tients with varied characteristics; therefore, it would be difficult to apply the findings obtained from this study to an entire cohort of NMIBC, particularly those showing high grade disease. In addition, the outcomes presented in this study strongly suggest the involvement of a switch from E-cadherin to N-cadherin in the molecular mechanism mediating intravesical recurrence; however, it would apparently be warranted to perform experimental studies using human NMIBC model systems to address the functional role of cadherin switching in intravesical recurrence following TUR.

In conclusion, we analyzed the expression profiles of E-cadherin and N-cadherin in NMIBC as predictors of intravesical recurrence following TUR, and demonstrated that patients with NMIBC characterized by negative E-cadherin as well as positive N-cadherin expression are significantly more likely to develop postoperative intravesical recurrence. These findings suggest that switching from E-cadherin to N-cadherin expression might be involved in the mechanism underlying intravesical recurrence of NMIBC.

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Clinical Trial Note

Watchful Waiting Versus Intravesical BCG Therapy for High-grade pT1 Bladder Cancer with pT0 Histology After Second Transurethral Resection: Japan Clinical Oncology Group Study JCOG1019

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A Phase III clinical trial has been started in Japan to determine the optimal treatment strategy for patients with high-grade pT1 bladder cancer who have pT0 histology after second transurethral resection. The aim of this trial is to demonstrate the non-inferiority of relapse-free survival (excluding Tis or Ta intravesical recurrence) for watchful waiting compared with intravesical bacillus Calmette–Guérin therapy for pT0 after second transurethral resection. Patients with high-grade pT1 bladder cancer at the first registration and pT0 after second transurethral resection at the second registration are randomized to either a watchful waiting arm or an intravesical bacillus Calmette–Guérin therapy arm. A total of 575 patients at the first registration and 260 patients at the second registration will be accrued for this study from 38 institutions over 5 years. The primary endpoint is relapse-free survival (excluding Tis or Ta intravesical recurrence), and the secondary endpoints are overall survival, metastasis-free survival with bladder preserved, annual proportion of intravesical relapse-free survival, annual proportion of T2 or deeper relapse-free survival, adverse events and serious adverse events.

Key words: bladder cancer – second transurethral resection – BCG – watchful waiting – Phase III clinical trial

INTRODUCTION

Bladder cancer is a common disease in urologic oncology. Non-muscle invasive bladder cancer (NMIBC) comprises about 70% of all bladder cancers. NMIBC consists of Ta, Tis and T1 bladder cancers. The main problems with treatment for NMIBC are recurrence and progression after transurethral resection of bladder tumor (TURBT). Above all, high-grade pT1 bladder cancer has a high risk for progression. Sylvester et al. (1) published risk tables for predicting recurrence and progression in Stage Ta and T1 bladder cancers and showed that T1 category and high-grade disease were the predominant risk factors for progression. In fact, some researchers have demonstrated that the 3-year relapse-free survival (RFS) rate of watchful waiting after initial

TURBT is approximately 40%, whereas that of intravesical bacillus Calmette–Guérin (BCG) therapy is approximately 70% for high-grade pT1 bladder cancer (2–5). The European Association of Urology (EAU) guidelines, therefore, advocate intravesical BCG therapy or total cystectomy as the standard treatments for bladder cancer in high-risk progression groups (6). Meanwhile, cystectomy is an invasive intervention and is generally considered to be a treatment option only for high-risk patients or poor BCG responders (6). Thus, intravesical BCG therapy is considered the first choice after TURBT for high-grade pT1 bladder cancer in clinical practice.

Jakse et al. (7) reported that residual tumors were observed in 27–62% of cases following second TUR after initial TURBT for high-grade Ta or T1 bladder cancer. It was recognized that one-time TURBT is insufficient for complete resection of bladder cancer and leads to an underdiagnosis of muscle invasive cancer. Based on this background, the practice of performing second TUR spreads widely. Actually, second TUR is the recommended therapy for high-grade Ta and T1 bladder cancers in the EAU guidelines (6). In addition, the National Comprehensive Cancer Network (NCCN) guidelines recommend repeat resection for any pT1 bladder cancers if the first TURBT does not allow adequate staging or if no muscle is observed in biopsy (8). Second TUR is currently recognized as the standard therapy for high-grade pT1 bladder cancer.

The diagnostic significance of second TUR is that it avoids the underdiagnosis of the first TURBT, but the treatment significance of second TUR is unknown. Before the concept of second TUR was proposed, the standard treatment for high-grade pT1 bladder cancer following TURBT was intravesical BCG therapy. A meta-analysis demonstrated the efficacy of intravesical BCG therapy in preventing recurrence and progression without second TUR (2). The recurrence rate of high-grade pT1 bladder cancer is 50–80% and the progression rate of high-grade pT1 bladder cancer is 30–60% when watchful waiting is selected after TURBT, but the recurrence rate of high-grade pT1 bladder cancer is 30–50% and the progression rate is 15–20% when intravesical BCG therapy is selected after TURBT (2–5,9–11). However, there is no evidence showing whether or not intravesical BCG therapy is necessary for patients with high-grade pT1 bladder cancer who have pT0 histology after second TUR. The current standard treatment for patients with high-grade pT1 bladder cancer who have pT0 histology after second TUR is intravesical BCG therapy. NCCN guidelines recommend intravesical BCG or mitomycin therapy when there is no residual tumor after second TUR. On the other hand, another opinion holds that pT0 status after second TUR carries minimal risk for recurrence or progression and that intravesical BCG therapy is overtreatment for these patients. It takes about 2 months to complete intravesical BCG therapy, and adverse events such as pollakisuria, macrohematuria and dysuria occur in almost all patients.

Based on this background, we began a multi-institutional Phase III trial (JCOG1019) to evaluate the non-inferiority in terms of RFS (excluding Tis or Ta intravesical recurrence) of a watchful waiting arm compared with an intravesical BCG therapy arm for patients with high-grade pT1 bladder cancer who have pT0 histology after second TUR.

The study protocol was designed by the Urologic Oncology Study Group (UOSG) of the Japan Clinical Oncology Group (JCOG), approved by the Protocol Review Committee of JCOG on September 2008 and activated on July 2011. This trial was registered at the UMIN Clinical Trials Registry as UMIN000006930.

PROTOCOL DIGEST OF THE JCOG 1019

Purpose

The aim of this study is to demonstrate the non-inferiority in terms of RFS (excluding Tis or Ta intravesical recurrence) of watchful waiting compared with intravesical BCG therapy for pT0 after second TUR after TURBT for high-grade pT1 bladder cancer.

Study Setting

This study is a multi-institutional open-label randomized Phase III trial.

Resources

This study is supported by a Health and Labour Sciences Research Grant for Clinical Cancer Research (H22-67) from the Ministry of Health, Labour and Welfare, Japan, and National Cancer Center Research and Development Funds (23-A-16 and 23-A-20).

Endpoints

The primary endpoint is RFS (excluding Tis or Ta intravesical recurrence), which is defined as days from randomization to first evidence of either intravesical recurrence of pT1 or deeper, distant metastasis, cystectomy or death from any cause, and censored at the latest day without events. Tis and Ta intravesical recurrence were excluded from the primary endpoint because Ta intravesical recurrence can be treated by TURBT and these recurrences are not critical. We considered adopting 'overall survival' (OS) or 'metastasis-free survival with bladder preserved' as the primary endpoint, but the prognosis of the study subjects is too good to evaluate by OS and the adaptation of cystectomy depends on a patient's preference or the general condition. Therefore, we selected 'RFS (excluding Tis or Ta intravesical recurrence)' as the primary endpoint because it is more objective and harder endpoint than 'metastasis-free survival with bladder preserved'.

The secondary endpoints are OS, metastasis-free survival with bladder preserved, annual proportion of intravesical RFS, annual proportion of T2 or deeper RFS, adverse events and serious adverse events. Tis or multiple Ta recurrence needs intravesical BCG therapy and survival without these recurrences might reflect a patient's benefit. The event of 'annual proportion of intravesical RFS' includes even Tis or Ta recurrence, so the influence with Tis or Ta recurrence will be evaluated by this endpoint.

