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Alpha-crystallin B chains enhance cell migration in basal-like 2 triple-negative breast cancer cells

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Triple-negative breast cancer (TNBC) can be divided into six subtypes. Among these subtypes, the basal-like 2 (BL2) subtype shows the lowest five-year survival rate and highest risk of metastasis. Alpha-crystallin B chains (αB-crystallin), a small heat shock protein that is known to be involved in breast cancer metastasis, is highly expressed in the basal-like subtype but not in the other non-basal subtypes. Thus, we hypothesized that αB-crystallin may be an important factor involved in the worse prognosis of the BL2 subtype compared with those of the other TNBC subtypes. Here, we examined the role of αB-crystallin in cell motility in two TNBC cell lines: HCC1806 (BL2 subtype) and, as control, MDA-MB-436 (mesenchymal stem-like subtype). HCC1806 showed greater cell migration capacity and a higher expression level of the gene encoding αB-crystallin (*CRYAB*) than did MDA-MB-436. Short interfering RNA–mediated silencing of *CRYAB* expression significantly reduced the cell migration capacity of HCC1806 cells, whereas it had no effect in MDA-MB-436 cells, indicating that αB-crystallin is essential for the migration of HCC1806 cells. Thus, high αB-crystallin expression may be a contributing factor to the poor prognosis of BL2 TNBC.

1. Introduction

Triple-negative breast cancer (TNBC) accounts for 15%–20% of all breast cancer cases. It is characterized by a lack of expression of hormone receptors and human epidermal growth factor receptor-2, which reduces its sensitivity to endocrine treatment and targeted therapies (Dent et al. 2007). This means there are few effective therapies currently available for the treatment of TNBC. Thus, compared with other types of breast cancer, TNBC patients show a shorter survival time, higher rate of mortality within five years after diagnosis, and higher distant metastasis rate (Dent et al. 2007; Lin et al. 2008). TNBC can be divided into six subtypes, with each subtype showing a different survival rate and different sensitivities to chemotherapy (Lehmann et al. 2011). Among these subtypes, patients with the basal-like 2 (BL2) subtype show the lowest five-year survival rate, whereas those with the mesenchymal stem-like (MSL) subtype show the highest five-year survival rate (Won and Spruck 2020). In addition, the BL2 subtype shows the shortest disease-free time and has the highest risk of metastasis (Masuda et al. 2013).

Alpha-crystallin B chains (αB-crystallin) is a small heat shock protein that is involved in a variety of cellular processes including cytoskeletal stabilization (Fittipaldi et al. 2015; Muchowski et al. 1999), the unfolded protein response (Ruan et al. 2011), and apoptosis (Dou et al. 2012; Kim et al. 2020). In addition, it is known to be associated with brain metastasis from clinical analyses of TNBC breast cancer patients (Malin et al. 2014), and its role of promotion metastasis is also proved in *in vivo* TNBC models (Voduc et al. 2015). However, the role of αB-crystallin in subtypes of TNBC is barely distinguished. In this regard, αB-crystallin is more highly expressed in the basal-like TNBC subtypes than in the other subtypes (Koletsa et al. 2014; Moyano et al. 2006). Thus, given the high risk of metastasis associated with the BL2 subtype, we hypothesized that αB-crystallin may play a role in the worse prognosis of the BL2 subtype compared with those of the other TNBC subtypes.

Here, we evaluated the difference of cell motility between HCC1806 cells (BL2 subtype) and, as a comparison, MDA-MB-436 cells (MSL subtype) to assess the malignant role of αB-crystallin in the BL2 subtype of TNBC.

2. Investigations, results and discussion

First, we used a wound healing assay to examine the difference in cell migration capacity between HCC1806 and MDA-MB-436 cells. In this assay, cell layers at confluence were damaged by scratching with a sterile micropipette tip; photomicrographs of the cell layers were recorded after scratching and relative wound healing area was calculated from the images. At 24 h after scratching, the relative

