Biochimica et Biophysica Acta 1813 (2011) 1803–1813

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

C/EBP ζ targets to neutrophil gelatinase-associated lipocalin (NGAL) as a repressor for metastasis of MDA-MB-231 cells

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ARTICLE INFO

Article history:
Received 4 February 2011
Accepted 23 June 2011
Available online 30 June 2011

Keywords:
Neutrophil gelatinase-associated lipocalin
Breast cancer
Metastasis
C/EBP ζ

ABSTRACT

Breast cancer is a leading cause of morbidity in women worldwide. neutrophil gelatinase-associated lipocalin (NGAL), a useful biomarker of ER negative (ER^-) breast cancer, promotes local tumor invasion and lymph node metastasis. We first identified the distinctive expression of NGAL in two breast cancer cell lines MCF7 and MDA-MB-231 cells, and then confirmed NGAL as a critical inducer of metastasis. Finally, the transcriptional factor CCAAT enhancer-binding proteins ζ (C/EBP ζ) was overexpressed in MDA-MB-231 cells. Consistent with the effect of NGAL knockdown, C/EBP ζ overexpression caused the significant changes that could prevent cell metastasis. C/EBP ζ overexpression induced a strong decrease in NGAL and matrix metalloproteinases (MMPs) expressions as determined by quantitative real time PCR and Western blotting. To identify the potential role of C/EBP ζ on regulating of NGAL in breast cancer, we established the dual-luciferase reporter assay for NGAL in MDA-MB-231 cells cotransfected with C/EBP ζ. Promoter reporter assays determined that C/EBP ζ directly repressed the human NGAL gene promoter activity by inhibiting the NGAL transcription. Taken together, this work identified that the C/EBP ζ overexpression downregulated NGAL to inhibit migration and invasion of breast cancer, which could be used as a novel strategy for breast cancer therapy.

1. Introduction

Breast cancer survival rate falls from 90% for localized to 20% for metastatic disease [1,2]. The metastasis of tumor cells causes 90% of human cancer deaths [3]. During the development of most types of human cancer, including breast carcinoma, primary tumor masses are always prone to spawn pioneer cells that move out, invade adjacent tissues, and thence travel to distant sites where they may succeed in founding new colonies [4]. Chemotherapy is the mainstay treatment for breast cancer patients with metastatic status. However, many patients exhibit resistance to chemotherapy that is either inherent or acquired during treatment [5].

NGAL (also referred to as Lipocalin 2, Lcn2) is a member of the lipocalin family. Lipocalins are small extracellular proteins that share the highly conserved structure of an 8-stranded antiparallel β barrel and have been shown to transport and present ligands, to bind to cell surface receptors, and to form macromolecular complexes, thereby playing important roles in cell regulation, proliferation, differentiation, migration, invasion and epithelial to mesenchymal transition (EMT) [6]. Estrogen exposure is one of the most well recognized risk factors for breast cancer. The expression of estrogen receptors (ER) in breast cancer suggests a role for these receptors in its pathogenesis and therapy [7]. NGAL is among those genes most highly associated with ER^- breast tumors for breast cancer progression, suggesting that NGAL is a new therapeutic target for the prevention and treatment of ER^- breast cancer [8].

As mentioned above, resistance to chemotherapy is a major problem facing breast cancer patients. A potential approach instead of chemotherapy to breast cancer is gene therapy. C/EBP ζ, a transcriptional factor, has been revealed to significantly downregulate in myeloid malignancies than normal controls [9]. It is a candidate tumor suppressor gene (TSG) that has been involved in the regulation of cellular growth and differentiation [10,11]. C/EBP ζ, also named DNA damage-inducible transcript 3 (DDIT3), belongs to a family of bZIP regulatory proteins containing two distinct domains: a basic region that binds to DNA, and an adjacent leucine-zipper region that enables homo- and hetero-dimerization of C/EBP proteins [12]. Members of the C/EBP family are known to heterodimerize among themselves, giving rise to different functional transcriptional complexes. Sequential expression of different C/EBP members was observed during the process of cell differentiation [13]. The disruption of these programs is oncogenic in several cellular contexts [14]. C/EBP ζ is proposed to act