Eligibility Criteria

Inclusion Criteria

Patients are included in this trial if they fulfill all of the following first registration criteria:

(i) Complete eradication of all visible tumors in the bladder by TURBT
  (a) Depth of TURBT: muscularis propria or deeper
  (b) Surgical specimens must contain muscularis propria
(ii) Histopathological diagnosis: Stage T1, high-grade urothelial carcinoma of the bladder
(iii) Aged between 20 and 85 years
(iv) Within 56 days from the date of TURBT
(v) ECOG performance status of 0 or 1
(vi) No history of administration of cyclophosphamide or methotrexate
(vii) No history of pelvic irradiation
(viii) No history of BCG intravesical therapy
(ix) No history of either bladder cancer (except for Tis Ta bladder cancer) or upper urinary tract cancer (ureteral cancer and/or renal pelvic cancer)
(x) Sufficient organ function
(xi) No strongly positive tuberculin reaction
(xii) Written informed consent

Patients receive second TUR after the first registration and are enrolled in the second registration if they fulfill all of the following second registration criteria:

(i) Histologically proven pT0 after second TUR
(ii) Negative or suspected positive urine cytology in two consecutive examinations (The classification of urine cytology is defined as negative, suspected positive and positive according to the General Rule for Clinical and Pathological Studies on Renal Pelvic, Ureteral and Bladder Cancer, first edition. Classes I and II are defined as negative, Class III is defined as suspected positive and Classes IV and V are defined as positive in the five-step evaluation.)
(iii) Within 28 days from the date of second TUR
(iv) Sufficient bone marrow function

Exclusion Criteria

Patients are excluded from the first registration if they meet any of the following criteria:

(i) Simultaneous or metachronous (within 5 years) double cancers
(ii) Infectious disease (including tuberculosis) to be treated
(iii) Body temperature of 38°C or higher
(iv) Positive anti-HIV antibody
(v) Women during pregnancy or breastfeeding
(vi) Psychiatric disease
(vii) Systemic and continuous steroid medication
(viii) History of severe brain ischemia or myocardial infarction within 6 months
(ix) History of systemic anaphylactoid reaction to BCG

There are no exclusion criteria at the second registration.

Randomization

After confirming the eligibility criteria, the first and second registrations are completed by telephone or fax or via the JCOG Data Center web site. At the second registration, patients are randomized to either the watchful waiting arm or the intravesical BCG injection arm by a minimization method that balance the arms in terms of institution, number of occurrences (initial or recurrence) and number of tumors (single or multiple).

Treatment Methods

Second TUR

Second TUR is performed from days 21 to 56 after the latest TURBT. Day 0 is defined as the day of the latest TURBT before the first registration. The resection area must include the entire scar from the latest TURBT as well as the surrounding area. The ureteral orifice and the internal urethral orifice are excluded from the resection area.

Intravesical BCG therapy

Intravesical BCG therapy is initiated within 28 days of the second registration. For the intravesical BCG therapy arm, Immunobladder® (80 mg/body) or Immucyst® (81 mg/body) is administered intravesically once a week for 8 weeks. Neither the change of the drug after the start of BCG therapy nor the dose reduction in BCG is permitted. After intravesical BCG therapy, patients are observed without any treatment until recurrence is observed.

Watchful Waiting

Patients allocated to the watchful waiting arm at the second registration are observed without any treatment until

recurrence is observed. Protocol completion is defined at the date of the second registration.

Follow-up

All enrolled patients are followed for at least 5 years. Blood and urine examinations are evaluated at least in the fourth and eighth courses during intravesical BCG therapy. For both arms, cystoscopy and urine cytology examinations are conducted every 3 months for the first 3 years, every 6 months for the next 2 years and every year after the 5th year. Abdominal computed tomography or magnetic resonance imaging is performed every year for the first 3 years and once during the 5th year.

Adverse events resulting from second TUR are evaluated for 30 days after the procedure. Adverse events related to BCG are evaluated every week during intravesical BCG therapy and every 3 months for the first 6 months. All adverse events are evaluated using Common Terminology Criteria for Adverse Events (CTCAE) ver. 4.0.

Protocol treatment is continued until progression, unacceptable toxicity or patient refusal.

Study Design and Satistical Analysis

This study is designed as a randomized Phase III trial to determine the non-inferiority of the watchful waiting arm in terms of RFS (excluding Tis or Ta intravesical recurrence) compared with the intravesical BCG therapy arm for patients with high-grade pT1 bladder cancer and pT0 after second TUR.

This study is designed with a two-stage registration. High-grade pT1 bladder cancer patients are registered at the first registration, while the second registration is performed when patients are diagnosed as pT0 at the time of second TUR. Patients enrolled at the first registration who do not proceed to the second registration will also be followed up for at least 5 years because there are few data for this population about the prevalence of residual tumors after first TURBT, adverse events, prognosis and clinical course after second TUR procedures.

The planned accrual period is 5 years, and the follow-up period is 5 years after the completion of accrual. The primary analysis is carried out at 3 years after accrual completion. The hazard ratio between treatment arms and its confidence interval, estimated by the Cox proportional hazard model stratified by number of tumors and number of occurrences, is used to test the non-inferiority of the watchful waiting arm in terms of RFS (excluding Tis or Ta intravesical recurrence). The significance level is set at 0.05 in a one-sided test because of the non-inferiority design of the study. Eighty-five events would be required to demonstrate, with a statistical power of 70%, that the watchful waiting arm is not inferior to the intravesical BCG therapy arm in terms of RFS (excluding Tis or Ta intravesical recurrence), with a non-inferiority margin of 10% in terms of 3-year RFS. Non-inferiority will be concluded if the upper limit of the confidence interval of the hazard ratio does not exceed the limit of 1.60, which is in accord with the non-inferiority margin. A sample size of 258 patients at the second registration is necessary to observe 85 events, considering the accrual and follow-up periods and an estimated 3-year RFS (excluding Tis or Ta intravesical recurrence) of 80% in both arms. We estimated that the number of T0 patients after second TUR would be 50% of the patients at the first registration, and there would be 10% ineligible patients at the secondary registration. Thus, the target sample size is set at 575 patients at the first registration and 260 patients (130 patients in each treatment arm) at the second registration.

Interim Analysis and Monitoring

We plan to conduct interim analyses twice during this study. The study might be terminated for futility, but not for efficacy, because the watchful waiting arm is unlikely to be superior to the intravesical BCG injection arm in terms of RFS. If the hazard ratio exceeds the non-inferiority margin of 1.60 (indicating that the watchful waiting arm is unexpectedly inferior to the intravesical BCG injection arm), the study will be terminated early for futility. In addition, if the 1-year intravesical RFS in the watchful waiting arm is $\leq$60%, if the 1-year T2 or deeper intravesical RFS in the watchful waiting arm is $\leq$90% or if the safety and/or efficacy of the intravesical BCG injection arm is much worse than expected, we will consider early termination of the study.

In-house monitoring will be performed every 6 months by the JCOG Data Center to evaluate study progress and to improve study quality.

PARTICIPATING INSTITUTIONS

The participating institutions (from north to south) are as follows: Hokkaido University Hospital, Sapporo Medical University Hospital, Hirosaki University Hospital, Tohoku University Hospital, Miyagi Cancer Center, Akita University Hospital, Yamagata University Hospital, Tsukuba University Hospital, Tochigi Cancer Center, National Defense Medical College Hospital, Chiba University Hospital, National Cancer Center Hospital, Keio University Hospital, Tokyo Jikei University School of Medicine, Teikyo University, Kitasato University, Niigata Cancer Center Hospital, Niigata University Hospital, Yamanashi University, Shinshu University, Shizuoka Cancer Center, Hamamatsu University School of Medicine, Nagoya University, Mie University, Kyoto University Hospital, Osaka Prefectural Hospital Organization Osaka Medical Center for Cancer and Cardiovascular Diseases, Kobe University, Nara Prefectural University, Tottori University, Shimane University, Yamaguchi University Hospital, Kagawa University, Shikoku Cancer Center, Kurume University, Kyushu

University Hospital, Harasanshin Hospital, Kumamoto University and Kagoshima University Hospital.

Conflict of interest statement

None declared.

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UROLOGIC ONCOLOGY

Original article

Functional role of LASP1 in cell viability and its regulation by microRNAs in bladder cancer☆

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Abstract

Objective: Our previous study demonstrated that fascin homolog 1 (FSCN1) might have an oncogenic function in bladder cancer (BC) and that its expression was regulated by specific microRNAs (miRNAs). Recently, LIM and SH3 protein 1 (LASP1) as well as FSCN1 have been reported as actin filament bundling proteins in the same complexes attached to the inner surfaces of cell membranes. We hypothesize that LASP1 as well as FSCN1 have an oncogenic function and that is regulated by miRNAs targeting LASP1 mRNA.

Methods: The expression levels of LASP1 mRNA in 86 clinical samples were evaluated by real-time RT-PCR. LASP1-knockdown BC cell lines were transfected by siRNA in order to examine cellular viability by XTT assay, wound healing assay, and matrigel invasion assay. We employed web-based software in order to search for candidate miRNAs targeting LASP1 mRNA, and we focused on miR-1, miR-133a, miR-145, and miR-218. The luciferase reporter assay was used to confirm the actual binding sites between the miRNAs and LASP1 mRNA.

Results: Real-time RT-PCR showed that LASP1 mRNA expression was higher in 76 clinical BC specimens than in 10 normal bladder epitheliums ($P < 0.05$). Loss-of-function studies using si-LASP1-transfected BC cell lines demonstrated significant cell viability inhibition ($P < 0.0005$), cell migration inhibition ($P < 0.0001$), and a decrease in the number of invading cells ($P < 0.005$) in the transfectants compared with the controls. Transient transfection of three miRNAs (miR-1, miR-133a, and miR-218), which were predicted as the miRNAs targeting LASP1 mRNA, repressed the expression levels of mRNA and protein levels of LASP1. The luciferase reporter assay demonstrated that the luminescence intensity was significantly decreased in miR-1, miR-133a, and miR-218 transfectants ($P < 0.05$), suggesting that these miRNAs have actual target sites in the 3′ untranslated region of LASP1 mRNA. Furthermore, significant cell viability inhibitions occurred in miR-218, miR-1, and miR-133a transfectants ($P < 0.001$).

