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EXPRESSION AND ROLES OF A XENOPUS HEAD-FORMING GENE HOMOLOGUE IN HUMAN CANCER CELL LINES

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ABSTRACT

Molecular mechanisms for both morphogenesis and carcinogenesis have frequently overlapped, and similar signaling pathways are often involved in these processes. Yamamoto *et al.* identified a novel protein that induces head formation in Xenopus (Yamamoto *et al. Cell,* 120, 223-225, 2005). This new protein, named Shisa, plays unique roles in head formation by suppressing the maturation processes of receptors for Wnt and FGF at the endoplasmic reticulum. Here, we have identified a human homologue of the *shisa* gene (*hu-shisa-2*), and analyzed its expression in various human cancer cell lines by real-time reverse transcription polymerase chain reaction. High levels of mRNA expression were observed in some neuroectoderm-derived human cancer cell lines and small cell lung cancer cell lines. Intracellular localization of hu-Shisa-2 protein was also analyzed, indicating that it is present in the endoplasmic reticulum. Over-expression of hu-Shisa-2 resulted in increased cell growth and invasion, suggesting that hu-Shisa-2 is involved in the evolution and/or progression of human cancers.

Key Words: Head, Wnt, Frizzled, Cancer, Xenopus

INTRODUCTION

Most signaling networks for the head formation of vertebrates are complex and delicate.[1)] During the early stage of embryonal formation, the interaction between various signaling molecules and their suppressor factors leads with the appropriate balance to correct architectures. Molecular mechanisms for morphogenesis and those for carcinogenesis frequently overlap, and identical signaling pathways are often involved in these processes.[2)] Namely, the same molecules are sometimes commonly used in both the evolution of cancers and the generation of tissues and organs.

Recently, Yamamoto *et al.* identified a novel protein that induces head formation in Xenopus.[3)]

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This new protein, named Shisa, plays crucial roles in the signal control needed to induce head formation by suppressing the maturation processes of receptors for Wnt and FGF at the endoplasmic reticulum (ER). This protein's modes of action in the regulation of signaling are quite unique, since it targets receptors for growth/differentiation factors in ER, and consequently regulates the intensity of transduced signals via those receptors.[3)]

A homology search of *shisa* gene resulted in the detection of a human homologue, *hu-shisa-2* with 85%. It was later found that there were four members in the xenopus *shisa* family, and that *hu-shisa-2* was actually an orthologue of xenopus *shisa-2*, suggesting that this gene also plays important roles in higher mammals such as humans and mice. In this study, the expression of *hu-shisa-2* gene in various human cancer cell lines was analyzed mainly by real-time reverse transcription-polymerase chain reaction (RT-PCR). High levels of mRNA were observed in some neuroectoderm-derived cancer cell lines. Furthermore, intracellular localization of hu-Shisa-2 protein and the effects of over-expression or knockdown of the gene on cancer properties were also analyzed, indicating that hu-Shisa-2 is mainly present in ER and promotes malignant phenotypes of cancer cells.

MATERIALS AND METHODS

Cell lines and tissue culture

Various human cancer cell lines were provided mainly by Dr. L. J. Old at Memorial Sloan-Kettering Cancer Center in New York. Most lung cancer cell lines were the gifts of Dr. T. Takahashi of the Division of Molecular Carcinogenesis at Nagoya University Graduate School of Medicine.[4)] Monolayer cell lines were maintained in Dulbecco's modified minimum essential medium (D-MEM) supplemented with penicillin and streptomycin and 10% fetal calf serum (FCS) in a CO_2 incubator at 37°C.

Isolation of cDNAs for hu-shisa-2

With the xenopus cDNA sequence of shisa gene (*X-shisa-2*), we looked for homologous genes expressed in the human body using BLAST search, and identified cDNA clones having homologies in about 85%. cDNA expression vectors containing the complete coding region in pCDNA3.1 were constructed with RT-PCR using primer sets as 5'-CAGGGTGGTGCCATGTGGGG-3' and 5'-CCCCTGCCTTCGTCTCCCTT-3'.

RNA extraction

Total RNA was extracted from various human cancer cell lines using TRIzol™ (Invitrogen, Carlsbad, CA) as described previously.[5)] The total RNA was dissolved in TE buffer, and both its quality and quantity were measured by spectrophotometer (Pharmacia, Uppsala, Sweden).

