Homeobox protein MSX1 inhibits the growth and metastasis of breast cancer cells and is frequently silenced by promoter methylation

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Abstract. Deregulation of msh homeobox 1 (MSX1) has been identified to be associated with multiple human malignant neoplasms. However, the association of the expression and biological function of MSX1 with breast tumorigenesis, and the underlying mechanism remain largely unknown. Therefore, the present study examined the expression and promoter methylation of *MSX1* in breast tumor cell lines, primary breast tumors and normal breast tissues using semi-quantitative, quantitative and methylation-specific reverse transcription-polymerase chain reaction. Colony formation assays, flow cytometric analysis, and wound healing and Transwell assays were used to assess various functions of MSX1. Western blot analyses were also conducted to explore the mechanism of MSX1. The results revealed that *MSX1* was broadly expressed in normal human tissues, including breast tissues, but was frequently downregulated or silenced in breast cancer cell lines and primary tumors by promoter methylation. Methylation of the *MSX1* promoter was observed in 7/9 (77.8%) breast cancer cell lines and 47/99 (47.5%) primary tumors, but not in normal breast tissues or surgical margin tissues, suggesting that tumor-specific methylation of *MSX1* occurs in breast cancer. Pharmacological demethylation reduced *MSX1* promoter methylation levels and restored the expression of *MSX1*. The ectopic expression of MSX1, induced by transfection with a lentiviral vector, significantly inhibited the clonogenicity, proliferation, migration and invasion of breast tumor cells by inducing G1/S cell cycle arrest and apoptosis. Ectopic *MSX1* expression also inhibited the expression of active β-catenin and its downstream targets c-Myc and cyclin D1, and also increased the cleavage of caspase-3 and poly (ADP-ribose) polymerase. In conclusion, *MSX1* exerts tumor-suppressive functions by inducing G1/S cell cycle arrest and apoptosis in breast tumorigenesis. Its methylation may be used as an epigenetic biomarker for the early detection and diagnosis of breast cancer.

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Introduction

Breast cancer has the highest mortality rate of the female cancers in developed and developing countries, and its incidence is steadily increasing (1,2). Genetic and epigenetic alterations in tumor-suppressor genes (TSGs) and oncogenes serve crucial roles in the development of human neoplasia (3). It has been recognized that the methylation of TSG promoters is frequently involved in numerous types of tumorigenesis, including breast cancer (4). The aberrant promoter methylation of certain TSGs has been identified in breast cancer by the present research group and other researchers, where the TSGs include *BRCA1*, *p16/CDKN2A*, *DKK3*, *cyclin D2*, *PLCD1*, *PCDH10* and *UCHL1* genes (5,6).

Homeobox proteins are essential transcriptional regulators that function in various developmental processes, including cell growth, proliferation, differentiation, cell-cell communication and the apoptotic pathway during pattern formation in embryogenesis (7). The abnormal expression of homeobox genes may cause an abnormal phenotype and cell growth (8). Msh homeobox 1 (*MSX1*) is a homeobox gene located in chromosomal region 4p16.1 (9). MSX1 interacts with β-catenin to inhibit the cell proliferation mediated by WNT signaling pathways (10). *MSX1* mutation is involved in the congenital lack of teeth (tooth agenesis or hypodontia), limb deficiency, craniofacial bone morphogenesis and cleft lip, but few studies have examined its role in tumorigenesis (11-16). Previous studies suggest that the deregulated expression of MSX1 is

involved in several human malignant neoplasms, including lung, gastric, ovarian and cervical cancers, as well as acute lymphoblastic leukemia. However, to the best of our knowledge, the expression pattern and biological functions of MSX1 in breast cancer have not yet been investigated (17-22).

In the present study, the expression and promoter methylation of *MSX1* in multiple breast cancer cell lines and primary tumors was examined, and the associations between *MSX1* methylation and the clinicopathological features of breast cancer patients were analyzed. The biological functions and underlying mechanisms of MSX1 in breast cancer were also investigated.

Materials and methods

Cell lines, tumor samples and normal tissues. Nine breast cancer cell lines provided by Professor Tao (Cancer Epigenetics Laboratory, Department of Clinical Oncology, State Key Laboratory of Oncology in South China, Sir YK Pao Center for Cancer and Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong and CUHK Shenzhen Research Institute, Hong Kong, China) were used in this study: BT549, MCF-7, MDA-MB-468, MDA-MB-231, SK-BR-3, T47D, YYC-B1, YCC-B3 and ZR-75-1. All the cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin/streptomycin at 37˚C in moist air containing 5% CO_2.

Normal human adult tissue RNA samples were purchased commercially (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA; EMD Millipore, Billerica, MA, USA; BioChain Institute, Inc., Newark, CA, USA). Primary breast cancer tumor tissues, matched adjacent non-malignant tissues and normal breast tissues were obtained from patients who had undergone primary surgery at the Department of Endocrinological and Breast Surgery, The First Affiliated Hospital of Chongqing Medical University (Chongqing, China), between January 2010 and March 2014. All samples were confirmed by pathologist physicians. The clinical and pathological data, including sex, age, tumor grade, tumor size, treatment and follow-up data, were available for the majority of the breast cancer samples. All patients signed informed written consent forms for participation in the study at initial clinical investigation. The present study was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (approval no. 2010/2012-23). A multifunctional user-friendly online tool, Gene Expression Based Outcome for Breast Cancer Online (GOBO; http://co.bmc.lu.se/gobo), was used to analyze the expression level of *MSX1* associated with molecular subtypes of breast cancer, ER status and histological grade as previously described (23).

Semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated from tissues and cells using TRI Reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Semi-quantitative RT-PCR was performed as described previously, using GAPDH as the control (24). The primer sequences used for PCR amplification are listed in Table I. RT-PCR was performed with 32 cycles for MSX1 and 23 cycles for GAPDH using Go-Taq Flexi DNA Polymerase (Promega Corporation, Madison, WI, USA).

5-Aza-2'-deoxycytidine (Aza) and trichostatin A (TSA) treatment. The MDA-MB-468, MCF-7 and ZR-75-1 cell lines were used for pharmacological demethylation. Briefly $1x10^6$ cells were treated with 10 mmol/l Aza for 72 h and then with 100 nmol/l TSA (both from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 24 h at 37˚C. The cells were then harvested for DNA and RNA extraction, and further analysis.

DNA bisulfite treatment and methylation-specific PCR (MSP). Genomic DNA was extracted from the tumors, normal tissues and cell pellets using the QIAamp DNA mini kit (Qiagen GmbH, Hilden, Germany). DNA bisulfite treatment and MSP were performed as previously described (25). The bisulfite-treated DNA was amplified using MSP with a methylated-*MSX1*-specific or unmethylated-*MSX1*-specific primer set. The primers used for MSP and bisulfite sequencing are listed in Table I. The methylated and unmethylated MSP primer sets target the same CpG sites and have been tested previously to confirm that they do not amplify any genomic DNA without bisulfite treatment, and are therefore specific. MSP was performed for 40 cycles to amplify the unmethylated and methylated gene using AmpliTaq Gold DNA Polymerase (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR products were analyzed on 2% agarose gel.