Conclusion: Our data indicate that LASP1 may have an oncogenic function and that it might be regulated by miR-1, miR-133a, and miR-218, which may function as tumor suppressive miRNAs in BC.

Keywords: Bladder cancer; LASP1; microRNA; miR-1; miR-133a; miR-218

1. Introduction

Bladder cancer (BC) is the fifth most common cancer in the general population and the second most common cancer of the genitourinary tract [1]. Although 70% to 80% of BC are classified as non-muscle-invasive BC at the initial treatment, approximately 70% of them recur. Among recurrent tumors, 10% to 15% proceed to muscle invasion and metastasis [2]. Therefore, it is crucial to find novel mechanisms involved in BC invasion and metastasis.

Our previous study demonstrated that fascin homolog 1 (FSCN1) might have an oncogenic function in BC and that it may be deeply associated with BC proliferation and invasiveness [3]. Recently, LIM and SH3 Protein 1 (LASP1) as well as FSCN1 have been reported as actin filament

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bundling proteins in the same complexes attached to the inner surfaces of cell membranes [4]. The LASP1 gene was initially identified by conducting searches in a cDNA library of breast cancer metastases. The gene was mapped to a region of human chromosome 17q21 that is altered in 20% to 30% of human breast cancers [5,6], suggesting that it could play a role in tumor development and metastasis of breast cancer. Although the exact cellular function of LASP1 is still not known, the protein has previously been reported to localize within multiple sites of the dynamic actin assembly, such as focal contacts, focal adhesions, lamellipodia, membrane ruffles, and pseudopodia [7]. However, little is known about the functional role of LASP1 in BC.

Our previous study revealed that FSCN1 was consistently regulated by microRNAs (miRNAs) (miR-133a and miR-145), which were down-regulated in BC [3]. MiRNAs are an abundant class of small noncoding RNAs, about 22 nucleotides in length, and they function as negative regulators (cleavage or translational repression) of gene expression by antisense complimentarily to specific mRNAs [8]. Although their biological functions remain largely unknown, recent studies suggest that miRNAs contribute to the development of various cancers. Regarding LASP1, It is not known whether its expression is regulated by specific miRNAs.

To investigate the functional roles of LASP1 in BC, we performed a loss-of-function study using BC cell lines. Furthermore, we did a search for all miRNAs predicted to target LASP1 mRNA by using the TargetScan algorithm. Interestingly, the TargetScan algorithms' results implied that several miRNAs, including miR-133a and miR-145, another target which was FSCN1 in our previous study, might be candidates targeting LASP1 mRNA. Among them, we focused on miR-133a, miR-145, and miR-218, which were listed as the down-regulated miRNAs in our previous screening [3,9]. We were also interested in miR-1, which was not a subject of the previous screening, because miR-1 and miR-133a clustered very closely (about 3,000 bp apart) at chromosome 18q11.2. We hypothesized that LASP1 is directly regulated by miR-1, miR-133a, miR-218, and miR-145 in BC. We performed a luciferase reporter assay to determine whether LASP1 actually has sites targeted by these miRNAs.

2. Materials and methods

2.1. Tissue samples

The tissue specimens were from 76 BCs patients who had undergone cystectomy or transurethral resection of BCs at Kagoshima University Hospital between 2001 and 2005. Patients' backgrounds and clinicopathologic characteristics are summarized in Table 1. These samples were staged according to the American Joint Committee on Cancer-Union Internationale Contre le Cancer (UICC) tumor-node-metastasis classification and were histologically graded [10]. Also used were 10 pathologically proven normal bladder epithelium (NBE) samples derived from organ-confined prostate cancer patients who underwent prostatectomy. Our study was approved by the Bioethics Committee of Kagoshima University; prior written informed consent and approval were given by the patients.

Table 1
Patients' characteristics

Bladder cancer (BC)	
Total number	76
Median age (range)	74 (48–100) years
Sex	
Male	49
Female	27
Stage	
pTa	16
pT1	31
pT2	10
pT3	7
pT4	5
Unknown	7
Grade	
G1	6
G2	49
G3	21
Operation	
Radical cystectomy	18
Partial cystectomy	1
TUR-Bt	53
Unknown	4
Recurrence	
Recurrence (+)	42
Recurrence (–)	29
Unknown	5
Normal bladder epithelium (NBE)	
Total number	10

2.2. BC cell lines and cell culture

We used 3 human BC cell lines; BOY was established in our laboratory from an Asian male patient aged 66 who had a diagnosis of stage III bladder cancer with lung metastasis [9]; T24 was obtained from American Type Culture Collection (ATCC); and KK47 was established in Kanazawa University from an Asian male patient aged 50 years who had a diagnosis of stage I bladder cancer; it was kindly provided to us by colleagues there. These cell lines were maintained in a minimum essential medium (MEM) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

2.3. Prediction of miRNA candidates targeting LASP1 mRNA

We employed the TargetScan program (release 5.1, April 2009; http://www.targetscan.org/) to investigate the pre-

dicted miRNA candidates targeting LASP1 mRNA. We also employed the TargetScan program to investigate the conserved sites where the seed regions of these miRNAs bind. The sequences of the predicted mature miRNAs were confirmed by miRBase (release 13.0, March 2009; http://microrna.sanger.ac.uk/).

2.4. Tissue collection and RNA extraction

A total of 86 samples (76 BC and 10 NBE) were immersed in RNAlater (Qiagen, Valencia, CA) and stored at −20°C until processing RNA extraction. Total RNA was extracted from frozen fresh tissues using an Isogen kit (Nippon Gene, Tokyo, Japan) in accordance with the manufacturer's protocol. The concentration of RNA was determined spectrophotometrically and their integrity was verified by gel electrophoresis. The RNA quality was checked in BioPhotometer (Eppendorf, Tokyo, Japan).

2.5. Quantitative real-time RT-PCR

Aliquots of total RNA (500 ng) were converted into cDNA by using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Quantitative TaqMan RT-PCRs were performed in a 20 μl reactive volume containing 1 μl of cDNA, 1 μl of TaqMan probes, and primers for LASP1, 8 μl H_2O, and 10 μl of TaqMan Universal PCR Master Mix (Applied). TaqMan qRT-PCRs were performed using the 7300 Real-Time PCR system (Applied Biosystems) under the following conditions: activation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, and annealing and extension at 60°C for 1 minute. TaqMan probes and primers for LASP1 (P/N: Hs00195978_m1; Applied Systems) were assay-on-demand gene expression products. We followed the manufacturer's protocol regarding the PCR conditions. All reactions were performed in triplicate, and a negative control lacking cDNA was included. For quantitative analysis of LASP1 mRNA, human GUSB (P/N: Hs99999908_m1; Applied Biosystems) served as an internal control, and the δ-δ CT methods were used to calculate the fold change. We used premium total RNA from normal human bladder (Clontech, Mountain View, CA) as a reference.

2.6. Mature miRNA and siRNA transfection

As previously described [4], the transfection of BC cell lines was accomplished with an RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA), Opti-MEM (Invitrogen) with 10 nM of mature miRNA molecules. Pre-miR and negative control miRNA (Applied Biosystems) were used in the gain-of-function experiments, whereas LASP1 siRNA (P/N: s8094; Applied Biosystems) and negative control siRNA (D-001810-10; Thermo Fisher Scientific, Waltham, MA) were used in the loss-of-function experiments. Cells were seeded under the following conditions: 8×10^5 in a 10 cm dish for protein extraction, 3,000 per well in a 96-well plate for the XTT assay, 2×10^5 per well in a 6-well plate for the wound healing assay, and 5×10^4 per well in a 24-well plate for the mRNA extraction, matrigel invasion assay, and luciferase reporter assay.

2.7. Cell viability, wound healing, and matrigel invasion assay

Cell viability was determined using an XTT assay (Roche Applied Sciences, Tokyo, Japan) performed according to the manufacturer's instructions. Cell migration activity was evaluated by wound healing assay. Cells were plated in 6-well dishes, and the cell monolayer was scraped using a micropipette tip. The initial gap length (0 hour) and the residual gap length 24 hours after wounding were calculated from Photomicrographs. A cell invasion assay was carried out using modified Boyden Chambers consisting of transwell-precoated matrigel membrane filter inserts with 8 μm pores in 24-well tissue culture plates (BD Biosciences, Bedford, MA). MEM containing 10% fetal bovine serum in the lower chamber served as the chemoattractant, and invading cells were fixed and stained using the Diff-Quick kit (Sysmex, Tokyo, Japan). All experiments were performed in triplicate.

