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Original

Analysis of miRNA Expression in Breast Cancer

Kentaro YATOMI[1)], Akiko SASAKI[1)], Mayumi TSUJI[1)],
Yuko UDAKA[1)], Yuko TSUNODA[2)], Eisuke FUKUMA[2)]
and Katsuji OGUCHI[1)]

Abstract: Triple-negative breast cancer (TNBC), lacking estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor (HER2) expression, is resistant to conventional therapies. Recent studies have focused on microRNAs (miRNAs) as novel molecular targets for treating TNBC because they modulate gene expression and tumor progression. In the current study, we analyzed the expression of selected miRNAs (miR-145 and miR-182) and tumor promoting factors such as Fascin and poly (ADP-ribose) polymerase (PARP) in human TNBC tissues and "Luminal A" breast cancer tissues, which express ER and PgR, but not HER2, as well as breast cancer cell lines including the triple-negative MDA-MB-231 and Luminal A MCF-7. The results showed that miR-145 and miR-182 were expressed in Luminal A breast cancer tissues and MCF-7 cells, but not in TNBC tissues and MDA-MB-231 cells. In contrast, Fascin and PARP proteins were highly expressed in TNBC and MDA-MB-231, but poorly expressed in Luminal A tissues and MCF-7 cells, indicating a negative correlation between expression of these miRNAs and that of the tumor promoting factors Fascin and PARP. The current study therefore suggests that miR-145 and miR-182 could be potential novel therapeutic targets for TNBC therapy.

Key words: breast cancer, triple negative, microRNA, Fascin, PARP

Introduction

Breast cancer is the most common cancer occurring in women. Patients with metastatic breast cancer may not survive the disease, and it is important to control tumor recurrence. The large number of etiological factors and the complexity of breast cancer treatments present a challenge for the prevention and treatment of this disease.

Breast cancer may be divided into subtypes based on gene expression analysis. This classification reflects the grade of malignancy and its susceptibility to medical treatment. Triple-negative breast cancer (TNBC) is a subtype that shows no estrogen receptor (ER), progesterone receptor (PgR), or HER2 (human epidermal growth factor receptor) gene expression. This form of breast cancer is not susceptible to hormone therapy or anti-HER2 treatment, and there is no established treatment that targets a specific molecule in such cases. Poly (ADP-ribose)

[1)] *Department of Pharmacology, Showa University School of Medicine,* 1-5-8 Hatanodai, Shinagawa-ku, Tokyo. 142-8555, Japan.
[2)] *Department of Breast surgery, Kameda Medical Center*

polymerase (PARP) is a potential breast cancer target molecule that, among other functions, plays a critical role in DNA repair. PARP inhibitors have therefore been studied as potential targeted therapies for patients with TNBC. Another potential new molecular target for therapeutic intervention in patients with ER-negative breast cancer is Fascin, which has shown striking upregulation in several human epithelial tumors including breast cancer[1–3].

MicroRNAs (miRNAs) are small non-coding genes that control gene transcription or protein translation and have been implicated in multiple regulatory roles in mammalian cells. These RNA molecules are also frequently repressed in various human malignancies and play a significant role in the pathogenesis of many cancers, including breast cancer[4–6]. The deletion of miRNAs that normally suppress the expression of one or more oncogenes may thus lead to carcinogenesis, tumor growth, and/or invasion. In recent years, "molecularly targeted therapy" aimed toward specific genes has attracted attention as a replacement for conventional treatment methods such as anticancer drug therapy and miRNAs may also be targets of such an approach including as prognostic or predictive biomarkers in TNBC patients.

This study aimed to explore the potential of miRNAs in regulating Fascin and PARP protein expression in TNBC.

Materials and Methods

Patient tissue specimens

Specimens of breast cancer from two patients were used for miRNAs analysis. One patient was diagnosed with TNBC [ER (−), PgR (−), HER (−)] and the other with luminal A type breast cancer [ER (+), PgR (+), HER2 (−), one case]; the latter cancer type is associated with high hormonal sensitivity and a good prognosis. The study was approved by the Institutional Review Board of the Kameda Medical Center (Table 1).