RT-quantitative PCR (RT-qPCR). Total RNA was purified from a panel of fresh, paired primary breast tissues (20 tumors and the corresponding adjacent tissues) using TRI Reagent as previously described (26). RT-qPCR with Maxima SYBR-Green/ROX qPCR Master mix (MBI Fermentas; Thermo Fisher Scientific, Inc.) was used to detect gene expression (Table I) according to the manufacturer' s protocol, using a HT7500 System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Melting-curve analysis and agarose gel electrophoresis of the PCR products were performed to verify the specificity of the PCR and the identity of the PCR products. Each experiment was performed in triplicate and the relative expression levels of MSX1 in the breast tissues were normalized to those of β-actin. Data were normalized using the $2^{-\Delta\Delta Cq}$ method (27).

Cell proliferation assay. MDA-MB-231 cells infected with LV-MSX1 or LV-empty were trypsinized and resuspended. The cells were then cultured in triplicate in 96-well plates at a density of $1x10^4$ cells/well in a volume of 100 μl DMEM and allowed to grow overnight. Cell viability was quantified with a Cell Counting kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan), in which only living cells are stained. After 24, 48 and 72 h, the medium was removed, and α-minimal essential medium (100 μl) containing 10 μl CCK-8 reagent was added to each well of precultured cells. Following incubation of the cells for 2 h at 37˚C, the optical density was determined at a wavelength of 450 nm with an automatic microplate reader. The results are the means of three independent experiments conducted over several days.

Table I. List of primers used in this study.

PCR		Sequence (5'-3')	Product size (bp)	PCR cycles	Annealing temperature
MSP	MSX1m3	GCGCCTCATCACATCAGCGC	116	41	60˚C
	MSX1m4	GCGATTTCTGATGCTGGCGC			
	MSX1u3	CAAGGCTAGTCATCATCAACCA	121	41	58˚C
	MSX1u4	CGCCTAGGGCTCAGTCCACCATGT			
RT-PCR	MSX1-F	CATTCGAATACCGGGGCCGACGA	176	32	55˚C
	MSX1-R	CGCCTAGGGCTCAGTCCACCATGT			
	GAPDH-F	CCTCAGTTGCCTAAACCA	202	23	55˚C
	GAPDH-R	CACTACCCTAAAGGTAACTA			
RT-qPCR	MSX1-F	CTGCTCGTCTCGTTAATGTGG	156	40	60˚C
	MSX1-R	TGCGCAAACTTACCCGTCT			
	β-actin-F	GGACACCGTAGCGTGCTTTGA	311	40	60˚C
	β-actin-R	CTTCGCTAAACGCCACCTGCTA			

PCR, polymerase chain reaction; MSP, methylation-specific PCR; RT, reverse transcription; qPCR, quantitative PCR; F, forward; R, reverse.

Colony formation assay. MDA-MB-231 and MDA-MB-468 cells infected by LV-MSX1 or LV-empty were plated at a density of 300 cells/well in 6-well plates and cultured for 14 days in normal culture medium. The cells were then washed twice with PBS, fixed with 10% buffered formalin, dried and stained with 2% crystal violet. The surviving colonies (≥50 cells/colony) were manually counted in four different fields of vision and the mean values calculated. Each treatment was tested in triplicate.

Wound healing assay. Cultured MDA-MB-231 cells infected with LV-MSX1 or LV-empty were evenly seeded in 6-well plates and allowed to reach 100% confluence in DMEM. A straight wound was induced by dragging a 20-μl pipette tip through the confluent cell monolayer. The cells were incubated and allowed to migrate in the medium. At 0, 12, 24 and 36 h after wounding, the plates were washed twice with PBS to remove the dead cells, and images were captured in four random fields at a magnification of x100 (Leica DMI4000B; Leica Microsystems, Ltd., Milton Keynes, UK). The rate of cell migration was quantified according to the percentage of repaired wound area, using Image Pro-Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA). Each experiment was performed in triplicate.

Flow cytometric analysis of the cell cycle. For cell-cycle analysis, MDA-MB-231 cells and MDA-MB-468 cells infected with LV-MSX1 or LV-empty were seeded in 6-well plates (1x10^6 cells/well) and incubated overnight at 37˚C in 5% CO_2. The cells were digested by trypsin at 48 h after infection and then centrifuged at 1,000 x g for 5 min at room temperature. The cells were washed twice with PBS, fixed in 70% ethanol at 4˚C for 2 h, and stained with propidium iodide (Sigma-Aldrich; Merck KGaA) for 30 min at 37˚C. The samples were evaluated with the BD FACSCanto and the results were analyzed using BD FACSDIVA software (version 4.1; BD Biosciences, San Jose, CA, USA). All experiments were performed in triplicate, and one representative figure is shown for each cell type.

Transwell cell migration and invasion assays. Cell migration and invasion assays were carried out using Transwell chambers (8 μm; Corning Incorporated, Corning, NY, USA). The cells were plated at a density of 2.5x10^5 with RPMI-1640 in the upper well of each Transwell chamber. For invasion assay, 100 μl of Matrigel were added in the upper well first. The lower compartment was filled with DMEM supplemented with 10% fetal bovine serum. Following incubation for 24 h at 37˚C with 5% CO_2 in a humidified atmosphere, the cells that had not migrated or invaded were removed from the upper surface of the filter with gentle swabbing. The cells on the lower surface of the filter were fixed in 4% paraformaldehyde, stained with 0.1% crystal violet staining solution for 15 min at room temperature, and counted by light microscopy at x100 magnification in 10 images taken in five random fields. The averages were then calculated. The experiment was performed in triplicate.

Western blot analysis. The infected cells were harvested and lysed in M-PER Mammalian Protein Extraction Reagent (Pierce, Thermo Fisher Scientific, Inc.) containing a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). The protein samples were incubated on ice for 10 min, and then centrifuged at 10,000 x g for 5 min at 4˚C to remove the cell debris. The protein samples were quantified by BCA Protein Assay kit (Thermo Fisher Scientific, Inc.), 20 μg protein were separated using 10% SDS-PAGE gel and then electrophoretically transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membrane was blocked with 5% skimmed milk in Tris-buffered saline with Tween-20 for 1 h at room temperature, and then incubated overnight at 4˚C with the following primary antibodies at the manufacturers' recommended dilutions: antibodies directed against β-catenin (#19807;

