SPANDIDOS PUBLICATIONS

CpG hypermethylation of *human four-and-a-half LIM domains 1* contributes to migration and invasion activity of human bladder cancer

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Abstract. We previously reported a simple technique that combines microarray data from clinical bladder cancer (BC) specimens with those from a BC cell line (BOY) treated with a pharmacologic demethylating agent (5-aza-dC). We focused on the *human four-and-a-half LIM domains 1 (FHL1)* gene which was selected on the basis of previous microarray data analysis. Because LIM domains provide protein-protein binding interfaces, *FHL* genes play an important role in cellular events, such as focal adhesion and differentiation, by interacting with the target protein as either a repressor or activator. We hypothesized that inactivation of the *FHL1* gene through CpG methylation contributes to cell viability including migration and invasion activity of human BC. After 5-aza-dC treatment, the expression levels of *FHL1* mRNA transcript markedly increased in all cell lines tested, as shown by real-time reverse transcription-polymerase chain reaction (RT-PCR). The methylation index of *FHL1* in our samples was significantly higher in 70 BC specimens than in 10 normal bladder epithelium (NBE) specimens (63.9±25.5 and 0.3±0.2, respectively; p=0.0066). Conversely, *FHL1* mRNA expression was significantly lower in the BC specimens than in the NBE ones (0.331±0.12 and 2.498±0.61, respectively; p=0.0011). In addition, significant inhibitions of wound healing (45.78±6.2, and 100±0, respectively; p=0.009) and of cell invasion (18.5±2.3 and 95.2±2.4, respectively; p=0.02) were observed in stable *FHL1*-transfected cells than in the control BC cells. In conclusion, we found that the mechanism of *FHL1* down-regulation in BC is through CpG hypermethylation of the promoter region. *FHL1* gene inactivation by CpG hypermethylation may thus contribute to migration and invasion activity of BC.

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Introduction

Bladder cancer (BC), one of the five most common malignancies in the US, is the second most common tumor of the genitourinary tract and the second most common cause of death in patients with urinary tract malignancies (1). As in other human cancers, loss of function of tumor suppressor genes and activation of oncogenes are two major alterations that promote BC initiation and progression (2). Our laboratory developed a simple technique that combines microarray data from clinical BC specimens with those from a BC cell line (BOY) treated with a pharmacologic demethylating agent (5-aza-dC) (3,4). As described in a previous report (4), we used it to identify the methylated gene profile in human BC; this profile contains 24 methylated genes. Among these genes, we focused on the *human four-and-a-half LIM domains 1 (FHL1)* gene, one of the down-regulated genes in the gene expression profile of human BC (3).

The *FHL* family genes are characterized by LIM domains. Because LIM domains provide protein-protein binding interfaces, *FHL* genes play an important role in cellular events, such as focal adhesion and differentiation, by interacting with the target protein as either a repressor or activator (5-7). The molecules associated with intercellular adhesion are possibly inhibited during tumor progression (8). The expression of the *FHL1* gene, located at human chromosome Xq26, is down-regulated in various types of malignancies including lung, prostate, breast, ovarian, colon, and thyroid cancer, brain tumors, renal cancer, liver cancer, melanoma, and gastric cancer (9-13).

Gene function can be altered by epigenetic mechanisms. Silencing of tumor suppressor genes by hypermethylation of CpG islands within the promoter and/or 5'-regions is a common feature of human cancer and is often associated

with partial or complete transcriptional blocking. Many tumor suppressor genes silenced by DNA methylation have been identified in various types of cancer including BC (14,15). However, little is known about the status of *FHL1* promoter methylation and the functional role of *FHL1* in BC. We hypothesized that inactivation of the *FHL1* gene through CpG methylation contributes to cell viability including migration and invasion activity of human BC. To test this hypothesis, we investigated the mRNA expression levels in BC cell lines and human BC tissue. Methylation status was determined by real-time quantitative methylation-specific polymerase chain reaction (PCR) in BC specimens. In addition, we established stable *FHL1*-transfected cells and compared their proliferation, migration, and invasion activities with those of the control BC cell line.