Real time-PCR

To synthesize cDNA, reverse transcription was performed using 4 μg of total RNA, oligo-dT primer, and SuperScript II RT™ (Invitrogen, Carlsbad, CA) in a total volume of 50 μl. Expression levels of *hu-shisa-2* mRNA were analyzed by SYBR Green-based real-time quantitative RT-PCR using DNA Engine Opticon2™ (Bio-Rad Laboratories) and DyNAmo™ kit (Finnzymes, Espoo, Finland). Following the real-time RT-PCR reaction with sequence-specific primers, a relative quantification of gene expression was analyzed by the $2^{-\Delta Ct}$ method[6)], and values of the expression levels of mRNAs were normalized with an internal control gene, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), examined in parallel. Primer sequences

used for real-time RT-PCR were, sense primer: 5'-CGTGTTT GTCGCCTTTATCA-3', antisense primer: 5'-ACGTTGTTCATGGTCCCTTC-3'.

Construction of expression vectors

To construct an expression vector for *hu-shisa-2* cDNA, we used pCMV-4B and pCMV-5A, attaching Flag tag or Myc tag, respectively, at the C'-terminus of the expressed protein as shown in Fig. 1. A cDNA insert cleaved by *Hind III* and *Xho I* was inserted at the *Hind III* and *Xho I* sites of pCMV-5A or pCMV-4B expression vectors, leading to the generation of the expression vectors, pCMV-5A/shisa-2 (myc-tag) or pCMV-4B/shisa-2 (flag-tag).

Transfection of hu-shisa expression plasmids and detection of hu-Shisa-2

For the over-expression of hu-Shisa protein in cells, the expression vector containing *hu-shisa-2* DNA with myc tag or pCMV-5A alone was transfected into HEK293T cells with Lipofectamine 2000™ (Invitrogen) according to the manufacturer's instructions. For detection of the expressed hu-Shisa-2 protein, Western immunoblotting was performed using cell lysates and a culture medium. Cell lysates containing approximately 50 µg of proteins were separated in SDS-PAGE, and then blotted onto PVDF membranes with a semi-dry type blotting apparatus (Biorad). Culture medium from the transfected cells was concentrated about 50-fold to serve for immunoblotting. The membranes were stained with anti-myc antibody and HRP-labeled anti-rabbit IgG second antibody (Cell Signaling Technology, Beverly, MA). Bands were finally visualized with the ECL™ detection system (PerkinElmer Life Science, Boston, MA).

Intracellular localization of hu-Shisa-2

To define the intracellular localization of hu-Shisa-2 protein, HEK-293T cells were transfected

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CLUSTAL W (1.83) multiple sequence alignment

XShisa-2   MWLE-----GSPLAVLAAVSFLLSVLAAA--QGSGEYCHGWLDAQAVWRDGFQCPERFDG
HShisa-2   MWGARRSSVSSSWNAASLLQLLLAALLAAGARASGEYCHGWLDAQGVWRIGFQCPERFDG
           **       .*. . :  :.:**:.* **  :.************.*** **********

XShisa-2   DDSTICCGKCELRYCCSSAEARLDQGVCNNDRQQGAPDHNRPDKDSPDSTAVPIYVPFLI
HShisa-2   GDATICCGSCALRYCCSSAEARLDQGGCDNDRQQGAGEPGRADKDGPDGSAVPIYVPFLI
           .*:*****.* *************** *:******* : .*.***.**.:**********

XShisa-2   VGSVFVAFIIVGSLVAICCCRCLRPKQEPQQSRAPGSNRLMETIPMISSASTSRGSSSRQ
HShisa-2   VGSVFVAFIILGSLVAACCCRCLRPKQDPQQSRAPGGNRLMETIPMIPSASTSRGSSSRQ
           **********:***** **********:********.**********.************

XShisa-2   SSTAASSSSSANSGARPPPTRSQTNCCLPEGAMNNVYVNMPTNFSVLNCQQATQLVQHQG
HShisa-2   SSTAASSSSSANSGARAPPTRSQTNCCLPEGTMNNVYVNMPTNFSVLNCQQATQIVPHQG
           ****************.**************:**********************:* ***