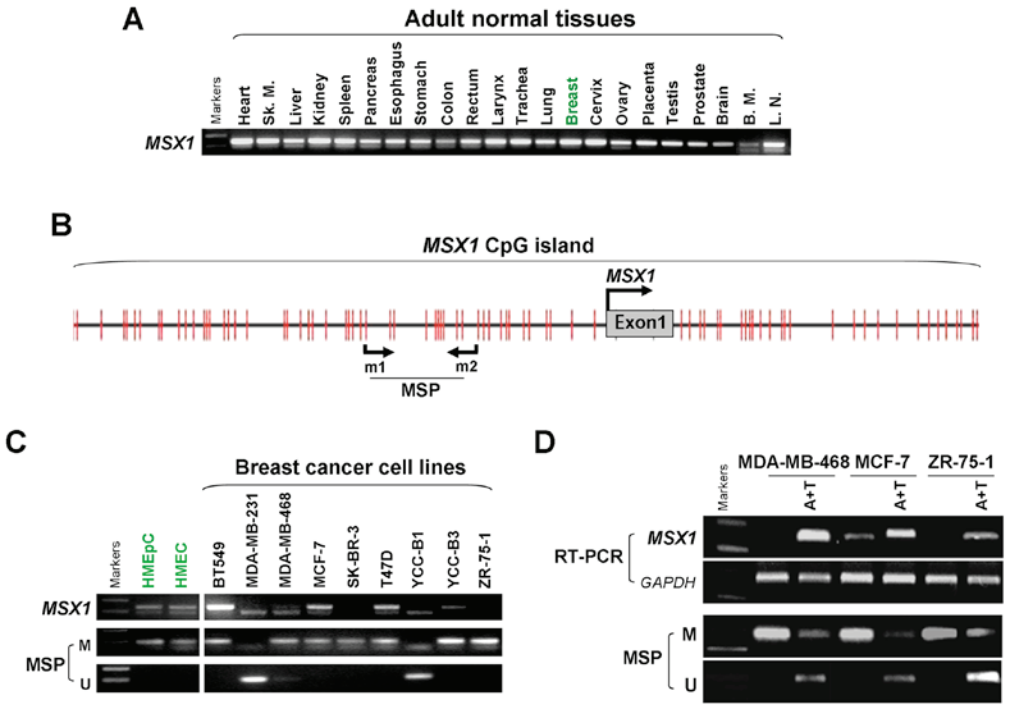

Figure 1. *MSX1* is downregulated by promoter methylation in breast cancer cell lines. (A) Analysis of *MSX1* expression in normal human adult tissues as measured by semi-quantitative RT-PCR, using GAPDH as the control. Sk.m, Sk. Muscle; B.M., Bone marrow; L.N., Lymph node. (B) Schematic structure of the *MSX1* promoter CpG island. The CpG sites are indicated by the short vertical lines. The MSP sites analyzed are shown. The transcription start site is indicated by a curved arrow. (C) *MSX1* is frequently silenced in breast cancer cell lines by promoter methylation, demonstrated with RT-PCR and MSP. (D) Pharmacological demethylation using A+T restored *MSX1* expression in *MSX1*-methylated/silenced breast cancer cell lines. MSX1, msh homeobox 1; RT-PCR, reverse transcription-quantitative polymerase chain reaction; MSP, methylation-specific PCR; M, methylated; U, unmethylated; A+T, 5-aza-2'-deoxycytidine plus trichostatin A.

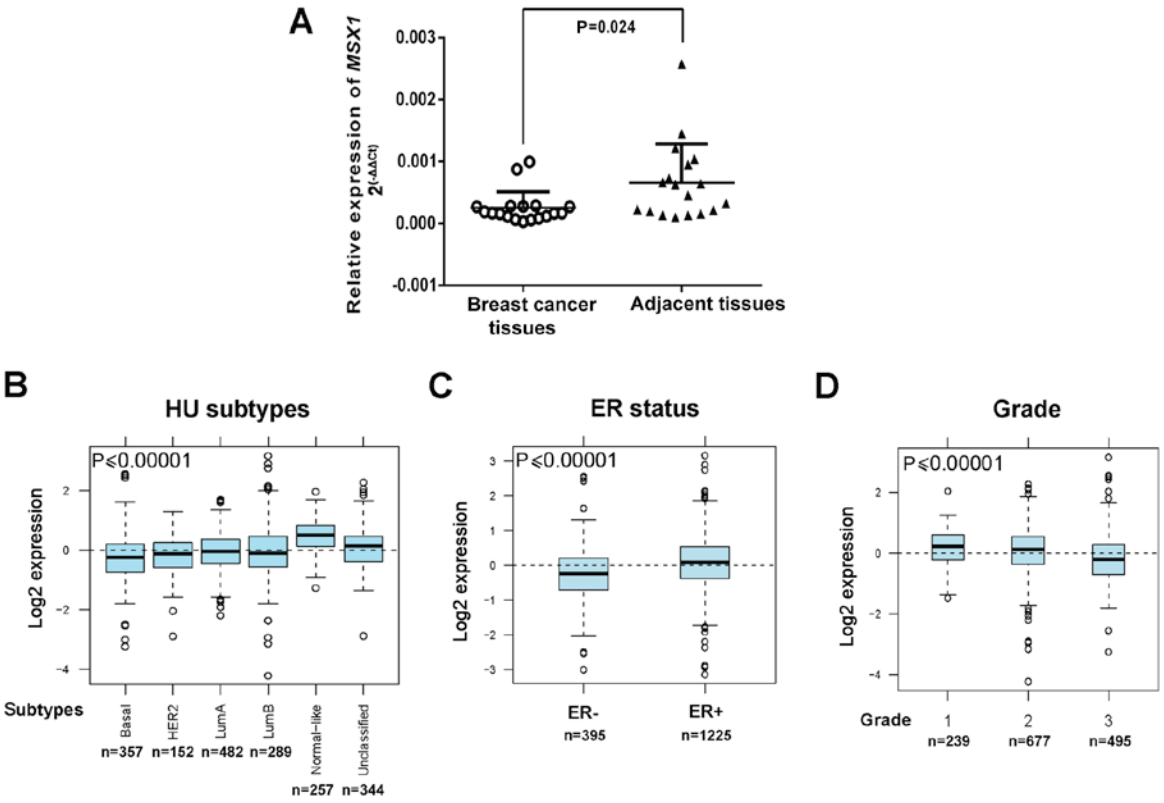

Figure 2. *MSX1* expression analysis in primary breast tissues and its association with clinicopathologic features. (A) MSX1 expression in primary breast tumor tissues detected by reverse transcription-quantitative polymerase chain reaction (B-D) Gene Expression-Based Outcome for Breast Cancer Online tumor analysis of MSX1 according to Hu gene expression subtypes (34), ER status and histological grade. MSX1, msh homeobox 1; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2.

Cell Signaling Technology, Inc., Danvers, MA, USA) active β-catenin (#4270; Cell Signaling Technology, Inc.), c-Myc (#1472-1; Epitomics; Abcam, Cambridge, UK), cleaved poly (ADP-ribose) polymerase (cleaved PARP; #9541), cleaved caspase-3 (#9661) (both from Cell Signaling Technology, Inc.) and cyclin D1 (CCND1; #1677-1; Epitomics, Abcam). The membranes were washed and then incubated with anti-mouse (#7076; Cell Signaling Technology, Inc.) or anti-rabbit (#7074; Cell Signaling Technology, Inc.) IgG, HRP-linked antibodies at a dilution of 1:3,000 at room temperature for 1 h. The protein bands were visualized using a Fusion FX5 system (Vilber Lourmat, Eberhardzell, Germany). The results were analyzed using ImageJ software (version 6.0; National Institutes of Health, Bethesda, MD, USA). GAPDH (sc-47724; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the endogenous protein for normalization.

