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The single N-glycan deletion mutant of soluble ErbB3 protein attenuates heregulin β1-induced tumor progression by blocking of the HIF-1 and Nrf2 pathway

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ABSTRACT

It has been well documented that activation of the ErbB3–PI3K–Akt pathway is implicated in tumor survival and progression. We previously demonstrated that the single N-glycan deletion mutant of soluble ErbB3 protein (sErbB3 N418Q) attenuates heregulin β1-induced ErbB3 signaling. The active PI3K–Akt pathway augments the nuclear accumulation of hypoxia inducible factor (HIF)-1α, which activates the transcription of many target genes and drives cancer progression. In this study, we focused on the effects of sErbB3 N418Q mutant on nuclear accumulation of HIF-1α. Pretreatment with the sErbB3 N418Q mutant suppressed heregulin β1-induced HIF-1α activation in MCF7 cells. Similar results were also obtained in other breast cancer cell lines, T47D and BT474. Interestingly, these suppressive effects were not observed with the sErbB3 wild type. In addition, pretreatment with the sErbB3 N418Q mutant suppressed the cell migration of MCF7 cells induced by heregulin β1. Furthermore, incubation with heregulin β1 also induced the nuclear accumulation of Nrf2, and this effect was also reduced by the sErbB3 N418Q mutant, but not the sErbB3 wild type. These findings indicated that the sErbB3 N418Q mutant suppressed malignant formation of cancer cells by blocking of the HIF-1α and Nrf2 pathways.

1. Introduction

Many studies have reported that intratumoral hypoxia triggers the activation of HIF-1. As such, HIF-1 activates the transcription of many target genes in cancer cells and is associated with a risk of metastasis and poor prognosis during cancer progression [1–4]. On the other hand, in cancer cells, activation of the PI3–Akt pathway in response to the growth signal plays an important role in tumor formation and progression [5]. Recent studies have indicated that the active PI3K–Akt pathway also augments the nuclear accumulation of HIF-1α under normoxic conditions [6]. Furthermore, the transcription factor NF-E2-related factor-2 (Nrf2), a master regulator of antioxidant and cytoprotective genes in response to oxidative stress, which relates to tumor cell proliferative signals, are downstream of PI3/Akt signaling [7].

Heregulin is an ErbB3/ErbB4 ligand, and regulates cell proliferation, differentiation, migration, apoptosis and survival [8]. Recent studies have indicated that heregulin β1 is often expressed in breast cancer tissues and associated with poor prognosis [9]. In addition, heregulin β1 induces activation of ErbB3–PI3K–Akt signaling, which promotes the process of epithelial–mesenchymal transition (EMT) [10]. Therefore, the ErbB3–PI3K–Akt signaling pathway has been considered a potential therapeutic target of breast cancer.

We have previously found that the single N-glycan deletion mutant of soluble ErbB3 protein (sErbB3 N418Q) has more suppressive effects on the heregulin β1-induced ErbB3 signaling pathway than the sErbB3 wild type. In addition, the combination of sErbB3 N418Q mutant with anti-cancer drug *Lapatinib* shows a significant synergistic effect on the suppression of cell proliferation [11]. Consequently, in this study, we examined whether the sErbB3 N418Q mutant attenuates malignant phenotype by suppressing the downstream signaling of heregulin β1 in a human breast cancer cell line, MCF7 cells.

Abbreviations: HIF-1, hypoxia inducible factor-1; Nrf2, NF-E2-related factor-2; sErbB, soluble ErbB.

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2. Materials and methods

2.1. Cell culture

MCF7 cells (a human breast cancer cell line) were obtained form RIKEN BRC (Ibaraki, Japan) and cultured in DMEM, supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Nacalai Tesque, Kyoto, Japan). T47D cells and BT474 cells (a human ductal cancer cell line) were obtained form American Type Culture Collection (ATCC) and cultured in DMEM or RPMI1640, supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were incubated at 37 °C under a humidified atmosphere of 5% CO_2 in air.

2.2. Purification of recombinant sErbB3s

Purification procedures have been reported previously [11]. Briefly, recombinant WT and N418Q mutant sErbB3s were produced in Lec3.2.8.1 cells using the Flp-In System (Invitrogen). The expressed myc-His tagged sErbBs were purified by a series of column chromatography on His Trap HP5 (GE-Healthcare), Mono Q (GE-Healthcare), an anti-myc-His peptide antibody column, and HiLoad Superdex 200 pg (GE-Healthcare) using the AKTA protein purification systems (GE-Healthcare).

