Epigenetic regulation of the potential tumor suppressor gene, *hLHX6.1*, in human cervical cancer

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Abstract. It is well known that the *Homo sapiens* LIM homeobox domain *6* gene (*hLHX6*), a putative transcription regulator, controls the differentiation and development of neural and lymphoid cells, particularly in the central nervous system. In this study, we investigated *hLHX6.1* (an isoform of *hLHX6*), which functions as a tumor suppressor gene in the cervix. Firstly, the methylation levels of the *hLHX6* and *hLHX6.1* promoters were investigated in 8 cervical cancer cell lines and human tissue samples with a distinctive degree of malignant transformation. In spite of the presence of multiple cytosine guanine dinucleotides (CpG islands) in 2 proximal promoters of the *hLHX6* and *hLHX6.1* genes, only the *hLHX6.1* promoters were found to be mostly hypermethylated and associated with transcriptional silencing by promoter methylation, whereas the *hLHX6* promoters were not. Methylation levels in the *hLHX6.1* promoter were also found to be strongly related to cervical cancer development. The level of *hLHX6.1* gene expression was found to be relatively high in normal cells, in which the *hLHX6.1* promoter was mostly unmethylated. However, the *hLHX6.1* gene expression was down-regulated or undetectable in cervical cancer cell lines and cancer tissues, in which the *hLHX6.1* promoter was hypermethylated. This epigenetic alteration in the *hLHX6.1* promoter begins at a relatively early stage, suggesting its potential as a biomarker for the early diagnosis and prevention of cervical cancer. Moreover, the overexpression of the *hLHX6.1* gene in cervical cancer cells suppressed the tumorigenic phenotype, as shown by soft agar colony formation and migration assays, suggesting that *hLHX6.1* could be a new tumor suppressor gene in the cervix.

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Abbreviations: hLHX6.1, *Homo sapiens* LIM homeobox domain 6.1 gene

Key words: methylation biomarker, tumor suppressor gene, cervical cancer, *Homo sapiens* LIM homeobox domain 6.1 gene

Introduction

Cervical cancer is the second most frequent malignant type of cancer worldwide and is still an important health issue for women. When infected by the human papillomavirus (HPV), a major cause of cervical cancer, the cervical epithelium develops an invasive cervical carcinoma via a multistep process (1-4). Multistep cervical carcinogenesis can be classified into 5 groups: Normal, cervical intraepithelial neoplasia (CIN) I (mild dysplasia), CIN II (moderate dysplasia), CIN III (severe dysplasia) and invasive cervical carcinoma (5-6). Persistent HPV infection accelerates the development of CINs by facilitating the dysregulation of cellular proliferation and the apoptotic process. In spite of its strong association with cervical cancer, HPV infection alone is not sufficient for the cervical epithelium to fully develop an invasive carcinoma. Additional accumulation of mutations in various genes is required before these premalignant lesions develop into invasive ones. These mutations include the overexpression of oncogenes or the repression of tumor suppressor genes. Among them, promoter hypermethylation is one of the main causes for the inactivation of the transcription of tumor suppressor genes (7-16). The epigenetic silencing of various genes by promoter hypermethylation is now recognized as a frequent event in the pathogenesis of many cancers, including cervical cancer. An abnormal pattern of DNA methylation occurs at specific genes in nearly all neoplasms, making DNA methylation of special interest as a tumor biomarker (16-18). High densities of CpG sites are found in many homeobox genes and some of them are found to be highly methylated (19). Homeobox genes encode transcription factors and play vital roles in embryogenesis, the differentiation of adult cells and related developmental processes (20). Among many homeobox genes, the human genome contains at least 12 LIM homeobox (*LHX)* genes encoding LIM homeo domain transcription factors. These genes usually have a

LIM domain in addition to a homeo domain. The LIM domain, a unique cytosine-rich zinc-binding domain, is used for the interaction with an LIM domain-binding protein (Ldb) that negatively regulates the transcriptional activity of many LHX proteins (21-22). Studies with mouse models and human patients have shown that LHX proteins play important roles in cytoskeletal organization, organ development and oncogenesis. LHX proteins are known to be involved in human diseases (23-25).

It is well known that the human *LHX6* gene, *hLHX6*, controls the differentiation and development of neural and lymphoid cells, particularly in the central nervous system (CNS) (26-30). The *hLHX6* gene is considered to be a putative transcription factor required for the expression of genes involved in interneuron migration and development. Two alternatively spliced transcript variants have been found for this gene, *hLHX6a* and *hLHX6b*. Besides these 2 transcripts, another isoform of *LHX6.1* was first found in a mouse model, as reported by Kimura *et al* (31). They suggested that the *LHX6.1* gene is closely related to the *LHX6* gene that is expressed predominantly in the developing CNS. They showed that LHX6.1 interacts with Ldb1 through tandem LIM-domains like other LHX proteins, implying the transcriptional regulation of *LHX6.1* by Ldb1 (31). In addition, *hLHX6s*, an alternative short isoform of the *hLHX6* gene, was identified by Estecio *et al* (32). However, the biological functions of this transcript variant have not yet been determined. While the *hLHX6* gene is significantly expressed in many tissues, the gene expression of *hLHX6s* has only been detected in a few tissues. In spite of these advanced studies, little information is available on the molecular mechanisms that regulate the transcription of the *hLHX6* and *hLHX6.1* genes. Particularly, gene regulation by hypermethylation on *hLHX6.1* gene expression has not been previously investigated in any cancers including cervical cancer. In the process of developing a methylation DNA biomarker for the early diagnosis of cervical cancer, we previously showed that the *hLHX6*-hypermethylated region, which includes the genomic sequences found between exons 4a and 5 of the *hLHX6s*, is a sensitive methylation-based molecular biomarker with increased sensitivity and specificity for the early diagnosis of cervical cancer (33). CpG islands are also found in 2 proximal promoters of the *hLHX6* and *hLHX6.1* genes. It is a well known fact that transcriptional silencing by promoter hypermethylation is an important regulatory mechanism in many cancer cells. These facts led us to further study the molecular mechanism and roles of the *hLHX6.1* gene in cervical cancer development.

In this study, we show that the *hLHX6.1* promoter is frequently hypermethylated in cervical cancer cells and that this epigenetic alteration of the *hLHX6.1* gene is associated with transcriptional silencing and cancer cell development. More importantly, our present study for the first time provides insight into the mechanism of *hLHX6.1* tumor suppression in cervical carcinogenesis.