2.8. Western blots

After 3 days of transfection, protein lysate (100 μg) was separated by NuPAGE on 4%–12% bis-tris gel (Invitrogen) and transferred into a polyvinylidene fluoride membrane. Immunoblotting was done with diluted (1:100) polyclonal LASP1 antibody (HPA012072; Sigma Aldrich, St. Louis, MO) and GAPDH antibody (MAB374; Chemicon, Temecula, CA). The membrane was washed and then incubated with goat anti-rabbit IgG (H + L)-HRP conjugate (Bio-Rad, Hercules, CA). Specific complexes were visualized with an echochemiluminescence detection system (GE Healthcare, Little Chalfont, UK).

2.9. Plasmid construction and dual-luciferase reporter assay

MiRNA target sequences were inserted between the XhoI–PmeI restriction sites in the 3′UTR of the hRluc gene in psiCHECK-2 vector (C8021; Promega, Madison, WI). Primer sequences for full-length 3′UTR of LASP1 mRNA and sites targeted by miR-1, miR-133a, miR-218, and miR-145 are summarized in Table 2. BOY cells were transfected with 5 ng of vector, 10 nM of miRNAs, and 1 μl of lipofectamine 2000 in a 100 μl Opti-MEM (Invitrogen). The activities of firefly and Renilla luciferases in cell lysates were determined with a dual-luciferase assay system (Pro-

Table 2
Primers sequence

Target sites	3′UTR position	Primers sequence (5′ to 3′)
Full-length 3′UTR of *LASP1* mRNA	1–2966	Forward GCGATCGCTCGAGACGACGGCTGGATGTACG
		Reverse CTACGTTTAAACTTGAGTCACCAGGAGAAAGATTC
miR-218	2080–2086	Forward GATCGCTCGAGCTAGGTGGAGGCAAGTGGAA
		Reverse GGCCGCTCTAGGTTTAAACGACTGGCTTGCCTTATCTGG
miR-1	29–35	Forward GATCGCTCGAGTACGTGGAGGCCATCTGAAC
		Reverse CTCTAGGTTTAAACACTGAAGAATGGACGGGTCA
	353–359	Forward GATCGCTCGAGTGGAATGGGAGACCTGTTG
		Reverse CTACGTTTAAACATCCCAGGAACCAAGAAGTG
miR-145	1581–1587	Forward TAGGCGATCGCTCGAGCAGGCATATGTTTCCCCATC
		Reverse TCTAGGTTTAAACAGCAGAGAGCAGGAACTGGA
miR-133a	2099–2105	Forward GATCGCTCGAGCTAGGTGGAGGCAAGTGGAA
		Reverse GGCCGCTCTAGGTTTAAACGACTGGCTTGCCTTATCTGG
	2522–2528	Forward TAGGCGATCGCTCGAGCAGCTCATTTAATCCCAGGAA
		Reverse GGCCGCTCTAGGTTTAAACGGGACATGAGAGGGAGAAAA

mega). Normalized data were calculated as the quotient of Renilla/firefly luciferase activities.

2.10. Statistical analysis

The relationship between 2 variables and the numerical values obtained by real-time RT-PCR was analyzed using the Mann-Whitney U test. The relationship among 3 variables and the numerical values was analyzed using the Bonferroni-adjusted Mann-Whitney U test. The analysis software was Expert StatView (ver. 4; SAS Institute Inc., Cary, NC); regarding the comparison test among three variables, a nonadjusted statistical level of significance of $P < 0.05$ corresponds to a Bonferroni-adjusted level of $P < 0.0167$.

3. Results

3.1. Quantitative real-time RT-PCR

Real-time RT-PCR showed that the mRNA expression level of LASP1 was higher in BCs than in NBEs (2.631 ± 0.339 and 1.000 ± 0.325, respectively, $P = 0.0311$, Fig. 1A). We found no correlation between LASP1 expression and clinicopathologic parameter (Supplemental Fig. 1, which can be found in the electronic version of this article).

3.2. LASP1 expression in BC cell lines and LASP1 silencing by siRNA transfection

LASP1 mRNA expression of the BC cell lines (BOY, KK47, and T24) was markedly higher than in the normal human bladder tissue in qRT-PCR (Fig. 1B). LASP1 knockdown was achieved following transient transfection with LASP1 siRNA duplexes (Fig. 1C).

3.3. Effect of LASP1 knockdown on cell viability, invasion, and migration activity in BC cell lines

To examine the functional role of LASP1, we performed loss-of-function studies using si-LASP1-transfected BC cell lines. The XTT assay revealed significant cell viability inhibition in the si-LASP1 transfectant compared with that in the untransfectant (mock) and the si-control transfectant (% of cell viability; BOY, 86.9 ± 2.0, 96.8 ± 1.1, 100.0 ± 1.4, respectively, $P < 0.0005$; and KK47, 88.5 ± 1.7, 98.0 ± 2.3, 100.0 ± 1.5, respectively, $P < 0.005$, Fig. 2A). However, no significant difference was observed in the si-LASP1 transfectants of T24 cell lines (% of cell viability; 100.4 ± 0.8, 95.2 ± 2.1, 100.0 ± 2.1). The wound healing assay also demonstrated significant cell migration inhibitions in the si-LASP1 transfectant compared with the counterparts (% of wound closure; BOY, 42.9 ± 3.1, 91.0 ± 1.5, 100.0 ± 2.7, respectively, $P < 0.0001$; and T24, 66.2 ± 2.0, 98.6 ± 1.6, 100.0 ± 3.1, respectively, $P < 0.0001$, Fig. 2B). The matrigel invasion assay demonstrated that the number of invading cell was significantly decreased in the si-LASP1 transfectant compared with the counterparts BOY, 19.0 ± 9.5, 148.8 ± 23.9, 132.3 ± 27.3, respectively, $P < 0.005$; and T24, 138.3 ± 10.6, 215.5 ± 11.8, 229.3 ± 4.8, respectively, $P < 0.0005$, Fig. 2C). We did not subject the KK47 cell line to these experiments because it showed focal growth and was not suitable for the experiments.

3.4. LASP1 as a target of post-transcriptional repression by miRNAs

The expression levels of LASP1 mRNA were significantly decreased in miR-1, miR-218, miR-133a, and miR-145 transfectants compared with the control transfectant ($P < 0.05$) (Fig. 3A), and the protein expression was markedly decreased in the transfectants (Fig. 3B). This data suggests that miR-1, miR-218, and miR-133a reduce LASP1 expression through cleavage or translational inhibition. Accord-

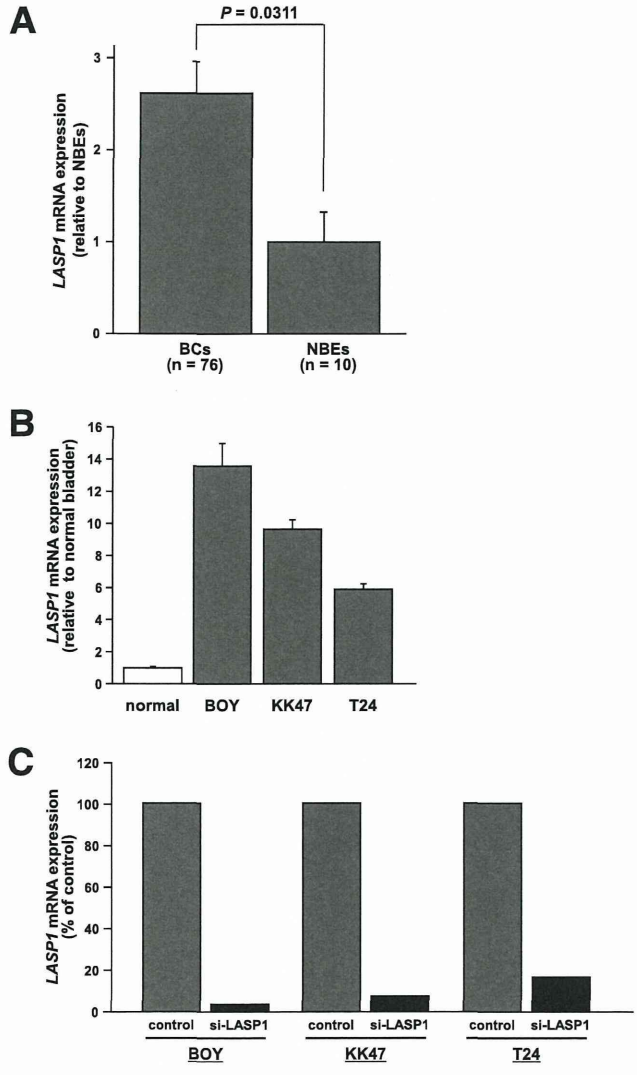

Fig. 1. mRNA expression levels of LASP1 in clinical samples and BC cell lines. (A) Real-time RT-PCR showed that the mRNA expression level of LASP1 in BCs was higher than that of the NBEs. (B) LASP1 expression in three BC cell lines (BOY, KK47, and T24) was evaluated by real-time RT-PCR. Premium total RNA from normal human bladder (Clontech) was used as a reference. (C) LASP1 expression following transfection with siRNA for LASP1 in three BC cell lines (BOY, KK47, and T24).

ingly, we performed a luciferase reporter assay to determine whether LASP1 mRNA actually has the target sites of these four miRNAs, as indicated by the TargetScan algorithm. We initially used the vector encoding full-length 3′UTR of LASP1 mRNA, and the luminescence intensity was significantly decreased in miR-218, miR-1, and miR-133a transfectants (Fig. 4A). Furthermore, to determine the specific sites targeted by the 4 miRNAs, we constructed vectors covering 2 conserved sites for miR-1 and miR-133a and 1 site for miR-218 and miR-145 (Fig. 4B). The luminescence intensity was significantly decreased at the 1 site targeted by miR-218 (position 2080–2086), 2 sites targeted by miR-1 (position 29–35 and 353–359), and 2 sites targeted by miR-133a (positions 2099–2105 and 2522–2528) (Fig. 4B). The luminescence intensity was not decreased at the 1 site targeted by miR-145 (position 1581–1587).

