p62 Regulates the Proliferation of Molecular Apocrine Breast Cancer Cells

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p62, also called sequestosome 1 (SQSTM1), is a multifunctional signaling molecule that affects cell proliferation. Recently, we found accumulation of p62 in apocrine carcinoma of the breast, however, the biological role of p62 expression in apocrine carcinoma still remains unclear. To investigate whether p62 might contribute to tumor cell proliferation in apocrine carcinomas, we used the MDA-MB-453 (androgen receptor-positive, HER2-type) and MFM223 (androgen receptor-positive, triple-negative type) breast cancer cell lines as models of molecular apocrine carcinoma. Both MDA-MB-453 and MFM223 showed strong and d high p62 protein expression than MCF7 cells (androgen receptor-negative, luminal A type). Knockdown of p62 resulted in significant reduction of the cell proliferative activity in both MDA-MB-453 ($P<0.01$) and MFM223 ($P<0.05$). In conclusion, p62 could contribute to cell proliferation and represent a therapeutic target in apocrine carcinoma.

Key words: p62, apocrine carcinoma, breast cancer

I. Introduction

Apocrine carcinoma of the breast is conventionally categorized as a special type of breast carcinoma because of its characteristic morphological features such as enlarged nuclei with prominent nucleoli and eosinophilic cytoplasm [11]. Farmer *et al.* proposed an identical cluster to molecular apocrine breast cancer that was characterized by androgen receptor (AR) gene expression in basal-like tumors [4]. In regard to the hormone receptor status, immunohistochemical studies have shown that apocrine carcinomas are predominantly estrogen receptor (ER)-negative and androgen receptor (AR)-positive [18], however, occasionally, ER-positive/AR-positive or ER-negative/AR-negative may also be encountered [17]. According to the current strategies for breast cancer treatment, conventional chemotherapy is available for ER-negative HER2-negative carcinoma, to which category most apocrine carcinomas belong, although AR is one of the candidate molecules for targeted therapy.

Recently, we found that overexpression of PGC1α and accumulation of p62 occurred significantly more frequently in human apocrine carcinomas than in non-apocrine carcinomas [5]. p62 is a multifunctional signaling molecule that affects cell proliferation. Several cancers, such as liver and lung cancers, are reported to show accumulation of p62, which is related to cancer cell proliferation [7, 8]. p62 expression was also investigated in some breast cancer cell lines [21], however, it remains unclear whether p62 expression might affect cell proliferation in apocrine carcinoma of breast cell lines.

To investigate whether p62 might contribute to tumor cell proliferation in apocrine carcinomas, we used the MDA-MB-453 and MFM223 breast cancer cell lines expressing AR, but not ER, as models of molecular apocrine carcinoma and performed p62 knockdown studies using siRNA.

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Table 1. *Antibodies used for the immunocytochemical analysis*

Antigen	Clone	Animal	Company	Dilution	Antigen retrieval
ER	1D5	Mouse	DAKO	×400	AC (pH 9.0)
HER2	polyclonal	Rabbit	DAKO	RTU (Hercep Test II Kit)	Boil (CB)
AR	AR441	Mouse	DAKO	×100	Boil (pH 9.0)
SQSTM1/p62	polyclonal	Mouse	Abcam	×2000	AC (CB)

AC, autoclave; AR, androgen receptor; CB, citrate buffer (pH 6.0); ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; RTU, ready to use.

II. Materials and Methods

Immunocytochemical analyses of MCF7, MDA-MB-453 and MFM223

MCF7, MDA-MB-453 and MFM223 (DS Pharma Biomedical, Osaka, Japan) were cultured in DMEM (Life Technologies, Carlsbad, CA, USA), L-15 (Life Technologies) and MEM (Life Technologies), respectively, containing 10% FBS (SAFC Biosciences, KS, USA), 100 U/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies). Immunocytochemical staining for ER, human epidermal growth factor receptor 2 (HER2), AR and p62 was performed on the cell blocks using the fibrin clot method (Table 1). The secondary antibodies used were as follows; Histofine LSAB-PO Multi (Nichirei, Tokyo, Japan) for p62, Histofine Simple Stain MAX PO (Nichirei) for ER and AR. 3,3'-diaminobenzidine was used as the chromogen. Counterstaining was performed with hematoxylin. The following positive controls were used: hepatocyte with Mallory body for p62, invasive ductal carcinoma cells for ER and HER2, prostate gland for AR. For the negative controls, treatment with the primary antibodies was omitted. Hematoxylin-eosin staining was also performed. The immunocytochemical staining results for ER and AR were assessed by estimation of the Allred score and total score (TS); a score of ≥3 was defined as positive [1]. HER 2 positivity was defined using the ASCO/CAP guidelines [22].