Materials and methods

Cervical cancer cell lines and human tissue samples. Eight cervical cancer cell lines were used for this study. C33A, CaSki, HeLa and SiHa cells were purchased from the American Type Culture Collection (USA). The other cell lines, SNU-17, -703, -1160 and -1299, were obtained from the Korean Cell Line Bank (KCLB, Korea). Each cell line was grown in one of the following different media: C33A, HeLa and SiHa cells in DMEM medium (WelGENE Inc., Korea), CaSki, SNU-703 and SNU-1299 cells in RPMI-1640 medium (Gibco BRL), and SNU-17 and SNU-1160 in AR5 medium (KCLB). All the media were supplemented with 10% fetal bovine serum (Gibco BRL) and 1% antibiotic-antimycotic solution (Gibco BRL). All the cells were cultured at 37˚C in a humidified atmosphere composed of 95% air and 5% CO_2. A total of 110 human tissue samples were kindly provided by Dr Chang-Jin Kim at the Soonchunhyang University Hospital (Cheonan, Korea). These tissue samples originated from cervical cancer patients, and their histological tumor grade and age is presented in Table I. The tissue samples for CIN diagnosis were prepared via micro-excision. Patients signed informed consent forms and the procedure for obtaining the tissue samples was approved by the institutional review board of the hospital clinic.

Reverse transcription (RT)-PCR. Following the manufacturer's instructions, total RNA was extracted from the cervical cancer cell lines or human tissue samples using the RNeasy mini kit (Qiagen). For reverse transcription, 1 μg RNA of each sample was subjected to cDNA synthesis using oligo(dT) primer and the RevertAid First Strand cDNA Synthesis Kit (Fermentas, Korea) according to the manufacturer's instructions. PCR amplification was performed using 10 ng cDNA, different sets of primers and AccuPower PCR PreMix (Bioneer, Korea). The nucleotide sequences of the primers and the conditions for gene amplification are shown in Table II. As the internal control, the 377-bp ß-actin gene products were amplified using pRT-ACTB-forward (F) and -reverse (R) primers. The amplification reaction was carried out using the GeneAmp PCR System 9700 from Applied Biosystems. The amplification products were electrophoresed on a 2% agarose gel stained with ethidium bromide. The band intensity was visualized and measured using a UV illuminator or a LAS-3000 imaging system.

Methylation-specific PCR (MSP) and bisulfite sequencing PCR (BSP) analyses. Genomic DNA was extracted from the cervical cancer cell lines or human tissue samples using the DNeasy Blood and Tissue Kit (Qiagen). A bisulfite treatment was conducted using 1 μg of genomic DNA at 55˚C for 16 h following the instructions included with the EZ DNA Methylation Kit (Zymo Research, CA, USA). For MSP analysis, the bisulfite-treated DNA samples underwent PCR amplification using 2 pairs of primers, which were designed to amplify unmethylated or methylated targets. The nucleotide sequence of each primer and the amplification conditions are shown in Table II. For all MSP analyses, the PCR mixtures contained 10X reaction buffer, dNTP mixture (1 mM), primers (final concentration of 10 pmole per reaction), 1 unit of HotStart prime Taq (Qiagen) and bisulfite-treated DNA. The amplification products were resolved in 2% agarose gels and stained with ethidium bromide. The methylation status was inferred by the presence or absence of

Table I. Human cervical tissue samples used in RT-PCR, MSP and BSP analyses.

Number[a]	Diagnosis samples[b] (age)				
	Normal[c]	CIN I	CIN II	CIN III	Carcinoma
1	08-3488-1d (48)	07-692 (44)	07-4215 (34)	07-852 (51)	7227 (75)
2	08-7568-1b (49)	07-949 (32)	07-4406 (27)	07-1631 (48)	12593 (42)
3	08-3782-1b (40)	07-1573 (40)	07-4556 (36)	07-1858 (56)	10931 (75)
4	08-7275-1a (43)	07-1888 (46)	07-4751 (37)	07-1854 (41)	6956 (59)
5	08-3665-1b (68)	07-1899 (45)	07-4926 (38)	07-2346 (43)	10919 (82)
6	08-5386-1a (44)	07-1857 (42)	07-5660 (39)	07-2914 (53)	8026 (71)
7	08-3513-1a (42)	07-1855 (43)	07-5881 (41)	07-8302 (25)	5739 (38)
8	08-3513-1b (NA)	07-2687 (22)	07-5908 (41)	07-9619 (38)	6851 (56)
9	08-3488-1b (NA)	07-2888 (25)	07-5929 (22)	07-10051 (72)	4321 (81)
10	08-5889-1a (45)	07-3349 (23)	07-6000 (27)	07-10432 (65)	5822 (46)
11		07-3596 (50)	07-6473 (48)	09-153 (41)	09-240 (65)
12		07-3651 (23)	07-6561 (32)	09-640 (35)	09-576 (65)
13		07-5594 (44)	07-6858 (32)	09-796 (57)	09-1183 (46)
14		07-6334 (36)	07-6859 (32)	09-875 (40)	09-1388 (41)
15		07-6474 (32)	07-7288 (36)	09-1877 (48)	09-1645 (48)
16		07-6439 (36)	07-7768 (27)	09-2986 (29)	09-2740 (49)
17		07-6644 (44)	07-8794 (47)	09-3072 (27)	09-2943 (68)
18		07-6665 (23)	07-9302 (29)	09-3431 (41)	09-3671 (62)
19		07-6697 (37)	07-9671 (26)	09-3670 (39)	09-3675 (60)
20		07-7713 (49)	07-9932 (32)	09-3613 (39)	09-4161 (40)
21		07-8301 (37)	07-10724 (50)		
22		07-8663 (26)	07-11282 (37)		
23		07-8899 (36)	07-12365 (28)		
24		07-12017 (24)	07-13050 (40)		
25		07-12230 (38)	07-562 (35)		
26		07-12320 (29)	07-708 (31)		
27		07-12412 (41)	07-879 (21)		
28		07-12620 (34)	07-915 (34)		
29		07-12766 (50)	07-934 (41)		
30		07-13172 (42)	07-1076 (25)		

[a]Number of cases examined. [b]Samples were collected from patients with different histological types of cervical cancer (different tumor grade or clinical stage in cervical carcinogenesis) (see Materials and methods). [c]Normal tissue samples are from tissue adjacent to tumor tissue. NA, not available

bands and its density was represented by the thickness of the bands. For BSP analysis, the bisulfite-treated DNA samples underwent a PCR reaction using the corresponding primer pairs, pBSP-LHX6-F/pBSP-LHX6-R for amplification of the *hLHX6* promoter and pBSP-LHX6.1-F/pBSP-LHX6.1-R for the *LHX6.1* promoter (Table II). All the BSP primers are designed to cover the transcriptional start site or be close to the transcriptional start site. The amplified PCR products were cloned into the pBlueScript-SK(+) vector using *Hin*dIII and *Eco*RI restriction enzymes and were transformed into DH5α competent cells. Plasmids purified from amphicillin-positive colonies were sequenced using the M13-F or -R primer by Solegent (Daejeon, Korea).

