Hair keratin KRT81 is expressed in normal and breast cancer cells and contributes to their invasiveness

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Received September 30, 2016; Accepted November 5, 2016

DOI: 10.3892/or.2017.5564

Abstract. Keratins are fibrous proteins. Hair keratins constitute hard structures such as the hair and nails, and cytokeratins have been used as markers of breast carcinoma. However, the expression and function of full-size hair keratin genes have not been previously demonstrated in breast cancer. We investigated the expression of the hair keratin, KRT81, and its function in human breast cancer and normal mammary epithelial cells. Western blotting showed full size 55-kDa KRT81 expression in the human breast cancer cell lines, MCF7, SKBR3 and MDA-MB-231, normal human mammary epithelial cells (HMEC), and non-neoplastic cells (MCF10A). Reverse transcription-polymerase chain reaction revealed that the full size KRT81, including its 5' region is expressed in breast cells. Immunohistochemical and immunofluorescence analyses showed that KRT81 was located in the cytoplasm. To investigate the function of KRT81, we knocked down KRT81 by siRNA in MCF10A cells. Microarray analysis revealed that the expression of genes related to invasion such as matrix metallopeptidase (MMP)9 was decreased. In KRT81-knockdown MDA-MB231 cells, zymography revealed a decrease in MMP9 activity, while scratch and invasion assays revealed that KRT81-knockdown decreased cell migration and invasion abilities. This is the first study showing that full size KRT81 is expressed in normal breast epithelial cells and breast cancer cells. Moreover, our results indicate that KRT81 contributes to the migration and invasion of breast cancer cells.

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Abbreviations: KRT81, keratin 81; MMP, matrix metallopeptidase; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Key words: breast cancer, hair keratin, invasion, KRT81, MMP9

Introduction

Keratin is an intermediate filament expressed in epidermal cells of specific regions. The large keratin multigene family is comprised of cytokeratins, which are differentially expressed in various types of epithelia. Cytokeratins have been extensively studied as breast cancer markers (1,2), and can be divided into the acidic type I and the basic-to-neutral type II cytokeratins. The intermediate filament network is formed through the obligatory association of equimolar amounts of type I and type II keratins (3). Hair keratins are expressed in hard-keratinized structures such as the hair and nails, and are believed to be structural proteins of the hair or nails that are not expressed in other organs such as the mammary gland. KRT81 is a type II hair keratin and one of the main hair proteins that is expressed in the hair cortex (4). However, it was reported that KRT81 is expressed in the human breast cancer cell line SKBR3 (5,6) and in metastatic lymph nodes of breast carcinomas (7), but not in normal breast epithelial cells. Furthermore, the expressed KRT81 was a 5'-truncated isoform (ΔHb1), and the full size protein was not expressed (5,6). However, its function remains unclear.

The matrix metalloproteinase (MMP) family includes more than 20 isoforms that modulate the extracellular milieu by degrading extracellular matrix proteins. MMPs are secreted by fibroblasts and tumor cells and are involved in tumor invasion and metastasis (8,9).

We previously reported that the hairless phenotype of the Hirosaki hairless rat (HHR) is due to the deletion of basic hair keratin genes, including KRT81 (10). HHR shows the involution by apoptosis of the mammary gland at an early stage of lactation (11), and is resistant to mammary tumors (12). A correlation between the incidence of breast cancer and an observed change in the X-ray diffraction pattern of hair from patients with breast cancer has been reported (13). Furthermore, X-ray diffraction of hair has the potential to provide a non-invasive test for the presence of breast cancer (14,5).

These previous studies suggest that breast cancer may express hair keratin, and hair keratins may have some function in breast cells. The aim of the present study was to investigate the expression and the function of hair keratin KRT81 in normal breast and breast cancer cells. We investigated

the expression of KRT81 using RT-PCR, western blotting and immunohistochemical analysis in human breast cancer cell lines, MCF7, SKBR3 and MDA-MB231, normal human mammary epithelial cells (HMECs) and non-neoplastic cells (MCF10A). To investigate the function of KRT81 in breast epithelial cells, we transfected MCF10A cells with siKRT81 and analyzed gene alterations using microarrays, Ingenuity® Pathway Analysis (IPA), and quantitative PCR (qPCR) to assess changes in gene expression. To investigate the effect of KRT81 on cell invasion, we performed zymography, scratch and invasion assays using the breast cancer cell line MDA-MB-231, which exhibits invasive properties (16). This is the first study on the expression of hair keratin KRT81 and its function in normal breast epithelial and cancer cells.