2.3. Heregulin β1 stimulation in vitro

MCF7 (4×10^5 cells/well DMEM containing 10% FBS) were grown in 6-well plates for 24 h followed by serum-starvation for 16 h prior to the stimulation by heregulin β1. MCF7 cells were incubated with or without sErbB3 (10 μg/ml) for 2 h, and then heregulin β1 (10 ng/ml, Millipore, Billerica, MA) was added into the indicated wells.

2.4. Preparation of nuclear extracts

Nuclear fractions were prepared by NE-PER nuclear and cytoplasmic extraction regents (Thermo scientific) following the manufacturer's instructions.

2.5. Western blot analysis

Cells were lysed in a cell lysis buffer (Cell Signaling Technology) with complete protease inhibitors (Roche). Protein extracts were subjected to 10% SDS–PAGE, transferred to PVDF membranes (Bio-Rad), and then probed with an anti-HIF-1α polyclonal antibody (1:1000; Novus Biologicals), an anti-Nrf2 polyclonal antibody (1:1000; H-300, Santa Cruz), an anti-pAkt polyclonal antibody (1:1000, Cell Signaling Technology), an anti-lamin B antibody (1:1000; C-20, Santa Cruz), and anti-β actin antibody (1:1000; Cell Signaling Technology). After incubation with a peroxidase-conjugated secondary antibody, immunoreactive bands were visualized using an enhanced chemiluminescence kit (BD Biosciences).

2.6. Wound healing assay

Wound healing and migration was assessed by ibidi's culture-inserts following the manufacturer's instructions (Ibidi, Martinsried, Germany). 70 μl/well MCF7 cell-suspension (2×10^5 cells/ml) was applied into each well and grown to confluence in a 10% FBS DMEM medium. After 16 h-serum starvation, the culture-insert was removed and filled with serum free medium, and then added heregulin β1. The cell migration was monitored at 24 h using a light microscope (Biozero, BZ8100, Keyence, Osaka, Japan). The gap distance was quantitatively evaluated using Adobe Photoshop CS6.

2.7. Statistical analysis

For comparison between more than two groups, and multiple comparisons, an ANOVA test was utilized by the Bonferroni multiple comparison test were used. The data were analyzed using GraphPad Prism 4 (GraphPad Software). Statistical significance was accepted at $P < 0.05$. The number of replicate samples per group (n) is specified in the figure legends.

3. Results

3.1. The sErbB3 N418Q mutant blocks heregulin β1-induced HIF-1α accumulation in MCF7 cells

We have previously demonstrated that of the sErbB3 N418Q mutant has ~2–3-fold increased suppressive effects on heregulin β1-induced ErbB3–PI3K–Akt signaling as compared with the sErbB3 wild type [11]. As agree with the former results, pretreatment of MCF7 cells with the sErbB3 N418Q mutant reduced heregulin β1-induced Akt phosphorylation more effectively, as compared the with the wild type (Fig. 1A). To determine whether sErbB3s suppressed the downstream signaling of Akt, we checked the nuclear accumulation of HIF-1α. As illustrated in the Western blotting analysis in Fig. 1B, 10 ng/ml heregulin β1 induced the nuclear accumulation of HIF-1α, which peaked at 6 h and was followed by a recovery to the control level at 12 h. We therefore determined the incubation time as 6 h. MCF7 cells were treated with the sErbB3s for 2 h and then incubated with heregulin β1