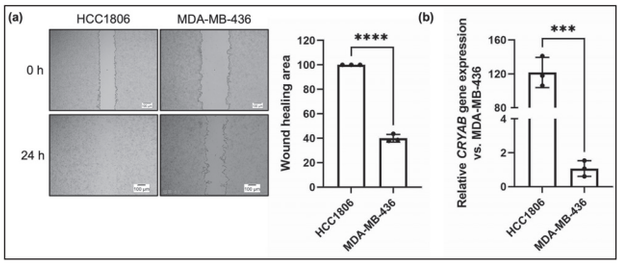

Fig. 1: HCC1806 cells show higher motility and a higher *CRYAB* expression level compared with MDA-MB-436 cells. (a) Cells were seeded in 24-well plates. After confluence was reached, a scratch was made in the cell monolayer. Images of the wound area at the beginning and end of a 24-h incubation period were recorded and the ImageJ software (National Institutes of Health) was used to calculate the relative healing area. (b) Total RNA was collected from HCC1806 and MDA-MB-436 cells, and the expression of *CRYAB* was quantified by real-time reverse-transcription polymerase chain reaction. *GAPDH* was used as the loading control. Data are presented as mean ± 1 S.D., n = 3, ***$P < 0.001$, ****$P < 0.0001$ by Student's *t*-test. Results are representative of two independent experiments.

wound healing area of the HCC1806 cells was significantly larger than that of the MDA-MB-436 cells, indicating that HCC1806 cells have a higher cell migration capacity than do MDA-MB-436 cells (Fig. 1a). When we examined the expression of *CRYAB*, the gene encoding αB-crystallin, in the two cell lines by means of real-time reverse-transcription polymerase chain reaction (real-time RT-PCR), the expression of *CRYAB* in HCC1806 cells was higher than that in MDA-MB-436 cells (Fig. 1b). Despite the 120 times higher mRNA expression in HCC1806 cells, their cell motility of is about 2.5 times greater than MDA-MB-436 cells. Although the precise mechanisms of αB-crystallin in cell migration have not been clarified, the ability of αB-crystallin to interact with intermediate filaments and microtubules for stabilization (Malin et al. 2016) suggests that the amount of *CRYAB* is not the only essential factor of cell motility in HCC1806. Thus, we consider that it is necessary to investigate the interaction between αB-crystallin and cell microtubule cytoskeleton for understanding the relationship between cell motility and expression level of *CRYAB*.

Next, to examine whether *CRYAB* contributes to cell migration, we used small interfering RNA (siRNA) to knockdown *CRYAB*. In both cell lines, we confirmed that *CRYAB* expression was significantly suppressed in cells treated with si*CRYAB* compared with that in cells treated with a siRNA negative control (Fig. 2a). When we repeated the wound healing assay in the cell lines after treatment with the two siRNAs, we found that the relative healing area of the HCC1806 cells was significantly inhibited in cells treated with si*CRYAB* compared with that in cells treated with the siRNA negative control (Fig. 2b). In contrast, although no significant difference was observed, knockdown of *CRYAB* also showed a decreasing tendency in MDA-MB-436 cells (Fig. 2b). It is probable that the originally low expression level in MDA-MB-436 cells reduced the effect of knockdown, and as a result, did not lead to a significant difference.

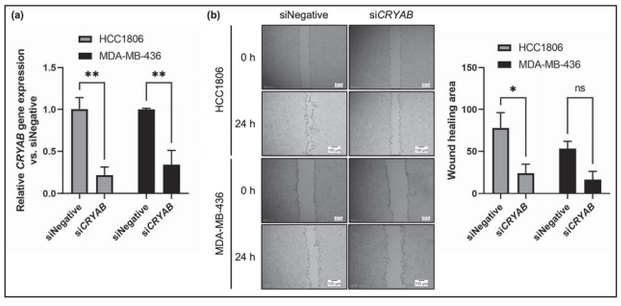

Fig. 2: *CRYAB* expression is needed for cell migration in HCC1806 cells. (a) After treatment with short interfering (si) RNA for 48 h, total RNA was collected from HCC1806 and MDA-MB-436 cells. The expression of *CRYAB* was quantified by real-time reverse-transcription polymerase chain reaction. *GAPDH* was used as the loading control. (b) Cells treated with siRNA were seeded in 24-well plates, and a scratch was made in the cell monolayer after reaching confluence. Images of the wound area at the beginning and end of a 24-h incubation period were recorded and the ImageJ software (National Institutes of Health) was used to calculate the relative healing area. Data are presented as mean ± 1 S.D., n = 3, $*P < 0.05$, $**P < 0.01$ by one-way ANOVA with post-hoc Tukey's honestly significant difference test. ns, not significant. Results are representative of two independent experiments.

This needs to be widely verified by comparing other BL2 cell lines with the other subtypes of TNBC cell lines, however, these findings indicate that high expression levels of *CRYAB* lead to a high cell motility in HCC1806 cells, BL2 subtypes. Considering that among the six subtypes of TNBC, patients with the BL2 subtype show a poor prognosis and the lowest pathologic complete response rate (Masuda et al. 2013), a high expression level of *CRYAB* in BL2 subtypes may increase the risk of metastasis and may thus be a potential drug target of inhibition cell motility.