Materials and methods

Clinical samples. A total of 70 pathologically proven BC specimens were obtained from BC patients who underwent TUR-Bt or radical cystectomy at Kagoshima University Hospital and affiliated hospitals between 2003 and 2008. Also obtained were ten normal bladder epithelium (NBE) specimens from patients without BC. The background and clinicopathological characteristics of the patients are summarized in Table I. Each tumor was staged and graded in accordance with the tumor-node-metastasis (TNM) staging system (16). The study was approved by the Institutional Review Board of our institution; written prior informed consent was obtained from all patients for use of their specimens and clinical and pathological data.

Cell culture and 5-Aza-dC treatment. The human BC cell lines (T24, UMUC and BOY) were maintained in MEM mixed with 10% FBS and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. To screen for epigenetic alterations of gene methylation, these cell lines were treated with a DNA methyltransferase inhibitor, 5-aza-dC (Sigma-Aldrich, St. Louis, MO). Cultured cells were harvested after seven days of exposure to 10 μM of 5-aza-dC, and total RNA was extracted from the cell lines before and after drug treatment.

Nucleic acid extraction. Genomic DNA was extracted from the BC and NBE specimens with a QIAamp tissue kit (Qiagen, Valencia, CA) after microdissection of 10-μm-thick paraffin-embedded sections. It was precipitated with ethanol. Total RNA was extracted from the clinical specimens using Isogen (Nippon Gene, Tokyo, Japan), following the manufacturer's protocol. Total RNA was extracted from the human BC cell lines with a QIAamp DNA/RNA Mini kit (Qiagen) according to the manufacturer's instructions. The concentration of nucleic acid was determined with a spectrophotometer, and the integrity was checked by gel electrophoresis.

cDNA preparation and real-time quantitative RT-PCR. First strand cDNA with 1 μg of total RNA was synthesized using random primers of high capacity cDNA reverse transcription kit (Applied Biosystems). Premium total RNA from normal human bladder (Clontech, Palo Alto, CA) was used as a normal bladder control. Gene-specific PCR products were continuously measured using an Applied Biosystems 7300 real-time PCR system, following the manufacturer's protocol. The first PCR step was a 10 min hold at 95°C followed by 45 cycles and the 45 cycles of a 15 sec denaturation step at 95°C and a 1 min annealing/extension step at 60°C. All reactions were performed in triplicate, and a negative control lacking cDNA was included. TaqMan® probes and primers for *FHL1* (P/N: Hs00793641_g1, Applied Biosystems) and *Glucuronidase ß* (*GUSB*) (P/N: Hs99999908_m1, Applied Biosystems) were the assay-on-demand gene expression products. The expression of *FHL1* mRNA was normalized to the amount of *GUSB* in the same cDNA using the standard curve method provided by the manufacturer.

Real-time quantitative methylation-specific PCR. For TaqMan quantitative methylation-specific PCR (QMSP), the fluorescent probe and primer sets were designed to hybridize to the amplified region of DNA (Fig. 1). Myogenic differentiation-1 (MYOD1) primer sequences were used as an internal control (4). The first PCR was amplified with universal primers (Pan-S; GGTTTGTTATTAAGGGGAGG and Pan-AS;

Table I. Patient characteristics.

Bladder cancer (BC)		
Total number	70	
Median age (range)	74	(49-100) yrs
Gender		
Male	46	
Female	24	
Stage		
Tis	1	
Superficial (pTa)	16	
Invasive (≥pT1)	53	
Grade		
G1	5	
G2	40	
G3	22	
Unknown	3	
Operation		
Cystectomy	8	
TUR-Bt	62	
Recurrence		
Recurrence	36	
Recurrence (-)	29	
Unknown	5	
Follow-up period (range)	513	(13-1440) days
Normal bladder epithelium (NBE)		
Total number	10	
Median age (range)	66	(58-76) yrs
Gender		
Male	8	
Female	2	

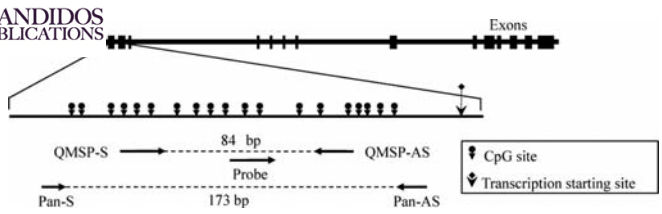

Figure 1. Schematic representation of location of CpG sites and primers designed within functional promoter of *FHL1* gene. First, universal PCR was performed with universal primers Pan-sense (S) and Pan-antisense (AS), which do not carry any CpG sites. Quantitative MSP was done using primer sets MSP-S and MSP-AS, and USP-S and USP-AS, respectively, and the universal PCR product was used as a template. For TaqMan QMSP, the fluorescent probe and primer sets were designed to hybridize to the amplified DNA region.

