. In addition, *c-Myc*, whose chromosomal location is at 8q24, regulates androgen-receptor mediated transcriptional signals and may also function in oncogenesis of prostate cancer.[24] In a breast cancer study, *Bmi-1* and *c-Myc* were transcriptionally repressed by *Mel-18*.[10,11] This observation is consistent with our study, where we found a significant negative correlation between the expression of *Mel-18* and *c-Myc* in prostate cancer. Further investigation will be necessary to understand the association between these *Mel-18* mediated tumor-suppressive and *c-Myc* mediated oncogenic signals in prostate cancer.

In conclusion, our study, together with recent research on Mel-18 in various cancers, suggests that Mel-18 functions as a tumor suppressor in prostate cancer. To our knowledge, this is the first study to evaluate the association among Mel-18 expression and genotype, and the clinical significance thereof, for patients with prostate cancer. We recognized, however, that no correction was made for multiple testing in the p values reported for genotype association and that this observation requires independent replication. Although further studies using other validation methods will be necessary to confirm the involvement of Mel-18 in the progression of prostate cancer, the present study provides important evidence indicating that Mel-18 is a possible therapeutic target, as well as a diagnostic marker for poor outcome in prostate cancer patients.

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ORIGINAL ARTICLE

Intravesical administration of γδ T cells successfully prevents the growth of bladder cancer in the murine model

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Abstract
Background Superficial bladder cancers are usually managed with transurethral resection followed by the intravesical administration of Bacillus Calmette-Guerin which requires major histocompatibility complex (MHC) class I expression on cancer cells. Since cancer cells often loose MHC expression, a novel immunotherapy such as MHC-unrestricted γδ T cell therapy is desired.
Objective To clarify the relationship between the expression of MHC class I and clinicopathological features in bladder cancer patients, and investigate the effects of the administration of intravesical γδ T cells on bladder cancer.
Methods Samples from 123 patients who had undergone either transurethral resection or radical cystectomies were examined for MHC expression and the relationship between this and the clinicopathological features was analyzed statistically. The in vitro and in vivo effects of γδ T cells expanded by zoledronic acid (ZOL) against several types of cancer cell line and an orthotopic bladder cancer murine model which was pretreated with ZOL were investigated.
Results MHC-diminished superficial bladder cancer was significantly more progressive than MHC-conservative bladder cancer ($P = 0.047$). In addition, there was a significant association between diminished MHC expression and poor disease free survival ($P = 0.041$) and overall survival ($P = 0.018$) after radical cystectomy. In vitro, all of the cell lines pretreated with 5-μM ZOL showed a marked increase in sensitivity to lysis by γδ T cells. Moreover, intravesical administration of γδ T cells with 5-μM ZOL significantly demonstrated antitumor activity against bladder cancer cells in the orthotopic murine model ($P < 0.001$), resulting in prolonged survival.
Conclusion The present murine model provides a potentially interesting option to develop immunotherapy using γδ T cells for bladder cancer in human.

Keywords Bladder cancer · Intravesical administration · Major histocompatibility complex · γδ T · Zoledronic acid

Abbreviations

BCG	Bacillus Calmette-Guerin
CIs	Confidence intervals
CIS	Carcinoma in situ
^{51}Cr	^{51} Chromium
CTL	Cytotoxic T-lymphocytes.
E/T	Effecter/target cell
IL-2	Interleukin-2
IVIS	In vivo imaging system
HRs	Hazard ratios
Luc	Luciferase
MHC	Major histocompatibility complex
NK	Natural killer
PBMCs	Peripheral blood mononuclear cells
SCID	Severe combined immunodeficiency
siRNA	Small interfering RNA

T. Yuasa and K. Sato contributed equally to the study.

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TUR Transurethral resection
ZOL Zoledronic acid

Introduction

Superficial bladder cancers, which comprise approximately 70% of bladder cancers at initial diagnosis are usually managed with transurethral resection (TUR), followed by the intravesical administration of agents such as mitomycin C, adriamycin, and Bacillus Calmette-Guerin (BCG) [1, 2]. Among these intravesical agents, BCG is considered to be the most effective for the eradication and prophylaxis of recurrent superficial bladder cancer, including carcinoma in situ (CIS) and residual cancers [1–3]. BCG is believed to initially attach to bladder surfaces that are coated with extracellular matrix protein and subsequently cause a local inflammatory reaction in the bladder mucosa, characterized by large numbers of T cells including CD4 positive helper T-lymphocytes, CD8 positive cytotoxic T-lymphocytes (CTL), and macrophages [4]. The CTL-mediated antitumor effect is one of the major contributors of BCG therapy; therefore, preservation of major histocompatibility complex (MHC) class I should be necessary for its efficacy. Indeed, Kitamura et al. [5] recently reported that expression of MHC class I molecules on tumor cells contributes significantly to the therapeutic effect of BCG immunotherapy on bladder cancer. Although information on MHC class I expression patterns in bladder cancer is limited, down-regulation of MHC class I molecules in tumor cells is thought to be an important mechanism of tumor escape from immune surveillance [5, 6].

One of the T-lymphocyte subsets, $\gamma\delta$ T cells, displays MHC-unrestricted cytotoxicity that is reminiscent of natural killer (NK) activity [7, 8]. Currently, $\gamma\delta$ T cells are considered to represent a promising new concept in immunotherapy. Recently, we reported that zoledronic acid (ZOL), the most potent bisphosphonate, induced a significant dose-dependent expansion of $\gamma\delta$ T cells both in vitro and in vivo, mainly to the Vγ 9Vδ 2 subset [9]. These observations have recently facilitated the development of novel auto-immunotherapeutic approaches using in vitro expanded $\gamma\delta$ T cells from patients and have already yielded encouraging preliminary results [10].

In the present study, we attempted to evaluate the use of MHC class I expression as a prognostic factor for bladder cancer and also investigated the use of intravesical $\gamma\delta$ T cells as a possible MHC-unrestricted therapeutic tool against bladder cancer. $\gamma\delta$ T cells, which express many of the NK receptors including inhibitory types that recognize HLA class I such as NK inhibitory receptors (KIR) [11]. This may be advantageous because, the disrupting interactions of KIR with their ligands on tumor cells may enhance antitumor responses mediated by $\gamma\delta$ T cells [12]. In addition, local administration may be more effective in treating cancer compared to systemic administration, owing to a favorable effecter/target cell (E/T) ratio. Indeed, some researchers have succeeded in performing local preclinical immunotherapy using $\gamma\delta$ T cells in subcutaneous and intraperitoneal tumor models [13, 14]. Here, we performed intravesical immunotherapy using a bladder orthotopic model, which is close to the observations in a clinical setting.

Materials and methods

Patients and human samples

All 123 patients underwent either TUR or radical cystectomy and simultaneous bilateral pelvic lymph node dissection at the Department of Urology, Akita University School of Medicine between 1995 and 2003. Samples were fixed in formalin, embedded in paraffin, and sectioned for use in microscopic analysis. Informed consent was provided according to the Declaration of Helsinki. Clinical and pathological data were obtained by retrospective chart review, as previously described [15]. The 1997 TNM classifications were used for tissue staging. Ta is a noninvasive papillary carcinoma and T1 means that the tumor invades sub epithelial connective tissue.

Animals, cell lines, and reagents

Approval for these studies was obtained from the institutional review board at Kyoto University Hospital. Specific pathogen-free 6–8-week-old male Balb/c severe combined immunodeficiency (SCID) mice were used (SLC, Kyoto, Japan). The human bladder cancer cell line UM-UC-3 and KU-7, the small cell lung cancer cell line SBC-5, the non-small lung cancer cell line A549, the fibrosarcoma cell line HT1080, and the mesothelioma cell line 211H were obtained from the American Tissue Type Culture collection (Rockville, MD). ZOL and interleukin-2 (IL-2) were obtained from Novartis Pharma AG (Basel, Switzerland) and Shionogi (Osaka, Japan), respectively. UM-UC-3 cells were stably transfected with the pGL3-control vector (Promega, Madison, WI) and pSV2Neo (the American Type Culture Collection) and denoted as UM-UC-3^{Luc}. These cells were maintained as described previously, to use for the in vivo imaging system (IVIS; Xenogen, Alameda, CA), which detects luciferase (Luc) expression [16]. We confirmed that there was no difference in in vitro proliferation between parental UM-UC-3 and UM-UC-3^{Luc}.