XShisa-2   QYLHPQFVGYAVPHDSVPMTPVPQFIDGLQGGYRQMQSPYPHSNPEQMMYPAVTV
HShisa-2   QYLHPPYVGYTVQHDSVPMTAVPPFMDGLQPGYRQIQSPFPHTNSEQKMYPAVTV
           ***** :***:* *******.** *:**** ****:***:**:*.** *******
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Fig. 1 Amino acid sequences of hu-Shisa-2 and XShisa-2 predicted from individual cDNAs.
Hu-shisa-2 was based on the sequence of NM_001007538, and xenopus Shisa-2 on that of NM_001092976.

with pCMV-5A/*shisa-2* and pDSRedN2-ER (*calreticulin*) as an ER marker. After co-transfection of two plasmids, cells were fixed with 4% paraformaldehyde and stained with an anti-myc antibody conjugated with FITC (Santa Cruz Biotechnology, Santa Cruz, CA). These preparations served for the analysis with a confocal microscope FV500™ (Olympus, Tokyo) as described previously.[7)]

Effects of hu-Shisa-2 over-expression on cell properties

To investigate the roles of hu-Shisa-2, stable transfectant cell lines were generated by the transfection of expression vectors into SK-LC-17, followed by selection with G418 (300 µg/ml). Two hu-Shisa-2-transfectant cells, Nos. 14 and 15, were established, and two vector control lines, VC1 and VC3, were also generated by transfection of the vector alone. Using these transfectant cell lines, we analyzed cell proliferation, invasion activity and cell motility. Cell proliferation was analyzed with an MTT assay as described elsewhere.[8)] Cell invasion was analyzed with a Boyden chamber filter overlayed with Matrigel™ (Becton Dickinson) as previously described.[4)] Cell motility was measured by the wound-healing scratching assay as described.[9)] Transfectant cells were cultured in regular medium containing no G418 for several days before these experiments.

Knockdown of hu-shisa-2 with siRNA

To further analyze the roles of hu-Shisa-2 in cancer cells, the effects of knockdown on cell proliferation and invasion were examined. Among three siRNAs (1:437, 2:705, 3:918), siRNA Shisa-2/1(437) was selected and transfected into a melanoma cell line, BD, with Lipofectamine-2000™ (Invitrogen), and used for the BrdU uptake assay and the invasion assay 24 h after the transfection.

RESULTS

Isolation of hu-shisa-2 cDNA and predicted protein structure

Using the xenopus cDNA sequence of shisa gene (*shisa-2*), we searched homologous genes expressed in the human body with BLAST search, and identified cDNA clones with homologies of about 85%. Then, cDNA clones containing an entire coding region were obtained with RT-PCR using primers covering the complete coding region of *hu-shisa-2*. Amino acid sequences of hu-Shisa-2 as well as xenopus *shisa-2* were depicted in Fig. 1. They showed about an 85% homology at the amino acid level. In particular, they exhibited high homology in the whole region except for the N'-terminus, as shown in Fig. 1.

Real time-PCR

Using total RNA extracted from various human cell lines, cDNAs were generated with an RT reaction. Expression levels of *hu-shisa-2* mRNA were analyzed by real-time quantitative RT-PCR. Resulting values of mRNA expression levels were normalized with *GAPDH*, and the results were shown in Fig. 2.

Among various human lung cancer cell lines, about 40% of small cell lung cancer lines showed high expression levels of *hu-shisa-2* mRNA, while there were no significant levels of mRNA in non-small cell lung cancer cell lines consisting of adenocarcinoma (11), squamous cell carcinoma (4) and large cell carcinomas (4). Next, we analyzed various neuro- ectoderm-derived cell lines, showing significant mRNA expression in 1 of 2 retinoblastoma lines, 1 of 4 neuroblastoma lines, and 3 of 9 melanoma lines. Interestingly, normal human melanocytes also showed fairly high level of mRNA.