Cell culture

We used human breast cancer cell lines MDA-MB-231 (Triple Negative) and human MCF-7 (Luminal A) derived from the American Type Culture Collection (ATCC). The culture medium contained 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin (GIBCO penicillin-streptomycin liquid; Invitrogen, CA, USA) in Dulbecco's modified Eagle's medium (Sigma, Deisenhofen, Germany). Cells were incubated at 37ºC with 5% carbon dioxide (Table 1).

miRNA extraction and PCR

Small RNA-enriched total RNA was isolated from total RNA using the RT^2 qPCR-Grade miRNA Isolation Kit (SABiosciences, Frederick, MD, USA). Nucleic acid concentration and purity were measured using UV spectrophotometry (A260/A280 $>$ 1.8) using a Nanodrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA). Reverse transcription and real-time PCR were performed using the RT^2 miRNA PCR Array (MIHS-109ZA, SABiosciences) and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Tokyo, Japan) with the following cycling conditions: 1 cycle at 95^0 for 15 minutes, 40 cycles of 94^0 for 15 seconds, 55^0

Table 1. Clinical laboratory findings and cell lines.

〈Clinical specimens〉 Characteristics	Triple negative	Luminal A
Histological type	ER (−), PgR (−), HER (−)	ER (+), PgR (+), HER2 (−)
Patient Age	37	47
Tumor size (cm)	1.2	1.8
Lymph node metastasis (N)	N0	N0
Nuclear variation	3	3
Stage	I	I
〈Cell lines〉 Characteristics	Triple negative	Luminal A
Histological type	ER (−), PgR (−), HER (−)	ER (+), PgR (+), HER2 (−)
Cell lines	MDA-MB-231	MCF7

ER : Estrogen receptor, PgR : Progesterone receptor, HER2 : human epidermal growth factor receptor 2

for 30 seconds, and 70^0 for 30 seconds. Data were analyzed using the ΔCt method.

ELISA for PARP and Fascin

Following centrifugation of 1×10^6 cells in 1.5-ml tubes, the pellet was washed in phosphate-buffered saline, and Cell Lysis Buffer 4 (80-1339 ; Enzo Life Sciences, Farmingdale, NY, USA) containing 1 mM PMSF (phenylmethylsulfonyl fluoride) and protease inhibitor cocktail (Sigma P8340, 0.5 μl/ml) was added. The sample was cooled on ice for 15 minutes and then re-centrifuged. Supernatant levels of fascin and PARP were then measured using the relevant enzyme-linked immunosorbent assay (ELISA) kit (E91757Hu ; Uscn Life Science, Wuhan, China and 4684-096 ; Trevigen, Inc, Gaithersburg, MD, USA, respectively) and a fluorospectrophotometer at $\lambda = 450$ nm.

Immunostaining

The cells were fixed in 10% formaldehyde and then incubated in non-specific blocking regent (X0909, Dako) for 5 minutes to block nonspecific staining. Sections were incubated with anti-rabbit PARP mAb (#9532, Cell Signaling Technology, Beverly, MA, USA) or anti-human fascin mAb (M3567, Dako) for 1 hour at room temperature, followed by a 30-minute incubation with anti-mouse secondary antibody (K4001, DakoCytomation EnVision System, HRP). Staining was visualized using chromogen 3,3-diaminobenzidine tetrahydrochloride (DAB) precipitation (K3466, DakoCytomation Liquid DAB substrate chromogen kit). Scoring of the staining was done on a 0～3 scale. Specimens with scores of 0 or 1+ (no or negligible membrane and nuclear staining in less than 0～30% of tumor cells) were considered immunonegative. Specimens that showed an intermediate (borderline) score of 2+ (weak to moderate membrane and nuclear staining in less than 30～60% of tumor cells) were considered equivocal. Specimens with PARP and Fascin scoring 3+ (strong complete nuclear and membrane staining in more than 60% of tumor