Treatment of 5'-aza-2'-deoxycytidine (DAC) and trichostatin A (TSA). Cells from 5 cervical cancer cell lines (HeLa, SiHa, SNU-17, SNU-703 and SNU-1299) were treated with a DNA demethylating agent of DAC (Sigma) and/or a histone deacetylase inhibitor of TSA (Sigma). The cells were plated onto 100-mm plates for 24 h before treatment. They were then treated with 1, 3, 5, or 10 μM of DAC for 24, 48, 72, or 96 h. The cells were also treated with 0.1, 0.3, 0.5, or 1 μM of TSA for 24 or 48 h.

Construction of pcDNA3-hLHX6.1. For the functional study of the hLHX6.1 protein, the *hLHX6.1* overexpressing vector, pcDNA3-hLHX6.1, was constructed as follows: The full

Table II. Oligonucleotide sequences and conditions for PCR analysis.

Primer name[a]	Primer sequence (5'-3')[b]	Amplicon size (bp)	Conditions[c]	Sources or references
pRT-LHX6-1-F	CGCGGACGTCCTTCACCGCGG	398	69˚C,	This study
pRT-LHX6-1-R	CCGGTTGGAGAGCGGCCCATCC		35 cycles	
pRT-LHX6-2-F	CCTTCACCGCGGAACAGCTGCAG	153	69˚C,	This study
pRT-LHX6-2-R	TATGACGCGCCCGGCAGTTTTGA		35 cycles	
pRT-LHX6.1-5'UTR-F	CCCTCCCCCAGGTGATGGCCCA	137	68˚C,	This study
pRT-LHX6.1-5'UTR-R	CTGACCCTCGTCCTTGTCCAGAGCT		35 cycles	
pRT-LHX6s-1-F	CCTCTGGCTTCTTCCCCTAC	316	60˚C,	(33)
pRT-LHX6s-1-R	ACTCCTCACCAGTGGACAGC		35 cycles	
pRT-LHX6s-2-F	GAGTTTCGGCCTCTCGGCTCAATAG	113	60˚C,	This study
pRT-LHX6s-2-R	TGGTAGGCGTTGCCGCGAGCTCTCC		35 cycles	
pMSP-UM-LHX6-F	GTAGTAGTTAGGGAGGTTGG	184	55˚C,	This study
pMSP-UM-LHX6-R	CAAAAAACCTCAAACTCAACAAA		35 cycles	
pMSP-M-LHX6-F	GTAGTAGTTAGGGAGGTCGG	185	55˚C,	This study
pMSP-M-LHX6-R	GAAAAACCTCGAACTCAACGA		35 cycles	
pMSP-UM-LHX6.1-F	AATTGTTTTATTAGAGAGATATTGT	151	58˚C,	This study
pMSP-UM-LHX6.1-R	ACAACAACTACTAAACTAACTCCACA		35 cycles	
pMSP-M-LHX6.1-F	AAATTGTTTTATTAGAGAGATATCGT	150	58˚C,	This study
pMSP-M-LHX6.1-R	ACGACGACTACTAAACTAACTCCG		35 cycles	
pBSP-LHX6-F	cgt*aagctt*GGGGGTTTTTTTAAGTTTGT[d]	255	58˚C,	This study
pBSP-LHX6-R	cta*gaattc*TTCTCATACTTCCAATACATAAACC		35 cycles	
pBSP-LHX6.1-F	cgt*aagctt*GGGTTTTAAATGTTTATTATAAAGTTAGGA	297	58˚C,	This study
pBSP-LHX6.1-R	cta*gaattc*CCTAACCAAATCCCCAAAAC		35 cycles	
pLHX6.1-*Bam*HI	ccgt*ggatcc*ATGGCCCAGCCAGGGTCCGGC	1112	58˚C,	This study
pLHX6.1-*Eco*RI	ccta*gaattc*TTAGTACTGAAAAAGGATGAC		35 cycles	
pRT-ACTB-F	AGGTCGGAGTCAACGGATTTG	377	58˚C,	This study
pRT-ACTB-R	GTGATGGCATGGACTGTGGT		21 cycles	

[a]F, forward primer; R, reverse primer; M, methylated-specific primers; UM, unmethylated-specific primers. [b]All sequences are shown in the 5'→3' direction. [c]Conditions are shown in the order of annealing temperature (˚C) and number of cycles. [d]Restriction enzymes are represented in italics and lower case letters. [d]Restriction enzyme sites are underlined.

length of *hLHX6.1* cDNA was amplified using the primer set, pLHX6.1-*Bam*HI/pLHX6.1-*Eco*RI (Table II), and the pME18SFL3 plasmid as a template. The pME18SFL3 plasmid containing the *hLHX6.1* cDNA [NITE Biological Resource Center (NBRC) clone no. AK313808] was obtained from the NBRC (www.nbrc.nite.go.jp/e/). Amplified PCR products were cloned into pcDNA3 using *Bam*HI and *Eco*RI restriction enzymes to generate the pcDNA3-hLHX6.1 plasmid.

Soft agar colony forming and wound healing migration assays. SiHa cells were transfected with 0.4 μg of pcDNA3-hLHX6.1 plasmid using the Lipofectamine™ reagent (Gibco) according to the manufacturer's instructions. A pcDNA3 plasmid without the *hLHX6.1* gene was also transfected as the control. For transient transfection, SiHa cells were treated with G418 and the clones were pooled. Overexpression of the hLHX6.1 protein was verified by Western blotting. For this process, the hLHX6 antibody was purchased from Santa Cruz (sc-81970, Santa Cruz Biotechnology). For the soft agar colony forming assay, the cells were then counted, diluted and seeded in duplicate at 50 cells per culture dish (6-well plate). The cells were incubated for 26 h at 37˚C. Colonies were allowed to grow for 13 days. They were counted after staining with 1% Giemsa solution. For the wound healing assay, SiHa cells ($1x10^5$) were plated onto 60-mm tissue culture dishes and allowed to create a confluent monolayer. Cells were grown for 48 h after transfection with pcDNA3 or pcDNA3-hLHX6.1. The cell monolayer was then scraped in a straight line to make a 'scratched wound' with a 0.2 ml pipette tip, and the cell debris was removed by washing the cells with phosphate-buffered saline. DMEM medium supplemented with 10% FBS and G418 were then added and the closure of the scratch was photographed at 0, 24 and 48 h.