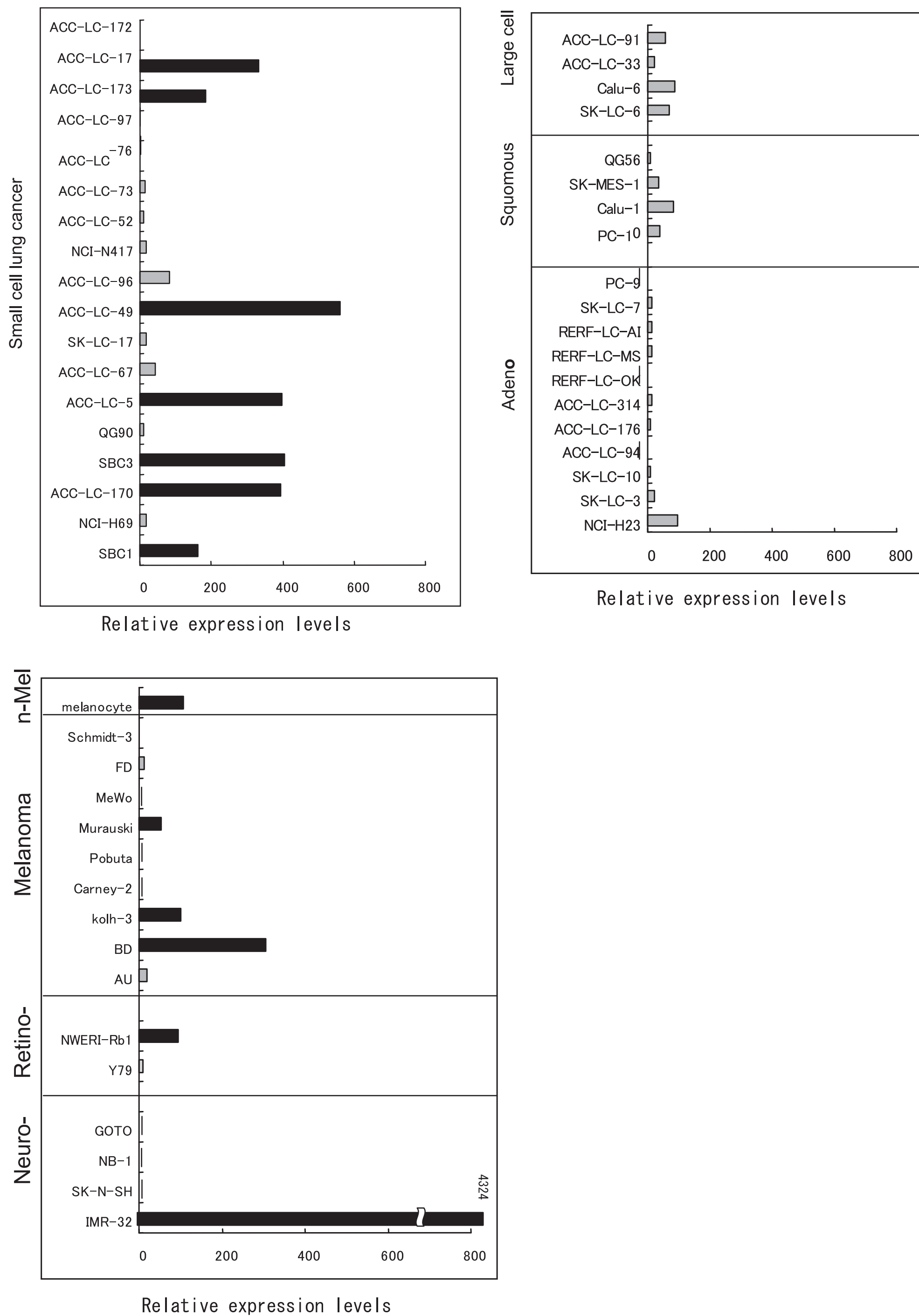

Fig. 2 Expression levels of *hu-shisa-2* mRNA as analyzed by real-time quantitative RT-PCR.
A, Expression levels of *shisa-2* mRNA in lung cancer cell lines were presented after being normalized with *GAPDH,* with the results shown as relative values to the result of Kohl-III as 100. B, Expression levels of *hu-shisa-2* mRNA in neuroectoderm-derived cell lines as analyzed in A.