ACAAAAACAACCAAATAAAAATAAC) using 3 μl of bisulfite-treated DNA in a total volume of 20 μl. The first PCR step was 10 min hold at 95˚C followed by 45 cycles of a 1 min denaturation step at 95˚C, a 1 min annealing step at 48°C, and a 1-min extension step at 72°C. A second round of nested QMSP was then carried out using 1 μl of the first PCR product with the same PCR conditions described above except for the annealing temperature (59°C). The PCR cycle was programmed up to 45 cycles, and all reactions were performed in duplicate. The primers and probe sequences were QMSP primers, 5'-GTCGTTGTAAGTTATCGGGTTTC-3' (sense), 5'-CTACGCACTCCCTAAAAACG-3' (antisense), and 5'-AGGTCGAAACGGCGTGG-3' (probe). Serial dilutions of CpGenome universal methylated DNA (Millipore, Tokyo, Japan) were used to construct a calibration curve. The methylation index was defined as the quantity of fluorescence intensity derived from *FHL1* promoter amplification divided by the fluorescence intensity from MYOD1 amplification, multiplied by 1000.

Immunoblotting. Total protein lysate was prepared with detergent lysis buffer in the presence of a protease inhibitor. Twenty micrograms of protein lysate was separated by NuPAGE on 4-12% bis-tris gel (Invitrogen, Tokyo, Japan) and transferred into a PVDF membrane. Immunoblotting was done with diluted (1/200) polyclonal FHL1 antibody (10991-1-AP, Protein Tech Group, Chicago, IL). After being washed, the membrane was incubated with goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad, Hercules, CA). Specific complexes were visualized with an echochemiluminescence detection system (GE Healthcare Bio-Sciences, Buckinghamshire, UK).

Construction of FHL1 expression vectors and transfection to BOY cells. The *FHL1* vector was constructed by inserting full-length *FHL1* cDNA into the Sse83871 and *Hin*dIII restriction site of the pBApo-CMV Neo™ vector (Takara Bio, Otsu, Japan). The *FHL1* and non-targeting (control) vector were transfected into BOY cells by calcium phosphate co-precipitation. The cells were then split and grown in selective medium with 1000 μg/ml of G418. After 2 weeks, G418-resistant colonies were chosen and expanded in medium containing 100 μg/ml of G418.

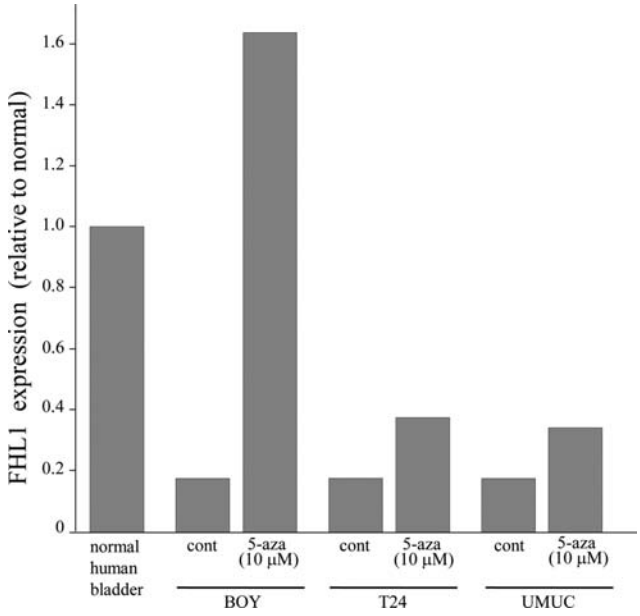

Figure 2. *FHL1* mRNA expression in BC cells before and after treatment with 5-aza-dC. Quantitative real-time RT-PCR demonstrated that mRNA transcript of *FHL1* gene was markedly increased after 5-aza-dC treatment especially in BOY cells. PCR conditions and primer sequences are described in Materials and methods section.