Statistical analysis. Statistical analyses were performed using the Student's t-test, χ^2 test and Fisher's exact test to determine the significance of differences between groups. For all tests, $p<0.05$ was considered to indicate a statistically significant difference. Results are presented as the mean ± standard deviation.

Results

Expression of MSX1 is reduced in breast cancer tissues and cells. The expression of *MSX1* was assessed in a panel of normal human adult tissues, including breast tissues, and breast cancer cell lines, using semi-quantitative RT-PCR (Fig. 1). *MSX1* was clearly expressed in all the normal human tissues, including normal breast tissue, at varying levels (Fig. 1A), but was frequently silenced or downregulated in breast cancer cell lines, with the exception of BT549 (Fig. 1C).

MSX1 expression was then examined at the mRNA level in primary breast tumors. qPCR demonstrated that *MSX1* mRNA was downregulated in the breast cancer tissues compared with the normal breast tissues ($p<0.05$; Fig. 2A). The GOBO online tool was also used to assess *MSX1* expression in 1,881 breast cancers. Gene-Set Analysis tumor demonstrated that the expression of *MSX1* differs among different subtypes of breast cancer (28). The most aggressive subtype, the basal-like subtype, exhibited the lowest MSX1 expression, whereas the least aggressive subtype, the normal-like subtype, exhibited the highest expression of *MSX1* ($p<0.00001$; Fig. 2B). The expression of *MSX1* was also reduced with a negative estrogen receptor (ER) status and a higher histological grade ($p<0.00001$; Fig. 2C and D). These results indicate that *MSX1* expression is frequently downregulated or silenced in breast cancer, and is associated with the malignant progression of breast cancer.

CpG methylation of the MSX1 promoter contributes to its silencing or downregulation in breast cancer. The involvement of promoter methylation in *MSX1* silencing in breast cancer was evaluated. Bioinformatic analysis revealed a typical CpG island spanning the proximal promoter and the exon 1 region of the *MSX1* gene (Fig. 1B). To determine whether promoter methylation leads to *MSX1* silencing, *MSX1* methylation in breast tumor cell lines was analyzed using MSP. The results demonstrated that *MSX1* was methylated in seven breast cancer cell lines (MDA-MB-231, MDA-MB-468, MCF-7, T47D, ZR75-1, SK-BR-3 and YCC-B3), consistent with its silencing, but not in another two cell lines (BT549 and YCC-B1) in which *MSX1* was unmethylated (Fig. 1C).

Pharmacological demethylation was conducted to test whether promoter methylation directly mediates the reduction of *MSX1* levels in breast cancer cells. Three cell lines (MDA-MB-468, MCF-7 and ZR-75-1) lacking MSX1 expression were treated with Aza and the histone deacetylase inhibitor TSA. Following treatment, the expression of *MSX1* in these cell lines was significantly increased compared with that prior to treatment, accompanied by decreased methylated alleles of *MSX1* (Fig. 1D). These results indicate that promoter methylation is responsible for *MSX1* silencing in breast cancer cells.

Table II. Promoter methylation status of *MSX1* in primary breast tumors.

Tissue	Samples (n)	MSX1 promoter		Methylation frequency (%)
		Methylated	Unmethylated	
Breast cancer	99	47	52	47/99 (47.5)
Breast cancer surgical-margin	8	0	8	0/8 (0)
Normal breast	13	0	13	0/13 (0)

MSX1, msh homeobox 1.

Frequent MSX1 methylation and its association with clinicopathological features in primary breast tumors. To ascertain whether *MSX1* methylation occurs in primary breast tumors, MSX1 methylation by MSP was examined in 99 primary breast tumor tissues, 8 surgical margin tissues and 13 normal breast tissues. Aberrant *MSX1* methylation was detected in 47 (47.5%) of the primary breast cancer tissues, but not in the surgical margin tissues or normal breast tissues (Fig. 3 and Table II). These results confirm the tumor-specific methylation of the *MSX1* promoter. The association of *MSX1* methylation with the clinicopathological features of breast cancer patients was also analyzed, where the clinicopathological features included age, tumor size, tumor grade, lymph-node metastasis, ER status, progesterone receptor status, human epidermal growth factor receptor 2 status and Ki67 status. However, no significant difference of MSX1 methylation status according to clinicopathological features of the patients was detected (Table III).

Ectopic expression of MSX1 suppresses breast cancer cell growth. MDA-MB-231 and MDA-MB-468 cells, which are MSX1-silenced, were infected with LV-MSX1 and LV-empty plasmids. MDA-MB-231 and MDA-MB-468 cells stably overexpressing MSX1 were successfully generated, which was confirmed by microscopy and RT-PCR (Fig. 4A and B). CCK-8

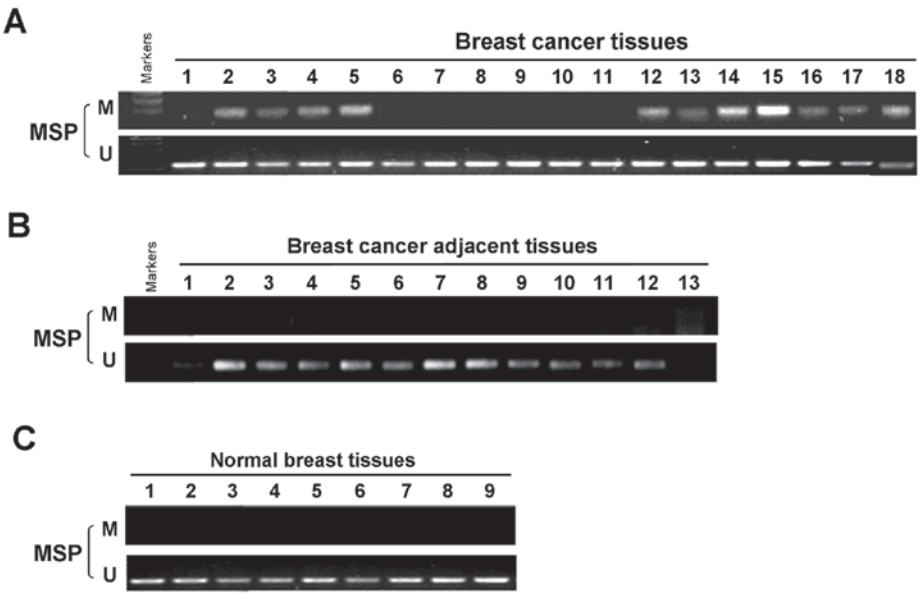

Figure 3. Representative MSP analysis of *MSX1* methylation in (A) primary breast tumors, (B) surgical margin tissues and (C) normal breast tissues. MSP, methylation-specific polymerase chain reaction; MSX1, msh homeobox 1; M, methylated; U, unmethylated.