Statistical analysis. Statistical analyses were carried out with the Statistical Package of the Social Sciences (SPSS) software. The association of the *hLHX6.1* promoter methylation with cervical carcinogenesis was determined using the Chi-square (or χ^2) test. Statistical significance was set at a P-value of <0.05.

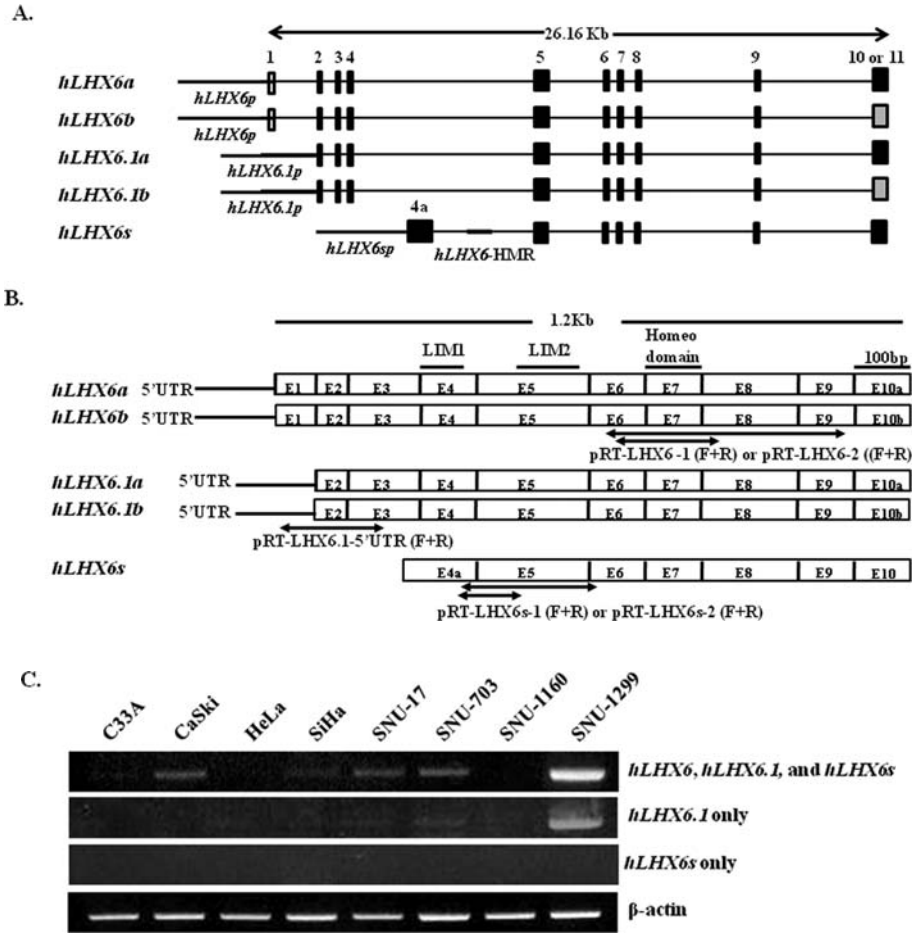

Figure 1. Expression of *hLHX6*, *hLHX6.1* and *hLHX6s* genes in 8 cervical cancer cell lines. (A) Genomic structure of the *hLHX6*, *hLHX6.1* and *hLHX6s* genes. Exons and introns are represented as black boxes and thin lines linking the boxes, respectively. The alternative splicing regions are indicated by grey boxes. Putative promoter regions of *hLHX6* isoforms are indicated by the bold lines. *hLHX6p*, *hLHX6* promoter; *hLHX6.1p*, *hLHX6.1* promoter; *hLHX6sp*, *hLHX6s* promoter. (B) Full-length mRNA products for *hLHX6*, *hLHX6.1* and *hLHX6s* are represented as a combination of exons and are indicated by boxes. Putative LIM and homeo domains are indicated. The arrows indicate the position of the primers used in the RT-PCR procedures. (C) The transcriptional level of each gene was measured by RT-PCR using different primer pairs: pRT-LHX6-1-F and -R for all transcripts of *hLHX6* isoforms, pRT-LHX6.1-5'UTR-F and -R for *hLHX6.1* only, and pRT-LHX6s-1-F and -R for *hLHX6s* only. ß-actin served as the internal control for the integrity of the cDNA.

Results

Transcriptional levels of hLHX6, hLHX6.1 and hLHX6s genes in 8 cervical cancer cell lines. The genomic structure of all known *hLHX6* isoforms (*hLHX6*, *hLHX6.1* and *hLHX6s*) is shown in Fig. 1A, in which they are mapped to chromosome 9p32. The full lengths of their cDNA are shown in Fig. 1B. The *hLHX6* and *hLHX6.1* transcripts basically contain 10 and 9 exons, respectively. Both genes have 2 types of alternatively spliced isoforms, *hLHX6a* and *hLHX6b*, and *hLHX6.1a* and *hLHX6.1b*. Nucleotide sequences were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). RT-PCR was employed to measure the transcriptional level of the *hLHX6*, *hLHX6.1* and *hLHX6s* genes in 8 cervical cancer cell lines using corresponding primer sets (see Materials and methods; Fig. 1B and C). The results showed that a relatively high level of gene expression was detected in the SNU-1299 cell line, whereas low levels were detected in other cell lines when the *hLHX6* isoforms were tested together (Fig. 1C). A similar expression level was detected when the *hLHX6.1* gene was tested alone. However, no transcript of the *hLHX6s* gene was detected in any of the cervical cancer cell lines. Therefore, we could not exclude the possibility that *hLHX6s* is not normally expressed in the cervix, as was also the case in the study by Estecio *et al* (32). This hypothesis was confirmed by testing for *hLHX6s* gene expression in normal cervix tissue samples (data not shown). On the whole, the results indicate that the transcription of the *hLHX6* and *hLHX6.1* genes is normally repressed in most cervical cancer cell lines.