Immunohistochemical staining

Immunohistochemical staining was performed by the conventional avidin–biotin–peroxidase complex method (ABC-Elite, Vector Labs), as described previously [16]. Anti-human MHC-1 monoclonal antibody EMR8-5 (Hokudo, Sapporo, Japan) was used at 1:100 dilution for the evaluation of patients samples. Sections were counterstained with hematoxylin and mounted. Normal mouse IgG was used as a negative control.

Immunohistochemical evaluation

All of the specimens were reviewed independently using light microscopy by investigators who were blind to the clinicopathologic data (TY and NT). Staining results were assessed in a semi-quantitative fashion by two independent investigators, as described previously [5]. Briefly, immunoreactivity for MHC class I was categorized on a scale of 0–3 as follows: 0, undetectable staining; 1, incomplete membrane staining in more than 20% of the tumor cells; 2, moderate to complete staining in the cytoplasm of the tumor cells; 3, complete membrane staining in more than 80% of the tumor cells [5]. MHC class I expression was then classified as negative (scores 0, 1, 2) or positive (score 3) [5].

Western blot analysis

Western blotting analysis was performed as described previously [17]. Equal amounts of protein extracts (50 μg) from peripheral blood mononuclear cells (PBMCs), UM-UC-3 or KU-7 were subjected to 12.5% polyacrylamide gel under denaturing conditions (SDS-PAGE) and then electroblotted onto a PVDF membrane (Millipore, Tokyo, Japan), as described previously [17]. Anti-human MHC-1 monoclonal antibody EMR8-5 with a 1:1,000 dilution was used as the primary antibody. Anti-human vinculin (Abcam, Tokyo, Japan) was used as a loading control.

Human $\gamma\delta$ T cell preparations and culture

Informed consent was obtained for the collection of peripheral blood from healthy volunteers. Human $\gamma\delta$ T cells were prepared as described previously [9]. Briefly, PBMCs were separated individually from blood samples donated from five healthy volunteers using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). PBMCs (1×10^5 cells) were stimulated with 5 μM of ZOL and cultured in 24-well round-bottom microtiter wells (Nunc, Wiesbaden, Germany) for 14 days at 37°C. On days 2, 6 and 10, 50 units/mL IL-2 were added to the culture. These ex vivo expanded $\gamma\delta$ T cells were enriched for the cytotoxicity assay both in vitro and in vivo using a magnetic activated cell sorting system (Miltenyi Biotech, Bergisch Gladbach, Germany) to exclude $\alpha\beta$ T cells, as described previously [9]. After the enrichment, the percentage of $\gamma\delta$ T cells was always more than 99.8% (data not shown).

In vitro cytotoxicity assay

The in vitro cytotoxicity of $\gamma\delta$ T cells from three healthy volunteers against SBC-5, HT-1080, UM-UC-3, 211H, and A549 was examined quantitatively using a standard ^{51}Chromium (^{51}Cr) releasing assay [9]. Briefly, 100-μl aliquots of each cell line that had been pretreated with 5-μM ZOL for 12 h were added to ^{51}Cr for the final 2 h and then washed 3 times. The cells were then incubated for 4 h with ex vivo expanded $\gamma\delta$ T cells at an E/T ratio 10:1, the supernatants were collected, and the radioactivity of ^{51}Cr released from target cells was measured in a gamma counter (Wallac, Gaithersburg, MD). The maximum ^{51}Cr release was determined in target cells treated with Triton X-100 at final concentration of 0.5%. The cytotoxicity was defined as the cell lysis percent according to the formula: cell lysis % = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] × 100.

In vivo effects of $\gamma\delta$ T cells in the orthotopic bladder cancer murine model

In order to establish the orthotopic bladder cancer models, Luc-labeled bladder cancer cells (2×10^6) were implanted into the murine bladder cavity via 24-gauge angiocatheters (Termo, Tokyo, Japan), as described previously [16]. Male BALB/c SCID mice were intravesically administered 1×10^7 UM-UC-3^{Luc} cells. Mice were randomized into four groups: (i) untreated mice, (ii) mice treated with 5-μM ZOL (200 μl), (iii) mice treated with 1×10^7 purified $\gamma\delta$ T cells and (iv) mice treated with 5-μM ZOL (200 μl) and 1×10^7 purified $\gamma\delta$ T cells. Randomization was performed 7 (first experiment) and 3 days (second experiment) after the cancer cell transplantation and each group contained 8 mice. 100 μL of 5-μM ZOL for 3 h and/or 1×10^7 $\gamma\delta$ T cells were administered for 5 sequential days from day 8 to day 12 for the first experiment and from day 4 to day 8 for the second experiment by the transurethral and intravesical method. The effect of $\gamma\delta$ T cells on UM-UC-3^{Luc} was monitored by IVIS (total number of photons), as described previously [18]. To examine how many of the $\gamma\delta$ T cells survive after 3-h incubation in the bladder, 1×10^7 $\gamma\delta$ T cells were administrated by the transurethral and intravesical method to intact bladders. $\gamma\delta$ T cell variability was examined after 3-h incubation in the bladders by the trypan blue dye exclusion method. In addition, to examine how $\gamma\delta$ T cells infiltrate healthy bladder epithelium and/or cancer

lesions, 1×10^7 $\gamma\delta$ T cells were also administrated by the transurethral and intravesical method to orthotopic bladder cancer mice models that had been 21 days earlier inoculated with 1×10^7 UM-UC-3^{Luc} cells. After treatment with ZOL and $\gamma\delta$ T cells, the bladders were dissected (each group, $n = 3$) and examined histologically after hematoxylin–eosin staining and immunohistochemically with anti-human CD3 (Novocastra Laboratories Ltd, Newcastle, UK) by light microscopy.

Statistical analysis

All data were entered into an access database and analyzed using Excel 2000 (Microsoft Co., Tokyo, Japan) and SPSS (version 10.0, SPSS, Inc., Tokyo, Japan) software; a probability (P) of <0.05 was required for statistical significance. Chi-square analysis was used for the variables of sex, age, configuration, grade, stage, and lymphatic and vascular involvement. Recurrence of the superficial cancers was defined as positive cytology or a pathologically proven tumor. Recurrence of invasive cancers was defined as clinically detected tumors, chiefly by imaging. Kaplan–Meier analysis was used to estimate the cumulative recurrence free survival, cause-specific survival, and overall survival rates, and the log-rank test was employed to correlate differences in patient survival with the staining intensity of MHC class I. Hazard ratios (HRs) and 95% confidence intervals (CIs) for disease free survival were assessed by the Cox proportional hazard regression model. The influence of the treatment on the growth of bladder cancers was analyzed by the Student's t test.

Results

Relationship between the expression of MHC class I and clinicopathological features in patients with superficial bladder cancer

We initially tested whether the immunohistochemical levels of MHC class I expression correlated with the clinicopathological features, recurrence and progression free survival of patients with superficial bladder cancer. The histologically high grade, T1, and CIS co-existence bladder cancers demonstrated diminished MHC class I expression which was significantly lower than that in the low grade, Ta, and free of CIS cancer patients (Fig. 1a; Table 1). Moreover, although the difference in recurrence free survival was not significant, patients with low MHC class I expression exhibited a significantly shorter progression free survival than patients with high MHC class I expression (Fig. 1b upper row; $P = 0.047$). In addition, of these patients, 22 underwent BCG instillation therapy. In general, patients with low MHC class I expression had a tendency to exhibit shorter progression free survival than the patients with high MHC class I expression, although this difference