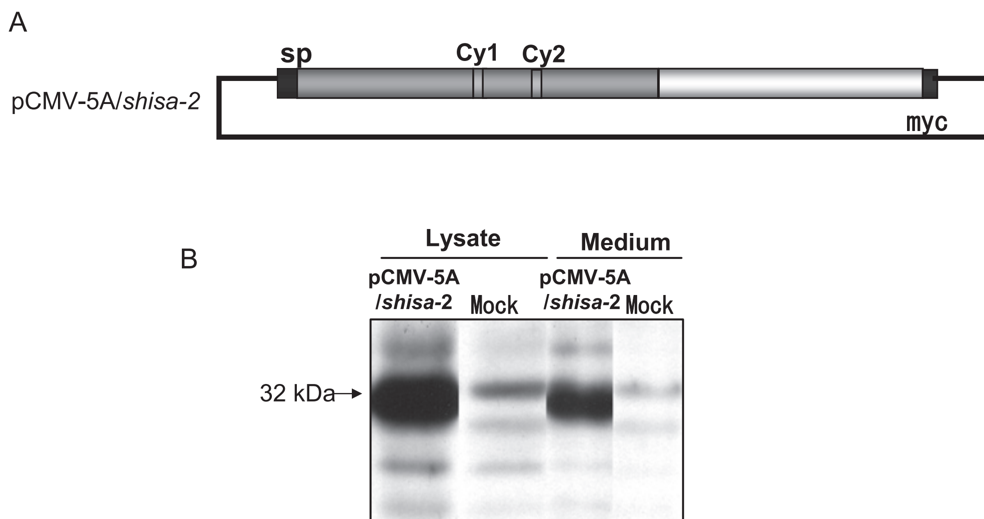

Fig. 3 Expression of hu-Shisa-2 in transfectant cells.
A, The cDNA expression vector pCMV-5A/*shisa-2* was constructed, and transfected into HEK-293T cells. Myc-tag was attached at the C-terminus. B, Immunoblotting was performed using cell lysates and spent culture medium. Detection of bands was performed with an anti-myc antibody and an HRP-labeled anti-rabbit IgG second antibody.

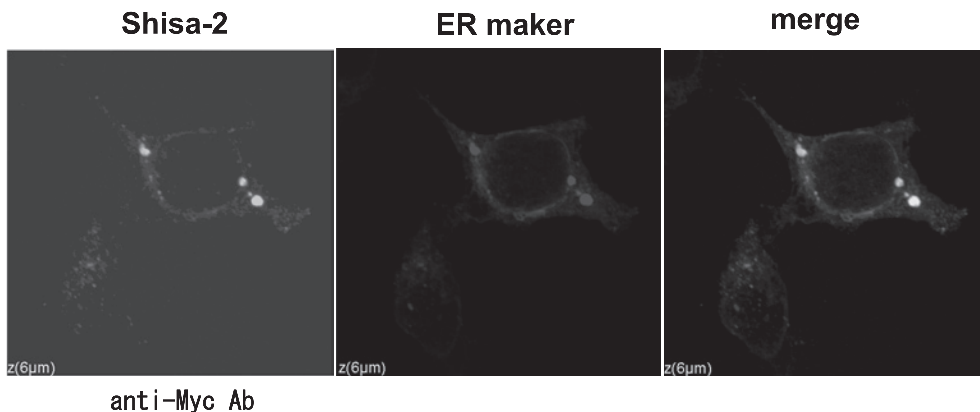

Fig. 4 Hu-Shisa-2 is localized in ER.
Intracellular localization of hu-Shisa-2 was examined using HEK-293T cells co-transfected with pCMV-5A/*shisa-2* and pDSRedN2-ER. Hu-Shisa-2 was detected in the cytoplasm relatively restricted to areas around nuclei. Hu-Shisa-2 staining was included in the location of the ER marker. Confocal immunofluorescent image of Shisa-2 (left), ER marker (center) and merge (right) are shown. Cells were stained with an anti-myc antibody for Shisa (green). An ER marker was calreticulin (red). Original magnification is x 400.

Expression of hu-Shisa-2 in transfected cells

To introduce hu-Shisa-2 in cultured cells, a cDNA expression vector, pCMV-5A/*shisa-2* (Fig. 3A), was transfected into HEK-293T cells. To detect the expressed hu-Shisa-2 protein, immunoblotting using cell lysates and spent culture medium was performed. Detection of bands with an anti-myc antibody revealed that a strong 32 kDa band was expressed in the cells transfected with pCMV-4B/*shisa-2* in both cell lysate and in culture medium, as shown in Fig. 3B.