Materials and methods

Cell culture. HMECs were purchased from PromoCell GmbH (Heidelberg, Germany). The MCF10A human breast epithelial cell line, and MCF7, SKBR3 and MDA-MB-231 human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). HMECs and MCF10A cells were routinely cultured using a Mammary epithelial cell growth medium kit at 37˚C in 5% CO_2. MCF7, SKBR3 and MDA-MB-231 cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37˚C in 5% CO_2.

siRNA transfection. The expression of rat KRT81 was blocked by transient transfection with KRT81 siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) using DharmaFECT Transfection Reagent (Thermo Scientific, Waltham, MA, USA). Approximately $2.5x10^5$ MCF10A or MDA-MB-231 cells were incubated with siRNA for 24-48 h before being used for subsequent assays. For the negative control experiments, MCF10A or MDA-MB-231 cells were transfected with Silencer® Negative Control #1 siRNA (Applied Biosystems, Foster City, CA, USA).

Western blot analysis. Western blotting was performed, according to the method described by Towbin *et al* (17). Proteins from HMECs, MCF10A, MCF7, SKBR3 and MDA-MB-231 cells were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (18) on 7.5% (w/v) polyacrylamide gels and electroblotted to Hybond nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). Blots were probed with anti-KRT81 antibody-C-terminal (ab192689; 1:1,000; Abcam, Cambridge, MA, USA) or anti-β-actin antibody (dilution 1:1,000; Cell Signaling Technology, Danvers, MA, USA) followed by horseradish peroxidase-conjugated anti-guinea pig IgG (dilution 1:2,000; Abcam) or anti-rabbit IgG (dilution 1:2,000; Cell Signaling Technology). Signals were generated with an ECL kit (GE Healthcare) according to the manufacturer's protocol.

Reverse transcription-polymerase chain reaction (RT-PCR) and qPCR. Total RNA was extracted from the HMECs, MCF10A, MCF7, SKBR3 and MDA-MB-231 cells using the RNeasy Mini kit (Qiagen, Tokyo, Japan). cDNA was reverse-transcribed from total RNA (200 ng) using the PrimeScript™ RT Master Mix (Takara, Tokyo, Japan). PCR was performed with Takara LA Taq® with GC Buffer (Takara) using specific primer pairs. PCR amplification consisted of 30 sec at 94˚C, 30 sec at 55-60˚C and 30 sec-2 min at 72˚C for 40 cycles. Gene-specific primers were designed according to known human sequences using the Primer3Plus software. The primers used were as follows (5'→3'): *KRT81* F-CCTGCGG ATCAGGATTTGGT (corresponding to exon 1) and R-AAGT GGGGGATCACACAGAG (corresponding to exon 9); *GAPDH*, F-AGAAGGCTGGGGCTCATTTG and R-AGGG GCCATCCACAGTCTTC. The RT-PCR products were subjected to electrophoresis on a 2% agarose gel and visualized with ethidium bromide. Levels of specific mRNAs were quantified by qPCR using SYBR-Green SuperMix (Bio-Rad Laboratories, Hercules, CA, USA). Transcript levels were normalized to that of *GAPDH* cDNA. The primers used were as follows (5'→3'): *KRT81*, F-AGGCTATGTGAAGGCATTGG (corresponding to exon 8) and R-AAGTGGGGGATCACAC AGAG (corresponding to exon 9); *GAPDH*, F-AGAAGGCT GGGGCTCATTTG and R-AGGGGCCATCCACAGTCTTC; *MMP9*, F-CACCTTCACTCGCGTGTAC and R-CATCTGC GTTTCCAAACCGAG. PCR specificity was ascertained by melting curve analysis. Relative gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ method.