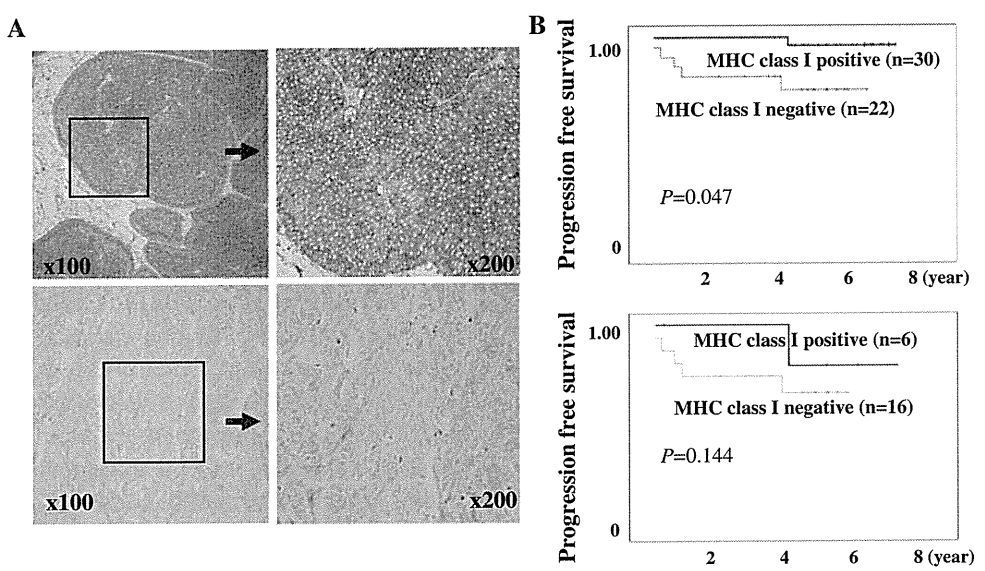

Fig. 1 MHC class I expression correlates with the progression of superficial bladder cancer. **a** MHC class I expression in the tissues of superficial bladder cancer was immunohistochemically analyzed by the conventional avidin–biotin–peroxidase complex method using the anti-human MHC class I monoclonal antibody EMR8-5 at a 1:100 dilution. A strong immune-reaction in low grade bladder cancers (*upper row*) and a weak and diminished immune-reaction in high grade bladder cancers (*lower row*) were typically observed. Nuclear staining was performed with Mayer's hematoxylin. **b** Comparison of the survival curves between patients with bladder cancers expressing high and low levels of MHC class I. Progression free survival curves for patients with superficial bladder cancer (*upper row*) and for those treated with intravesical BCG (*lower row*)

Table 1 Relationship between the level of MHC class I expression and clinicopathological features of the patients with superficial bladder cancer

	Total ($n = 52$)	High ($n = 30$)	Low ($n = 22$)	P value
Age (mean SD)	68.1 (15.4)	67.1 (16.9)	69.4 (13.4)	0.98
Sex				
Male	41	23	18	0.65
Female	11	7	4	
Number of tumors				
CIS	11	3	8	0.50
Solitary	12	7	5	
Multiple	27	20	7	
Grade				
Well/mod	31	23	8	0.0034
Poor	21	7	14	
Depth of invasion				
CIS	11	3	8	0.0013
Ta	27	24	3	
T1	14	3	11	
Recurrence				
Negative	31	18	13	0.95
Positive	21	12	9	
Progression				
Negative	47	29	18	0.072
Positive	5	1	4	
Follow-up in month (mean SD)	43.0 (28.3)	44.9 (31.5)	40.3 (24.5)	0.56

was not statistically significant (Fig. 1b lower row, $P = 0.144$).

Relationship between the expression of MHC class I and clinicopathological features in patients with invasive bladder cancer

Next, we tested whether immunohistochemical levels of MHC class I expression were related to various clinicopathological features and survival rates in patients with invasive bladder cancer. The lymph node involvement and lymphatic invasive bladder cancers demonstrated diminished MHC class I expression by immunohistochemistry, compared to the non-lymph node involvement and non-lymphatic invasive cancers (Fig. 2a; Table 2). Moreover, the patients with low MHC class I expression experienced a significantly shorter disease free survival time and overall survival than patients with high MHC class I expression (Fig. 2b upper and lower row, $P = 0.041$, 0.018, respectively). A Cox proportional hazard regression analysis indicated that perivesical invasion ($\geq$pT3a), high grade, lymph node involvement (pN+), and MHC diminishing were associated with poor disease free survival. However, a multivariate analysis using all of these clinicopathological and molecular factors indicated that only lymph node involvement was a significantly unfavorable prognostic factor independent of other factors ($P = 0.021$). These results suggest that bladder cancers with diminished MHC class I expression are biologically aggressive and treatment-resistant tumors.

Ex vivo-expanded $\gamma\delta$ T cells show cell dose-dependent cytotoxic activity against various cancer cell lines

In order to overcome MHC class I deletion, we investigated the use of $\gamma\delta$ T cells as a potential therapeutic tool. We investigated the cytotoxic activity of ex vivo-expanded $\gamma\delta$ T cells at an E/T ratio of 10:1 against various cancer cell lines. The average cytotoxicity of the $\gamma\delta$ T cells from three healthy volunteers with and without pretreatment of 5 μM of ZOL was 75.2 and 32.4% for SBC-5 small cell lung cancer cells, respectively, 37.3 and 15.9% for HT-1080 fibrosarcoma cells, respectively, 52.2 and 14.8% for UM-UC-3 bladder cancer cells, respectively, 55.0 and 34.2% for 211H mesothelioma cells, respectively, and 30.4 and 13.2% for A549 non-small cell lung cancer cells, respectively (Fig. 3a). The differences in cytotoxicity between cells with no pretreatment and those pretreated with a low dose ZOL were significant for all of the cells examined ($P < 0.001$ for all cells). Thus, $\gamma\delta$ T cells kill various cancer cell lines and a low dose of ZOL can augment their cytotoxic effect.

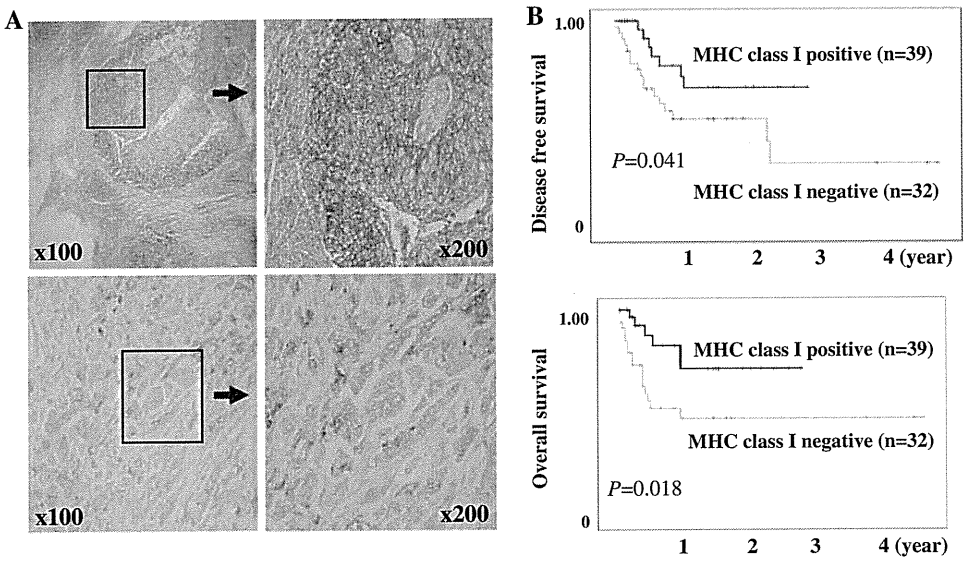

Fig. 2 MHC class I expression correlates with the progression of invasive bladder cancer. **a** MHC class I expression in the tissues of invasive bladder cancer was immunohistochemically analyzed. A strong immune-reaction in moderate grade bladder cancers (*upper row*) and a weak and diminished immune-reaction in high grade bladder cancers (*lower row*) were typically observed. Micropapillary growth pattern of the high grade bladder cancers also demonstrated a diminished immune-reaction (*lower row*). Numbers indicate original magnifications. **b** Comparison of the survival curves between patients with bladder cancers expressing high and low levels of MHC class I. Disease free survival curve (*upper row*) and overall survival curve (*lower row*) for invasive bladder cancer after radical cystectomy

$\gamma\delta$ T cells in the bladder

A total of 86.5 ± 6.3% of $\gamma\delta$ T cells survived after 3-h incubation in the bladder ($n = 5$). UM-UC-3 which was used for in vivo experiments revealed less MHC class I expression than normal human PBMCs and KU-7 cells (Fig. 3b). $\gamma\delta$ T cells massively infiltrated tumor in the bladder, while few $\gamma\delta$ T cells infiltrated the healthy bladder epithelium (Fig. 4a, b). These findings suggested that the treatment of 100 μL of 5-μM ZOL for 3 h and/or 1×10^7 $\gamma\delta$ T cells might show in vivo growth inhibitory effects in the orthotopic murine bladder cancer models.