Abbreviations: NGAL, neutrophil gelatinase-associated lipocalin; EMT, epithelial to mesenchymal transition; ER, estrogen receptors; TSG, tumor suppressor gene; DDIT3, DNA damage-inducible transcript 3; ESCC, oesophageal squamous cell carcinoma; CM, conditioned medium; NHE1, Na^+/H^+ exchanger 1; MMPs, Matrix metalloproteinases; pHi, intracellular pH; pHe, extracellular pH; IL, interleukin; 2-MEO, 2-mercaptoethanol

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doi:10.1016/j.bbamcr.2011.06.010

as a dominant negative inhibitor of other C/EBPs [15]. And subsequent studies demonstrate that C/EBP ζ expression is essential for the activation of a number of genes induced in the endoplasmic reticulum stress response for cell apoptosis [16]. C/EBP ζ has also been demonstrated to induce a decrease in tumor marker *NGAL* gene mRNA expression in human fibrosarcoma cell line HT 1080[17]. Elevated NGAL expression has been shown in different human tumors including breast [18], lung [19], colorectal [20], ovarian [21], pancreatic cancer [22], oesophageal squamous cell carcinoma (ESCC) [23] and hematopoietic malignancies [24]. Based on these findings, we hypothesize that C/EBP ζ is potential as a therapeutic gene for breast cancer treatment, and the overexpression of C/EBP ζ could downregulate NGAL expression to inhibit the metastasis of breast carcinoma.

Therefore, in this paper, the potential role of C/EBP ζ on metastasis was studied in MDA-MB-231 ER^- clones, in which C/EBP ζ was stably overexpressed, thereby resulting in a sustained downregulation of NGAL. The approach revealed that C/EBP ζ inhibited cell migration and invasion evidently by counteracting NGAL expression. These findings strongly support the concept of gene therapy that manipulation of C/EBP ζ might be beneficial to the breast cancer therapy depending on the downregulation of NGAL protein via the transcriptional regulation on NGAL promoter.

2. Materials and methods

2.1. Cell culture and reagents

HeLa, MCF7, MDA-MB-231 cells were grown in DMEM (Life Technologies, Rockville, MD), and Jurkat, K562 cells in RPMI 1640 (Life Technologies). The medium was supplemented with 10% fetal bovine serum (Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). Cells were grown at 37 °C in a humid atmosphere with 5% CO_2.

The following antibodies were used for Western blotting: anti-β-actin (Chemicon, USA), anti-human NGAL (R&D Systems, USA), anti-human NGAL (Abcam, UK), anti-human NGAL (Santa Cruz, USA), and anti-human C/EBP ζ (DDIT3) (Santa Cruz, USA).

Enhanced Chemiluminescence Reagent Plus (ECL) reagents were from Santa Cruz Biotechnology (Santa Cruz, USA). Cariporide were purchased from Sigma (Shanghai, China). FH535 and BAY117082 were purchased from Sigma (Beyotime, Shanghai, China).

2.2. RNA isolation and real time PCR

RNA isolation, DNase treatment, and RT-total RNA were isolated using Trizol (Invitrogen, Grand Island, NY), treated with DNase I (Invitrogen, Grand Island, NY), and 2 μg RNA were reverse-transcribed using Superscript II RT (Invitrogen, Grand Island, NY) following the manufacturer's instructions in a total volume of 20 μl.

Primers for real time PCR were designed using Primer premier software 5.0. Human β-actin primers used as an internal control were 5′-CCA CGA AAC TAC CTT CAA CTC C-3′ (forward) and 5′-ACT CGT CAT ACT CCT GCT TGC T-3′ (reverse; 272 bp). Human NGAL primers were 5′-CAA GGA GCT GAC TTC GGA AC-3′ (forward) and 5′-TAC ACT GGT CGA TTG GGA CA-3′. Human C/EBP ζ primers were (forward primer) 5′-CAG AAC CAG CAG AGG TCA CA-3′ and (reverse primer) 5′-GCT GTG CCA CTT TCC TTT C-3′. Real time PCR was performed with TransGen SYBR Green PCR kit (TransGen Biotech, China) on the ABI Prism 7500 Fast Sequence Detection System. Thermal cycling conditions were 95 °C for 10 s, followed by 40 cycles of 5 s at 95 °C, and 40 s at 60 °C. PCR reactions were performed in a total volume of 20 μl, containing 2 μl of sample cDNA, 0.2 μM of each primer, and the SYBR Green PCR kit following the manufacturer's instructions. Each test was amplified in three different wells in one experiment.