3.5. Effect of miR-218, miR-1, and miR-133a transfection on cell viability activity in BC cell lines

We performed gain-of-function studies using the miRNA transfectants to investigate the functional role of miR-218, miR-1, and miR-133a. The XTT cell viability assay showed significant cell viability inhibition in miR-218, miR-1, and miR-133a transfectants compared with the controls from BOY, KK47, and T24 cell lines (BOY, 84.1 ± 1.7, 55.3 ± 1.0, 65.8 ± 1.5, 100 ± 1.1, respectively, $P < 0.0001$; KK47, 96.2 ± 0.9, 92.6 ± 1.9, 72.8 ± 1.4, 100 ± 1.8, respectively, $P < 0.001$; T24, 85.4 ± 0.9, 49.5 ± 1.0, 62.7 ± 1.6, 100 ± 1.6, respectively, $P < 0.0001$, Fig. 5). However, no significant difference was found in the miR-218- transfected KK47 cell line.

4. Discussion

Our previous study demonstrated that FSCN1 might have an oncogenic function in BC [3]. LASP1 is the same actin-binding protein as FSCN1. Cell migration and controlled assembly and disassembly of focal adhesions are highly integrated multistep processes and a central feature of the molecular pathology of cancer [11]. To date, more than 50 different adhesion proteins that regulate the rate and organization of actin polymerization and focal adhesion turnover in protrusions have been identified. Overexpression of LASP1 is associated with increased cellular proliferation in different cancers, including human colorectal adenocarcinoma [12]. Down-regulation of LASP1 using RNAi resulted in suppression of cell proliferation in human breast and ovarian cancer [13,14]. Recent literature indicates that LASP1 may be a target gene for p53 [15]. Consistent with these previous findings, we have shown that the LASP1 is highly expressed in human BC and that it reduces cell viabilities in si-LASP1-transfected BOY and KK47 cell lines. These results suggest that LASP1 has an oncogenic function through cell viability, migration, and invasion activity in BC. However, in the si-LASP1-transfected T24 cell line, there were significant differences in migration and invasion activity but not in cell viability, suggesting that cell viability does not depend on LASP1 expression in some phenotypes. We found no correlation between LASP1 expression and clinicopathologic parameters. The expression levels of LASP1 mRNA were relatively higher in low stage and low grade BCs, suggesting that increased expression of LASP1 might be an early event in bladder carcinogenesis. The use of a stable LASP1 knockdown system in vivo and the development of pharmacologic inhibitors of LASP1 will be instrumental in furthering our understanding of the role of LASP1 dynamics in human BC. We found that some discrepancies between protein and mRNA expression levels

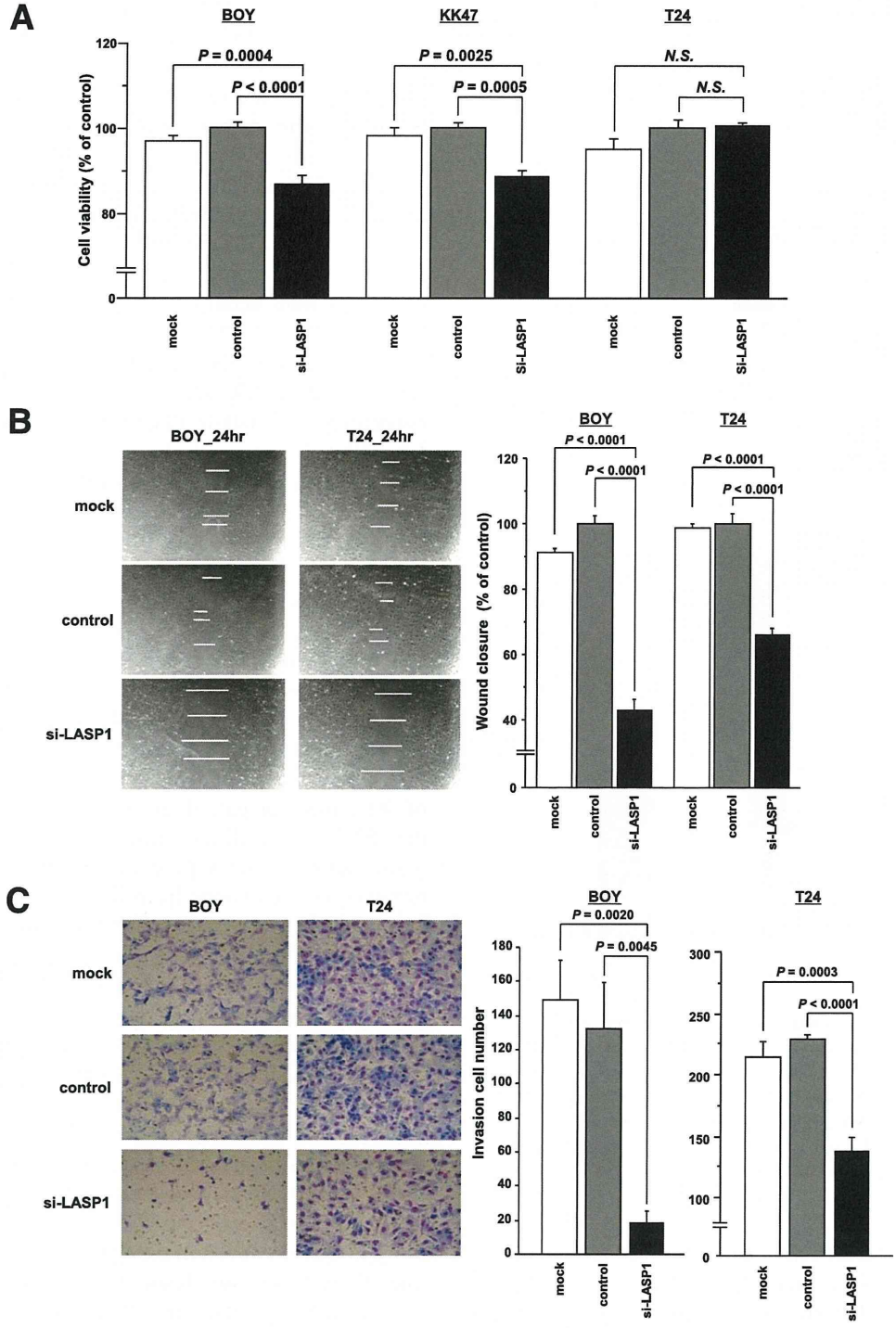

Fig. 2. LASP1 knockdown effect on BC cell viability as revealed by siRNA transfection. (A) siRNA for LASP1 transfected BOY and KK47 cells showed a significant decrease in cell viability in comparison with negative control siRNA-transfectant or mock (untransfectant). (B) si-LASP1 inhibits monolayer wound healing efficacy of BC cell lines. Phase micrographs of BOY and T24 cell lines 24 hours after monolayer wounding are on the left. Quantification of cell migration using the monolayer wound healing assay is on the right. (C) si-LASP1 inhibits BOY and T24 cell invasion activity through Matrigel. Phase micrographs of invading BOY and T24 cell lines are on the left. Both cell lines were transfected with siRNA directed against either LASP1 or negative control siRNA-transfectant. Quantifications of cell invasion are shown in the right panel. Shown is the mean (± SEM) of eight randomly selected 200× magnification fields. (Color version of figure is available online.)

occurred in some cell lines (Fig. 3). These results suggest that cell line-specific post-translational ubiquitination or proteolysis might decrease the protein expression levels of LASP1. However, we believe that our data still show a trend toward to repression of LASP1 expression by the miRNAs.