In vivo growth inhibition of human bladder cancer in orthotopic murine models by transurethral administration of ex vivo-expanded human $\gamma\delta$ T cells

We introduced UM-UC-3^{LUC} cells into the bladder, and observed their bioluminescence by IVIS. Bioluminescence was not detectable the following day, but was faintly detected 3 days later. In the initial experiment (the first experiment), we randomized these mice on day 8 after transplantation and administered the respective intravesical agent for 5 sequential days starting on day 9. There was no difference between these groups, possibly due to a low E/T ratio. In the second experiment, we divided the mice into 4 groups at day 3 after transplantation. We investigated the growth inhibitory effect of the human $\gamma\delta$ T cells with or without a low dose of ZOL (5 μM) in vivo. Figure 4c shows typical images at day 21 taken by IVIS and the growth curves of the respective transplanted cancers in murine bladder (Fig. 4d). Although equivalent numbers of cancer cells were injected, cancer growth rates differed among the treatment groups. Photon emissions from mice treated with both human $\gamma\delta$ T cells and a low dose of ZOL were significantly lower than those from mice in the non-treatment groups ($P < 0.001$). Moreover, the photon emissions from mice treated with human $\gamma\delta$ T cell and a low dose of ZOL were significantly lower than those of the mice from either the $\gamma\delta$ T cells alone or a low dose of ZOL alone ($P = 0.048$, $P < 0.001$, respectively).

The mucosal surfaces of the $\gamma\delta$ T cell treated murine bladders did not show apparent injury and there were no microscopic differences in the non-cancerous bladder mucosa among the treated and non-treatment mice (Fig. 4a). We found no differences in the body weight among the groups of mice. Furthermore, the mice treated with the combination of $\gamma\delta$ T cells and a low dose of ZOL showed apparently better survival compared with the non-treated, $\gamma\delta$ T cell alone or a low dose of ZOL alone treatment groups (Fig. 4e). The median survival time was 31, 58, and 44 days for mice not treated, treated with $\gamma\delta$ T cell alone, and treated with a low dose of ZOL alone, respectively. The median survival time was not reached by mice treated with the combination of $\gamma\delta$ T cell and a low dose of ZOL until the end of the experiment.

A combination of $\gamma\delta$ T cells and a low dose of ZOL inhibited the growth of bladder cancers not only in vitro,

Table 2 Relationship between the level of MHC class I expression and clinicopathological features of the patients with invasive bladder cancer

	Total ($n = 71$)	High ($n = 39$)	Low ($n = 32$)	P value
Age (mean SD)	67.0 (12.6)	67.4 (14.2)	66.4 (11.0)	0.96
Sex				
Male	55	33	22	0.11
Female	16	6	10	
Configuration				
Papillary	14	9	5	0.52
Non-papillary	53	29	24	
Flat	4	1	3	
Number of tumors				
Solitary	24	12	12	0.41
Multiple	43	26	17	
Flat	4	1	3	
Grade				
Well/mod	10	8	2	0.085
Poor	61	31	30	
Depth of invasion				
≤T2	26	15	11	0.72
>T3	45	24	21	
Lymph node involvement				
Negative	18	6	12	0.027
Positive	47	30	17	
Unknown	6	3	3	
Lymphatic invasion				
Negative	31	22	9	0.017
Positive	40	17	23	
Venous invasion				
Negative	41	26	15	0.093
Positive	30	13	17	
Follow-up in month (mean)	28.7 (25.8)	27.3 (20.0)	30.2 (30.2)	0.67

but also in vivo, and the duration of survival was also significantly prolonged by these treatments without any severe adverse effects in the murine model.

Discussion

In this study, we demonstrated that a loss of MHC class I expression in bladder cancer is a poor prognostic factor both for the progression of superficial cancers and the survival of invasive bladder cancers. Approximately 40–90% of human cancers derived from various MHC class I positive tissues have been reported to be MHC class I deficient [6]. Bladder cancers exhibiting a down-regulation of MHC class I expression acquire the ability to escape from T cell-mediated immune responses, consequently resulting in tumor development, progression, and a poor outcome; these observations are in line with other recent reports [5, 6, 19]. Kitamura et al. [5] also clearly show that the expression of MHC class I molecules on bladder cancer cells contributes significantly to the therapeutic effect of BCG immunotherapy and to recurrence free survival of superficial bladder cancer. In contrast, Sharma et al. [20] reported that the presence of intratumoral CD8-positive tumor-infiltrating cytotoxic cells, not MHC class I expression, was significantly associated with clinical outcome among patients with invasive bladder cancer. These controversies might come from the sensitivity of the antibody against human MHC class I. However, their study is also in line with our observation that the CTL-mediated tumor immune microenvironment plays an important role in the tolerance of BCG therapy.

More than 50% of bladder cancers will recur intravesically, and 20–30% of the these cancers will develop to a higher grade and/or stage within the first 5 years of treatment, and progress to local invasive cancers [20]. In order to avoid progression to an invasive cancer, we have investigated the use of small interfering RNA (siRNA) targeting

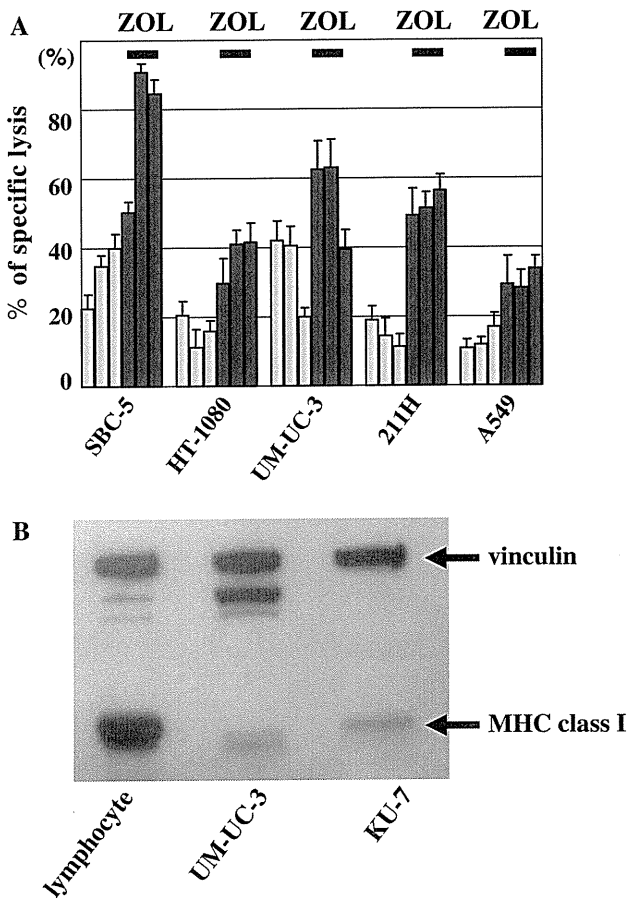

Fig. 3 In vitro combined effects of $\gamma\delta$ T cells with ZOL on various cancer cells and their MHC class I expression. **a** Augmentation of the cytotoxic activity of the ex vivo-expanded $\gamma\delta$ T cells by a low dose of ZOL (5 μM) in various cancer cell lines. In vitro cytotoxicity of $\gamma\delta$ T cells from 3 healthy volunteers against SBC-5, HT-1080 UM-UC-3, 211H, and A549 was examined quantitatively by a standard ^{51}Chromium (^{51}Cr) releasing assay. Each value indicates the mean ± SEM ($n = 6$). **b** MHC class I expressions of human normal PBMNCs, UM-UC-3 and KU-7. The data shown are representative of 3 independent experiments

the *polo like kinase-1 (PLK-1)* gene as a novel therapeutic approach against bladder cancer [16]. We have demonstrated that intravesical administration of *PLK-1* targeted siRNA/cationic liposomes inhibited cancer growth in murine orthotopic bladder cancer models and that the transurethral siRNA therapy could overcome the drug delivery system problem of siRNA delivery.

Molecular targeted therapies are very promising especially for the hematological disorders such as Abl tyrosine kinase inhibitors for chronic myeloid leukemia [21]. However, the efficacy of molecular targeted therapy for solid malignancy remains to be unsolved. A recent topic for urological oncology is the success of sunitinib, which is a novel specific inhibitor of the receptor tyrosine kinases, and anti-angiogenic agents for metastatic renal cell cancer. Although progression free survival was longer and response rates were higher in patients with metastatic renal cell cancer who received sunitinib than in those receiving interferon-α, complete remission was seldom seen even in patients who received sunitinib [22]. The pathogenesis of solid malignancies such as bladder cancer is complicated; therefore, the efficacy of simple gene target therapy is doubtful. We attempted to perform immunotherapy in combination with molecular targeted therapy. We investigated the use of the anti-cancer action of $\gamma\delta$ T cells, which represent a minor subset of human peripheral T cells (1–10%) and contribute to the host immune defense in a different way to CTLs [8].