The methylation status of hLHX6 and hLHX6.1 promoters in 8 cervical cancer cell lines. It has been reported that transcriptional silencing by promoter hypermethylation is an important regulatory mechanism in many tumors. We therefore examined the DNA methylation status of the *hLHX6* and *hLHX6.1* promoters. Putative CpG islands in 2 promoter regions of the *hLHX6* and *hLHX6.1* genes were predicted using the MethPrimer program (http://www.urogene.org//methprimer) (34), with the default setting (%GC>50%, ObsCpG/ExpCpG>0.6). A much higher frequency of CpG

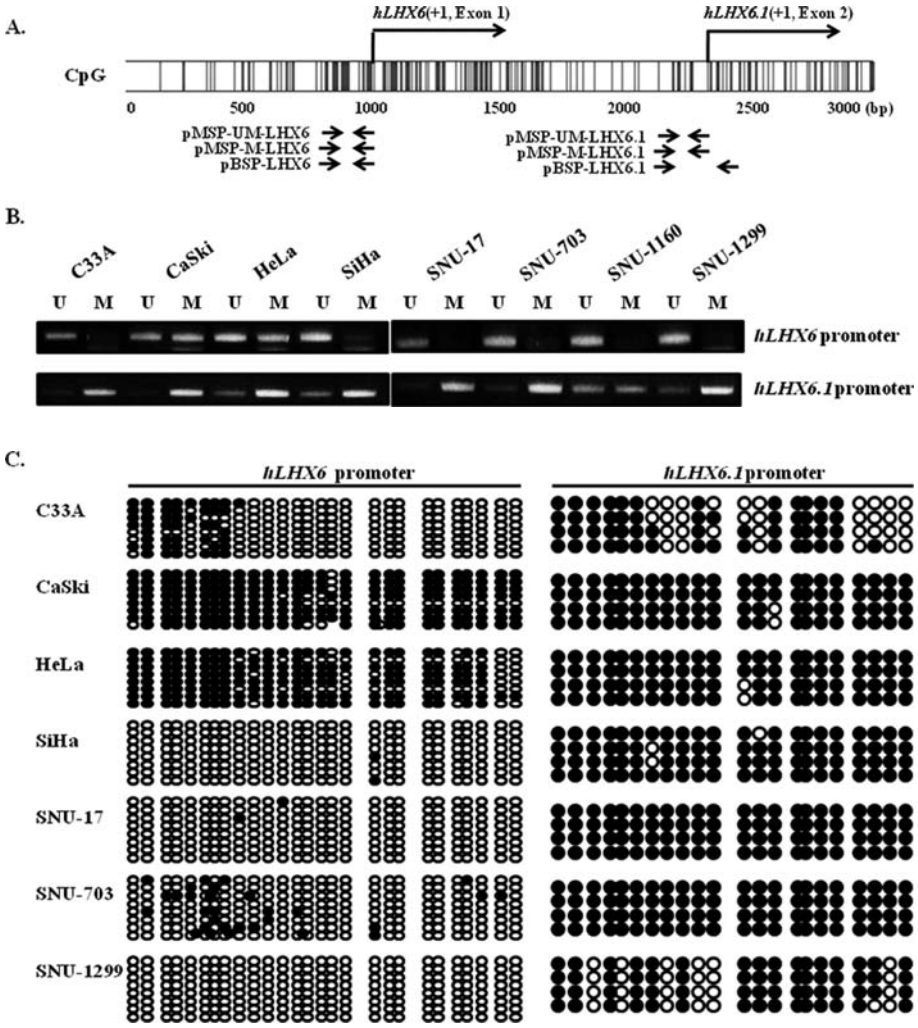

Figure 2. Methylation status of CpG sites in the 2 GC-rich promoters of the *hLHX6* and *hLHX6.1* genes in 8 cervical cancer cell lines. (A) Putative CpG sites in the genomic DNA containing the *hLHX6* and *hLHX6.1* genes. CpG dinucleotides are shown as short vertical lines. Numbers indicate the positions relative to the translation start site of the *hLHX6* and *hLHX6.1* genes. Short thin arrows indicate the positions of the primers used in the MSP and BSP assays. (B) MSP analysis of the *hLHX6* and *hLHX6.1* promoters in 8 cervical cancer cell lines. U and M represent PCR products amplified by primers specific to unmethylated or methylated DNA, respectively. (C) BSP analysis of the *hLHX6* and *hLHX6.1* promoters in 7 cervical cancer cell lines. For each cell line, the methylation status of CpGs in each promoter region is shown as 8 clones for *hLHX6* and 4 clones for *hLHX6.1*. Each row represents an individual cloned allele that was sequenced following sodium bisulfite DNA modification. Circles represent CpG sites and their spacing accurately reflects the CpG density of the region. Unmethylated and methylated cytosines are represented as white and black circles, respectively.

dinucleotides was found in the *hLHX6* and *hLHX6.1* promoters compared to the *hLHX6s* promoter (Fig. 2A). The CpG island searcher program revealed CpG islands spanning the whole region of the genes, *hLHX6* and *hLHX6.1* (data not shown). Considering the high frequency of CpG sites in the *hLHX6* and *hLHX6.1* promoters and the low transcriptional level of the *hLHX6* and *hLHX6.1* genes, it is likely that the low expression of the *hLHX6* and *hLHX6.1* genes in cervical cancer cells results from DNA methylation in the *hLHX6* and *hLHX6.1* promoters. To test this hypothesis, MSP and BSP assays were employed to investigate the methylation status in the *hLHX6* and *hLHX6.1* promoters (see Materials and methods). Initially, MSP assay was performed using primers designed to amplify unmethylated or methylated DNA targets. The MSP results showed that only unmethylated bands were detected in the *hLHX6* promoters of most cervical cancer cell lines except for the CaSki and HeLa cells, in which both unmethylated and methylated PCR products were amplified with similar densities (Fig. 2B). Methylated PCR products were dominantly amplified from the *hLHX6.1* promoters (Fig. 2B). These results suggest that the *hLHX6.1* promoter is preferentially targeted for DNA methylation in cervical cancer cell lines. The accuracy of the MSP method in detecting methylated DNA was verified by BSP assay. The bisulfited DNA samples were amplified using corresponding primer sets for *hLHX6* and *hLHX6.1* (see Materials and methods). Consistent with the results from the MSP assay, the BSP analysis demonstrated that the *hLHX6.1* promoters were hypermethylated in all tested cell lines although relatively low levels of methylation were found in C33A and SNU-1299 cell lines. The *hLHX6* promoters were not methylated in most cell lines but were found to be hypermethylated in the CaSki and HeLa cell lines (Fig. 2C).