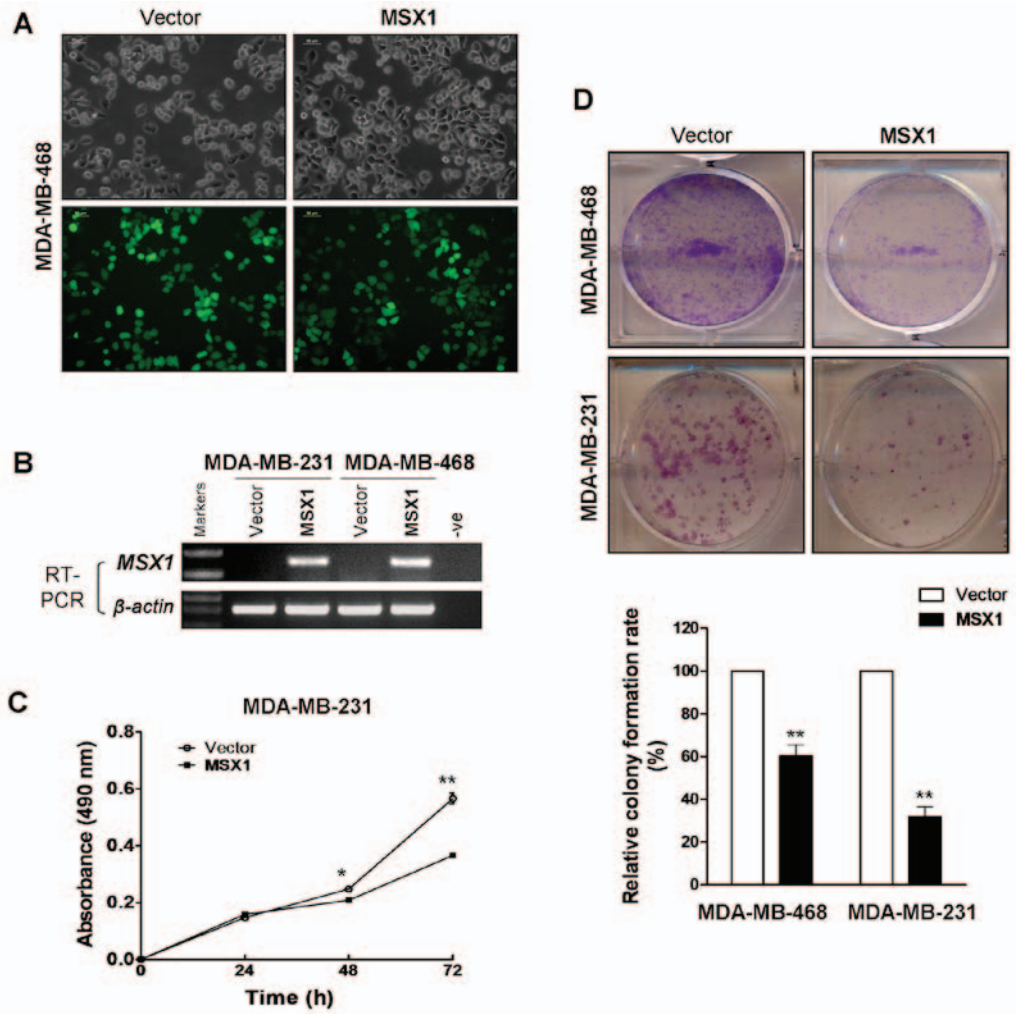

Figure 4. MSX1 inhibits breast cancer cell growth and clonogenicity. (A) Breast tumor cells infected with LV-MSX1 or control lentivirus by light and fluoresence microscopy (magnification, x200). (B) MSX1 expression was measured by RT-PCR in vector- and MSX1-infected MDA-MB-231 and MDA-MB-468 cells. (C) The proliferative capacity of the MDA-MB-231 breast cancer cell line was lower than that of the vector-infected control cells at 48 and 72 h following infection with MSX1, as detected using a Cell Counting kit-8 assay. (D) Effects of MSX1 overexpression on colony formation in MDA-MB-231 and MDA-MB-468 cells. Representative results and quantitative analysis of the colony numbers in three replicates presented as the mean ± standard deviation. $^{*}p<0.05$ and $^{**}p<0.01$ vs. the vector control. MSX1, msh homeobox 1; RT-PCR, reverse transcription-polymerase chain reaction.

Table III. Clinicopathologic features of 99 breast cancer patients according to MSX1 methylation status.

Clinicopathological features	No. of patients	MSX1 promoter		P-value
		Methylated	Unmethylated	
Age (years)				
≥60	26	15	11	0.36
<60	68	29	39	
Unknown	5	3	2	
Tumor stage				
I	28	17	11	0.18
II	34	16	18	
III	28	9	19	
Unknown	9	5	4	
Tumor size (cm)				
<2	23	13	10	0.53
≥2, ≤5	64	29	35	
>5	7	2	5	
Unknown	5	3	2	
Lymph node metastasis				
Positive	47	20	27	0.35
Negative	45	22	23	
Unknown	7	5	2	
Estrogen receptor status				
Positive	53	24	29	0.85
Negative	33	17	16	
Unknown	13	6	7	
Progesterone receptor status				
Positive	42	18	24	0.73
Negative	43	22	21	
Unknown	14	7	7	
HER2 status				
Positive	67	34	33	0.56
Negative	19	7	12	
Unknown	13	6	7	
Ki67 status				
<14%	38	19	19	0.91
>14%	29	13	16	
Unknown	32	15	17	

MSX1, msh homeobox 1; HER2, human epidermal growth factor receptor 2.

cell proliferation and colony formation assays were performed to clarify the function of MSX1 in the proliferation of breast cancer cells. Cell viability was significantly reduced at 48 and 72 h after the infection of the MDA-MB-231 cells with MSX1 ($p<0.05$ and $p<0.01$, respectively; Fig. 4C). The colony formation assay demonstrated that MSX1-infected MDA-MB-231 and MDA-MB-468 cell colonies were reduced by 40 and 65% compared with those of the respective control cells ($p<0.01$; Fig. 4D). These results suggest that MSX1 inhibits the proliferation of breast cancer cells.