2.3. Western blotting

Secreted proteins (secreted NGAL protein included) were separated from the conditioned medium (CM) by fast ultrafiltration using Amicon® Ultra-15 centrifugal filter devices (Millipore, USA) according to the manufacturer's protocol, intracellular proteins were isolated by RIPA lysis buffer (RIPA: 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris (pH 8.0)) with protease inhibitors , and 1 mM PMSF (Sigma, USA). The lysates were cleared using centrifugation at 12,000 rpm for 15 min at 4 °C. Protein samples were completely denatured by boiling for 10 min at 100 °C in the presence of 2% SDS and 10% 2-MEO (2-mercaptoethanol). The denatured samples were then separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, USA). The membranes were blocked for 1 h with 5% skimmed milk in PBS and then incubated first with primary antibodies and then horseradish peroxidase-conjugated secondary antibodies for 2 and 1 h, respectively. Specific proteins were visualized with enhanced chemiluminescence detection reagent and determined by densitometric analysis with a Lynx video densitometer (Biological Vision, Inc., San Mateo, CA).

2.4. Short hairpin RNA-mediated RNA interference studies

Three independent hairpins targeting to NGAL were developed using software from Ambion. These hairpins were synthesized and cloned into the eukaryotic vector pSilencer U6 (Ambion, Inc, USA) and then transfected into MDA-MB-231 cells. Infected cells were selected by hygromycin 48 h postinfection for at least 2 weeks and stable clones were obtained. Inhibition of NGAL expression was measured by quantitative PCR as well as by Western blotting using a rat anti-NGAL antibody. For RNA interference studies, cells transfected with the pSilencer vector that expresses a scrambled control siRNA were the negative control.

2.5. Plasmids for overexpression and promoter studies

Human C/EBP ζ was generated by PCR from a human normal cDNA library and then cloned into the plasmid pTARGET ™/Neo, which was then used to generate clones encoding the full-length protein (aa 1–169). A 1221 bp, 500 bp and 236 bp fragments of the human NGAL promoter region (−1137 to +84; forward primer, 5′-TACTCGAG-CAAGCAGCACGTAGGCAGAG-3′), (−416 to +84; forward primer, 5′-AACTCGAGCAGGAAACAGCACATGATCT-3′), (−152 to +84; forward primer, 5′-TACTCGAGCTGTCTTGCCCAATCCTGAC-3′, and reverse primer, 5′-ATAGATCTTCAG GGCCGAGGAAGCAGGC-3′) were PCR amplified with above corresponding primers, using genomic DNA as template. The restriction enzyme digested PCR fragments were cloned in Xho I/Bgl II-restricted pGL3-basic (Promega, Madison, WI), resulting in pGL3-ngal 1208, pGL3-ngal 500, pGL3-ngal 236 plasmids for luciferase reporter assay. For reporter assay, the fragment of NGAL promoter from −1137 to −185 with C/EBP ζ potential interaction sites deleted was PCR amplified and cloned into the pGL3-promoter (Promega, USA) as a negative control (designated as pGL3-control), the primers for PCR were: forward, 5′-TACTCGAGCAAGCAGCACG-TAGGCAGAG-3′, and reverse, 5′-ATAGATCTACCTCTGGCAGGGA-CAACG-3′.

2.6. Cell transfection and dual-luciferase reporter assay

MDA-MB-231 cells (1.00×10^5 cells) were transfected with 500 ng of the indicated reporter plasmid together with 2.5 ng of the internal control plasmid phRL-TK using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's protocol. Cells were lysed 24 h after transfection. To analyse the responses to pharmacological inhibitors of Na^+/H^+ exchanger 1 (NHE1) selective inhibitor