On the whole, these results indicate that DNA methylation is common in the *hLHX6.1* promoter but not in the *hLHX6*

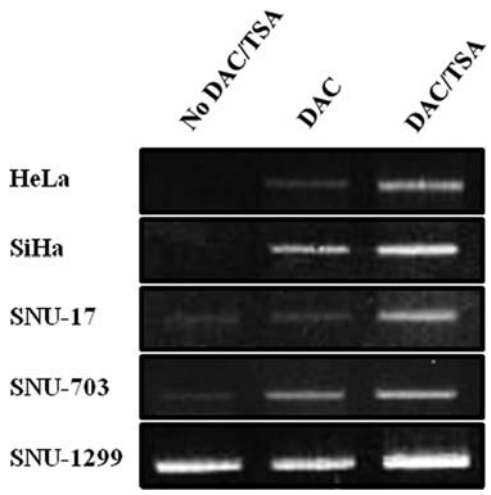

Figure 3. Effect of the demethylating agents, DAC and/or TSA on *hLHX6.1* gene expression. RT-PCR was carried out using cDNA from each cell line which was subjected to different concentrations of the drug treatments and pRT-LHX6-2-F and -R primers (see Materials and methods).

promoter of cervical cancer cell lines. The RT-PCR, MSP and BSP analyses showed an inverse relationship between *hLHX6.1* gene expression and promoter methylation. The transcriptional levels of the *hLHX6.1* gene were found to be low in cervical cancer cell lines that have a hypermethylated *hLHX6.1* promoter. Therefore, the effect of the *hLHX6.1* promoter methylation on its gene expression was further investigated.

Recovery of hLHX6.1 gene expression by treatment with DAC and TSA. Our results implied that hypermethylation in the *hLHX6.1* promoter could be responsible for the transcriptional silencing of the *hLHX6.1* gene in cervical cancer cell lines. To test this hypothesis, *hLHX6.1* gene expression was measured after treatment with DAC and/or TSA (see Materials and methods). Consistent with our hypothesis, *hLHX6.1* gene expression was reactivated by drug treatment in the HeLa, SiHa, SNU-17 and SNU-703 cell lines (Fig. 3). Treatment with DAC increased *hLHX6.1* expression in most tested cell lines. Treatment with DAC and TSA together significantly increased *hLHX6.1* gene expression (Fig. 3). As expected, no significant change was observed in the SNU-1299 cell line, in which considerable expression was detected both before and after the treatment. These results suggest that *hLHX6.1* promoter hypermethylation is associated with the transcriptional silencing of the *hLHX6.1* gene in cervical cancer cell lines.

Inverse relationship between hLHX6.1 promoter methylation and hLHX6.1 gene expression during multistep cervical carcinogenesis. The *hLHX6.1* promoter hypermethylation in cervical cancer cell lines suggested the possibility of the same situation *in vivo*. Therefore, this investigation was extended to human tissue samples that were collected from 5 different stages of cervical cancer development: A normal cervix, CIN I, II and III, and invasive carcinoma. Initially, the methylation level of both the *hLHX6* and *hLHX6.1* promoters was evaluated in a total of 110 cervical tissues samples. The MSP analysis showed that unmethylated PCR products were dominantly amplified from *hLHX6* promoters in all tissue samples (Fig. 4A). In contrast, the methylation level in the *hLHX6.1* promoter increased as normal cells developed into cancer cells. Non-methylated or partially methylated *hLHX6.1* promoters were found in normal and CIN I tissue samples, whereas a high degree of methylation was observed in CIN III and invasive carcinoma (Fig. 4A). We validated the reliability of MSP analysis by performing BSP assay. Certain *hLHX6* and *hLHX6.1* methylation-negative or -positive cervical cancers were selected for BSP analysis. Five or 8 clones from representative samples were sequenced to evaluate the methylation levels of the *hLHX6* and *hLHX6.1* promoters. None of the tissue samples exhibited hypermethylation of the *hLHX6* promoter (Fig. 4B). Very low levels of methylation were found in the *hLHX6.1* promoter from normal, and CIN I and II tissue samples, whereas high levels of methylation were observed in CIN III and invasive carcinoma cells (Fig. 4B). Importantly, abnormal DNA methylation in the *hLHX6.1* promoter occurs at CIN I, a relatively early stage of multistep carcinogenesis. This finding suggests that *hLHX6.1* promoter methylation is correlated with cervical cancer development. This fact proposes the potential clinical application of *hLHX6.1* methylation as an important molecular biomarker in the early diagnosis and prevention of cervical cancer. *hLHX6.1* gene expression was also measured in normal and cervical cancer tissues to test whether the *hLHX6.1* expression level is related to cervical cancer development (Fig. 4C). RT-PCR was used to check the *hLHX6.1* gene expression level using pRT-hLHX6-2-F and -R primers. The *hLHX6.1* transcript was well expressed in normal tissue samples while it was undetectable in most invasive carcinoma cells, where a high degree of methylation was found in the *hLHX6.1* promoter (Fig. 4C). An unexpected observation was 2 PCR products of different sizes, a relatively large PCR product and the expected PCR product, which was considered to be an alternatively spliced transcript (Fig. 4C). However, this requires additional testing.

Overall, the *hLHX6.1* gene expression is shown to be frequently silenced in cervical cancer cells. DNA methylation analyses and RT-PCR revealed an inverse relationship between the *hLHX6.1* promoter methylation and its gene expression. Furthermore, hypermethylation of the CpG-rich *hLHX6.1* promoter was only found in carcinoma cells and not in normal cervical tissues, suggesting that it most likely represents a tumor-associated event that occurs during cervical cancer development. This fact implies that the *hLHX6.1* gene could be a potential tumor suppressor gene. Therefore, our hypothesis was further tested using functional assays.

