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Research Article

A homeobox protein, NKX6.1, up-regulates interleukin-6 expression for cell growth in basal-like breast cancer cells

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ABSTRACT

Among breast cancer subtypes, basal-like breast cancer is particularly aggressive, and research on the molecules involved in its pathology might contribute to therapy. In this study, we found that expression of NKX6.1, a homeobox transcription factor, is higher in basal-like breast cancer than in other subtypes. In loss-of-function experiments on basal-like breast cancer cell lines, NKX6.1-depleted cells exhibited reduced cell growth. Because cytokine interleukin-6 (IL-6) is expressed in basal-like breast cancer, and increases cell growth, we analyzed expression levels of *IL6*, an IL-6 gene, and observed reduced *IL6* expression in NKX6.1-depleted cells. In a reporter assay, *IL6* promoter activity was reduced by loss of NKX6.1 function. A pull-down assay showed that NKX6.1 binds to the proximal region in *IL6* promoter. These results indicate that NKX6.1 directly up-regulates *IL6* expression. To investigate further, we established cells with forced expression of IL-6. We observed that exogenous IL-6 expression restored the reduced cell growth of NKX6.1-depleted cells. Furthermore, orthotopic xenografts showed that NKX6.1-depleted cells lost the capacity for tumor formation. We therefore conclude that NKX6.1 is a factor for IL-6-regulated growth and tumor formation in basal-like breast cancer. Our findings facilitate profound understanding of basal-like breast cancer, and the development of suitable therapy.

1. Introduction

Breast cancer is the most common cancer in women [1]. It is classified into five subtypes, based on differences in gene expression patterns: luminal A; luminal B; human epidermal growth factor receptor 2 (HER2)-enriched; normal breast-like; and basal-like [2]. Among these, basal-like breast cancer is particularly aggressive, with the worst prognosis [3].

Basal-like breast cancer is characterized by the strong expression of basal markers such as cytokeratin 5/6 and 17 [2, 4, 5]. It is associated with up-regulation of mesenchymal markers, such as vimentin, smooth muscle actin, and N-cadherin, and reduced expression of epithelial markers, such as E-cadherin [6–8].

Transcription factors determine and maintain cellular characteristics through regulation of gene expression. Some transcription factors have been identified and analyzed with the aim of improving our understanding of the nature of basal-like breast cancer [9–12]. Although we now know about this cancer's molecular properties, we do not yet fully understand it, and the transcription factors responsible for basal-like breast cancer pathology still remains to be identified and investigated.

NKX homeobox protein is a family of transcription factors that possess a DNA binding domain [13], which amino acid sequence is conserved among family members. The NKX family of genes has been found to play a role in various types of cancer. For example, NKX2.1 functions as a proto-oncogene, and is closely associated with lung cancer [14–16]. NKX2.8 expresses in liver cancer, and regulates the expression of α-Fetoprotein, a fetal oncogene [17]. Loss of NKX3.1 function is an initiating event in prostate carcinogenesis [18]. NKX6.1 is a metastasis suppressor that regulates the epithelial-mesenchymal transition in cervical cancer [19]. With respect to breast cancer, some studies of the role of NKX family members in luminal breast cancer have been conducted [20,21], but none have focused on the basal-like type. Given that NKX family members are involved in the pathologic functions of various cancers, it is reasonable to surmise that there might be as yet unrecognized NKX factor(s) active in basal-like breast cancer.

Interleukin-6 (IL-6) is a cytokine that plays a role in various biological activities, including cell growth and differentiation. In basal-like breast cancer, the level of IL-6 secretion is high, and the IL-6/Janus kinase (JAK) 2/signal transducer and activator of the transcription 3 (STAT3) pathway is active in regulating cell growth [22,23]. In this pathway, IL-6 binds to IL-6 receptor and activates JAK2 tyrosine kinase. Activated JAK2 proteins facilitate phosphorylation of STAT3. Phosphorylated-STAT3 (pSTAT3) is then translocated into the nucleus to regulate genes involved in apoptosis and

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proliferation. The IL-6/JAK2/STAT3 signaling pathway is therefore a potential target for cancer therapy [24–27].

Since *IL6*, an IL-6 gene, expression is involved in breast cancer cell growth, some studies have focused on the regulation of *IL6* expression in these cells. The epigenetic status of *IL6* expression differs between *IL6*-low luminal and *IL6*-high basal-like breast cancer cells [28]. The *IL6* promoter of basal-like breast cancer is demethylated, and this epigenetic activation is regulated by the IL-6 autocrine loop [29]. In luminal breast cancer, estrogen receptor, a nuclear receptor, inhibits *IL6* expression [30]. However, specific regulators for *IL6* expression in basal-like breast cancer have not been fully understood.

In this study we found that expression of NKX6.1, also known as NKX6A, is up-regulated in basal-like breast cancer, compared to other breast cancer subtypes. We show that NKX6.1 regulates *IL6* expression by binding to the *IL6* promoter to promote cell growth. In addition, NKX6.1 is involved in tumor formation. These results improve our understanding of basal-like breast cancer pathology, and might contribute to future therapy for this type of cancer.

2. Materials and methods

2.1. Cell culture and animal experiment

The breast cancer cell lines MCF10A, MCF-7, T-47D and MDA-MB-231 were obtained from American Type Culture Collection (Manassas, VA, USA). SUM159 cells were obtained from Asterand (Detroit, MI, USA). The colon cancer cell lines HCT116 and LoVo were obtained from RIKEN BioResource Center (Tsukuba, Japan).