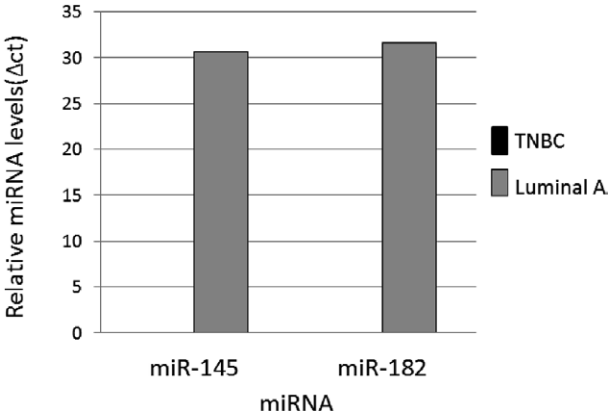

Fig. 1. miRNA expression levels in TNBC and luminal A breast cancer clinical samples. The X axis represents miRNA and the Y axis represents computed tomography.

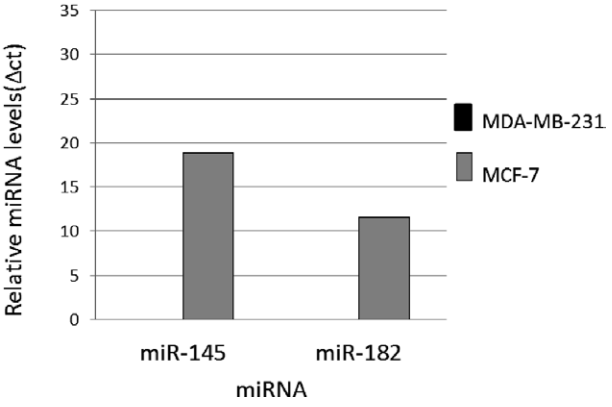

Fig. 2. miRNA expression levels in MDA-MB-231 and MCF-7 cell lines. Neither miRNA was expressed in the MDA-MB-231 cells.

cells) were considered immunopositive.

Statistical analysis

Data were tested for statistical significance using ANOVA, with significance set at $P < 0.05$.

Results

Analysis of miRNA expression

Clinical specimens

Fig. 1 shows the results of miRNA gene expression analysis in the clinical specimens. No miRNA-145 or miRNA-182 expression was detected in the TNBC specimens, while the Luminal A samples had miRNA-145 and miR-182 ΔCT values of 30.59 and 31.56, respectively.

Cell lines

Fig. 2 shows the results of gene expression analysis of miRNA in the cell lines. MDA-MB-231 cells showed no miRNA-145 or miRNA-182 expression, while the ΔCT values for miRNA-145 and miR-182 in the MCF-7 cell line were 18.92 and 11.5, respectively.

Immunohistochemistry

Clinical specimens

Immunostaining for Fascin was positive in the TNBC tissue samples (positive rate 80%, score 3); however only a Fascin positive cytoplasmic signal was present in the luminal A tissue samples (positive rate 10%, score 1) (Fig. 3A). The TNBC tissue samples also showed a positive nuclear signal for the protein PARP (positive rate 60%, score 2), while the luminal A breast cancer tissue samples were PARP immunonegative (positive rate 10%, score 1) (Fig. 3C).