MSX1 induces the G1/S cell-cycle arrest and apoptosis of breast cancer cells. To investigate the mechanism underlying the growth-inhibitory effect of MSX1 on breast cancer cells, the cell cycle status of breast cancer cells was examined using flow cytometry. The number of cells arrested in the G1/S phase increased significantly in the MSX1-expressing MDA-MB-231 and MDA-MB-468 cells compared with the respective control cells, which was accompanied by a significant reduction in S-phase cells ($p<0.01$; Fig. 5A). Western blot analysis revealed upregulated expression levels of cleaved caspase-3 and cleaved

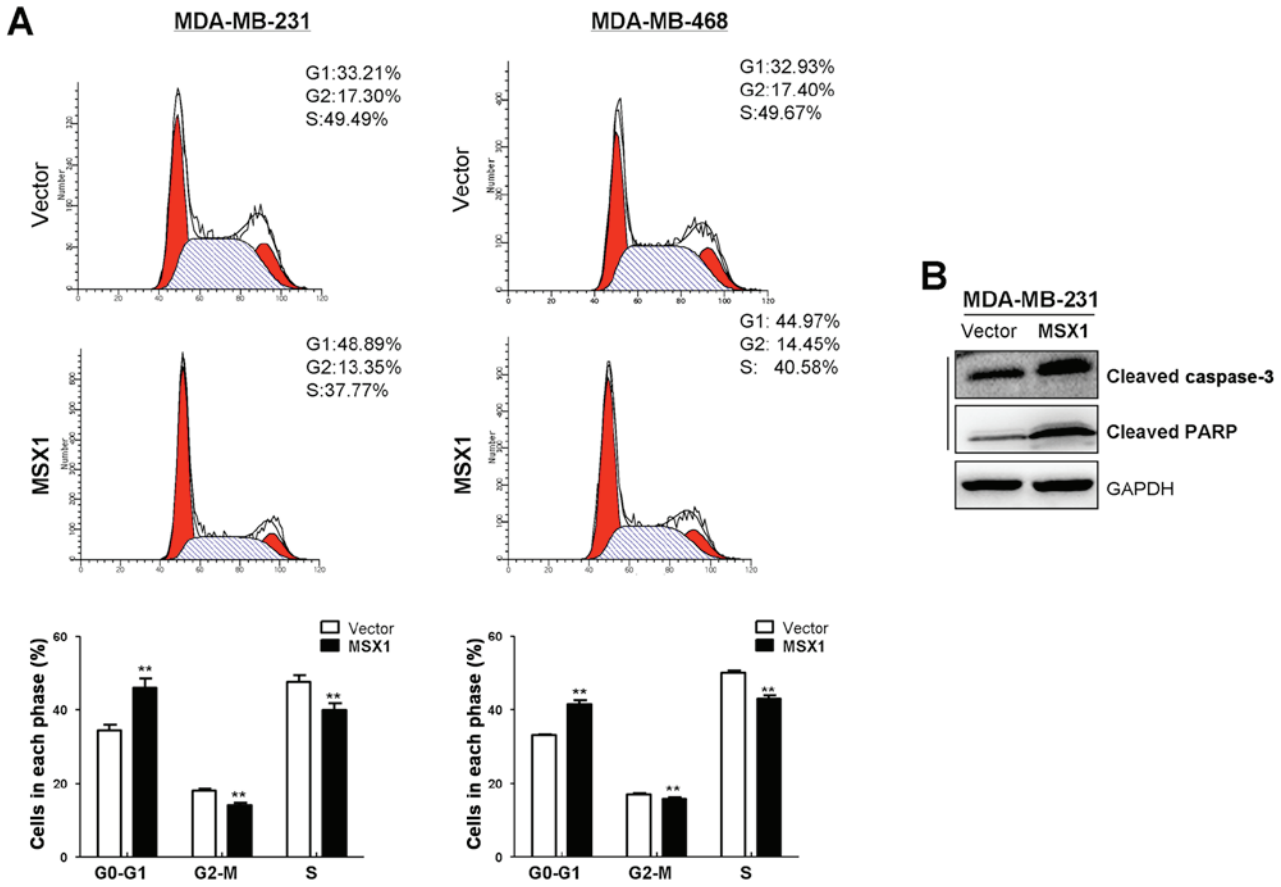

Figure 5. Ectopic expression of MSX1 induces cell cycle G1/S arrest and apoptosis. (A) Flow-cytometric analysis of MDA-MB-231 and MDA-MB-468 cells demonstrated an increased percentage of cells in the G1/S phase in MSX1-expressing cells compared with the respective controls. Representative results and quantitative analysis of the results are shown. $^{**}p<0.01$ vs. the vector control. (B) Western blots showing upregulation of the apoptotic markers cleaved caspase-3 and cleaved PARP in MSX1-expressing MDA-MB-231 cells. MSX1, msh homeobox 1; PARP, poly (ADP-ribose) polymerase.

PARP in the MSX1-expressing MDA-MB-231 cells compared with the control cells infected with empty vector. These results indicate that MSX1 causes G1/S cell cycle arrest and apoptosis in breast cancer cells.

MSX1 inhibits breast tumor cell migration and invasion. Wound-healing and Transwell assays were performed to assess the effects of MSX1 expression on the migration and invasion of breast tumor cells. In the scratch wound-healing assay, the percentage wound closure of a confluent monolayer of MSX1-expressing MDA-MB-231 cells was significantly lower compared with that of control cells at 12 and 14 h post-wounding ($p<0.05$ and $p<0.01$, respectively; Fig. 6A). In the Transwell assays, the numbers of migrating and invading cells were significantly reduced in the MSX1-overexpressing cells compared with the control cells, suggesting that MSX1 inhibits breast cancer cell migration and invasion ($p<0.01$; Fig. 6B and C).

MSX1 reduces the expression of β-catenin and its downstream target genes. As the β-catenin signaling pathway is critical in the regulation of cell proliferation, whether MSX1 affects this pathway in breast cancer cells was investigated. The expression of active β-catenin was examined using western blotting. The expression levels of active β-catenin and its downstream targets c-Myc and CCND1 were reduced in the MSX1-expressing MDA-MB-231 cells compared with the control cells (Fig. 6D). These results indicate that MSX1 reduces the levels of active β-catenin and its downstream target genes, and thus may antagonize the WNT/β-catenin signaling pathway.

Discussion

In the present study, it was demonstrated that *MSX1* is expressed in normal breast tissues, but frequently methylated and silenced in breast cancer cell lines and primary tumor tissues. *MSX1* was methylated in 77.8% of the breast cancer cell lines and 47.5% of the primary breast tumors that were tested, but not in surgical margin tissues or normal breast tissues. Pharmacological demethylation restored the expression of *MSX1*. The present study also demonstrated that MSX1 inhibits breast cancer cell proliferation, migration and invasion by inducing G1/S cell-cycle arrest and apoptosis, through the downregulation of β-catenin activity. These findings indicate that MSX1 acts as a functional tumor suppressor in breast cancer.