Immunofluorescence staining. Cells were seeded onto a 4-well Slide & Chamber (Watson, Japan) and incubated at 37˚C for 24 h, fixed immediately in 4% paraformaldehyde, and permeabilized in 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 5 min. The slides were then incubated with the anti-KRT81 antibody-C-terminal (ab192689; dilution 1:100) at 4˚C overnight, followed by incubation with the secondary antibody, Alexa Fluor 647 anti-guinea pig IgG (ab150187; dilution 1:500) (both from Abcam) for 30 min at room temperature. Nuclear staining and mounting were performed with Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Images were captured using a fluorescence microscope FSX100 (Olympus, Tokyo, Japan).

Histological analysis and immunohistochemistry. Breast cancer specimens, including normal areas, were obtained from patients at the time of surgery. The archival specimens were obtained from the Department of Pathology and Bioscience at Hirosaki University. The sections (4-μm) were mounted onto silane-coated slides. Immunohistochemistry was automatically performed using the Ventana XT System Discovery® (Roche, Basel, Switzerland). Briefly, tissue sections were treated with protease I (Roche) at 37˚C for 16 min for antigen retrieval. The following primary antibody was used: KRT81 polyclonal antibody (11342-1-AP; dilution 1:25; ProteinTech, Manchester, UK), and sections were incubated at 37˚C for 32 min. The tissue sections were then incubated at 37˚C for 20 min with a universal secondary antibody (Roche). The site of peroxidase binding was determined using DISCOVERY DABMap Detection kit (Roche). Sections were then counterstained with Hematoxylin II (Roche) for microscopic examination. As a negative control, non-immune γ-globulin was used instead of the antibody. The specimens were observed and photographed using a fluorescence microscope FSX100 (Olympus). The present study was

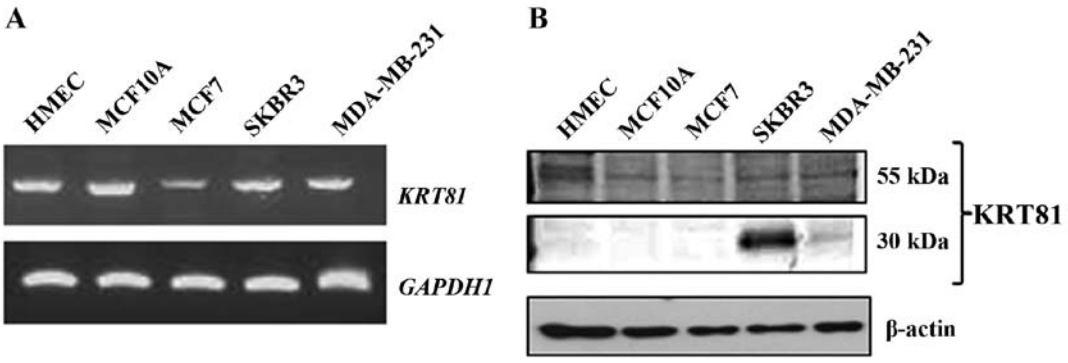

Figure 1. Expression of KRT81 mRNA and protein in normal breast epithelial and breast cancer cell lines. (A) RT-PCR was performed using the specific primers and mRNA isolated from each cell line. *GAPDH* mRNA was used as the control. (B) Western blotting to detect KRT81 or β-actin in each cell line. Proteins were resolved by SDS-PAGE and then blotted with an anti-KRT81 or anti-β-actin antibody.

approved by the Committee for Medical Ethics of Hirosaki University (Hirosaki, Japan). Informed consent was obtained from all patients prior to the beginning of the study.

Gene expression analysis by microarray. Total RNA was extracted from the siRNA-transfected or control MCF10A cells using the RNeasy Mini kit. One microgram of RNA was used to produce biotin-labeled complementary RNA (cRNA). The labeled and fragmented cRNA was subsequently hybridized to the SurePrint G3 Human Gene Expression microarray (8x60 K version 2; Agilent Technologies Inc., Santa Clara, CA, USA). Labeling, hybridization, image scanning and data analysis were performed at Bio Matrix Research Inc. (Chiba, Japan). The MCF10A microarray dataset is available at http://www.ncbi.nlm.nih.gov/geo under accession code GSE85236. Genes with 2-fold or greater upregulation following siRNA transfection were analyzed using the Qiagen Ingenuity® Pathway Analysis (IPA®) software (version 18030641). The z-score algorithm was utilized to decrease the possibility of false-positive results, where $z \geq 2$ indicated that transcript expression was significantly increased and $z \leq -2$ indicated that the expression was significantly decreased.