Our previous study also demonstrated that miR-145 and

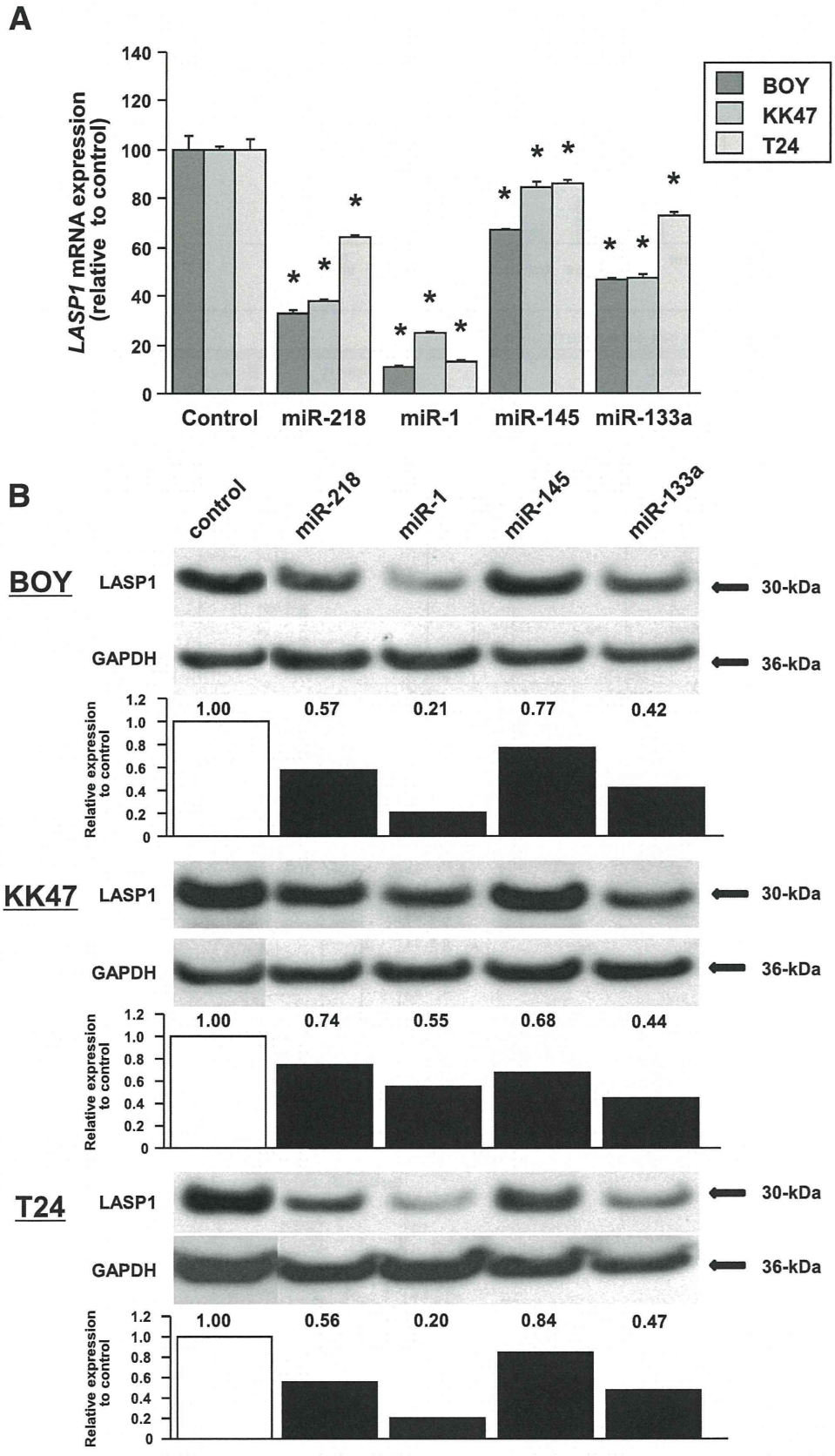

Fig. 3. Regulation of LASP1 expression in the down-regulated miRNA transfectants. (A) LASP1 mRNA expressions after transfection of miRNAs for 24 hours. LASP1 mRNA expressions were repressed in miR-218-, miR-1-, and miR-133a transfectants. $*P < 0.05$. (B) LASP1 protein expressions after transfection of miRNAs for 72 hours. LASP1 protein expressions were repressed in miR-218-, miR-1-, and miR-133a transfectants.

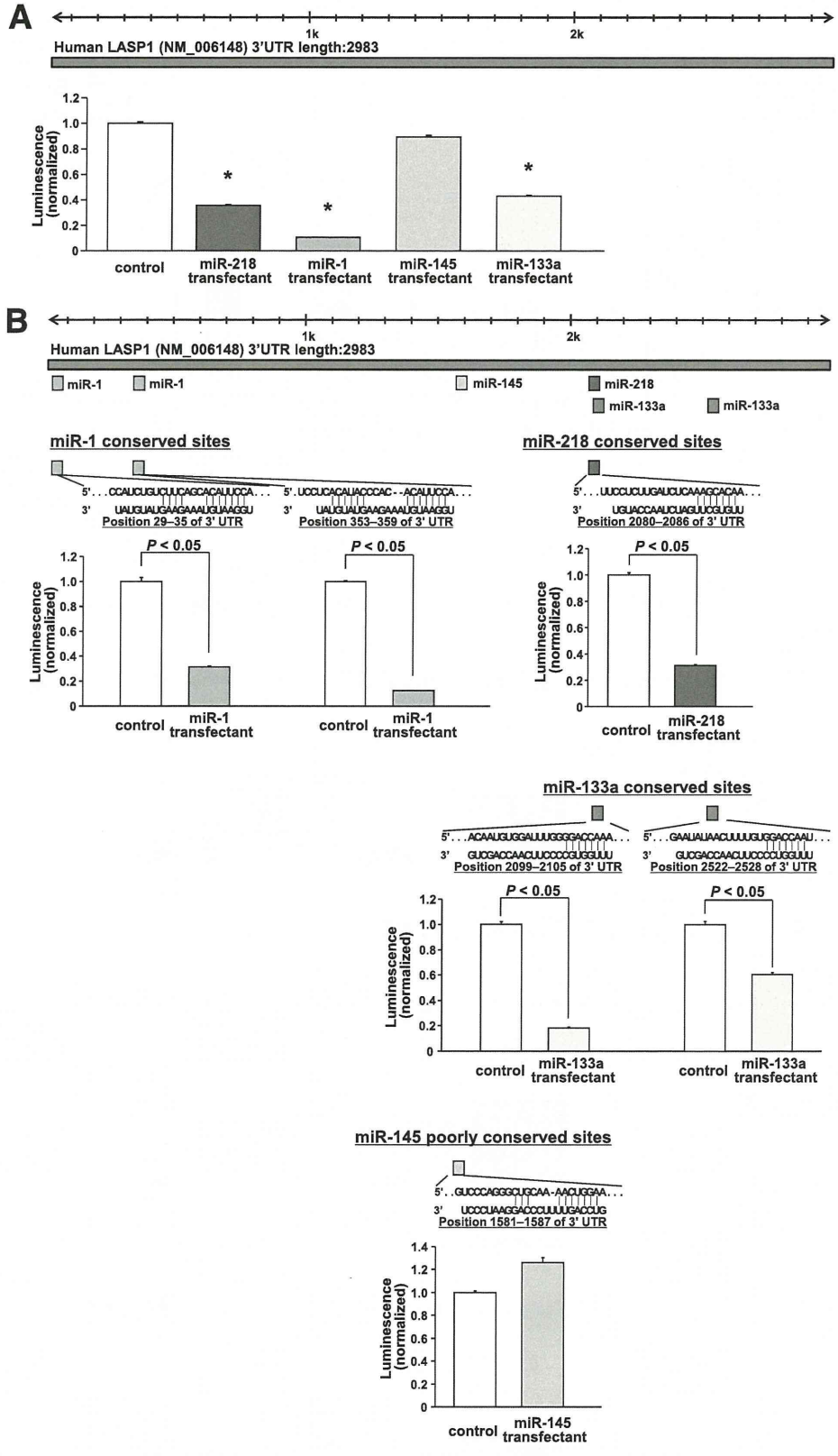

Fig. 4. MiR-218, miR-1, miR-145, and miR-133a binding sites in 3′UTR of LASP1 mRNA. (A) Luciferase reporter assay using the vector encoding full-length 3′UTR of LASP1 mRNA. BOY cells were transfected with 5 ng of vector and 10 nM of microRNAs. The Renilla luciferase values were normalized by firefly luciferase values. *$P < 0.05$. (B) Luciferase reporter assays using the vectors encoding putative conserved target sites of 3′UTR of LASP1 mRNA identified with the TargetScan database: two conserved sites for miR-1 and miR-133a, and one site for miR-218, and one poorly conserved site for miR-145.