The epigenetic inactivation of TSGs, including promoter methylation, histone modification and RNA interference, serves an important role in tumor initiation and progression (30). To the best of our knowledge, the present study is the first to demonstrate that *MSX1* is widely expressed in normal adult tissues, including mammary tissue, but frequently down-regulated or totally silenced in breast cancer cell lines and

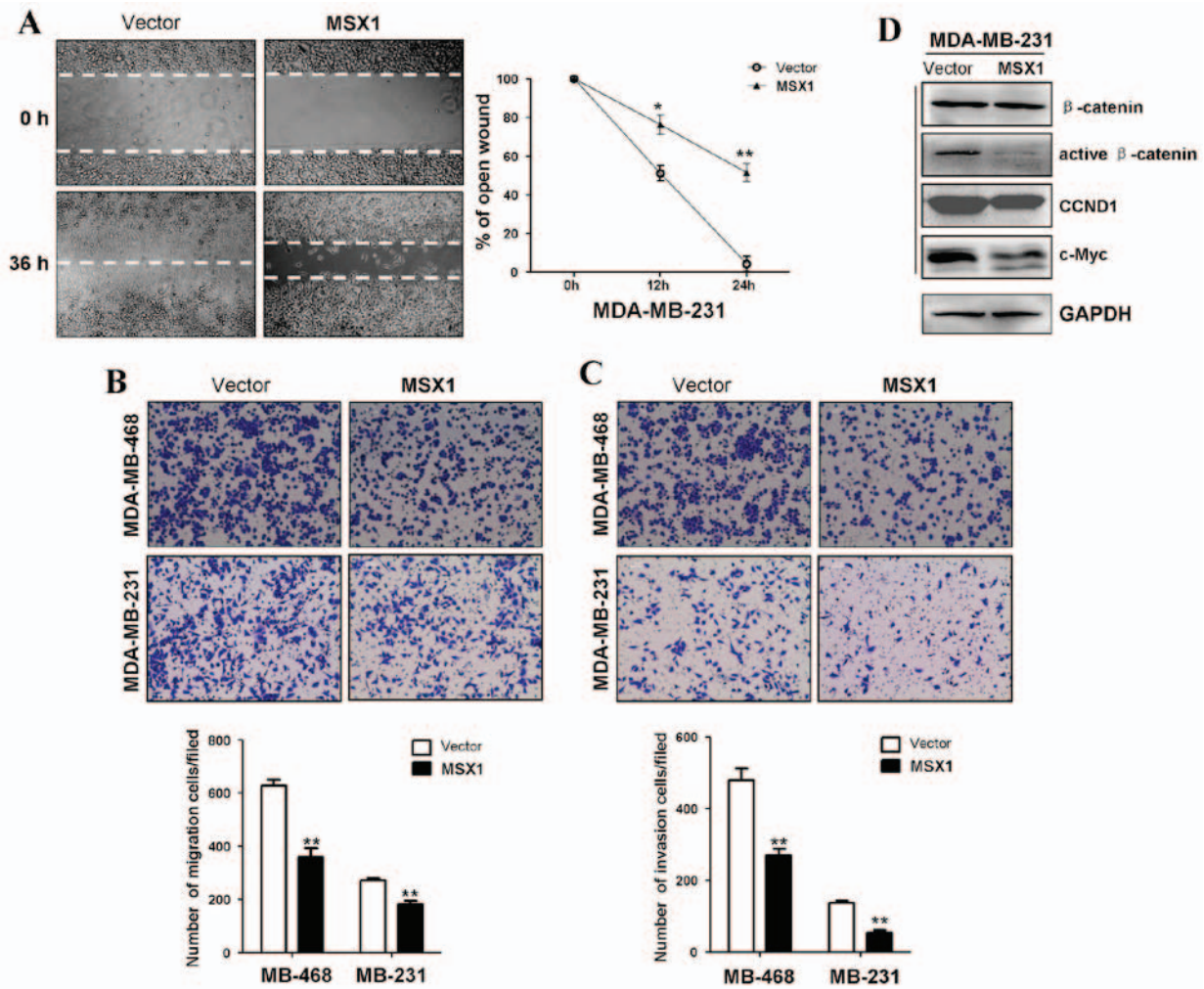

Figure 6. Ectopic expression of MSX1 inhibits cancer cell migration and invasion. (A) The effect of MSX1 on cell migration was assessed by a wound-healing motility assay. Representative images of wound healing at 0 and 36 h after wound scratch are shown. The percentage of wound healing compared with that of the respective control at each time-point is indicated. Transwell assays indicate that the ectopic expression of MSX1 inhibited the (B) migration and (C) invasive potential of MDA-MB-231 and MDA-MB-468 cells. Photographs show cells that have travelled through the membrane, and quantified data presenting the percentage of migrated or invasive cells are shown. (D) Western blot analysis of expression of β-catenin and its target genes, CCND1 and c-Myc. *p<0.05 and **p<0.01 vs. the vector control. MSX1, msh homeobox 1; CCND1, cyclin D1.

primary tumors, and that this is frequently accompanied by its methylation. However, no *MSX1* primer methylation was detected in certain breast cancer cell lines (MDA-MB-231 and YCC-B1) in which *MSX1* was silenced, indicating that other epigenetic alterations, such as histone modification, may be an alternative mechanism contributing to *MSX1* silencing, in addition to promoter CpG methylation.

The WNT signaling pathway is an evolutionarily conserved signaling pathway that is important in the regulation of numerous fundamental cellular processes, including cell proliferation, migration, stemness and tumorigenesis, with a wide range of biological activities (31). The WNT/β-catenin signaling pathway has frequently been implicated in the development of various cancers, and serves an important role in tumor initiation and progression (7,11,32). MSX1 has been shown to induce the expression of the WNT-pathway-antagonistic genes Dickkopf 1-3, and secreted frizzled-related protein 1, thus deregulating the WNT signaling pathway in neuroblastoma cells (7). In the present study, the ectopic expression of MSX1 was observed to reduce the expression of active β-catenin and its downstream target genes c-Myc and CCND1 in breast tumor cells, and induce G1/S cell cycle arrest and apoptosis, thus inhibiting tumor cell proliferation and metastasis, which is consistent with a previous study of glioblastoma (33). MSX1 also interacts with p53 and inhibits tumor growth by inducing apoptosis (18). Indeed, the present study indicated that MSX1 induced the apoptosis of breast cancer cells by upregulating cleaved caspase 3 and cleaved PARP.

The association of the *MSX1* gene with breast cancer and its characteristics has previously been reported. The MSX1 gene was observed to be associated with an increased risk of breast cancer in a Polish population and may be considered as an early marker of the disease (11). In the present study, the tumor-specific methylation of *MSX1* was observed in breast cancer, although no significant correlation between its methylation status and the clinicopathological features of primary breast cancer tumors, including clinical stage, lymph-node metastasis and ER status was detected, which requires further confirmation in a study with a larger sample size. Future studies are also required to investigate whether circulating

methylated *MSX1* in the serum or in combination with other methylated TSGs can be used to detect early breast cancer.

In conclusion, the present study provides the first evidence that *MSX1* is frequently downregulated or silenced in breast cancer by promoter CpG methylation. MSX1 acts as a functional TSG in breast carcinogenesis through the inhibition of active β-catenin. The methylation of *MSX1* could be used as an epigenetic biomarker for the early detection and diagnosis of breast cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YiY and JF detected the cell function, CL and LL performed the DNA bisulfite treatment and MSP examination, YuY and WP finished the western blot experiment and wrote the paper, GR designed the experiment and finished the figures. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (approval no. 2010/2012-23). All patients signed informed written consent forms for participation in the study at initial clinical investigation.