Protein expression analysis of p62 in MCF7, MDA-MB-453 and MFM223

Cell lysates were prepared in 3 wells from a 24-well plate using sodium dodecyl sulfate (SDS) buffer. Each protein expressions of p62 were measured by western blot analysis, as described below.

Transfection of siRNA for p62 in MDA-B-453 and MFM223 cells

MDA-MB-453 and MFM223 cells were seeded at 4×10^4 cells per well in 24-well plates and 6×10^3 cells per well in 96-well black plates 24 hr before the siRNA transfection experiments. MDA-MB-453 and MFM223 were transfected with siRNA specific for p62 (siGENOME SMART pool, M-010230-00-0005, Life Technologies) in 24- and 96-well plates, according to the manufacturer's transfection protocol. siGENOME Non-Targeting siRNA

(D-001210-01-05, Life Technologies) was used as the non-targeting siRNA control (NTC). The final siRNA concentration was 10 nM. Twenty-four hours after the transfection, the transfection medium was changed to each complete medium, followed by culture for another 48 hr. The efficiency of the siRNA treatment was measured by quantitative RT-PCR analysis and western blot analysis, as described below.

Quantitative RT-PCR analysis for p62 mRNA in culture cell

Total RNA was extracted with Trizol reagent (Life Technologies) using two wells of a 24-well plate. cDNA synthesis was performed using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Tokyo, Japan), according to the manufacturer's protocol. RT-PCR analysis was performed in the ABI StepOnePlus Real-Time PCR System (Life Technologies) using a 96-well tray with 10 μl of a reaction mixture comprising 5 μl TaqMan Fast Advanced Master Mix (Life Technologies), 0.5 μl each of the TaqMan probes (p62: Hs00177654_m1, β-actin: Hs01060665_g1), 3.5 μl of double-distilled water and 10 ng of the cDNA template. PCR was performed by preheating at 95°C for 10 min, followed by 45 cycles of 95°C for 1 sec and 60°C for 20 sec. Each experiment was performed in duplicate. Relative quantitation was performed by the ΔΔCt method and the expression level of p62 mRNA was normalized to the β-actin expression level.