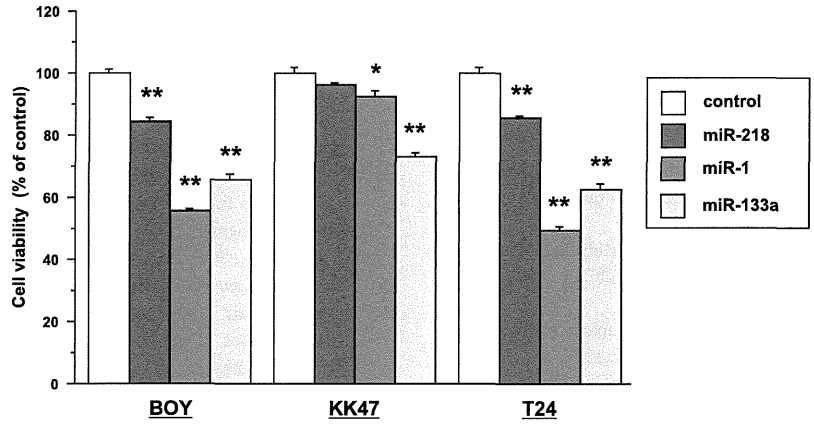

Fig. 5. Effect of cell viabilities in miR-218-, miR-1-, and miR-133a transfectants by the XTT assay. Cell viability inhibitions were observed in BOY, KK47, and T24 cell lines transfected with the miRNAs. $*P < 0.001$, $**P < 0.0001$.

miR-133a directly target FSCN1 [3]. The results of the web-based software implied that LASP1 mRNA was a common target of these 2 miRNAs. It has been reported that FSCN1 and LASP1 proteins localized in the filopodia and lamellipodia of various types of cells, and both of them were involved in the dynamics of actin filaments [4]. It is interesting that both were regulated by common miRNAs. However, we found that LASP1 was actually targeted by miR-133a but not by miR-145. Regarding this, other factors, which the target algorithm could not predict, might be associated with regulation of gene expression by miRNAs. Previous reports showed that miR-133a is down-regulated in colorectal cancer and SCC of tongue [16,17]. We also found that miR-133a has a critical role in regulating oncogenic FSCN1 in BC [3]. MiR-218 is also down-regulated in prostate, gastric, and cervical cancer [18–20]. Martinez et al. described that human papillomavirus type 16 reduces the expression of miR-218 in cervical carcinoma cells [20]. MiR-1 is down-regulated in colon, liver, and lung cancer, and in head and neck squamous cell carcinoma [20–24]. However, the functional role of these miRNAs has not been fully elucidated. Our study suggests that these miRNAs may have tumor suppressive functions through binding and cleaving LASP1 mRNA, but these phenomena might be limited to some BC cell lines. Further investigations using other cancer cell lines are necessary to clarify whether these phenomena is critical for cancer development. It is interesting that miR-1 and miR-133a clustered on the same chromosomal loci (18q11.2) have been found in a region of frequent losses in BC in comparative genomic hybridization (CGH) studies [25]. Chhabra et al. showed that overexpression of miR-23a, miR-27a, and miR-24-2 clusters induced apoptosis in human embryonic kidney cells [26]. It is plausible that miR-1 and miR-133a clusters function as tumor suppressors through down-regulating LASP1 and FSCN1, and that the cluster is often deactivated in BC under chromosomal deletion. Our data suggest that up-regulation of miR-133a and miR-1 clusters could be a new therapeutic strategy for BC.

In summary, LASP1 knockdown in BC cell lines results in decreased migration, suggesting that LASP1 plays a key role in BC invasiveness. LASP1 might have an oncogenical function in BC, and miR-1, miR-133a, and miR-218 might function as tumor suppressors through repression of LASP1 in BC. We have demonstrated a strategy to address the mechanisms of cancer development through functional miRNAs in BC.

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Supplementary Material

Supplementary material associated with this article may be found in the online version at doi:10.1016/j.jasms.2010.05.008

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Significance of Uracil/Tegafur for Preventing Intravesical Recurrence of Non-Muscle Invasive Urothelial Carcinoma of the Bladder

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Key Words

Non-muscle invasive urothelial carcinoma of the bladder • Uracil/tegafur • Recurrence • Angiogenesis • γ-butyrolactone

Abstract

Background: The objective of this study was to assess the role of uracil/tegafur (UFT) and its metabolite γ-butyrolactone (GBL), a potent inhibitor of angiogenesis, in the prevention of intravesical recurrence in patients with non-muscle invasive urothelial carcinoma of the bladder (NMIUCB). ***Patients and Methods:*** This study included 48 patients with NMIUCB following complete transurethral resection who were randomly divided into 27 receiving UFT therapy (group A) and 21 without any adjuvant therapies (group B). Serum levels of GBL, vascular endothelial growth factor, basic fibroblast growth factor, platelet-derived growth factor and interleukin-8 were measured. ***Results:*** There was no significant difference in the intravesical recurrence-free survival between groups A and B. Despite the lack of significant differences in serum levels of vascular endothelial growth factor, basic fibroblast growth factor, platelet-derived growth factor and interleukin-8, serum GBL in group A was significantly greater than in group B. Multivariate analysis identified tumor size as an independent predictor of intravesical recurrence irrespective of the other factors examined. ***Conclusions:*** Despite the significant induction of GBL, adjuvant UFT therapy failed to show a preventive effect on intravesical recurrence of NMIUCB. Therefore, we should consider enhancing the anti-angiogenic effect of GBL using an alternative administration schedule of UFT.

Introduction

Approximately 80% of patients with newly developed urothelial carcinoma of the bladder are pathologically diagnosed as having non-muscle invasive tumors that are limited to the urothelium or infiltrate no deeper than the lamina propria. Complete transurethral resection (TUR) of the visible tumor burden is currently regarded as the standard approach for patients with non-muscle invasive urothelial carcinoma of the bladder (NMIUCB), and the prognosis of such patients is generally favorable, achieving 5-year survival rate greater than 80% [1]. Several previous studies, however, reported that intravesical recurrence following TUR occurred in 30 to 80% of patients with NMIUCB [1–3]. Therefore, intensive efforts have been made to develop adjuvant therapy that can effectively prevent postoperative intravesical recurrence of NMIUCB.

To date, intravesical therapy has been widely performed to delay or prevent intravesical recurrence of NMIUCB following TUR. Commonly used agents for intravesical instillation include immunotherapy with bacille Calmette-Guérin and chemotherapy with mitomycin C, adriamycin, epirubicin and gemcitabine [4, 5]. Shelly et al. [5] recently carried out a systemic review of randomized trials associated with intravesical therapy against NMIUCB, and concluded that an immediate post-TUR instillation of a chemotherapeutic agent is effective in reducing intravesical recurrence, and further intravesical induction as well as maintenance therapy with bacille Calmette-Guérin are recommended for high-risk

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patients. However, there has been no reliable evidence that any agent for intravesical therapy is able to improve overall survival. Furthermore, several investigators suggested that the effectiveness of the routine instillation of an intravesical agent is likely to remain unresolved pending more critical evaluations, and considering important issues associated with this therapy, such as complications and cost [6, 7].

The combination of uracil and tegafur at a molecular ratio of 4:1, also referred to as UFT, is an orally available anticancer agent. Uracil inhibits the degradation of fluorouracil to inactivate the metabolite through biochemical modulation, and therefore, UFT exerts its activity more specifically within tumor tissues than that in normal tissues [8]. To date, there have been a number of studies showing the efficacy of UFT against several types of malignant tumors, including bladder cancer [9–14]. In this study, therefore, a randomized clinical trial was conducted in order to assess the significance of the adjuvant administration of UFT for patients who underwent TUR and were subsequently diagnosed as having NMIUCB. In this trial, we also analyzed the impact of UFT therapy on changes in the serum level of its metabolite γ-butyrolactone (GBL), a potent inhibitor of angiogenesis [15], in addition to those of major angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and interleukin-8 (IL-8) [16].

Patients and Methods

The population of this study consisted of patients who were at least 18 years old and underwent complete TUR of pathologically documented NMIUCB. Key eligibility criteria for this study included an Eastern Cooperative Oncology Group performance status of 0 or 1, adequate hematologic, coagulation, hepatic, renal and cardiac functions, and the absence of perioperative anticancer therapy. Patients were ineligible if they had carcinoma in situ or T1G3 disease and a history of malignant disease during the preceding 12 months. Written informed consent to enter this study was obtained from all patients, and the study design was approved by the Research Ethics Committee of our institution.

A total of 48 patients who were judged to be eligible were included and randomly assigned to either the adjuvant therapy group (group A) or the control group (group B). In group A, 2 weeks after TUR, UFT was orally given at a dose of at least 300 mg/d for 12 months, while no adjuvant therapy was performed in group B. Toxicities associated with the administration of UFT were graded according to the NCI Common Toxicity Criteria, and if an adverse event greater than grade 1 occurred, the dose of UFT was reduced. Postoperative follow-up examinations were carried out as follows: cystoscopy and urinary cytological examination were performed every 3 months for 3 years after TUR, and then every 6 months until 5 years after TUR, and intravenous pyelography was performed every 6 months until 3 years after TUR and then annually until 5 years after TUR. On the detection of tumors or hyperemic mucosa by cystoscopy and/or positive findings on urinary cytology, transurethral biopsy of the abnormal region and/or TUR of the tumor were carried out.

To assess the effects of UFT therapy on its metabolite and major angiogenic factors, serum samples were obtained from all patients approximately 4 weeks after their inclusion in this trial, and the sample collection in group A was also done at 2 hours after the administration of UFT. Serum levels of GBL were determined by employing gas chromatography mass spectrometry as previously described [17]. For the measurement of serum concentrations of VEGF, bFGF, PDGF and IL-8, commercially available sandwich enzyme-linked immunoassay kits were employed according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN, USA).