Consent for publication

Not applicable.

Competing interests

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. CA Cancer J Clin 65: 87-108, 2015.
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. CA Cancer J Clin 61: 69-90, 2011.
3. Taby R and Issa JP: Cancer epigenetics. CA Cancer J Clin 60: 376-392, 2010.
4. Esteller M: Epigenetics in cancer. N Engl J Med 358: 1148-1159, 2008.
5. Hoque MO, Prencipe M, Poeta ML, Barbano R, Valori VM, Copetti M, Gallo AP, Brait M, Maiello E, Apicella A, *et al*: Changes in CpG islands promoter methylation patterns during ductal breast carcinoma progression. Cancer Epidemiol Biomarkers Prev 18: 2694-2700, 2009.
6. Xiang TX, Yuan Y, Li LL, Wang ZH, Dan LY, Chen Y, Ren GS and Tao Q: Aberrant promoter CpG methylation and its translational applications in breast cancer. Chin J Cancer 32: 12-20, 2013.
7. Revet I, Huizenga G, Koster J, Volckmann R, van Sluis P, Versteeg R and Geerts D: MSX1 induces the Wnt pathway antagonist genes DKK1, DKK2, DKK3, and SFRP1 in neuroblastoma cells, but does not block Wnt3 and Wnt5A signalling to DVL3. Cancer Lett 289: 195-207, 2010.
8. Shah N and Sukumar S: The Hox genes and their roles in oncogenesis. Nat Rev Cancer 10: 361-371, 2010.
9. Bhatlekar S, Fields JZ and Boman BM: HOX genes and their role in the development of human cancers. J Mol Med (Berl) 92: 811-823, 2014.
10. McGinnis W and Krumlauf R: Homeobox genes and axial patterning. Cell 68: 283-302, 1992.
11. Sliwinski T, Synowiec E, Czarny P, Gomulak P, Forma E, Morawiec Z, Morawiec J, Dziki L, Wasylecka M and Blasiak J: The c.469+46_56del mutation in the homeobox MSX1 gene - a novel risk factor in breast cancer? Cancer Epidemiol 34: 652-655, 2010.
12. Saadi I, Das P, Zhao M, Raj L, Ruspita I, Xia Y, Papaioannou VE and Bei M: Msx1 and Tbx2 antagonistically regulate Bmp4 expression during the bud-to-cap stage transition in tooth development. Development 140: 2697-2702, 2013.
13. Nassif A, Senussi I, Meary F, Loiodice S, Hotton D, Robert B, Bensidhoum M, Berdal A and Babajko S: Msx1 role in craniofacial bone morphogenesis. Bone 66: 96-104, 2014.
14. Bendall AJ and Abate-Shen C: Roles for Msx and Dlx homeoproteins in vertebrate development. Gene 247: 17-31, 2000.
15. Nakatomi M, Wang XP, Key D, Lund JJ, Turbe-Doan A, Kist R, Aw A, Chen Y, Maas RL and Peters H: Genetic interactions between Pax9 and Msx1 regulate lip development and several stages of tooth morphogenesis. Dev Biol 340: 438-449, 2010.
16. Ceyhan D, Kirzioglu Z and Calapoglu NS: Mutations in the MSX1 gene in Turkish children with non-syndromic tooth agenesis and other dental anomalies. Indian J Dent 5: 172-182, 2014.
17. Dunwell TL, Hesson LB, Pavlova T, Zabarovska V, Kashuba V, Catchpoole D, Chiaramonte R, Brini AT, Griffiths M, Maher ER, *et al*: Epigenetic analysis of childhood acute lymphoblastic leukemia. Epigenetics 4: 185-193, 2009.
18. Park K, Kim K, Rho SB, Choi K, Kim D, Oh SH, Park J, Lee SH and Lee JH: Homeobox Msx1 interacts with p53 tumor suppressor and inhibits tumor growth by inducing apoptosis. Cancer Res 65: 749-757, 2005.
19. Yamashita S, Tsujino Y, Moriguchi K, Tatematsu M and Ushijima T: Chemical genomic screening for methylation-silenced genes in gastric cancer cell lines using 5-aza-2'-deoxycytidine treatment and oligonucleotide microarray. Cancer Sci 97: 64-71, 2006.
20. Bonds J, Pollan-White S, Xiang L, Mues G and D' Souza R: Is there a link between ovarian cancer and tooth agenesis? Eur J Med Genet 57: 235-239, 2014.
21. Baylin SB and Ohm JE: Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? Nat Rev Cancer 6: 107-116, 2006.
22. Park J, Park K, Kim S and Lee JH: Msx1 gene overexpression induces G1 phase cell arrest in human ovarian cancer cell line OVCAR3. Biochem Biophys Res Commun 281: 1234-1240, 2001.
23. Ringner M, Fredlund E, Hakkinen J, Borg A and Staaf J: GOBO: gene expression-based outcome for breast cancer online. PLoS One 6: e17911, 2011.
24. Xiang T, Li L, Yin X, Yuan C, Tan C, Su X, Xiong L, Putti TC, Oberst M, Kelly K, *et al*: The ubiquitin peptidase UCHL1 induces G0/G1 cell cycle arrest and apoptosis through stabilizing p53 and is frequently silenced in breast cancer. PLoS One 7: e29783, 2012.
25. Klaus A and Birchmeier W: Wnt signalling and its impact on development and cancer. Nat Rev Cancer 8: 387-398, 2008.
26. Giusti AF, O' Neill FJ, Yamasu K, Foltz KR and Jaffe LA: Function of a sea urchin egg Src family kinase in initiating Ca^{2+} release at fertilization. Dev Biol 256: 367-378, 2003.
27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408, 2001.

28. Chesi M and Bergsagel PL: Epigenetics and microRNAs combine to modulate the MDM2/p53 axis in myeloma. Cancer Cell 18: 299-300, 2010.
29. Clevers H: Wnt/beta-catenin signaling in development and disease. Cell 127: 469-480, 2006.
30. Veeman MT, Axelrod JD and Moon RT: A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. Dev Cell 5: 367-377, 2003.
31. Seidensticker MJ and Behrens J: Biochemical interactions in the wnt pathway. Biochim Biophys Acta 1495: 168-182, 2000.
32. Moon RT, Kohn AD, De Ferrari GV and Kaykas A: WNT and beta-catenin signalling: Diseases and therapies. Nat Rev Genet 5: 691-701, 2004.
33. Tao H, Guo L, Chen L, Qiao G, Meng X, Xu B and Ye W: MSX1 inhibits cell migration and invasion through regulating the Wnt/β-catenin pathway in glioblastoma. Tumour Biol 37: 1097-1104, 2016.
34. Hu Z, Fan C, Oh DS, Marron JS, He X, Qaqish BF, Livasy C, Carey LA, Reynolds E, Dressler L, *et al*: The molecular portraits of breast tumors are conserved across microarray platforms. BMC Genomics 7: 96, 2006.