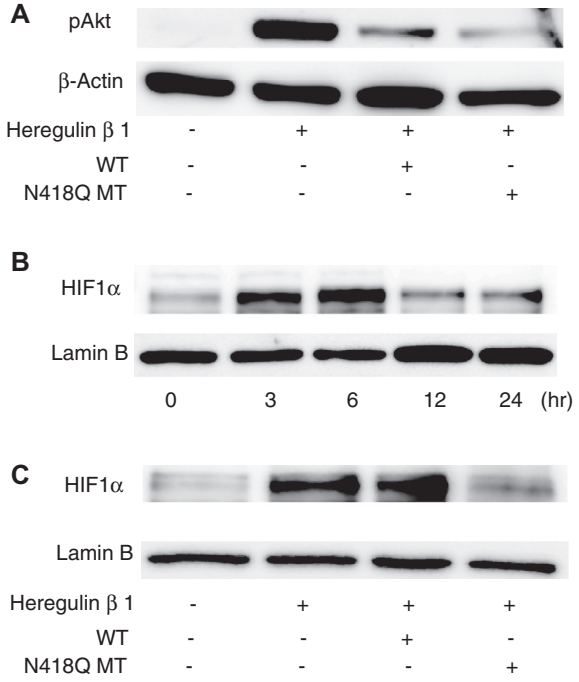

Fig. 1. The sErbB3 N418Q mutant inhibited HIF-1α accumulation induced by heregulin β1 in MCF7 cells. (A) MCF7 cells were serum-staved for 16 h, incubated with the wild type or the sErbB3 N418Q mutant for 2 h, and stimulated with 10 ng/ml heregulin β1 for 10 min. The cell lysates (10 μg of protein) was subjected to Western blot analysis. Data are representative of three experiments. (B) Time course of nuclear accumulation of HIF-1α stimulated by heregulin β1 in MCF7. After serum starved for 16 h, MCF7 cells were stimulated by heregulin β1 (10 ng/ml) for 4–24 h. HIF-1α in nuclear fractions (10 μg of protein) were subjected to Western blot analysis. The nuclear HIF-1α-accumulation reached a maximum level at 6 h. Data are representative of three experiments. (C) MCF7 cells were serum-staved for 16 h, incubated with the wild type or the sErbB3 N418Q mutant for 2 h, and stimulated with 10 ng/ml heregulin β for 6 h. Data are representative of three experiments.

for 6 h. Pretreatment of MCF7 cells with the sErbB3 N418Q mutant suppressed heregulin β1-induced HIF-1α activation. This suppressive effect was not observed in the sErbB3 WT, suggesting that the sErbB3 N418Q mutant, but not the sErbB3 wild type, can inhibit heregulin β1 induced-HIF-1α accumulation in MCF7 cells.

3.2. The sErbB3 N418Q mutant attenuates the nuclear accumulation of HIF-1α in T47D and BT474 cells

We next used T47D and BT474 cells in order to determine whether the sErbB3 N418Q mutant regulated HIF-1α accumulation in other type of breast cancer cells. Our recent study indicated that the sErbB3 N418Q mutant also suppressed heregulin β1-induced ErbB3–Akt signaling in T47D and BT474 cells [11]. As shown in Fig. 2, heregulin β1 also induced HIF-1α accumulation in T47D and BT474 cells, and that was attenuated by the pretreatment with the sErbB3 N418Q mutant, as observed in MCF7 cells.

3.3. The sErbB3 mutant N418Q suppressed heregulin β1–induced cell migration

A recent study has indicated that the heregulin β1 induced cancer cell activation through the ErbB3–PI3K–Akt pathway, leading to cell migration in MCF7 cells [9]. Furthermore, many studies have indicated that HIF-1α activation is related to cancer metastasis [3,12]. Therefore, the attenuation of HIF-1α activation by the sErbB3 N418Q mutant prompted us to examine whether the sErbB3 N418Q mutant could interfere with heregulin β1 induced cell migration. To check cancer cell migration activity, we performed a wound healing assay. As shown in Fig. 3, heregulin β1 promoted cell motility in MCF7 cells at 24 h, however pretreatment with the sErbB3 N418Q mutant significantly reduced cell motility. These data suggest that the sErbB3 N418Q mutant suppressed heregulin β1 induced-cancer cell migration probably through the inhibition effect of ErbB3–PI3K–Akt signaling and the HIF-1α activation pathway.

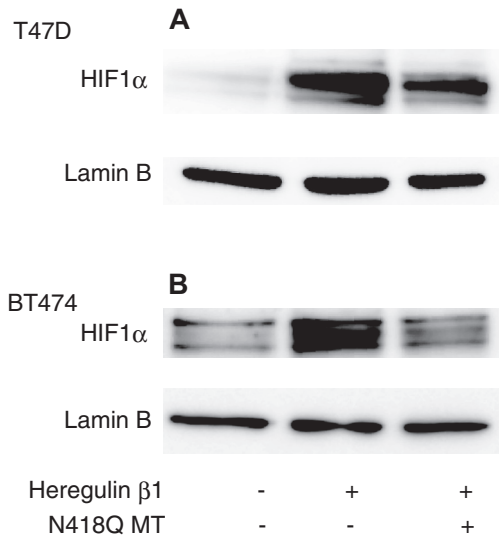

Fig. 2. The sErbB3 N418Q mutant suppressed heregulin β1-induced HIF-1α accumulation in T47D cells and BT474 cells. T47D (A) and BT474 (B) cells were serum-staved for 16 h, incubated with the N418Q sErbB3 mutant for 2 h, and stimulated with 10 ng/ml heregulin β1 for 6 h. HIF-1α in nuclear fractions (10 μg of protein) was analyzed by Western blot analysis. Data are representative of three experiments.