Western blot analysis for p62 protein in culture cell

siRNA-transfected cell lysates were prepared in 3 wells from a 24-well plate using SDS and electrophoresed on an SDS–10% polyacrylamide gel. The separated proteins were transferred to a nitrocellulose membrane using the iBlot Dry Blotting System (Life Technologies). p62 and β-actin protein were detected by the iBind Western System (Life Technologies) according to the manufacturer's protocol. The first antibodies used were as follows: 0.5 μg/ml of mouse monoclonal anti-p62 antibody (clone 2C11, Abcam) and 0.1 μg/ml of mouse monoclonal anti-β-actin antibody (clone 8226, Abcam). The second antibody used was 2 μl/ml of horseradish peroxidase-conjugated anti-mouse IgG (Dako, Glostrup, Denmark). The chemiluminescence was detected with Light-Capture (Atto, Tokyo, Japan) and quantified by the CS Analyzer (Atto). The signal intensity of the p62 protein band was normalized to the β-actin expression level.
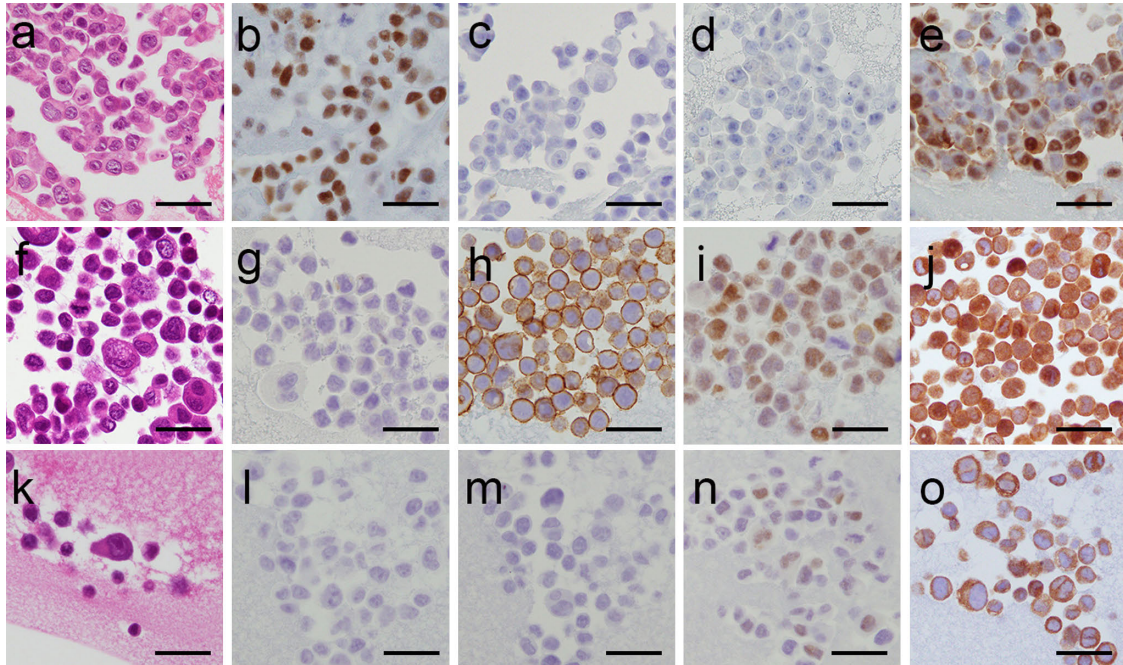
Fig. 1. Immunocytochemical examination of MCF7 (**a–e**), MDA-MB-453 (**f–j**) and MFM223 (**k–o**) cells using cell blocks. (**a, f, k**) HE staining. (**b, g, l**) immunocytochemical staining for ER. (**c, h, m**) immunocytochemical staining for HER2. (**d, i, n**) immunocytochemical staining for AR. (**e, j, o**) immunocytochemical staining for p62. Original magnification 400×. Bars=50 μm.

Cell proliferation assay

BrdU incorporation assay was carried out in the cells grown in 96-well black plates using Cell Proliferation ELISA, BrdU, chemiluminescence (Roche, Indianapolis, USA), in accordance with the manufacturer's instructions. The reaction products were quantified using a microplate reader (Tecan infinite 200, Tecan Japan, Kanagawa, Japan) from measurements replicated ten times. BrdU incorporation was estimated by mean relative light units/seconds ±standard error (SE).

Statistical analysis

The p62 mRNA level expression and BrdU assay values were analyzed using the Mann-Whitney U test. Statistical analysis was performed using Stat Mate III (ATMS, Tokyo, Japan). P values of less than 0.05 were considered as denoting statistical significance.

III. Results

Hormonal and p62 characteristics of the MCF7, MDA-MB-453 and MFM223 cells

Immunocytochemical examination confirmed that the MCF7 cells were ER-positive, HER2-negative and AR-negative, MDA-MB-453 cells were ER-negative, HER2-positive and AR-positive, and the MFM223 cells were ER-negative, HER2-negative and AR-positive (Fig. 1b–d, g–i, l–n). Half of the MCF7 cells showed positive staining for p62, while MDA-MB-453 and MFM223 cells showed diffuse positive staining for p62 (Fig. 1e, j, o).

Protein expression of p62 analyses of MCF7, MDA-MB-453 and MFM223

The p62 protein expressions of MDA-MB-453 and MFM223 were higher than that of MCF7 (0.81, 0.64 and 0.45, respectively, Fig. 2b).