Differences between groups A and B were compared using the chi-square test or unpaired-t test. Intravesical recurrence-free survival rates were calculated using the Kaplan–Meier method, and the difference was determined by the log-rank test. The prognostic significance of certain factors was assessed by employing the Cox proportional hazards regression model. All statistical calculations were performed using Statview 5.0 software (Abacus Concepts, Inc., Berkley, CA, USA), and probability (p) values less than 0.05 were considered significant.

Results

Of the 48 eligible patients, 27 and 21 were assigned to groups A and B, respectively. There were no significant differences in several clinicopathological parameters between groups A and B (table 1).

During the observation period of this study (median 24 months, range 11 – 42 months), intravesical recurrence was detected in 14 patients in group A (51.9%) and 9 patients in group B (42.9%). There was no significant difference in intravesical recurrence-free survival between these 2 groups (fig. 1).

Adverse events associated with adjuvant UFT therapy were observed in 6 (22.2%) of the 27 patients assigned to group A, including gastrointestinal symptoms in 5 and thrombocytopenia in 1. However, interruption of UFT therapy was not required in any patient, while dose reduction from 600 to 300 mg/d was necessary in 2 patients with gastrointestinal symptoms corresponding to grade 2.

As shown in figure 2A, mean serum levels of GBL in groups A and B were 37.5 and 21.1 ng/ml, respectively, and there was a significant difference in the serum GBL level between these 2 groups. However, there were no significant differences in serum concentrations of VEGF, bFGF, PDGF and IL-8 between groups A and B (fig. 2B–E).

Table 1. Patient characteristics in group A (n = 27) and B (n = 21)

	Group A	Group B	p
Age, years	64.8 ± 9.7	72.5 ± 9.0	0.14
Gender			0.96
Male	23	18	
Female	4	3	
Multiplicity			0.72
Solitary	14	12	
Multiple	13	9	
Size, cm			0.78
Less than 3	19	14	
3 or greater	8	7	
History of upper urinary tract caner			0.47
Positive	9	5	
Negative	18	16	
Grade			0.65
1	8	5	
2	19	19	
T Stage			0.78
Ta	21	17	
T1	6	4	

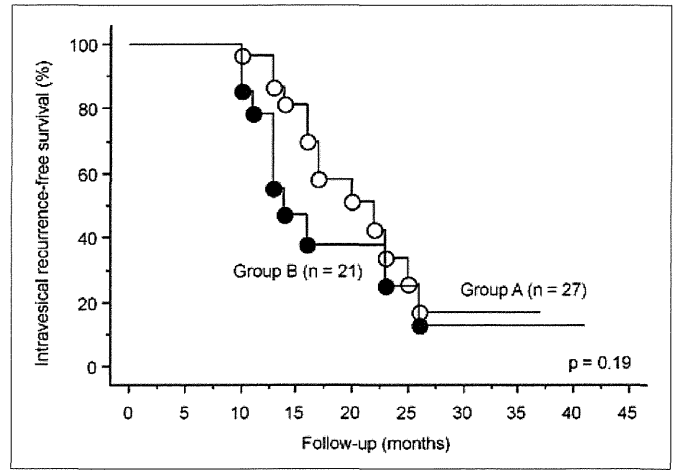

Fig. 1. Intravesical recurrence-free survival of patients with NMI-UCB in groups A and B.

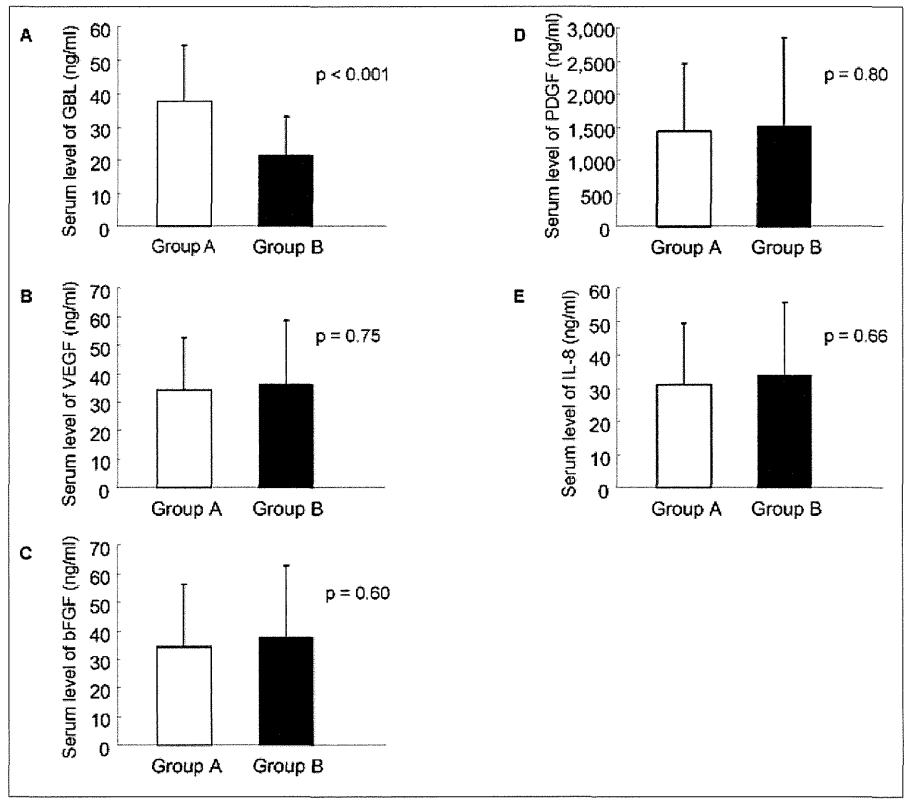

Fig. 2. Comparison of serum levels of **A**: GBL; **B**: VEGF; **C**: bFGF; **D**: PDGF; **E**: IL-8 between groups A and B.

Table 2. Univariate and multivariate analysis of the associations between various parameters and intravesical recurrence-free survival

	Univariate analysis		Multivariate analysis	
	Hazard ratio	p	Hazard ratio	p
Age, <70 vs. ≥70 years	1.04	0.42		
Gender, male vs. female	0.97	0.88		
Multiplicity, solitary vs. multiple	2.07	0.031	1.22	0.2
Size, <3 vs. ≥3 cm	2.23	0.015	1.98	0.033
History of upper urinary tract cancer, positive vs. negative	1.5	0.2		
Grade, 1 vs. 2	1.44	0.29		
T stage, Ta vs. T1	1.54	0.12		
Adjuvant UFT therapy, yes vs. no	0.83	0.46		
Serum GBL level, high vs. low[a]	0.79	0.35		
Serum VEGF level, high vs. low[a]	0.94	0.89		
Serum BFGF level, high vs. low[a]	1.11	0.78		
Serum PDGF level, high vs. low[a]	1.07	0.81		
Serum IL-8 level, high vs. low[a]	0.96	0.86		

[a]Determined based on the median value of each molecule as a cut-off point.

To analyze factors significantly related to postoperative intravesical recurrence, univariate and multivariate analyses were performed. Multiplicity and tumor size were identified as significant predictors on univariate analysis, however, the remaining factors, including adjuvant therapy and serum levels of GBL, VEGF, bFGF, PDGF and IL-8, had no significant impact on intravesical recurrence. In addition, tumor size appeared to be independently associated with intravesical recurrence on multivariate analysis (table 2).

Discussion

To date, a number of investigators have evaluated the usefulness of intravesical instillation therapy in order to delay or prevent intravesical recurrence of NMIUCB following TUR; however, there are several limitations regarding this therapy [4–7]. For example, intravesical instillation itself involves a comparatively invasive procedure and usually takes approximately 1 hour to keep an agent in the bladder. In addition, it has been well documented that more than 10% of patients who receive intravesical therapy experience complications, including uncommon but severe adverse events [6]. Also, from an oncological viewpoint, there has been no report of intravesical therapy leading to a significant improvement of overall survival in patients with NMIUCB [4, 5]. Collectively, these findings suggest that it is necessary to develop an effective adjuvant therapy with an orally available agent following TUR of NMIUB.

UFT, the combination of uracil and tegafur in a 4:1 molecular ratio, is an oral anticancer agent showing selective activity, because biochemical modulation by uracil enhances fluorouracil concentration more specifically in tumor tissues than that in normal tissues [8]. A number of studies have reported the usefulness of UFT for a wide variety of malignant diseases, such as lung, breast, gastric and prostate cancer [9–11]. For bladder cancer, the efficacy of UFT has been widely investigated as a therapeutic as well as prophylactic agent, and some of these studies have demonstrated that treatment with UFT is tolerable and effective in delaying progression and preventing recurrence in patients with bladder cancer [12–14]. Considering these findings, the significance of adjuvant UFT therapy in the prevention of intravesical recurrence in patients undergoing TUR for NMIUCB was evaluated.

In this series, there were no significant differences in several clinicopathological parameters between the treatment and control groups. In addition, there was no significant difference in the intravesical recurrence-free survival between these 2 groups. To our knowledge, there has been only one study evaluating the efficacy of UFT