3.4. The sErbB3 N418Q mutant blocks heregulin β1-induced Nrf2 accumulation in MCF7 cells

Current studies indicated that ErbB2 mediated tumorigenesis is associated with the activation of Nrf2 [13], and the activation of PI3K–Akt pathway induces the nuclear accumulation of Nrf2 [7]. We examined the nuclear accumulation of Nrf2 in MCF7 cells, and the results indicated that it was reduced by the sErbB3 N418Q mutant, but not the sErbB3 wild type (Fig. 4). These findings suggested that the sErbB3 N418Q mutant also suppressed the activation of Nrf2 pathway.

4. Discussion

Heretofore, many studies have indicated that intratumoral hypoxia evokes the HIF-1α accumulation. Additionally, HIF-1α activates the transcription of hundreds of target genes in hypoxic cancer cells, which may underlie the malignant phenotype in tumors [3,12]. Furthermore, increased nuclear accumulation of HIF-1α is associated with tumor metastasis and poor prognosis in patients with breast cancer [1–3,14,15]. In addition, HIF-1α-levels are regulated by PI3K–Akt related-proliferation signals through ErbB receptors under non-hypoxic conditions [4–6,9,16]. Because the accumulation of HIF-1α promotes malignant potential in cancer cells regardless of the oxygen environments, we examined whether sErbB3 can suppress heregulin β1 induced-HIF-1α nuclear accumulation. Indeed, the sErbB3 N418Q mutant attenuated the nuclear accumulation of HIF-1α induced by heregulin β1 in breast cancer cell lines MCF7, T47D and BT474 (Figs. 1C and 2). These results suggest that the sErbB3 N418Q mutant may reduce ErbB3-driven tumor progression through the blockade of HIF-1α activation.

It is well known that HIF-1α is linked to the invasion and metastasis of cancer [3,12]. In addition, investigators have previously shown that heregulin β1 induces cancer cell migration through PI3K–Akt signaling [9]. As the sErbB3 N418Q mutant attenuates both transcriptional activity of HIF-1 and induced the PI3K–Akt signaling induced by heregulin β1, we examined whether the sErbB3 N418Q mutant inhibits heregulin β1 induced cell migration by performing a wound healing assay. Our data also identified that heregulin β1 induced MCF7 cell migration, and treatment of MCF7 cells with the sErbB3 N418Q mutant significantly inhibited heregulin β1 induced cell migration (Fig. 3). Migration of cancer cells is critical for tumor invasion and metastasis [17,18]. Thus, our results suggest that the sErbB3 N418Q mutant may have suppressive effects on the cell growth and the tumor malignancy.

Another transcription factor Nrf2, plays a central role in the cytoprotective response to oxidative stress. In addition, Nrf2 is expressed on various types of cancer including breast cancer, and its expression induces drug resistance and metabolic reprogramming [7], and the PI3K/Akt pathway is essential for the activation of Nrf2 [7,16,19]. As we expected, heregulin β1-induced the nuclear accumulation of Nrf2, and pre-incubation of the sErbB3 N418Q mutant reduced heregulin β1 induced-Nrf2 nuclear accumulation in MCF7 cells (Fig. 4). These results imply that the regulation of Nrf2 is another therapeutic target of interest in terms of the treatment of cancer.