MCF10A cells were maintained with DMEM/F12 containing 5% horse serum, 50 μg/mL EGF, 500 ng/mL hydrocortisone, 100 ng/mL cholera toxin and 10 μg/mL insulin. MCF-7 cells were cultured with RPMI-1640 medium containing 1 nM estradiol and 10% fetal bovine serum (FBS). T-47D and MDA-MB-231 cells were cultured with RPMI-1640 containing 10% FBS. HCT116 cells were cultured with DMEM containing 10% FBS. SUM159 cells were maintained in Ham's F-12 nutrient mixture containing 5% FBS, 5 μg/mL insulin, 1 μg/mL hydrocortisone and 10 mM HEPES. LoVo cells were maintained with Ham's F-12 nutrient mixture containing 10% FBS. In drug selection for infectants, 1 μg/mL puromycin or 10 μg/mL blasticidin S was used. For the xenograft assay, 1×10^6 cells were suspended in 40 μL of serum-free medium, mixed with equal amount of Matrigel (Corning, 356237, Bedford, MA, USA), and transplanted into the mammary fat pad of six-week-old NOD/SCID mice. In the same mouse, control and NKX6.1 knockdown cells were injected at the right and the left sides, respectively. The animal experiments in this study were approved by the Animal Research Committee of Kyoto University. All animals were maintained according to the Guide for the Care and Use of Laboratory Animals (National Institute of Health Publication).

2.2. Quantification of mRNA level

The primers used in this study are listed in Supplementary Table S1. For RNA-seq analysis and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis, RNA samples were extracted using TRIzol (Life Technologies, 15596026, Carlsbad, CA, USA) and PureLink RNA micro kits (Life Technologies, 12183-016). For RNA-seq analysis, total RNA samples from 3 independent extractions were pooled, and used for a cDNA library for analysis with HiSeq2500 system (Illumina, San Diego, CA, USA). Aligned data was normalized by RPKM method [31] and quantified with Strand NGS software (Strand Life Sciences, Aurora, CO, USA). For each qRT-PCR sample, 1 μg of total RNA was used for cDNA synthesis with SuperScript III (Life Technologies, 18080051). A 1:10 dilution of synthesized cDNA solution was used as the template in each qRT-PCR reaction with FastStart Universal SYBR Green Master (Roche, 4913850001, Mannheim, Germany). The StepOne Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used to run the qRT-PCR analyses. Amplification of *EF1A1* was used as an internal control. The following conditions were used: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 1 min. The relative mRNA levels and standard deviations were calculated according to the manufacturer's instructions. The cycle threshold value normalized by the *EF1A1* value was used for statistical analysis.

2.3. Immunoblotting

Rabbit anti-NKX6.1 antibody (1:500; Sigma, HPA036774, St. Louis, MO, USA), rabbit anti-phospho-STAT3 Tyr705 (D3A7) antibody (1:1000; Cell Signaling technology, X9145, Danvers, MA, USA), rabbit anti-STAT3 (D3Z2G) antibody (1:1000; Cell Signaling Technology, 12640), rabbit anti-IL-6 antibody (1:1000; Cell Signaling Technology, 12153), rabbit anti-FLAG antibody (1:500; Sigma, F7425) and mouse anti-beta actin antibody (1:5000; Abcam, ab6276) were used for primary reactions. The Easy-Western-II detection system (Beacle, BCL-EZS21, Kyoto, Japan) was used instead of secondary antibody for rabbit IgG. We used goat anti-mouse IgG antibody conjugated to a peroxidase (1:50,000; Pierce biotechnology, 31340, Rochford, IL, USA) to detect beta-actin signals. Images were obtained with Ez-Capture II (ATTO, Tokyo, Japan) and ImageSaver5 Software (ATTO).

2.4. Construction of plasmid vectors

For NKX6.1 knockdown, we constructed an shRNA expression system. We used a lentiviral vector, pLKO.1 (Addgene, 8453), which has the human U6 promoter. The sequences of shRNAs are listed in Supplementary Table S2. To knockout the NKX6.1 gene, we used a lentiviral vector for the CRISPR/Cas9 system, lentiCRISPR v2 (Addgene, 52961). The target sequence for GFP control is 5′-CAACGTCTATATCATGGCCG-3′, and that for NKX6.1 knockout is 5′-TGGGCCGGTACCCCAAGCCG-3′.

To construct a FLAG-tagged gene, we inserted a synthesized double strand oligo, 5′-CACCATGGGCGGCGGCGGCTCCGACTACAAGGACGACGACGACAAGTGA-3′, into the cloning site of pENTR vector (Life Technologies, K2400-20, USA). The inserted sequence has an *Nco*I site, a GGGGS linker-FLAG coding sequence and an opal terminator. We cloned the *IL6* cDNA into the *Nco*I site using the In-Fusion HD cloning system (Takara Bio, Z9645N, Otsu, Japan). We then subcloned the FLAG or IL6-FLAG gene into the lentiviral vector, pLenti 6.3 (Life Technologies, V533-06).

To analyze *IL6* promoter activity, we cloned a fragment of the upstream region of the *IL6* transcription start site into the upstream region of the minimal promoter (miniP) of the pGL4.30 vector (Promega, Madison, WI, USA). The *IL6* promoter-miniP fragment was subcloned into the region between the *Cla*I - *Spe*I sites of the pLenti 6.3 vector. Mutations were introduced by In-Fusion system (Takara).

2.5. Growth assay

Cells were plated in 24-well plates at a density of 5×10^4 cells/well on day 0. On day 4, cells were trypsinized and resuspended in the medium as single cells. Four samples were collected from each well, and each sample was mixed with the same quantity of trypan blue solution. Viable cells were counted manually under a microscope. We calculated the mean of the four samples from each well as the cell number for that well, and analyzed four wells for each experimental group.