Gelatin zymography. The MDA-MB-231 cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. After 24 h, the cells were transfected with siKRT81 or Silencer® Negative Control #1 siRNA in serum-free medium. After 48 h, the serum-free conditioned medium was harvested by centrifugation at 1,500 rpm for 5 min. Gelatin zymography was performed as previously described (19,20).

Scratch wound healing assay. The effects of KRT81 on cell migration were examined using the scratch wound healing assay with KRT81-silenced and control MDA-MB-231 cells. Briefly, $2x10^5$ cells were seeded onto 60-mm cell culture dishes. After cells reached ~70% confluence as a monolayer, they were transfected with siKRT81. After 24 h, the surface of the dishes was scratched linearly with a 200-μl pipette tip. After 24 h, images were captured using a fluorescence microscope CKX41 (Olympus). The cells that migrated were counted/field. Results were obtained from 3 independent experiments with 9 fields.

Invasion assay. The invasiveness of MDA-MB-231 cells treated or not with siKRT81 was assessed using Transwell chambers with 8-μm pore size membrane (CytoSelect™; Cell Biolabs, Inc., San Diego, CA, USA). Before the invasion assay, MDA-MB-231 cells were transfected with the siKRT81 and cultured for 24 h in another culture dish. In the upper compartment of the chamber, ~$1.5x10^5$ cells (treated or not with siKRT81)/insert were added into the culture medium without serum, and 500 μl of culture medium with 10% FBS were added to the lower well of the invasion chamber. The cells were incubated at 37˚C in 5% CO_2 for 24 h. Cells were then washed, fixed and stained with cell stain solution. Non-migrating cells were removed from the upper surface of the Transwell membrane with a cotton swab. Cells were counted with a fluorescence microscope FSX100. Results were obtained from 3 independent experiments with 9 fields.

Statistical analysis. Experiments were conducted at least 3 times in duplicate or triplicate. Results are presented as the mean ± standard error of the mean (SEM). Statistical significance was determined using Student's t-test. $p<0.01$ was considered statistically significant.

Results

KRT81 expression in normal breast epithelial and cancer cells. While northern blot analysis revealed that the human truncated form of KRT81 is expressed in breast cancer SKBR3 cells, but not in normal mammary glands (5,6), no study has examined other breast cells by RT-PCR. Therefore, to determine the expression of KRT81 in breast cells, RT-PCR was performed using specific primers (forward primer located in exon 1 and reverse primer located in exon 9). Expression of KRT81, including the 5' region, resulting in a 1,674-bp product, was detected in all analyzed cells (Fig. 1A). To examine whether KRT81 protein is expressed in breast cancer cells, western blotting was performed with a KRT81 antibody. A 55-kDa protein was detected in all breast cell lines. Furthermore, a 30-kDa protein was detected in the SKBR3 and MDA-MB-231 cell lines (Fig. 1B). To examine the location of KRT81, we performed immunofluorescence staining using an anti-KRT81 antibody. The immunofluorescence staining revealed that KRT81 was expressed in the cytoplasm in all