In this study, we initially demonstrated that $\gamma\delta$ T cells, proliferated by bisphosphonates, demonstrated active anti-cancerous effects against various cancer cells in vitro; this is consistent with previous reports [23]. Kato et al. [23] clearly demonstrated that bladder cancer cells can efficiently present bisphosphonate and pyrophosphomonoester compounds to $\gamma\delta$ T cells, inducing specific proliferation and interferon-γ production. Internalization of bisphosphonates by cancer cells led to rapid inhibition of farnesyl pyrophosphate (FPP) synthase, resulting in intracellular accumulation of isopentenyl pyrophosphate (IPP) upstream of FPP synthase in the mevalonate pathway (Supplemental Fig. 1) [7, 24]. Accumulated IPP acts as a powerful danger signal that activates the immune response and as such might represent a novel target for tumor immunotherapy [7, 9]. We have reported that the tumor killing mechanism of $\gamma\delta$ T cells depends mainly on direct contact involving a perforin-dependent cytolytic pathway [9, 25]. Consequently, we advanced to in vivo experiments.

Here, we have demonstrated that intravesical administration of $\gamma\delta$ T cells with a low dose of ZOL inhibited cancer growth in a murine orthotopic bladder cancer model. Previously, we have confirmed that a high dose of intravesical ZOL resulted in growth inhibition of bladder cancer without any severe side effects [26]. Most current immunotherapeutic approaches such as BCG therapy, which is currently the most effective agent for bladder cancer treatment aim to induce an antitumor response via stimulation of the adaptive immune system, which is dependent on MHC-restricted CTLs. In contrast, $\gamma\delta$ T cells from healthy volunteers, which were proliferated ex vivo, were able to function in an MHC-unrestricted manner and thus, MHC-unrestricted $\gamma\delta$ T cells represent a promising new concept in immunotherapy. The initial failure of the first experiment was probably due to an insufficient E/T ratio; therefore, in the second experiment we administrated $\gamma\delta$ T cells earlier. Improvement of the E/T ratio gave us satisfactory results. Although we have not yet investigated whether patient's $\gamma\delta$ T cells behave similarly as those from healthy volunteers, patient's $\gamma\delta$ T cells might have antitumor effects. Kobayashi

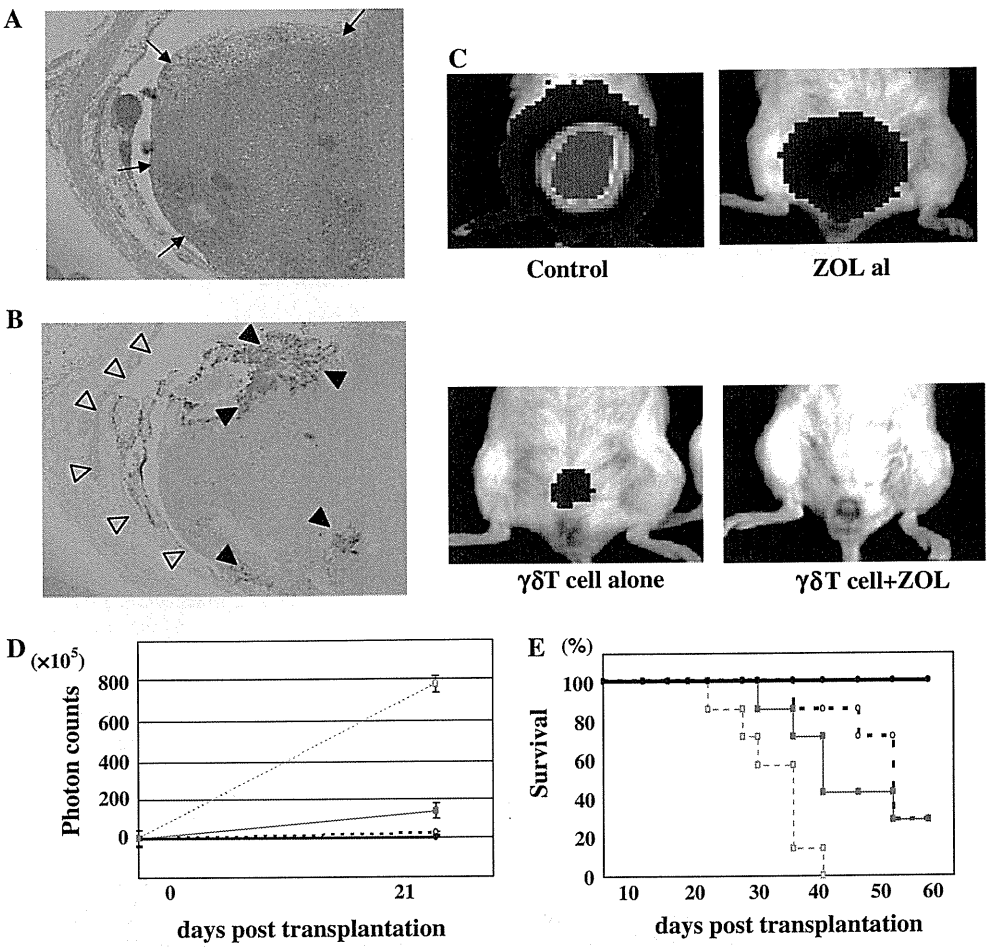

Fig. 4 In vivo effects of intravesical administrated γδ T cells. After 3-h incubation with ZOL and γδ T cells, the bladders was occupied by a large tumor (↑) were dissected and examined histologically with hematoxylin-eosine staining (**a**) and immunohistochemically with anti-human CD3 antibody (**b**). Although the region of healthy bladder epithelium (*triangle*) was not infiltrated by γδ T cells, the tumor was massively infiltrated by γδ T cells indicated by human CD3 positivity (*filled triangle*). Original magnification ×16. The data shown are representative of 3 independent experiments. **c** In vivo effects of intravesical γδ T cells in the orthotopic bladder cancer murine model. Typical images of the respective mice not treated or treated with a low dose of ZOL alone, γδ T cells alone, or γδ T cells and a low dose of ZOL, were observed by IVIS. **d** The growth curves of orthotopically transplanted UM-UC-3^{LUC} were measured by IVIS. The anti-cancerous effect of intravesical ex vivo-expanded γδ T cells was demonstrated in vivo: no treatment, *open square*; treatment with a low dose of ZOL alone, *closed square*; treatment with γδ T cells alone, *open circle*; treatment with γδ T cells and a low dose of ZOL, closed circle. **e** The survival curves of mice not treated or treated with γδ T cells alone, a low dose of ZOL alone, or γδ T cells and a low dose of ZOL. Survival of the orthotopic mice was improved by the intravesical administration of ex vivo-expanded γδ T cells: no treatment, *open square*; treatment with a low dose of ZOL alone, *closed square*; treatment with γδ T cells alone, *open circle*; treatment with γδ T cells and a low dose of ZOL, *closed circle*

et al. [27] reported that adoptive immunotherapy using in vitro-activated autologous γδ T cells induced antitumor effects in patients with advanced renal cell carcinoma after radical nephrectomy. Taken together, we hypothesize that a combination of molecular targeted therapy such as PLK-1 siRNA and this novel MHC class I-unrestricted immunotherapy may overcome the progression of bladder cancer.

In conclusion, we believe that the antitumor effect of intravesical autologous γδ T cells is an attractive tool for use as a novel immunotherapy and may cause a breakthrough in clinical applications of cell therapeutics. The efficacy and safety of intravesical γδ T cells should be verified by early phase clinical trials.

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Editorial Comment

Editorial Comment to docetaxel-based combination chemotherapy with zoledronic acid and prednisone in hormone refractory prostate cancer: Factors predicting response and survival

Docetaxel is a microtubule-targeted tubulin-polymerizing agent that has come to occupy a central role in the treatment of various cancers, both in early and advanced settings. In 2004, two landmark trials, namely TAX 327 and SWOG 99-16, showed for the first time that docetaxel-based chemotherapy improves the survival of metastatic castration-resistant prostate cancer (CRPC) patients.[1,2] However, compared with mitoxantrone, the median improvement in survival was only ~2.5 months, which underscores the need for better therapies, in particular the need for docetaxel partners that deliver superior outcomes. Zoledronic acid is a highly potent nitrogen-containing bisphosphonate that has been shown to reduce skeletal complications in patients with bone metastases arising from various solid cancers, including prostate cancer.[3] Bone metastasis occurs in the majority of patients with advanced prostate cancer and is a clinically significant issue that affects the management of these patients.[1,2] Accumulated evidence has revealed that zoledronic acid and docetaxel act synergistically or additively in inhibiting the growth of various malignancies, including leukemia, lung cancer, bladder cancer, prostate cancer, and breast cancer.[4] Thus, the chemotherapeutic combination consisting of docetaxel, prednisolone, and zoledronic acid may provide superior outcomes and should be studied.