### Knockdown of p62 mRNA in the cultured cells

Next, the MDA-MB-453 and MFM223 breast cancer cells were transfected with siRNA reagents targeting p62, or NTC as control. Twenty-four hours after transfection with p62 siRNA, the amount of p62 mRNA in the MDA-MB-453 and MFM223 cells was found to be significantly decreased as compared to that in the NTC cells (Fig. 3a). The protein expression levels were also decreased in both MDA-MB-453 and MFM223 cells transfected with p62 siRNA as compared to those in the NTC cells (Fig. 3b, c).

### Cell proliferation assay in the cultured p62-knockdown cells

We examined the cell growth of both MDA-MB-453 and MFM223 cells by the BrdU incorporation assay. Both MDA-MB-453 and MFM223 cells transfected with p62 siRNA showed significantly reduced cell proliferative activity as compared to NTC cells ($P<0.01$ and $P<0.05$ in the two cell lines, respectively) (Fig. 4a, b).

IV. Discussion

Based on the results of microarray studies using human breast cancer tissues, Farmer *et al.* defined molecular apocrine breast tumors as being characterized by AR
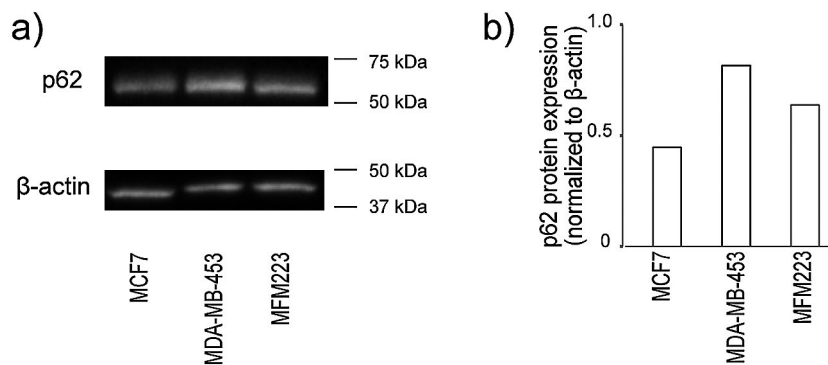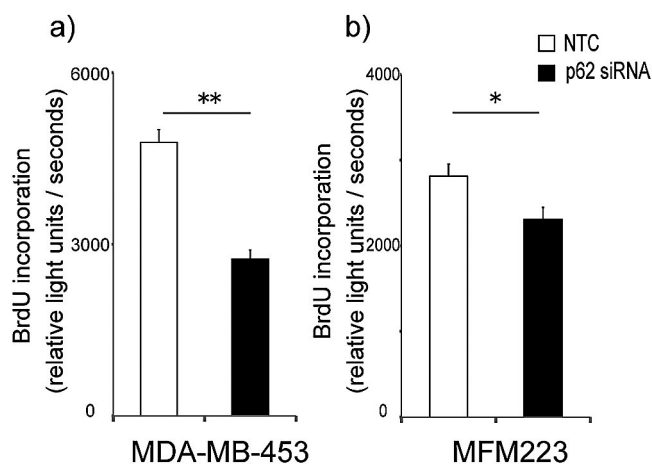
Fig. 2. (**a**) p62 protein expression of MCF7, MDA-MB-453 and MFM223 were detected by Western blot analysis. (**b**) p62 protein was quantified by densitometry with standardization to the β-actin expression.
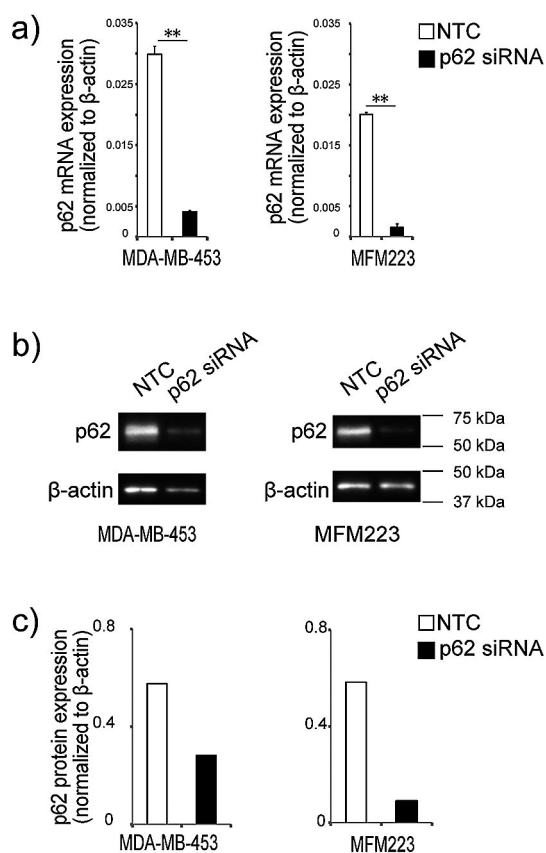
Fig. 3. Knockdown of p62 using the siRNA transfection method in MDA-MB-453 and MFM223 cells. (**a**) The p62 mRNA expression level standardized to the β-actin expression level is shown. (**b**) p62 protein was detected by Western blot analysis, and quantified by densitometry with standardization to the β-actin expression (**c**). Statistical significance was evaluated by the Mann-Whitney U test. **; $P<0.01$. NTC; non-targeting control.