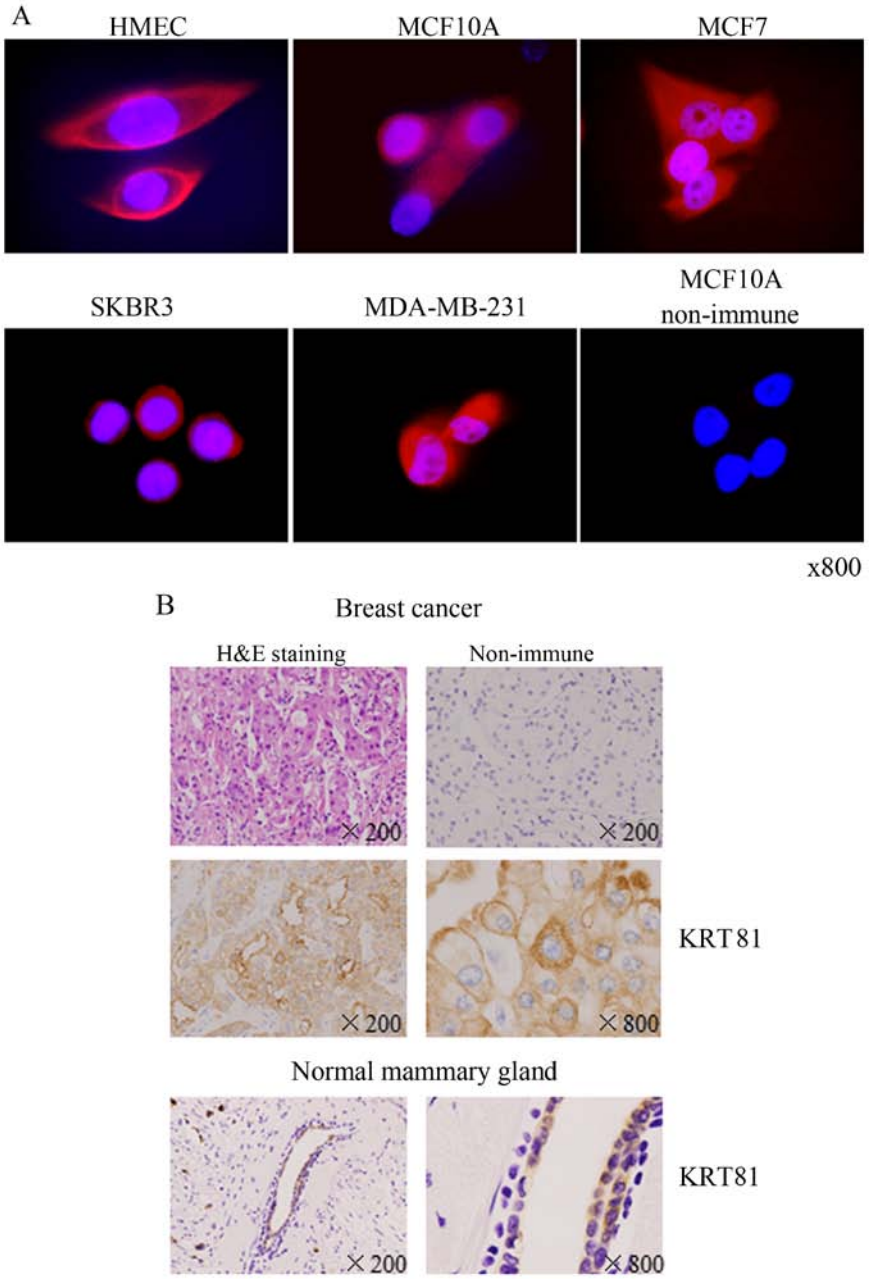

Figure 2. Localization of KRT81 in normal breast epithelial and breast cancer cells. (A) Normal human mammary epithelial cells (HMECs) and MCF10A, and breast cancer cell lines, MCF7, SKBR3 and MDA-MB-231, were stained with an anti-KRT81 antibody (Red Alexa Fluor 647). The cells were mounted with Vectashield Mounting Medium with DAPI to visualize the nuclei. As a negative control, non-immune γ-globulin was used instead of the antibody. Images are at a magnification of x800. (B) Breast tissue sections from patients with breast cancer were stained with H&E or an anti-KRT81 antibody. Images are at a magnification of x200 (H&E) and x200 or x800 (anti-KRT81 antibody or non-immune γ-globulin).