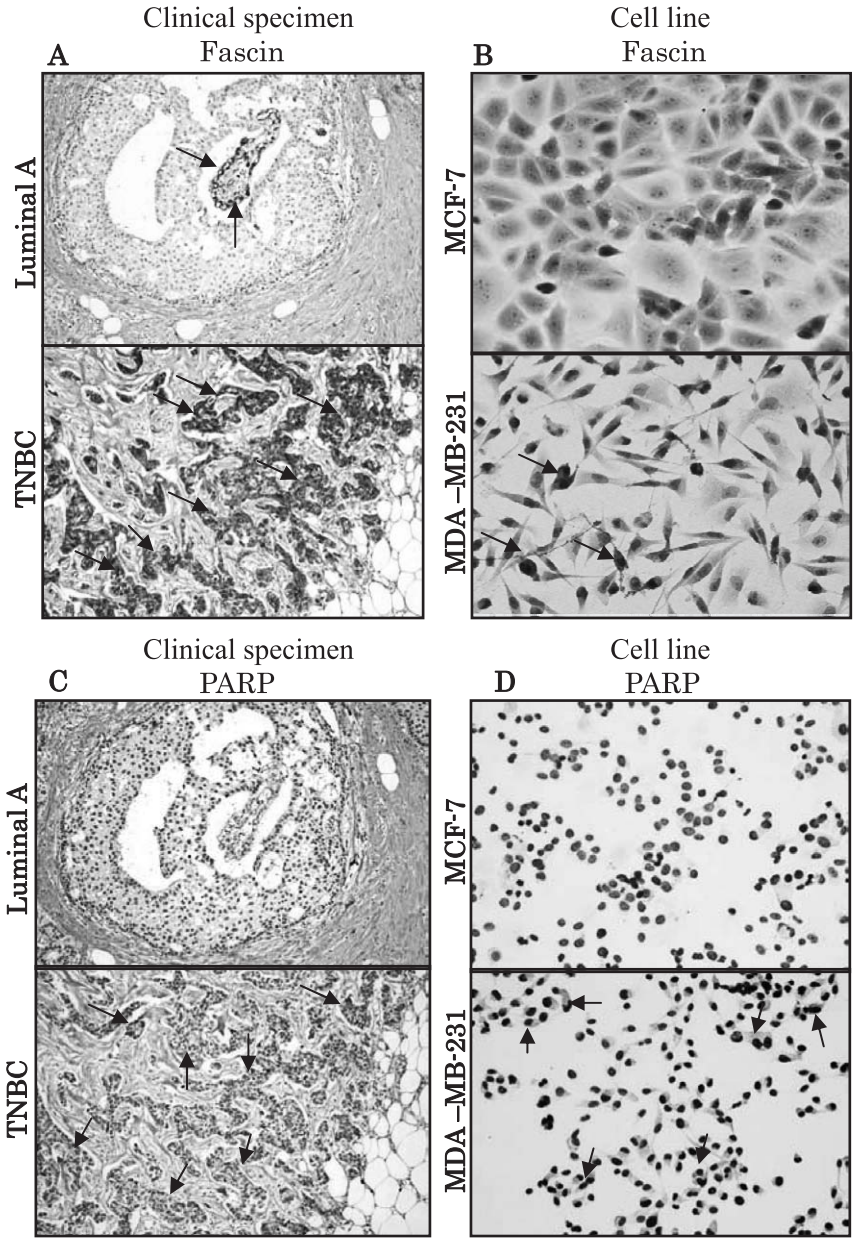

Fig. 3. Immunohistochemical staining of Fascin and PARP in TNBC (MDA-MB-231) and luminal A breast cancer tissue samples. (A) Immunohistochemistry of clinical specimens showing positive Fascin staining. (B) Immunohistochemistry of cell lines showing weak cytoplasmic positivity for fascin (brown) in the MDA-MB-231 cells. (C) Immunohistochemistry of clinical specimens showing positive PARP staining. (D) Immunohistochemistry of cell lines showing weak cytoplasmic positivity for PARP (brown) in the MDA-MB-231 cells. Images were taken at 200 × magnification.

Cell lines

The MDA-MB-231 cells showed a positive cytoplasmic signal for Fascin immunostaining (positive rate 40%, score 2) and positive nuclear PARP expression (positive rate 60%, score 2).