Hu-Shisa-2 is localized in ER

To define the intracellular localization of hu-Shisa-2 protein, HEK-293T cells were co-transfected with pCMV-5A/*shisa-2* and pDSRedN2-ER. As shown in Fig. 4, hu-Shisa-2 was detected in the cytoplasm in relatively restricted areas around nuclei. When this was superimposed upon the fluorescence pattern of an ER marker (calreticulin), hu-Shisa-2 staining was distributed throughout the marker location, suggesting that hu-Shisa-2 was present in at least some ER compartment.

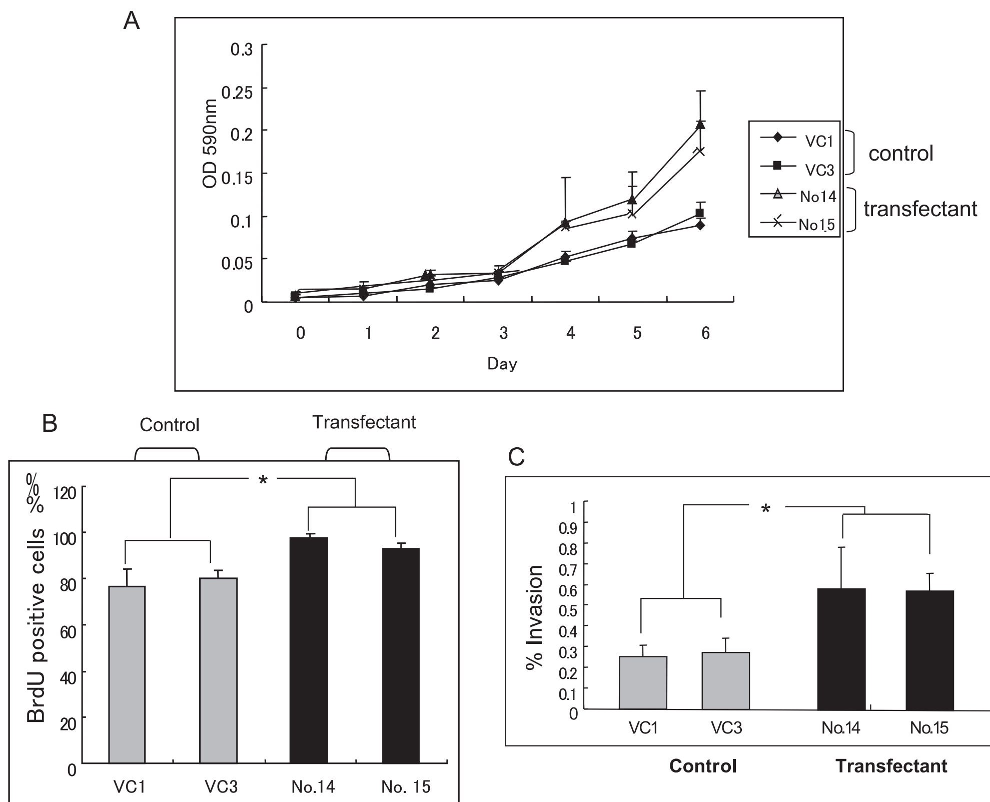

Fig. 5 Hu-Shisa-2 expression enhances cell proliferation and invasion.
A, Cells were seeded in 48-well plates, and cell growth was quantified by assessing the reduction of MTT to formazan. The absorbance at 590 nm was measured using the plate reader ImmunoMini NJ-2300 (System Instrument). B, for BrdU uptake, cells grown in a 60-well plates were incubated in the presence of BrdU for 14 h and then fixed with acid-ethanol for 30 min. The cells were immunostained with anti-BrdU antibody and Alexa Fluor 546-conjugaed secondary antibody (Molecular Probes). The BrdU-positive cells were observed by fluorescence microscopy, and the percentage of BrdU-positive cells was calculated. The transfectants also showed a significantly increased incorporation of BrdU. *, $p < 0.05$
Invasion activity of the transfectants was analyzed using a Boyden chamber. Matrigel was diluted with ice-PBS (250 μg/ml), and 0.6 ml was added to each Falcon 3093 filter (polyethylene Terephthalate membrane, 8-mm pore size, 23.1-mm diameter) and left to be polymerized overnight. The membrane was reconstituted with serum-free medium. The lower chamber (6-well plate, Falcon 3502) was filled with the culture medium without serum. Cells (1×10^6/well) were added to serum-free medium in the upper chamber and incubated for 18 h; cells on the surface of the filter were then stained with Giemsa and counted under a microscope. The ratios of cell numbers in the lower chamber/total cell number applied (in percent) are shown. VC1 and VC3 are the vector controls, and Nos. 14 and 15 are the *shisa-2* gene transfectant cells. Results when the lower chamber was filled with serum-free medium are shown. *, $p < 0.002$