XTT assays. *FHL1*-transfected, control, and wild-type BOY cells were transferred to 96-well plates ($3x10^3$ cells/well) and incubated for 72 h. A 50 μl solution of XTT (0.3 mg/ml) containing 1.25 μM of PMS was then added to each well. The absorbance at 492 nm was measured after another 4 h of incubation at 37°C.

Wound healing assay. *FHL1*-transfected, control, and wild-type BOY cells were initially seeded uniformly onto 60 mm culture plates. An artificial 'wound' was carefully created at 0 h by using a P-20 pipette tip to scratch the subconfluent cell monolayer. Microphotographs were taken at 0 and 12 h. Quantitative analysis of the percentage of wound healing was calculated using the distance across the wound (n=5) at 0 and 12 h, divided by the distance measured at 0 h for each cell line. The experiment was repeated twice in sets of two, independently.

Invasion assay. 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). Two days after transfection with *FLH-1*, $5x10^4$ BOY cells were placed in the top of the chamber in serum-free DMEM, and the bottom chamber was filled with DMEM containing 10% FBS as a chemoattractant. After 22 h of incubation in 5% CO_2 humidity at 37°C, non-invading cells were removed from the upper filter surfaces, and the filters were washed, fixed, and stained using a Diff-Quick kit (Sysmex, Kobe, Japan). Four randomly selected fields, magnification x200, were photographed, and the number of invading cells in each was counted. The experiment was repeated twice in sets of two, independently.

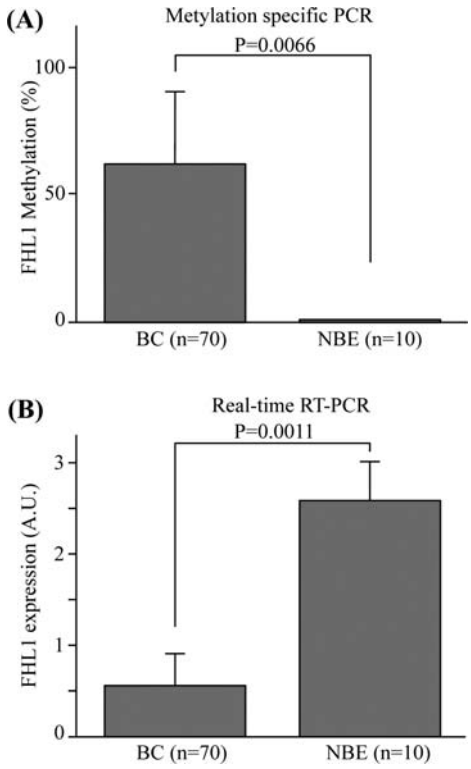

Figure 3. *FHL1* methylation index and mRNA expression of BC and NBE specimens. (A) *FHL1* methylation index was significantly higher for BC (p=0.0066). Methylation index was defined as described in Materials and methods section. (B) *FHL1* mRNA expression was significantly lower in BC (p=0.0011).

Statistical analysis. The relationship between two groups and the numerical value obtained by real-time RT-PCR was analyzed using the Mann-Whitney U test. The relationship between the BC and NBE specimens with *FHL1* methylation status was carried out using the χ^2 test. The analysis software was Expert StatView (version 4, SAS Institute Inc., Cary, NC), and the differences of $p<0.05$ were considered statistically significant.

Results

Validation of altered FHL1 mRNA expression in BC cell lines before and after 5-aza-dC treatment. The three BC cell lines (T24, UMUC and BOY) were subjected to real-time RT-PCR experiments to evaluate the mRNA expression levels of *FHL1* (Fig. 2). Before 5-aza-dC treatment, the *FHL1* mRNA expression levels in the BC cell lines were down-regulated and were lower than those of the normal human bladder. After 5-aza-dC treatment, the *FHL1* mRNA expression was up-regulated in the BC cell lines, and it was markedly higher in the BOY cells, which had been subjected to cDNA microarray analysis for obtaining the methylated gene profile in BC. Hence, the pattern of *FHL1* mRNA expression by 5-aza-dC treatment was validated by both real-time RT-PCR and cDNA microarray analysis (4).