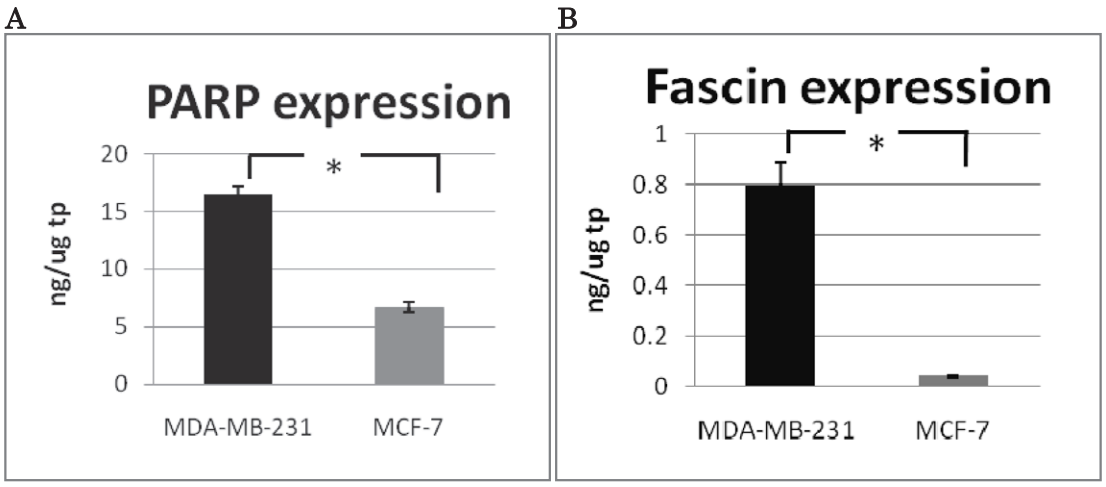

Fig. 4. Increased levels of (A) PARP and (B) Fascin protein in MDA-MR-231 and MCF-7 cells as determined by ELISA. $^{*}P < 0.05$

In contrast, MCF-7 cells were immunonegative for both signals (Fig. 3D).

ELISA measurements on cell lines

By ELISA, PARP expression levels were 16.4 ng/μg protein in the MDA-MB-231 cells and 6.68 ng/μg protein in the MCF-7 cells ($P < 0.001$), while Fascin expression levels were 0.8 ng/μg protein in the MDA-MB-231 cells and 0.04 ng/μg protein in the MCF-7 cells ($P = 0.0002$) (Fig. 4).

Discussion

In recent years, various reports focused on the relationship between breast cancer and miRNAs, which are small non-coding RNAs of 20 to 25 nucleotides that bind to the 3'terminal untranslated regions of mRNAs and thereby inhibit their translation into proteins. By the methods of literature search, aberrant expressed miRNAs were collected. In MCF-7 and MDA-MB-231 cell lines, miR-339-5p is involved with metastasis and cellular infiltration by breast cancer cells, while miR-125b, miR-182, and miR-183 have been proposed as factors in anti-oncogenesis and anti-metastasis mechanisms[7]. Additionally, in a chemotherapy trial using mice, concurrent use of miR-145 and anticancer-drug 5-FU enhanced the anticancer effect of either anticancer drug used alone[8].

In this study, we analyzed miRNA expression in luminal A breast cancer and TNBC using the relevant cultured cells and clinical specimens. TNBC accounts for 15%～20% of breast cancer cases and carries a poor prognosis; however, there is no molecular target established for these ER/PgR/HER (−) tumors. Distant metastasis to soft tissues and brain is also common, contributing to the low survival rate in patients with TNBC. Elucidation of molecular markers for TNBC is therefore critically needed to improve patient outcomes[9].

MiR-145 controls the expression of fascin-1, c-myc, SMAD2/3, and IGF1R, all of which are proteins controlling tumor growth factors in breast cancer cell lines and breast cancer tissue[10, 11].