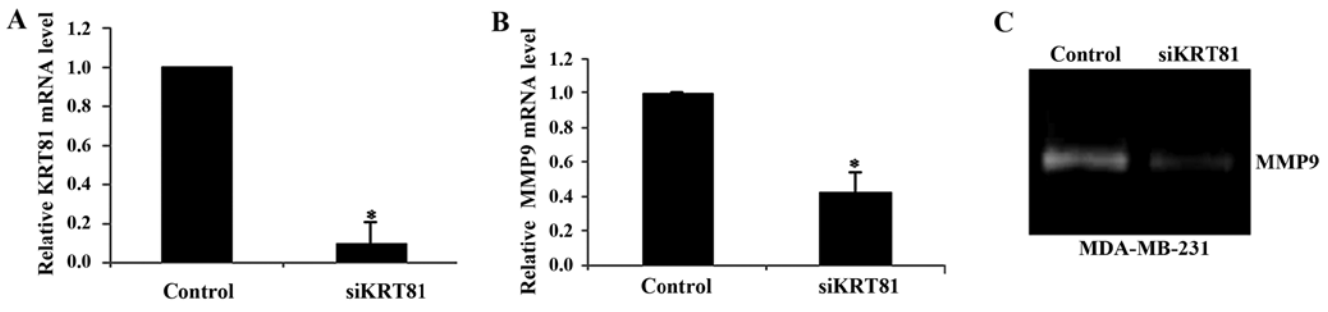

Figure 3. KRT81 knockdown results in a decrease in MMP9 expression in breast cancer MDA-MB-231 cells. MDA-MB-231 cells were transfected with KRT81 siRNA or a Silencer® Negative Control #1 siRNA and cultured for 48 h. (A) *KRT81* and (B) *MMP9* mRNA expression was evaluated by qPCR; *p<0.01 vs. the control. (B) Gelatin zymography of supernatants from the control and siKRT81-transfected MDA-MB-231 cells.

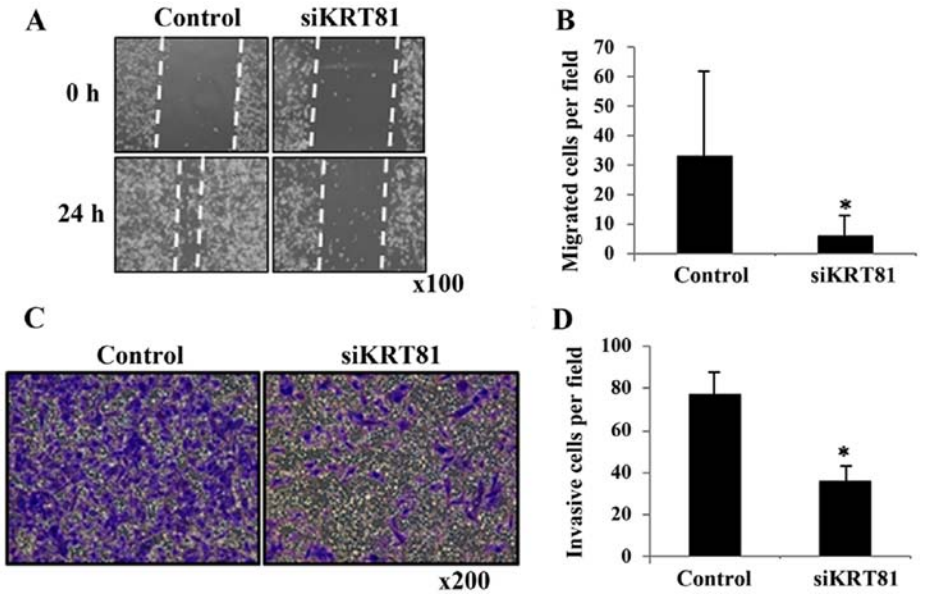

Figure 4. KRT81 knockdown inhibits the migration and invasion of MDA-MB-231 cells (A) MDA-MB-231 cells were transfected with KRT81 siRNA or a Negative Control #1 siRNA and cultured. After 48 h, they were scratch-wounded and cultured for 24 h. (B) Cells that migrated/field; *p<0.01 vs. the control. (C) MDA-MB-231 cells were transfected with KRT81 siRNA or a negative control #1 siRNA and cultured. After 24 h, an invasion assay was performed as described in 'Materials and methods'. (D) Invasive cells/field; *p<0.01 vs. the control.

breast cell lines (Fig. 2A). KRT81 expression in breast cancer tissue from patients was examined by immunohistochemistry with an anti-KRT81 antibody. KRT81 was expressed in the cytoplasm of ductal epithelial cells in breast cancer and normal areas (Fig. 2B). Staining with non-immune γ-globulin was negative. These results indicated that the full size and truncated form of KRT81 are expressed in human normal breast epithelial and cancer cells.