In this issue of the *International Journal of Urology*, Nayyar *et al.* describe their prospective study analyzing the efficacy and safety of the combination of docetaxel and zoledronic acid for CRPC.[5] While the patients in this study had been treated with at least two regimens of hormonal therapy, including combined androgen blockade using luteinizing hormone-releasing hormone (LH-RH) analog and bicalutamide and anti-androgen withdrawal, and a second line hormonal therapy with fosfestrol or diethylstilbestrol, none had been given flutamide or estramustine phosphate. The purpose of chemotherapy for patients with CRPC is to prolong their survival and improve their quality of life. While the observations made by Nayyar *et al.* should be considered as preliminary, because the observation period is rather short and the median overall survival has not yet been reached, an objective PSA response (i.e., a decrease in PSA levels of more than 50%) was seen in 26/44 cases (59.1%) without any severe adverse effects being observed. In addition, 17/44 cases (38.6%) expired with a median overall survival of 15 months. These observations suggest that the combination of docetaxel and zoledronic acid could prolong the survival of CRPC patients.[5] I look forward to the final results of this study and the analyses of the factors that are predictive of response and survival. In

particular, it would be of interest to see the results of single nucleotide polymorphism analyses, if possible.

Although adding zoledronic acid to docetaxel could be a relatively safe and effective treatment option for CRPC patients, the optimal treatment time, the optimal treatment order, and the optimal therapeutic partner with docetaxel remain to be determined. In addition, the efficacy of novel agents, including the endothelin-A receptor agonist atrasentan, the small molecule angiogenesis inhibitors, and other new agents, is currently under investigation in clinical trials. Further investigations are necessary to resolve these clinically relevant issues.

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Clinical significance of a single nucleotide polymorphism and allelic imbalance of matrix metalloproteinase-1 promoter region in prostate cancer

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Abstract. Matrix metalloproteinase-1 (MMP-1) is associated with cancer invasion and metastasis. The *2G* allele of the polymorphic site in the *MMP-1* promoter was demonstrated to have a higher transcription activity than the *1G* allele. Allelic imbalance (AI) at 11q22 harboring the *MMP-1* is frequently observed in various cancers and may be associated with an advanced disease. We conducted a case-control study to determine the association of the *MMP-1* genotype with susceptibility to prostate cancer involving 283 prostate cancer patients and 251 controls. Furthermore, AI, retention allele of the *MMP-1* promoter, and MMP-1 protein expression were analyzed in 77 prostate cancer specimens. The *MMP-1* promoter polymorphism was associated with neither susceptibility nor progression of prostate cancer. Tumors with *1G/2G* and *2G/2G* genotypes had a significantly higher MMP-1 expression level compared to those with *1G/1G* genotype (P=0.006 and 0.013, respectively). AI at 11q22 was observed in 13 (40.6%) of 32 informative cases. Retention of the *1G* and *2G* alleles were observed in 4 and 9 cases, respectively. AI was significantly associated with the Gleason score (P=0.003) and pathological stage (P=0.022). In addition, retention of the *2G* allele showed a significant association with the pathological stage (P=0.026). AI at 11q22 region, retention of the *2G* allele, specifically appeared to be involved in the progression of prostate cancer. However, the presence of the *2G* allele of the *MMP-1* promoter polymorphism itself seems to influence neither the susceptibility nor the progression of prostate cancer.

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Introduction

Tumor invasion or metastasis is one of the most crucial events for determining the outcome of cancer patients. In the first step of these events, several proteinases such as serine proteinase urokinase plasminogen activator and matrix metalloproteinases (MMPs) degrade the extra-cellular matrix (ECM), so that the tumor cells can migrate to the stromal tissue through the basement membrane (1). MMP-1, a member of MMP family, is abundantly expressed in many types of cancer cells and adjacent stromal fibroblasts (2-5), and digests various elements of the ECM including collagen types I, II, II, VII, VIII, X, and XI (6). The overexpression of MMP-1 has been shown to be associated with tumor progression and poor outcomes for cancers of the digestive system and melanoma (3,5,7,8).

The expression of *MMP-1* is mainly regulated by activated protein-1 (AP-1) transcription factor that mediates signal transduction from cytokines and growth factors such as interferons, interleukins, epidermal growth factor, and fibroblast growth factor (9). In the promoter region of the *MMP-1*, three AP-1 binding sites are located at -72, -186, and -1062 bp from the transcription start site. Insertion of an extra guanine residue adjacent to the AP-1 binding site at -1062 bp creates a new binding site for ETS transcription factor at -1602 bp (5'-*AAGGAT*-3'; *2G*), and both the AP-1 and ETS cooperatively enhance the expression of *MMP-1* (10). AP-1 binding to the -1062 bp acts as a repressor of transcription when the polymorphic site has only one guanine residue (5'-*AAGAT*-3'; *1G*) (11).

Recent epidemiological studies have demonstrated almost consistent results regarding the biological function of the *MMP-1* promoter polymorphism and its association with the susceptibility or progression in several cancer types (4,12-23). The *2G* allele of the polymorphism has been reported to be related with increased risk of colorectal, lung, endometrial, ovary, kidney, and bladder cancers (4,12-20), while the *2G* allele was associated with tumor progression or patient survival but not with susceptibility of uterus, stomach, and colorectal

cancers (21-23). However, the results of most studies are consistent with each other regarding the risk associated with the *2G* allele. Meanwhile, 11q22 harboring the *MMP-1* gene exhibits amplification in esophageal, colorectal, cervical, and prostate cancers (24-27). Determining which of the polymorphic alleles is amplified and how the amplified allele affects the expression of MMP-1 or phenotypes of cancers is also of interest. In prostate cancer, immunohistochemical and *in situ* hybridization analysis have detected MMP-1 in cancer cells as well as in normal adjacent and prostatic intraepithelial cells (28). However, a previous study that evaluated the effect of the polymorphism on prostate cancer found no association between MMP-1 and susceptibility or metastatic status of prostate cancer (29). There have been few studies focusing on the clinical implication of MMP-1 expression, promoter polymorphism, and allelic imbalance (AI) of 11q22.

We hypothesized that the *1G/2G* polymorphism or AI of the *MMP-1* promoter region affects the development and/or progression of prostate cancer. To validate this hypothesis, we first conducted a case-controlled study to examine the effect of the polymorphism on the risk and progression of prostate cancer, and then investigated how the genotype and AI of the region affected the malignant potential, invasiveness of the tumors, and protein expression of MMP-1.

Materials and methods

Subjects. We studied a series of 524 registered subjects, including 283 patients with prostate cancer and 251 control males at the Akita University Medical Center and related community hospitals in Akita prefecture, who agreed to participate in this study and provided blood specimens. Prostate cancer patients were selected from April 1997 to December 2003 and control subjects were selected from March 1998 to September 2001.

For all patients with prostate cancer, histological evaluation was performed on specimen obtained by transrectal needle biopsy or transurethral resection of the prostate for voiding symptoms. The clinical or pathological stage of prostate cancer at the time of diagnosis was determined by reviewing the medical records based on the Tumor-Node-Metastasis system. Prostate cancer was classified into stage A (T1a-bN0M0), stage B (T1c-2N0M0), stage C (T3-4N0M0), or stage D (T1-4N1M0-1 or T1-4N0-1M1) by the modified Whitmore-Jewett system. In patients who underwent radical prostatectomy, the final pathological stage was applied and in patients who did not undergo radical prostatectomy, clinical stage was applied. Pathological grading of PCa was determined according to the General Rule for Clinical and Pathological Studies on Prostate Cancer by the Japanese Urological Association and the Japanese Society of Pathology, which is mostly based on the WHO criteria and the Gleason score. All pathological grading was based on needle biopsy specimens in stages B-D patients and surgical specimens in stage A patients. Well, moderately, and poorly differentiated carcinomas generally correspond to Gleason scores of 2-4, 5-7, and 8-10, respectively. In the present study, because the two grading systems were individually used by local pathologists, the tumor grading system was newly categorized as follows: low grade cancer included well-differentiated or Gleason 2-4 carcinomas, intermediate grade cancer included moderately differentiated or Gleason 5-7 carcinomas, and high grade cancer included poorly differentiated or Gleason 8-10 carcinomas. No definitive pathological grade could be determined in 24 patients due to inadequate information or inappropriate classification by local pathologists.