Functional study of hLHX6.1 by soft agar colony formation and migration assays. This study shows that the methylation level of the *hLHX6.1* promoter increases as normal cells develop into cervical cancer, in which *hLHX6.1* gene expression is repressed, partly due to promoter hypermethylation. It is well known that the transcription of many key tumor suppressor genes is found to be inactivated by promoter hypermethylation in many cancer cells. These facts imply that *hLHX6.1* could act as a tumor suppressor gene in

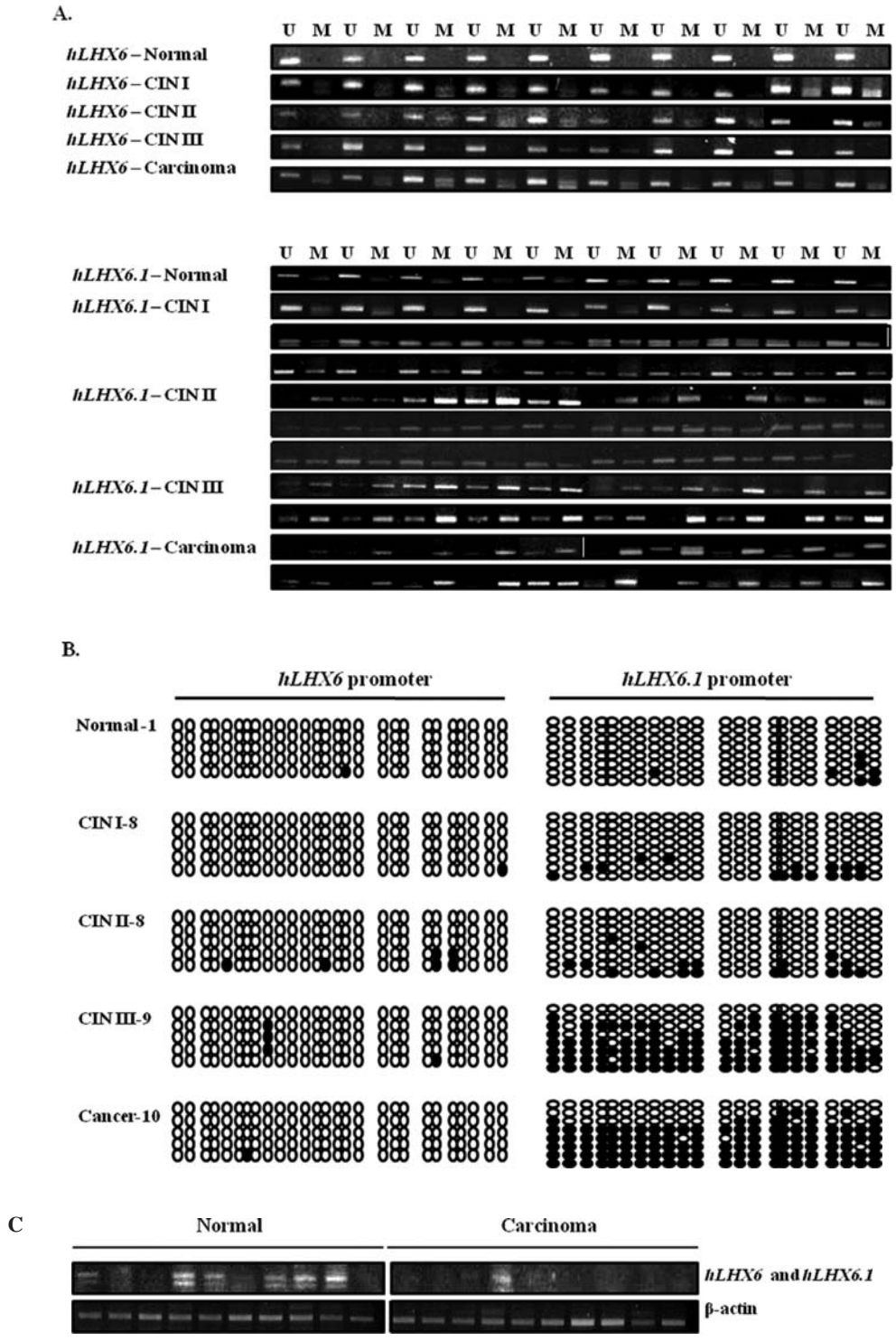

Figure 4. Methylation status of the *hLHX6* and *hLHX6.1* promoters and *hLHX6.1* gene expression in human tissue samples. (A) MSP assays of the *hLHX6* and *hLHX6.1* promoters in 110 tissue samples. U indicates PCR products amplified by primers specific to unmethylated DNA, whereas M represents PCR products amplified by primers specific to methylated DNA. (B) BSP assays of the *hLHX6* and *hLHX6.1* promoters in each representative tissue sample. Representative *hLHX6* and *hLHX6.1* methylation-negative or -positive cervical cancers were selected from MSP analysis as indicated by the numbers. For each sample, the methylation status of CpGs is shown as 5 or 8 clones for the *hLHX6* and *hLHX6.1* promoter regions, respectively. White and black circles indicate unmethylated and methylated CpGs, respectively. (C) RT-PCR analysis demonstrating *hLHX6.1* gene expression in normal and invasive cervical carcinoma tissues. ß-actin gene was used as the internal control.

the cervix. Therefore, soft agar colony formation assay was used to examine the tumor suppression ability of the *hLHX6.1* gene (see Materials and methods). Fig. 5A shows the number of G418-resistant colonies arising from cells transfected with the control expression vector (pcDNA3) or the *hLHX6.1* overexpressing vector (pcDNA3-hLHX6.1). The *hLHX6.1* overexpressing SiHa cells showed a significant reduction in cell size and number compared to the control cells