KRT81 knockdown downregulates the expression of invasion-related genes in MCF10A cells. To analyze the function of KRT81 in mammary epithelial cells, we transfected MCF10A cells with a *KRT81* siRNA, and whole transcript profiling was performed by microarray analysis. IPA was performed to investigate the functional relationships between sets of genes with modified expression levels. Table I shows that invasion-related genes such as tumor necrosis factor (*TNF*), *MMP9* and Lipocalin 2 (*LCN2*) were downregulated in the siKRT81-transfected cells.

KRT81 knockdown decreases the migration and invasion abilities of MDA-MB-231 cells. Since MCF10A cells are non-invasive, we investigated the effect of KRT81 on cell invasion using the invasive breast cancer cell line MDA-MB-231. qPCR analysis revealed that KRT81 knockdown decreased *KRT81* and *MMP9* expression at the mRNA level to 0.1- and 0.5-fold of that of the control cells, respectively (p<0.01; Fig. 3A and B). Gelatin zymography revealed that MMP9 activity was decreased in the siKRT81-transfected cells (Fig. 3C). To investigate whether KRT81 affects migration and invasion, we performed scratch and invasion assays using MDA-MB-231 cells. siKRT81 knockdown decreased cell migration to 0.2-fold (p<0.01; Fig. 4A and B) and invasion to 0.5-fold (p<0.01; Fig. 4C and D). These results demonstrated that KRT81 contributes to breast cancer cell migration and invasion.

Table I. Invasion-related genes in breast cancer cell lines.

Gene symbol	Gene name	Fold-change[a]
JUN	Jun proto-oncogene	0.48
ARF6	ADP-ribosylation factor 6	0.46
SP100	SP100 nuclear antigen	0.45
HPSE	Heparanase	0.45
MMP7	Matrix metallopeptidase 7	0.39
MMP9	Matrix metallopeptidase 9	0.45
MMP10	Matrix metallopeptidase 10	0.40
MMP13	Matrix metallopeptidase 13	0.14
HIF1A	Hypoxia inducible factor 1	0.44
TNF	Tumor necrosis factor	0.42
LCN2	Lipocalin 2	0.38
BMP2	Bone morphogenetic protein 2	0.38
PTGS2	Prostaglandin-endoperoxide synthase 2	0.29
FAS	Fas (TNF receptor superfamily, member 6)	0.24
TNFSF10	Tumor necrosis factor superfamily, member 10	0.09

[a]Expression level in MCF10A cells transfected with KRT81 siRNA/negative control siRNA.

Discussion

Hair is produced by hair follicles. The embryonic mammary glands and hair follicles are both derived from the ventral ectoderm; mammary glands are skin appendages and their development depends on a number of common fundamental developmental pathways (21,22). Therefore, we speculated

Table II. EZH2-regulated genes in MCF10A cells.

Gene symbol	Gene name	Fold-change[a]
TNF	Tumor necrosis factor	0.42
RARRES3	Retinoic acid receptor responder 3	0.14
PTGS2	Prostaglandin-endoperoxide synthase 2	0.29
NCOA7	Nuclear receptor co-activator 7	0.46
LCN2	Lipocalin 2	0.38
KRT81	Keratin 81	0.15
IL6	Interleukin 6	0.19
IL24	Interleukin 24	0.40
CXCL10	C-X-C motif chemokine ligand 10	0.05
C15orf48	Chromosome 15 open reading frame 48	0.20

[a]Expression level in MCF10A cells transfected with KRT81 siRNA/negative control siRNA. z-score of EZH2 was -2.61. EZH2, zeste 2 polycomb repressive complex 2 subunit.

that KRT81 expressed in breast cells may have some, yet, unknown functions. In the present study, we demonstrated the role of KRT81 in the migration and invasion of breast cancer cells.