In this current study, expression of miR-145 as indicated by the miRNA levels was 30.59 in Luminal A breast cancer samples and 18.92 in MCF-7 cell lines. However, TNBC tissue and the MDA-MB-231 cell line showed no miR-145 expression. Fascin-1 protein is an actin-binding protein expressed specifically in human neurons (both during embryonic development and into maturity), follicular dendritic cells in lymphoid tissues, the stratum basale in the epidermis, and mesenchymal and vascular endothelial cells[12, 13]. Furthermore, Fascin expression patterns are similar in the embryo to those encountered during the onset of cancer in tissues. Fascin expression was demonstrated in the highly malignant and invasive tumors, endocervical adenocarcinoma, intraoral melanoma, and cancer of the uterus or lung, and it is currently used as a prognostic marker in patients with oral squamous cell carcinoma[14]. In a mouse knockdown model, miR-145 adjusts the migration of breast cancer cells, hence it may be useful as a marker for tumor invasiveness and also as an antimetastatic breast cancer treatment[11]. In the current study, there were higher levels of miR-145 expression in the luminal A breast cancer clinical specimens and cultured cell linesthan in the TNBC equivalents, while the expression of fascin protein was controlled in the MCF-7 cell line. However, despite miR-145 not being expressed in the clinical specimens of TNBC, Fascin protein was expressed at a higher level in MDA-MB-231 cells than in MCF-7 cells. While miRNA controls protein expression, the expression of miR-145 was not associated with the regulation of Fascin expression in TNBC in this study. Immunostaining for Fascin in the clinical specimens of TNBC showed a diffuse cytoplasmic signal throughout 80% of the tissue. In contrast, the luminal A clinical samples showed cytoplasmic Fascin expression across only 10% of the whole tissue. Thus, there was high Fascin expression in tissues with low miR-145 expression, suggesting miR-145 and fascin protein expression may be useful together as a prognostic marker in TNBC.

Breast cancer patients have shown many mutations of the BRCA1 gene, which is involved in DNA repair. The enzyme PARP repairs damaged DNA in TNBC by adding ADP-ribose residues to the protein posttranslationally using NAD as a substrate. There are 17 different types of PARP enzyme, and poly-ADP-ribose (PAR) glycohydrolase (PARG) is one that restricts the length of the PAR polymer and repairs DNA[15]. PARP inhibitors cause a decrease in PARP activity, by combining with, and stabilizing, the truncated DNA termination. Therefore, PARP may also be a useful molecular target in the treatment of TNBC, and indeed, Phase Ⅲ clinical trials are currently underway with PARP inhibitors[16, 17]. An miRNA that controls PARP expression is miR-182[18], thus we also analyzed the relationship between PARP expression and miR-182 levels in clinical specimens and cultured cells in this study. PARP protein expression in MCF-7 cells was significantly lower than that in MDA-MB-231 cells, while miR-182 expression also showed the highest expression in the clinical specimens of luminal A breast cancer tissue and MCF-7 cells. Immunostaining showed PARP expression surrounding the nuclear membrane of carcinoma cells across 20% of the whole tissue in the clinical specimens ; however, it was not expressed by MCF-7 cells. Moreover, although the PARP expression in the MDA-MB-231 cell line was significantly higher than in MCF-7 cells, miR-182 expression in MDA-MB-231 cells was lower than in MCF-7 cells, and miR-182 was not expressed in the luminal A clinical specimens.

Immunostaining using PARP antibody showed expression in 70% of the nuclear membrane of carcinoma cells in the whole tissue as well as in MDA-MB-231 cells.

Since miRNA resides in an exosome of the cell membrane, it is stable in blood. Furthermore, miRNA is stable even in hot or acid conditions, or with freeze thawing[19]. Hence, miRNA is convenient for study and for use in clinical applications. The relevance of miR-145 and Fascin protein expression as well as miR-182 and PARP protein expression in TNBC is apparent from this study, further confirming that miR-145 and miR-182 may be useful novel molecular targets in TNBC.

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