Hu-Shisa-2 expression enhances cell proliferation and invasion

The transfectant cell lines showed increased cell growth when compared with the vector control cells as analyzed by MTT assay (Fig. 5A). When BrdU uptake was compared between the transfectant cells and controls, the transfectants also showed a significantly increased incorporation of BrdU (Fig. 5B). They also exhibited a higher rate of cell invasion activity than the control cells, as seen in Fig. 5C. On the other hand, hu-Shisa-2-expressing cells showed an almost equivalent motility to that of the control cells (data not shown).

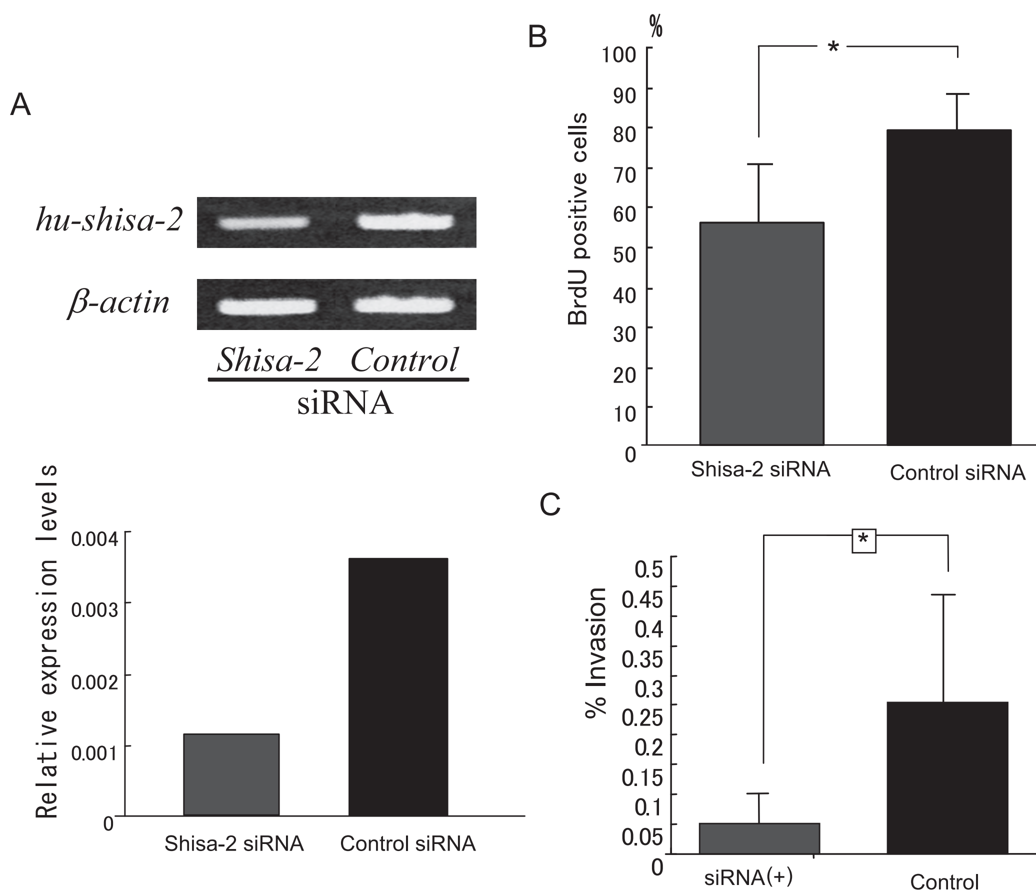

Fig. 6 Knockdown of hu-Shisa-2 resulted in suppression of cell proliferation and invasion.
A, Expression of the *shisa-2* mRNA level after knockdown (real time-RT-PCR). BD cells were transfected with siRNA of shisa-2 by lipofectamine 2000™. After 24 h, RT-PCR was performed. Hu-shisa-2 mRNA levels were strongly suppressed by the siRNA. *Upper,* depicts bands in an agarose gel with RT-PCR. *Lower*, a graph to show band intensity in the upper panel. B, 24 h after the transfection, BD cells were incubated in the presence of BrdU as described in the legend for Fig. 5, and analyzed using the cell proliferation kit according to the manufacturer's instructions. BrdU-positive cells were observed by fluorescence microscopy, and the percentage of such cells was calculated. *, $p < 0.01$. C, Knockdown of hu-Shisa-2 also resulted in a marked suppression of invasive activity, which was analyzed as described in the legend for Fig. 5. Results when the lower chamber was filled with serum-free medium. *, $p < 0.04$

Knockdown of hu-Shisa-2 resulted in suppression of cell proliferation and invasion

To confirm the roles of hu-Shisa-2 in cells, an siRNA-mediated knockdown was attempted using a melanoma cell line BD. *Hu-shisa-2* mRNA levels were strongly suppressed by the siRNA, as shown in Fig. 6A. When cell growth was examined using hu-Shisa-2-knockdown cells with BrdU assay, siRNA-treated cells underwent a significant suppression of BrdU uptake, as shown in Fig. 6B. Knockdown of hu-Shisa-2 also resulted in a marked suppression of the invasion activity, as shown in Fig. 6C. On the other hand, cell motility was not affected by the knockdown of hu-Shisa-2 (data not shown).

DISCUSSION