Fig. 4. Cell proliferation assay in p62-knockdown culture cells. Cell proliferation of MDA-MB-453 (**a**) and MFM223 (**b**) cells were examined by the BrdU incorporation assay. Data represent mean relative light units/seconds±standard error (SE). Statistical significance was evaluated by the Mann-Whitney U test. *; $P<0.05$, **; $P<0.01$. NTC; non-targeting control.

gene expression [4]. MDA-MB-453, MFM223, SKBR3, SUM190 and SUM225 are considered as molecular apocrine cell models [3, 6].

p62, also called sequestosome 1 (SQSTM1), is a multifunctional signaling molecule affecting cell prolife-

ration that is selectively disaggregated by autophagy [9, 10, 14, 16]. p62 overexpression leads to activation of NFkB through the TRAF6 signaling pathway or Nrf2/KEAP1 signaling pathway, resulting in cancer cell growth [9, 10, 15]. We previously showed that overexpression of p62 was significantly higher in human apocrine carcinomas than in non-apocrine carcinomas [5]. We selected two molecular apocrine cell lines, MDA-MB-453 (AR-positive, HER2-type) and MFM223 (AR-positive, triple-negative type), which show different expression of HER2 [20] and also used MCF7 cells as non-apocrine carcinoma control. Our immunocytochemical study showed that MDA-MB-453 and MFM223 cells showed strong and diffuse expression of p62 than MCF7 cells. The quantity of p62 protein of MDA-MB-453 and MFM223 cells was higher than that of the MCF7 cells in western blot analysis. While the expression of p62 has been examined in several breast cancer cell lines such as MDA-MB-231, MCF7, BT-20, HCC1428 and

HCC1806 [19, 21], no studies have focused on the role of p62 expression in apocrine carcinoma cells. This is the first report showing p62 expression and the effect of p62 knock-down in the molecular apocrine cancer cell lines MDA-MB-453 and MFM223. Several cancers have been reported to show accumulation of p62, which is known to be involved in the proliferation of cancer cells [7, 13]. Recently, Li *et al.* suggested the possibility of p62 as an oncogene in a renal cancer cell line [12]. These observations have led to the speculation of p62 as one of the possible candidate therapeutic targets in breast carcinoma.

It is intriguing that the inhibitory effect p62 knock-down on cell proliferation was more prominent in MDA-MB-453, which was AR-positive and HER2-positive, than in MFM223, which was AR-positive and triple-negative. It was reported that p62 facilitates tumorigenesis in HER2-expressing breast cancer cells via the PTEN/PI3K/Akt axis, WNT/β-catenin signaling pathway, NFkB pathway, and NRF2/KEAP1 axis [2]. We observed that HER2-positive human carcinomas showed significantly higher mRNA expression levels of p62 than HER2-negative carcinomas (unpublished data). One possible explanation for the different reaction of the two cell lines to p62 inhibition is that MDA-MB-453 shows HER2 expression, suggesting that the cell growth in this cell line may be more dependent on multiple signaling pathways related to p62.

In summary, the present study demonstrated the high p62 protein expression level of the cultured molecular apocrine carcinoma cells, MDA-MB-453 and MFM223, and speculated that p62 could contribute to cell proliferation in apocrine carcinomas of the breast.

V. Acknowledgments

VI. References

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