Methylation status of FHL1 gene promoter and FHL1 mRNA expression in human BC specimens. We used QMSP and real-time quantitative RT-PCR to evaluate the methylation status of the *FHL1* promoter region and the expression levels of *FHL1* mRNA. The methylation index of the BC specimens was significantly higher than that of the NBE ones (63.9±25.5 and 0.3±0.2, respectively; p=0.0066) (Fig. 3A). In contrast, the expression level of *FHL1* mRNA in the BC specimens was significantly lower than that in the NBE ones (0.331±0.12 and 2.498±0.61, respectively; p=0.0008) (Fig. 3B). These results indicate that *FHL1 mRNA* expression might be down-regulated by methylation of the *FHL1* promoter region. We found no relationship between the clinicopathological parameters and the *FHL1* methylation index or mRNA expression (data not shown).

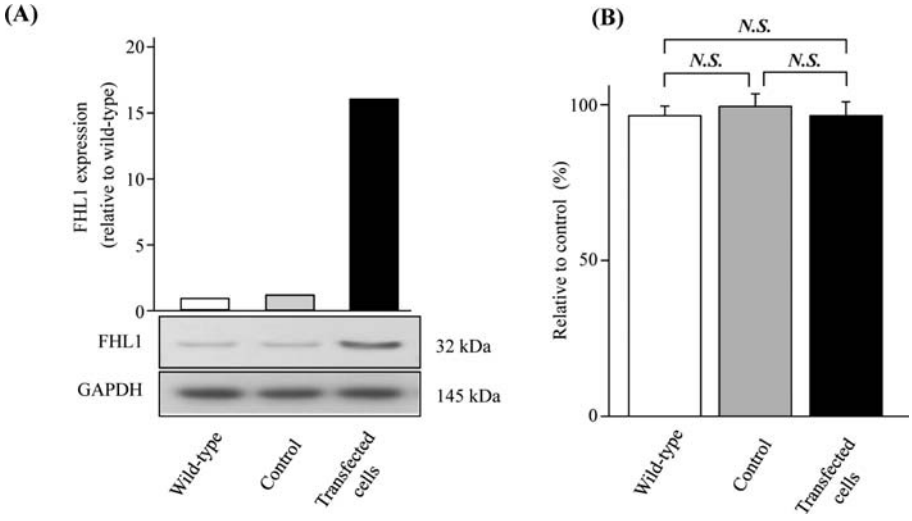

Figure 4. Effect of *FHL1* transfection on cell growth. (A) Expression level of *FHL1* mRNA was more than 16-fold higher in *FHL1*-transfected cells than in the control and the wild-type cells (upper; real-time RT-PCR). Western blot analysis revealed high expression levels of FHL1 in *FHL1*-transfected cells compared to control and wild-type cells (lower). (B) Cell growth determined by XTT assay. There was no significant difference in anchorage-dependent cell growth for *FHL1*-transfected, control, and wild-type BOY cells.