The male controls comprised 251 volunteers without any apparent voiding symptoms. They were selected randomly from the native Japanese population attending community-based medical check-ups. They were all tested for total serum PSA levels (the Tandem-R assay), and those with abnormal total PSA levels (4.0 ng/ml or more) were omitted from the study. Written informed consent was obtained from all the subjects. The present study was approved by the Institutional Review Board of the Akita University School of Medicine, Akita, Japan.

Genotyping of the MMP-1 promoter polymorphism. DNA was extracted from the collected blood samples of each subject using a QIAamp Blood Kit (Qiagen, Hilden, Germany). The PCR was performed in a final volume of 15 μl containing 20 ng genomic DNA, 5 pmol forward primer (5'-GTT ATG CCA CTT AGA TGA GG-3'), 5 pmol reverse primer (5'-CTT GGA TTG ATT TGA GAT AAG-3'), 0.2 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 1.0 mM $MgCl_2$, and 0.5 unit of Ampli-Taq Gold DNA polymerase (Perkin-Elmer, Branchburg, NJ). Initial denaturation at 94°C°C for 30 sec, annealing at 55°C, and extension at 72°C for 30 sec with final extension at 72°C for 7 min. The forward primer was labeled with a fluorescent dye, HEX, to examine the size of PCR products with an autosequencer (ABI 310, Applied Biosystems, Foster, CA) as described previously (30). The size of the PCR products was determined in comparison with an internal ROX 400-size standard (Applied Biosystems) and analyzed using GENESCAN software v3.1 (Applied Biosystems) (Fig. 1A). The 72-bp and 73-bp fragments of amplified DNA are equivalent to *1G* allele and *2G* allele, respectively. The validity of the analysis was confirmed by direct sequencing of several PCR samples using ABI PRISM 310 DNA sequencer (Applied Biosystems).

Analysis of allelic imbalance (AI). AI of the polymorphic site of *MMP-1* promoter was analyzed using DNA pairs obtained from the tumor and peripheral blood samples in a subgroup of 77 patients who underwent radical prostatectomy. Pathological diagnosis was confirmed by microscopic examination of hematoxylin and eosin (H&E)-stained sections. Regions of >80% tumor density were marked on H&E-stained slides to be used as guidelines for microdissection. The target foci were microdissected using a 20-gauge needle, comparing the slide with the H&E staining in the same position. DNA was extracted using a DEXPAT kit (Takara Biomedical Inc., Shiga, Japan), precipitated in cold ethanol with sodium acetate and Pellet Paint NF, Co-Precipitant (Novagen, Madison, WI), and resuspended in Tris-EDTA buffer. Peripheral blood and corresponding prostate tissue DNA samples were simultaneously amplified and both the PCR products were analyzed using ABI PRISM 310 DNA sequencer as described above. AI was determined by measuring the signal imbalance between the opposing alleles. Presence of AI was detected when one

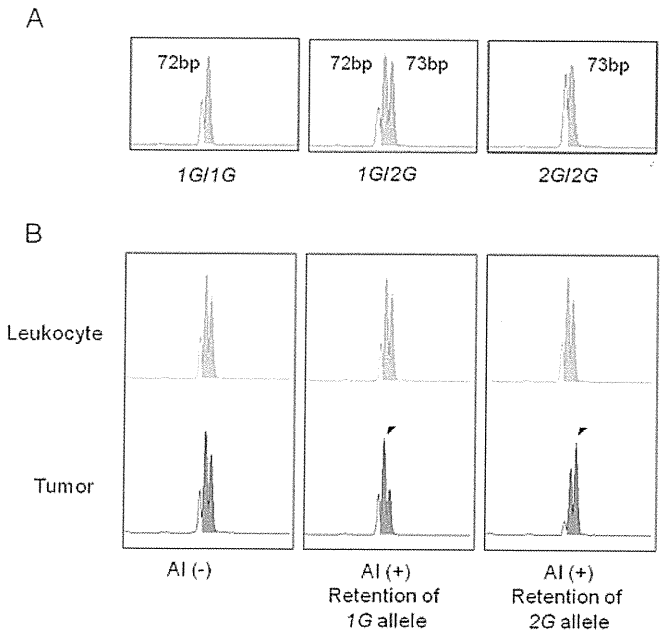

Figure 1. Genotyping the *MMP-1* promoter insertion polymorphism using autosequencer and GENESCAN software are illustrated (A). *1G* and *2G* alleles are identified as 72-bp and 73-bp PCR amplified DNA fragments, respectively. Allelic imbalance (AI) is determined when one of the two signal peaks in a PCR product from the prostate tumor DNA was <70% of that from the corresponding peripheral blood (leukocyte) DNA. Representative results for AI analysis, no AI, retention of the *1G* allele, and retention of the *2G* allele (from the left to the right), are shown (B). Arrows indicate retention alleles.

of the signal peaks in a PCR product from prostate tumor DNA was <70% of that from the corresponding peripheral blood DNA on the GENESCAN software (Fig. 1B). Although the AI was reportedly attributed to amplifications of the 11q22 region containing the *MMP-1* promoter, an allele that had a significantly higher signal peak was determined as a retained allele because the absolute copy number of the gene was not determined in this study.

Immunohistochemical analysis of MMP-1 expression. Seventy-seven prostate specimens obtained at radical prostatectomy were subject to immunohistochemical analysis. The specimens were fixed in 10% buffered formalin and embedded in paraffin. Individual paraffin blocks containing cancer lesions with representative Gleason scores were selected from each specimen. Tissue sections (5 μm) were de-paraffinized in xylene and rehydrated through a graded ethanol series. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 15 min. The sections were boiled in 0.01 M citric acid (pH 6.0) to retrieve the antigen and non-specific binding was blocked with 5% goat serum for 10 min. After washing, mouse monoclonal anti-human MMP1 antibody (F-67; Daiichi Fine Chemical Co., Ltd., Toyota, Japan) at 1:1000 was applied and incubated at 4°C overnight. After washing in PBS, secondary antibody conjugated with horseradish peroxidase (EnVIsion system; Dako, Japan Inc., Tokyo, Japan) was applied, followed by incubation at room temperature for 30 min. After washing in PBS again, tissue sections were developed with diaminobenzidine (DAB; Nichirei Biosciences Inc., Tokyo, Japan) and counterstained with hematoxylin.

The expression of MMP-1 was classified into four categories according to the staining intensity (i.e., negative, weak, intermediate, and strong) (Fig. 2). Assessment was performed by two independent observers unaware of clinical information. In the statistical analysis, patients with Gleason score of 8 or more were compared with those with a Gleason score of 7 or less, because patients with a Gleason score of 8 or more had a greater chance of recurrence after radical prostatectomy than those with a Gleason score of 7 or less (31). Similarly, pathological categorization of tumor stage was dichotomized as T3a or less versus T3b or more, because previous studies have demonstrated that patients with pT3b or more tumor stage had a significantly greater chance of recurrence than those having pT3a or less tumor stage (31).

Table I. Demographic data of subjects analyzed in the cohort study.

	Cases	Controls
Total number	283	251
Mean age (years ± SD)	72.0±8.3	70.0±7.6
Tumor stage		
A	24	
B	101	
C	49	
D	109	
Tumor grade[a]		
Low	49	
Intermediate	114	
High	96	
Unknown	24	

[a]Low, Gleason score 2-6; intermediate, 7; high, 8-10.

Statistical analysis. The data were analyzed by SSPS version 16.0J software (SSPS Inc., Chicago, IL). Hardy-Weinberg equilibrium analyses were performed to compare the observed and expected genotype frequencies using the χ^2 test. The age-adjusted odds ratio (aOR) and 95% confidence interval (CI) for the relative risk of prostate cancer in each genotype were determined by multiple logistic regression analysis with the inclusion of age. The relationships between tumor stage and AI status, and between the genotype and MMP-1 expression level were analyzed by the χ^2 test. A probability of <0.05 was required for statistical significance.

Results

Association of the MMP-1 promoter polymorphism with a risk and progression of prostate cancer. The demographics of the study subjects are shown in Table I. The mean age of male controls and prostate cancer patients was 70.0±7.6, 72.0±8.3, respectively. The numbers of *1G/1G*, *1G/2G*, and

Table II. Genotype distribution and logistic regression analysis of the *MMP-1* promoter polymorphism.