Although, the sErbB3 wild type has approximately 25–50% suppressive effects of the sErbB3 mutant on the ErbB3–Akt signaling induced by heregulin β1 [11] (Fig. 1A), this suppressive effect failed to affect the nuclear accumulation of HIF-1α (Fig. 1B) and Nrf2 (Fig. 4). However, the sErbB3 N418Q mutant could inhibit the nuclear accumulation of HIF-1α and Nrf2. We assume that the difference of the extent of suppressive effects of the sErbB3 N418Q mutant and the wild type may be crucial in influencing to the nuclear accumulation of HIF-1α and Nrf2 induced by heregulin

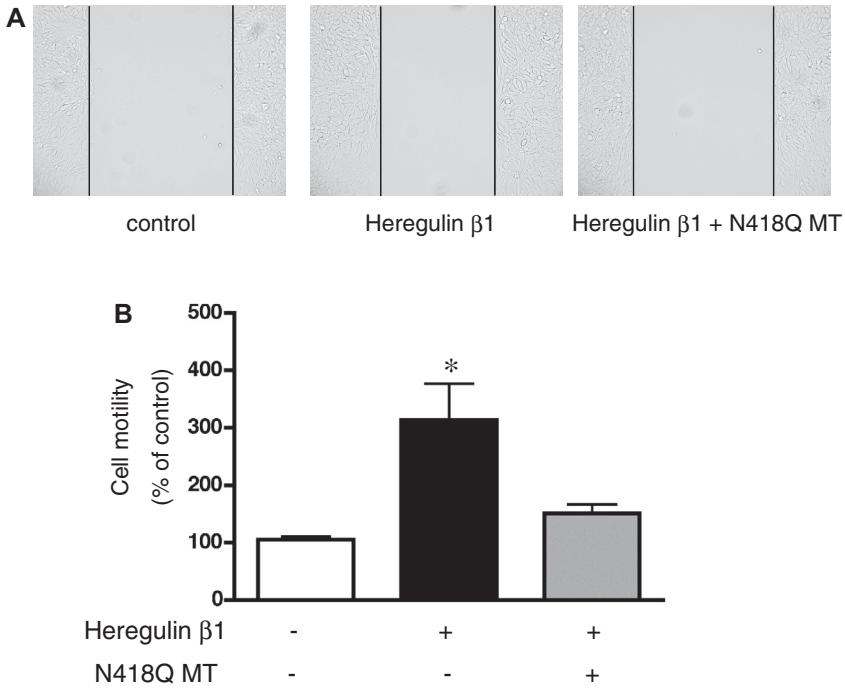

Fig. 3. Heregulin β1-induced cell motility was attenuated by the sErbB3 N418Q mutant in MCF7 cells. The cell motility of MCF7 was assessed by ibidi's culture inserts for a wound healing and migration assay. (A) MCF7 cells were serum-staved for 16 h, incubated with wild type or the sErbB3 N418Q mutant for 2 h, and stimulated with 10 ng/ml heregulin β1. After 24 h stimulation, pictures were taken using a light microscope, with ×200 magnification. (B) To identify cell motility, the results were presented as the relative percentage of cell motility compared with control cells. $^{*}P < 0.05$ vs. control. The assays were repeated four times independently.

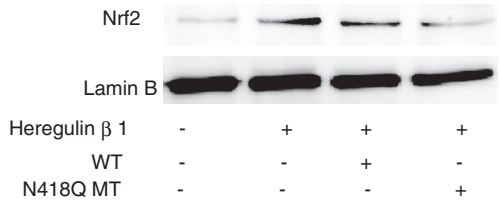

Fig. 4. The sErbB3 N418Q mutant inhibited Nrf2 accumulation induced by heregulin β1 in MCF7 cells. MCF7 cells were serum-staved for 16 h, incubated with the wild type or the sErbB3 N418Q mutant for 2 h, and stimulated with 10 ng/ml heregulin β for 6 h. Nrf2 in nuclear fractions (5 μg of protein) was analyzed by Western blot analysis. Data are representative of two experiments.

β1. These results therefore suggest that the sErbB3 N418Q mutant, but not the wild type, may reduce ErbB3-driven tumor progression by blocking of the HIF-1 and Nrf2 pathway.

In conclusion, our study demonstrated that the sErbB3 N418Q mutant can suppress heregulin β1-induced the nuclear accumulation of HIF-1α and Nrf2, and attenuate cell migration in breast cancer cells. Breast cancer is the most common malignancy among women worldwide [20,21]. In the clinical course of the tumor metastasis, the activation of HIF-1α and Nrf2 is a key regulator of the malignant phenotype of breast cancer. Thus, the present findings suggest the possibility that the sErbB3 N418Q mutant can suppress the cell growth and the malignant alteration of cancer cells mediated by ErbB3, and may be a new potential therapeutic application for breast cancer.

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