A previous study revealed that ΔHb1, but not the full size KRT81, was expressed in the breast cancer cell line, SKBR3, but not in normal breast cells using northern blotting, and that ΔHb1 lacked the 270 amino terminal residues (5,6). However, the present study revealed that the full size (exon 1-9) *KRT81* mRNA and the 55-kDa KRT81 protein were expressed not only in breast cancer cells, but also in normal breast cells (Fig. 1A and B). In western blot analysis, the 55-kDa KRT81 protein was detected in all breast cell lines, but the major band detected in the SKBR3 cells was ΔHb1 (30-kDa), suggesting that the amount of hair keratin proteins is low in breast cells. Furthermore, the amount of full size KRT81 was very low in typical breast cells, and the detection of ΔHb1 such as in SKBR3 cells appears rare. Immunohistochemistry revealed that KRT81 is located in the cytoplasm of breast cancer cells similar to cytokeratins, which suggests that KRT81 may have a function related to the cytoskeleton. However, recent studies proposed that cytokeratins not only function in the cytoskeleton, but also function as regulators of transcription factors and KRT17 may also be localized in the nucleus (23,24). Furthermore, ΔHb1 was detected not only in breast cancer, but also in colon or nasopharyngeal carcinoma. It is thought that ΔHb1 may inhibit some of the functions of the keratin cytoskeleton or interfere with transcription regulation (25). Thus, KRT81 may have other functions in breast cells.

The siRNA-mediated *KRT81*-knockdown in MCF10A cells decreased the expression of invasion-related genes, including *LCN2* and matrix metalloproteinases. LCN2 promotes mammary tumor formation and progression by upregulating matrix metalloproteinase expression (26). These alterations suggest inactivation of the NF-κB pathway, since *LCN2* and *MMP9* expression is regulated by NF-κB (27,28). However, microarray and IPA analysis suggested that *KRT81* knockdown did not alter the NF-κB pathway (data not shown), except for TNF expression, which was decreased (Table I). TNF is a known regulator of MMPs and LCN2 (29-31). In the present study, TNF, MMP9 and LCN2 expression was decreased in the siKRT81-treated MCF10A cells. Additionally, the expression of several TNF signaling-related genes, including Fas cell surface death receptor, tumor necrosis factor superfamily member 10, and TNF receptor superfamily member 14, was decreased (data not shown). While the function of hair keratins has not been reported, the functions of some keratins and TNF have been clarified. For example, keratin 8/18 and keratin 17 interact with the TNF receptor 1 (TNFR1)-associated death domain protein (TRADD), a death adaptor essential for TNFR1-dependent signaling. These keratins may attenuate TNF-induced apoptosis through association with TRADD. Furthermore, TNF regulates rat Krt23 (32) or human KRT15 (33). These studies suggest that KRT81 may also regulate TNF-dependent functions.

Furthermore, we performed an upstream regulator prediction analysis using IPA. The IPA analysis revealed that the predicted upstream regulator in siKRT81-treated MCF10A cells was the histone-lysine N-methyltransferase enzyme, enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), as EZH2 (z-score, -2.61) and the downstream genes of EZH2 were inhibited (Table II). Since EZH2 has been linked to cancer invasion or growth (34), recent studies have proposed that EZH2 may be useful as a therapeutic target (35,36). Furthermore, it is known that EZH2 regulates the expression of KRT81, but its mechanism is unclear (37). Since KRT81 silencing downregulated EZH2-downstream genes such as *LCN2* and *MMP9,* KRT81 may be involved in EZH2 activation.

In conclusion, this is the first study on KRT81 gene expression in normal and breast cancer cells, suggesting that KRT81 is involved in breast cancer migration and invasion. Collectively, the findings of the present study revealed that KRT81 is expressed in clinical specimens from patients with breast cancer. Indeed, it was reported that a KRT19 fragment in serum can be used as a marker of liver and breast cancer (38,39), and KRT81 microRNA is associated with the risk and survival of patients with non-small cell lung cancer (40-42). Therefore, KRT81 may be used as a biomarker for patients with breast cancer once we clarify its biological significance.

Acknowledgements

The present study was supported in part by the JSPS KAKENHI (grant no. 20770096) and a Hirosaki University Institutional Research Grant for Young Scientists.

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