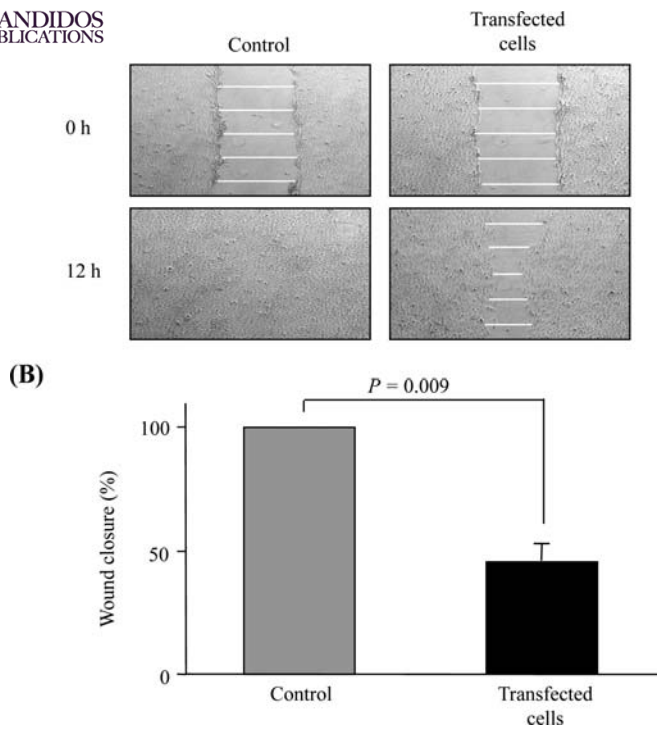

Figure 5. Effect of *FHL1* transfection on cell migration. (A) Representative photographs of cell migration activity from the wound healing assay. Wound was completely closed at 12 h after scratching in control cells whereas it was still open in *FHL1*-transfected cells. (B) Percentage of wound closure corres-ponding to distance between wound edges at 12 h in five randomly chosen regions (mean ± SD) relative to distance at time 0 h for each cell.

Identification of FHL1 expression in transfected cells and effect of FHL1 overexpression on cell proliferation. Because we found an extremely low expression of *FHL1* mRNA and a marked retrieval of the gene expression after 5-aza-dC treatment in the BOY cells (Fig. 2), we established stable *FHL1*-transfected BOY cells. The expression level of *FHL1* mRNA was more than 16-fold higher in the transfected cells than in the control and wild-type cells (Fig. 4A, upper). Western blot analysis demonstrated that the expression levels of the *FHL1*-transfected cells were high (Fig. 4A, lower). For gain-of-function experiments, we first performed XTT cell growth assay using *FHL1*-transfected, control, and wild-type BOY cells. We found no significant difference in anchorage-dependent cell growth among the three groups from three independent assays (Fig. 4B).

Effect of FHL1 transfection on cellular migration and invasion. To evaluate cellular migration, we performed wound healing assay using *FHL1*-transfected and control BOY cells. As shown in Fig. 5A, the cell migration ability of the *FLH1*-transfected cells was less than that of the control cells. Two independent wound healing assays showed significant wound healing inhibition after 12-h incubation in the *FHL-1* transfected cells compared to the control cells (45.78±6.2 and 100±0, respectively; $p<0.009$) (Fig. 5B). Next, we evaluated the invasive capacity of the BC cell lines using an *in vitro* invasion assay since penetration of extracellular matrix and basement membrane by cancer cells is a key step in tumor invasion. As shown in Fig. 6A, there were fewer invading cells in the *FHL1*-transfected cells than in the control cells. The number of invading cells was significantly lower in the *FHL1*-transfected cells than in the control cells (18.5±2.3 and 95.2±2.4, respectively; $p=0.02$) (Fig. 6B).

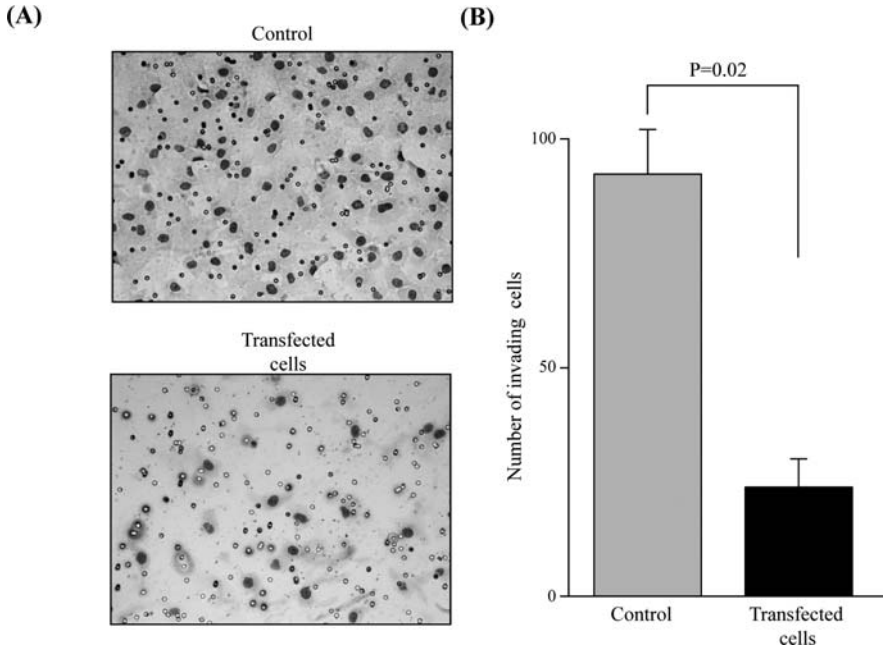

Figure 6. Effect of *FHL1* transfection on cell invasion. (A) Representative photographs of cell invasion activity from the Matrigel invasion assay. Number of invading cells was lower in *FHL1*-transfected cells than in control cells. (B) Number of invading cells counted in four random fields (mean ± SD). *FHL1*-transfected cells had significantly fewer invading cells.