To be able to use αB-crystallin as a drug target, it will be important to elucidate the molecular mechanism underlying αB-crystallin–mediated cell motility. Although the metastatic role of αB-crystallin has been explored in clinical studies (Koletsa et al. 2014; Voduc et al. 2015) and in an orthotopic TNBC model (Malin et al. 2014), the molecular interactions of αB-crystallin within cells remain to be examined. The motility of TNBC cells is induced via the epithelial–mesenchymal transition (Petruk et al. 2021), and the epithelial-mesenchymal transition of TNBC cells requires unfolded protein signaling (Feng et al. 2014; Santamaría et al. 2019). αB-Crystallin is an effector of the unfolded protein response in endothelial cells (Ruan et al. 2011). Therefore, studies to explore the epithelial-mesenchymal transition markers and cytoskeleton-related proteins expressed by the various TNBC subtypes are needed to elucidate the involvement of αB-crystallin in TNBC cell migration.

Together, the present findings show that *CRYAB* is highly expressed in HCC1806 cells and that expression of this gene is essential for cell migration in HCC1806 cells. This suggests that *CRYAB* could be an important factor underlying the worse prognosis associated with the BL2 subtype of TNBC.

3. Experimental

3.1. Cell lines and cell cultures

Two breast adenocarcinoma cell lines-HCC1806 and MDA-MB-436-were purchased from the American Type Culture Collection (Manassas, VA, USA). HCC1806 cells were cultured in RPMI-1640 (Wako, Osaka, Japan) and MDA-MB-436 cells were cultured in D-MEM (Wako). Both culture media were supplemented with 10% (v/v) fetal bovine serum (Biosera, Nuaille, France) and 1% (v/v) penicillin-streptomycin-amphotericin B suspension (×100) (Wako Pure Chemical Industries, Osaka, Japan). Cells were maintained at 37 °C under 5% CO_2 and at >95% humidity.

3.2. Real-time RT-PCR analysis

Total RNA was extracted from cells by using a FastGene RNA Basic Kit (Nippon Genetics, Tokyo, Japan) and reverse transcribed to cDNA by using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). A PCR mixture containing the above cDNA as the template, primers for *CRYAB* (forward, 5′-GCACTTCTCCCCAGAGGAAC-3′; reverse, 5′-CCATTCACAGTGAGGACCCC-3′) and *GAPDH* (forward, 5′-GAAGGTGAAGGTCGGAGTC-3′; reverse, 5′-GAAGATGGTGATGGGATTTC-3′) (Eurofins Genomics, Tokyo, Japan), and GeneAce SYBR qPCR Mix α Low ROX (Nippon Genetics) was prepared, and real-time RT-PCR was performed using a CFX-384 system (Bio-Rad Laboratories, Hercules, CA, USA).

3.3. Transient transfection of small interfering RNA (siRNA)

Cells were transfected with 10 nM stealth siRNA against *CRYAB* (5′-CCCUCUCACCAUUACUUCAtt-3′ and 3′-UGAAGUAAUGGUGAGAGGGtc-5′) or stealth siRNA negative control with medium GC content (Invitrogen, Carlsbad, CA, USA). The transfection was executed by adding lipofectamine RNAiMAX transfection reagent (Invitrogen) to the cell culture medium for 48 h.

3.4. Wound healing assay

Cells were seeded at 1.5×10^5 cells/well without drugs in 24-well flat plates (Thermo Fisher Scientific) and incubated overnight. After incubation, a scratch was made in the cell layer by using a sterile micropipette tip. The cell layer was then washed with phosphate-buffered saline and incubated for a further 24 h in culture media without fetal bovine serum. Images of the scratch area were acquired at the beginning and end of the 24-h incubation period. The images were recorded via an all-in-one fluorescence microscope (IX71N-22, Olympus, Tokyo, Japan) and were analyzed by using ImageJ software (version 1.53k, National Institutes of Health; imagej.nih.gov/ij/index). The reduction rate of the scratch area was calculated as follows:

Relative healing area = (wound area after scratch − wound area after incubation) / (wound area after scratch) × 100%.

3.5. Statistical analysis

Statistical analyses were conducted using Graph Pad Prism Mac version 9.0 (GraphPad Software, La Jolla, CA; www.graphpad.com). Data are presented as mean ± 1 S.D. Student's *t*-test was used in Fig. 1. One-way analysis of variance (ANOVA) followed by post-hoc Tukey's honestly significant difference test was used to compare groups in Fig. 2.

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