Xenopus *shisa-1* gene was isolated from Spemann's organizer as a head-forming gene by Yamamoto *et al.*[3] Injection of the mRNA in a Xenopus fetus resulted in the formation of another head, and the disruption of its mRNA expression caused the loss of head formation.[3] This gene was quite unique in having no known homologous gene. The most interesting feature of Shisa-1 was its unique mode of action in the induction of head formation, i.e., Shisa-1 protein trap Wnt receptor Frizzled (Fz) and FGF receptor in the ER, and inhibit surface expression of those receptors. Consequently, caudalization was blocked, leading to the induction of head formation.[3] There have been a number of regulatory mechanisms for the Wnt signals reported so far.[10] However, regulation of the Wnt signaling by controlling the maturation of Wnt receptors has not yet been reported, and Shisa-1 was a quite novel molecule as a regulator of Wnt signal.

As shown in the study of xenopus Shisa-1, hu-Shisa-2 was mainly present in the ER, and excreted in the culture medium of the transfectatnt cells. Whether it binds to Fzs and FGFRs remains to be investigated. Although intracellular localization in the ER and excretion outside of cells were observed for hu-Shisa-2 as for XShisa-1, hu-Shisa-2 may have different properties from that of XShisa-1. It has become clear that there are several members of *shisa* in both xenopus and mammals, and Xshisa-1 and hu-Shisa-2 might not be orthologs of each other. Hu-shisa-2 is closer to Xshisa-2 than to Xshisa-1, and Xshisa-2 behaves slightly differently from Xshisa-1.[11] Namely, Shisa-2 of Xenopus was reported to play roles in the maturation of somatic precursors and in transition to the segmental fate.[12]

Recently, knockout mice of genes belonging to the *shisa* family were generated, and main phenotypes found in individual mutant mice were reported.[11] None of them showed abnormal head formation or over-growth/growth retardation. Therefore, Shisa-1, which is present only in Xenopus, and not in humans or mice, might play a unique role in mammals distinct from Shisa family members, i.e., a role in head formation. In turn, an over-expression of hu-Shisa-2 resulted in an increase of cancer properties such as cell proliferation and invasion. Thus, the results obtained in this study seem to contradict the reported role of XShisa-1 that suppressed Wnt/Fz signaling. If hu-Shisa-2 suppresses Fz expression as the Xenopus homologous gene product does, the targeted Wnt signal should be inhibitory. Certainly, it is also known that some combination of Wnt/Fz might generate negative signals in lung cancer cells.[13] To clarify this issue, effects of hu-Shisa-2 on the canonical pathway measured by LEF/TCF promotor remain to be investigated,[14] and experiments to that end are now in the preparation.

The roles of Wnt/Fz signaling in cancer cells, and those of Shisa family members in human cancers need to be clarified. In order to address these questions, the issue of whether hu-Shisa-2 binds to FZs will be a crucial one.

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