Discussion

The four-and-a-half LIM (FHL) proteins are characterized by four complete LIM domains preceded by an N-terminal half LIM domain. LIM domains are cysteine-rich zinc finger motifs involved in a wide range of protein-protein interactions (17). Through interaction with cellular proteins, FHL proteins regulate cellular processes, including proliferation, differentiation, apoptosis, adhesion, migration, transcription, and signal transduction (18-22). Among these *FHL* family genes, we focused on *FHL1*, which was selected on the basis of two types of microarray analysis (3,4). Although the down-regulation of *FHL1* has been found in several types of cancer (9-13), the expression level and molecular function of *FHL1* in BC have not been clarified. Since many tumor suppressor genes silenced by DNA methylation have been identified in primary BC samples (14,15), we hypothesized that *FHL1* expression is down-regulated by promoter hypermethylation in BC cells and that *FHL1* functions as a tumor suppressor in BC cells.

In this study, we found that *FHL1* was significantly down-regulated at the mRNA level in the human BC tissue samples and that the methylation index was significantly higher in BC specimens than in NBE ones. Treatment with demethylation agent (5-aza-dC) restored *FHL1* mRNA expression in the BC cell lines. These results suggest that the down-regulation of *FHL1* expression in BC cell lines and human BC tissue correlates with CpG hypermethylation of the *FHL1* promoter. This is in accordance with our initial hypothesis that the *FHL1* promoter region is hypermethylated in BC. Since there was no apparent relationship between the clinicopathological parameters and the *FHL1* methylation index or mRNA expression, promoter methylation and down-regulation of *FHL1* might be involved in the initial stage of tumorigenesis (23).

We also hypothesized that *FHL1* functions as a tumor suppressor in BC. To examine the function of the *FHL1* gene, we established stable *FHL1*-transfected cells and compared their proliferation, migration, and invasion activities with those of the control BOY cell line, which was transfected with an empty vector. Cell proliferation assay revealed no difference in cell growth ability between the *FHL1*-transfected cells and the control cells. Shen *et al* demonstrated that *FHL1*-transfected cells have lower activity of non-anchored cell growth but not of anchored cell growth (9). Although the difference in the cell growth mechanism between anchored and non-anchored cells is unknown, our finding is similar to theirs. Although *FHL1* restoration resulted in no significant difference on anchored cell growth, we demonstrated that it significantly inhibited cell migration and invasion. These results strongly indicate that *FHL1* functions as a tumor suppressor in BC cells. Ding *et al* demonstrated that transfection of hepatocellular cancer cell line HepG2 with *FHL1* inhibited cell growth and decreased anchorage-independent colony formation whereas *FHL1* knockdown by siRNA increased cell growth and colony formation (17). They also found that FHL proteins ultimately increased the expression of growth inhibitor genes, such as the CDK inhibitor, *p21*, and that they decreased the expression of the growth-promoting gene, *c-myc* (17). In addition, *FHL1* interacts with estrogen receptors and inhibits breast cancer cell growth (24,25). Moreover, estrogen receptors are highly expressed in BC and contribute to BC cell proliferation (26,27). Thus, previous and current findings lead us to think that *FHL1* might have a tumor suppressive function through interaction with p21, c-myc, and estrogen receptors in BC. Further study is necessary to test this hypothesis.

In conclusion, we have shown that *FHL1* is frequently down-regulated in BC cell lines and clinical BC tissue. The mechanism of *FHL1* down-regulation in BC is through CpG hypermethylation of the promoter region. In addition, *FHL1* restoration causes suppression of BC cell migration and invasion. These results suggest that *FHL1* functions as a tumor suppressor gene in BC cells and that restoration of *FHL1* might offer a new therapeutic approach for the treatment of BC patients.

Acknowledgements

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