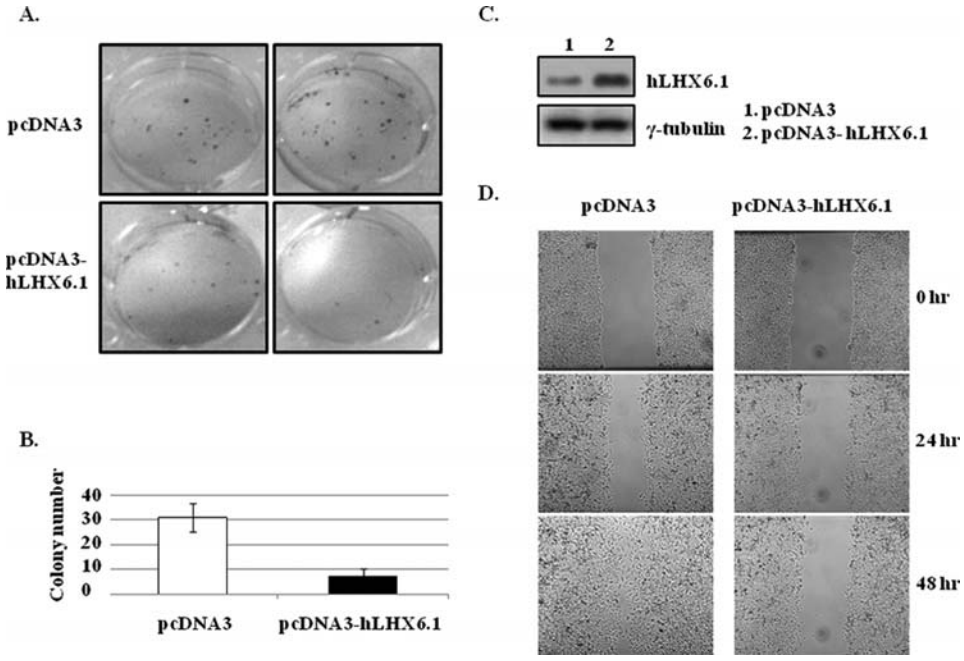

Figure 5. Functional study of *hLHX6.1* in SiHa cervical cancer cells. (A) Soft agar colony-forming assays. SiHa cervical calls were transfected with pcDNA3 or pcDNA3-LHX6.1 vectors and the transfected cells were plated with 50 cell numbers per 6-well plate for 13 days. (B) Suppression of cervical cancer colony formation by the pcDNA3-hLHX6.1 expression vector. (C) Western blotting verifying the overexpression of the *hLHX6.1* gene. (D) Phase micrographs of SiHa cells at various times after monolayer wounding. The pcDNA3- or pcDNA3-hLHX6.1-transfected SiHa cells were scratched and the closure of the scratch was photographed at the indicated times. This experiment was repeated 3 times and the closest result average is presented.

(Fig. 5A and B). The *hLHX6.1* overexpression suppressed the colony-forming ability by at least 75% ($P<0.01$). These results suggest that *hLHX6.1* exerts a tumor suppressive effect on cervical cancer cells. The pcDNA3-hLHX6-transfected SiHa cells were able to express comparable levels of hLHX6.1 protein, which was confirmed by Western blot analysis (Fig. 5C). In order to investigate whether *hLHX6.1* expression is involved in cervical cancer cell migration, we performed a wound healing migration assay. The pcDNA3 transfected SiHa cells migrated and covered the scratch after 48 h, whereas a significant area of the scratch remained uncovered in the pcDNA3-hLHX6.1-transfected SiHa cells, suggesting that the overexpression of the *hLHX6.1* gene inhibits the wound healing of SiHa cells (Fig. 5D). The exogenous expression of *hLHX6.1* in the SiHa cell line exhibiting hypermethylation in the *hLHX6.1* promoter significantly decreased motility *in vitro*, indicating the involvement of *hLHX6.1* in cell migration. On the whole, these findings show that the *hLHX6.1* expression could affect cell growth and migration in cervical cancer cells.

Discussion

Our investigation into the methylation level of the *hLHX6* and *hLHX6.1* promoters showed that only the *hLHX6.1* promoter was preferentially hypermethylated in cervical cancer cells despite the presence of multiple CpG sites in both promoters. The hypermethylation of the *hLHX6.1* promoter is crucial for its transcriptional silencing. It was of special interest that the *hLHX6.1* gene expression was detected in normal human cervical tissue cells despite the fact that it is a well-known brain region-specific gene product. Unexpectedly, the *hLHX6* gene expression level was also repressed despite the fact that its promoter was not methylated in most cervical cancer cell lines. This result implies that the *hLHX6* gene expression could be under the control of another transcriptional regulation system, rather than epigenetic regulation by promoter methylation. Studies on the *hLHX3* gene have shown that the cell-specific expression of 2 transcripts of *hLHX3* is driven by the 2 different regulatory systems of epigenetic and genetic mechanisms (35). Considering that they belong to the same family, it is plausible to assume that the *hLHX6* gene expression could be regulated in a similar manner. In other words, the *hLHX6* gene expression could be regulated by a transcriptional factor in a genetic regulation, while *hLHX6.1* gene expression is under the control of epigenetic regulation. However, we could not exclude the possibility that *hLHX6.1* could be regulated by both genetic and epigenetic regulations. Hence, attempts to predict the possible transcription factors involved in the genetic regulation of the *hLHX6* and *hLHX6.1* genes, were made using the TRANSFAC database (http://www.genome.ad.jp). Putative motifs of MZF1 and Sp1 were found in both *hLHX6* and *hLHX6.1* promoters. The binding sites of MZF1, Sp1 and AP-4 were dominantly found in the *hLHX6* promoter and the binding sites of MZF1, Sp1, USF and E2F1 were found in the *hLHX6.1* promoter (data not shown). Our data show the role of *hLHX6.1* as a potential tumor suppressor gene in the cervix. Other LHX genes have also been implicated in tumorigenesis. Choi *et al* showed that inactivated *LHX8* reduces the transcription of the pro-apoptotic genes, *Bax*, *Casp2* and *Casp3* in mouse oocytes

(36). Thus, *hLHX6.1* could function as a tumor suppressor gene by regulating the activity of the pro-apoptotic protein in the cervix. Therefore, the transcriptional levels of the *Bax* and *Casp2* genes were measured in *hLHX.6.1* overexpressing HeLa and SiHa cervical cancer cells. However, no significant difference was detected between the control and *hLHX6.1* overexpressing cells at the transcriptional level (data not shown). Vladimirova *et al* showed that the *LHX9* gene is frequently silenced in pediatric malignant astrocytomas by hypermethylation and that this epigenetic alteration is involved in glioma cell invasiveness and migration (25). This is consistent with our data that *hLHX6.1* overexpressing cells suppressed cell migration (Fig. 5). These results imply that *hLHX6.1* could play a significant role in cell migration. They also showed that the exogenous expression of *LHX9* in glioma cell lines did not directly affect cell proliferation. In contrast, according to a colony formation assay, our present results show that *hLHX6.1* overexpressing cells suppress cell proliferation (Fig. 5). On the whole, these discrepant results imply that *hLHX6.1* could play a different role in cancer cells despite the fact that it belongs to the same LHX family.

Our study led us to conclude the following: Firstly, that the transcription of both the *hLHX6* and *hLHX6.1* genes is repressed in cervical cancer cells. However, their gene expression is under the control of different regulatory systems. Transcriptional silencing by promoter methylation is associated only with the *hLHX6.1* gene expression, not with the *hLHX6* gene expression in cervical cancer cells. Secondly, that the methylation level in the *hLHX6.1* promoter increases as normal cells develops into cervical cancer. This epigenetic alteration begins at a relatively early stage, suggesting its potential as a biomarker for the early diagnosis of cervical cancer, and thirdly, that *hLHX6.1* functions as a tumor suppressor gene in the cervix. This study on *hLHX6.1* provides insight into the mechanism of *hLHX6.1* tumor suppression.

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