	Genotype		aOR[a] (95% CI)	P-value	Genotype		aOR[b] (95% CI)	P-value
	1G/1G	*1G/2G* + *2G/2G*			*1G/1G* + *1G/2G*	*2G/2G*		
Control (%)	33 (13.1)	218 (86.9)	1.000		133 (53.0)	118 (47.0)	1.000	
Prostate cancer (%)	35 (12.4)	248 (87.6)	1.040 (0.622-1.739)	0.880	157 (55.5)	126 (44.5)	0.934 (0.662-1.317)	0.697
Tumor stage								
A + B	18 (14.4)	107 (85.6)	1.000		69 (55.2)	56 (44.8)	1.000	
C + D	17 (10.8)	141 (89.2)	1.418 (0.696-2.887)	0.336	88 (55.7)	70 (44.3)	0.988 (0.616-1.586)	0.961
A + B + C	22 (12.6)	152 (87.4)	1.000		93 (53.4)	81 (46.6)	1.000	
D	13 (11.9)	96 (88.1)	1.061 (0.510-2.209)	0.874	64 (58.7)	45 (41.3)	0.833 (0.513-1.353)	0.461
Tumor grade								
Low	8 (16.7)	41 (83.3)	1.000		28 (57.1)	21 (42.9)	1.00	
Intermediate + High	25 (11.9)	185 (88.1)	1.467 (0.615-3.499)	0.388	118 (56.2)	92 (43.8)	1.043 (0.555-1.959)	0.896
Low + Intermediate	23 (14.1)	140 (85.9)	1.000		89 (54.6)	74 (45.4)	1.000	
High	10 (10.4)	86 (89.6)	1.439 (0.650-3.187)	0.369	57 (59.4)	39 (40.6)	0.824 (0.493-1.379)	0.824

[a]Age-adjusted odds ratio (aOR) are expressed against *1G/1G* genotype. [b]aOR are expressed against *1G/1G* + *1G/2G* genotypes.

2G/2G genotype in the control group were 33 (13.1%), 100 (39.7%), and 118 (46.8%), respectively, whereas those in prostate cancer group were 35 (12.4%), 122 (43.1%), and 126 (44.5%), respectively, demonstrating no statistical significance (P=0.746). The frequencies of the *1G* and *2G* in the control group were 166 (33.1%) and 336 (66.9%), whereas those in the prostate cancer group were 192 (33.9%) and 374 (66.1%), respectively. There was no statistically significant difference in the allelic frequency between the two groups (P=0.768). The observed genotype frequency of the polymorphism in the control group did not significantly differ from the expected frequencies according to the Hardy-Weinberg equilibrium (data not shown). The genotype distribution of the *MMP-1* promoter polymorphism and results of logistic regression analyses are summarized in Table II. Age-adjusted logistic regression analysis showed no significant association between the genotypes and the risk of prostate cancer in either the dominant (*1G/1G* vs. *1G/2G* or *2G/2G*) or the recessive (*1G/1G* or *1G/2G* vs. *2G/2G*) models. Next, the associations between the genotypes and the tumor stage were analyzed using both dominant and recessive models. There were no significant differences between stage A/B (localized) and stage C/D (invasive), stage A/B/C (non-metastatic) and stage D (metastatic), between low and intermediate/high grade, or between low/intermediate and high grade and low grade cancer.

Association of AI of the MMP-1 promoter region with pathological stage or tumor grade of prostate cancer. Association of AI with pathological T status or tumor grade is summarized in Table III. Of the 77 prostate cancer specimens, an AI was evaluated in 32 informative cases (41.6% of all the cases), of which 13 (40.6% of informative cases) had an AI. Two (13.3%) of the 15 cases with a Gleason score of 7 or less had an AI, whereas 11 (64.7%) of the 17 cases with a Gleason score 8 or more had an AI. Similarly, 7 (29.2%) of 24 cases with pT3a or less disease had an AI, while 6 (75.0%) of 8 with pT3b or more disease had an AI. Among 13 cases with an AI, retention of *1G* and *2G* allele was observed in 4 (30.8%) and 9 (69.2%) cases, respectively. The association of the AI status was significant in association with a higher Gleason score (P=0.003) and a higher pathological tumor stage (P=0.022). Although a retention allele showed no statistically significant association with a Gleason score (P=0.522), the frequency of the *2G* allele retention significantly increased with tumor invasiveness (P=0.026) (Table III).

Association of MMP-1 expression with the MMP-1 promoter polymorphism and AI of the MMP-1 promoter region. In 77 patients who underwent radical prostatectomy, we evaluated the relationship between the *MMP-1* promoter polymorphism and MMP-1 expression levels (Table IV). In immunohistochemical analysis of MMP-1, negative, low, moderate, and high expression was observed in 3 (3.9%), 14 (18.2%), 39 (50.6%), and 21 (27.3%) cases, respectively. Tumors with *1G/2G* and *2G/2G* genotypes had a significantly higher MMP-1 expression level compared to those with *1G/1G* genotype (P=0.006 and 0.013, respectively), whereas there was no significant difference in the expression level between the *1G/2G* and *2G/2G* genotypes (P=0.581). AI status of

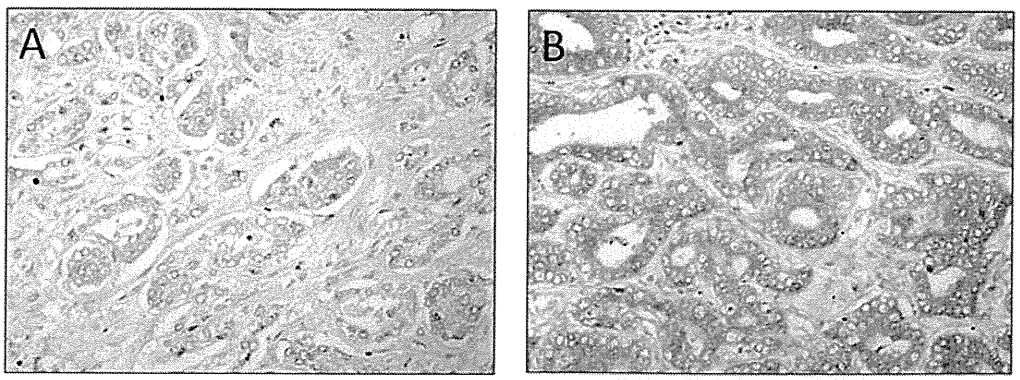

Figure 2. Representative immunohistochemical stainings demonstrating weak (A) and strong (B) expression of MMP-1.

Table III. Association of allelic imbalance and retention allele of the *MMP-1* promoter region with Gleason score and pathological stage.

	Total	Informative case	Allelic imbalance		P-value	Retention allele		P-value
			-	+		*1G*	*2G*	
Gleason score								
≤7	44	15	13	2		1	1	
≥8	33	17	6	11	0.003	3	8	0.561
T status								
≤pT3a	55	24	17	7		4	3	
≥pT3b	22	8	2	6	0.022	0	6	0.026

Table IV. Association of MMP-1 expression with the polymorphism and allelic imbalance in the *MMP-1* promoter region.

	Genotype			Allelic imbalance	
IHC[a]	*1G/1G*	*1G/2G*	*2G/2G*	-	+
Negative	1 (11.0)	1 (3.1)	1 (2.8)	1 (9.1)	0 (0.0)
Weak	4 (44.5)	4 (12.5)	6 (16.7)	3 (18.2)	1 (7.6)
Moderate	4 (44.5)	16 (50.0)	19 (52.8)	10 (45.6)	6 (46.2)
Strong	0 (0.0)	11 (34.4)	10 (27.8)	5 (27.3)	6 (46.2)
P-value		0.006[b]	0.013[b]		0.156
			0.581[c]		

[a]IHC, immunohistochemistry; [b]vs. *1G/1G*; [c]vs. *1G/2G*.

tumors was not significantly associated with the MMP-1 expression level (P=0.156) (Table IV).

Discussion

The association of the *2G* allele of the polymorphism with a higher MMP-1 expression has been reported in several cancer types (4,12,21,32). Immunohistochemical or gene expression analyses of surgical specimens showed that ovarian, cervical, and endometrial cancers with the *2G* allele had a higher expression of MMP-1 than those with the *1G/1G* genotype (4,12,21), and tongue cancers with the *2G/2G* genotype showed a higher MMP-1 expression than those with the *1G* allele (32). Our study demonstrated that prostate cancer with the *2G* allele also had a significantly higher MMP-1 expression level than that with *1G/1G* genotype, the observation being consistent with previous studies. The increased expression of MMP-1 under the existence of the *2G* allele was confirmed by the luciferase assay using constructs with either *1G* or *2G* at the promoter region (-1602 bp) in fibroblast, melanoma, and breast cancer cell lines (10,11). In these cells, the *2G* construct resulted in a