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Egr-1 is required for neu/HER2-induced mammary tumors

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ABSTRACT

Egr-1 is known to function mainly as a tumor suppressor through direct regulation of multiple tumor suppressor genes. To determine the role of Egr-1 in breast tumors *in vivo*, we used mouse models of breast cancer induced by HER2/neu. We compared neu-overexpressing Egr-1 knockout mice (neu/Egr-1 KO) to neu-overexpressing Egr-1 wild type or heterozygote mice (neu/Egr-1 WT or neu/Egr-1 het) with regard to onset of tumor appearance and number of tumors per mouse. In addition, to examine the role of Egr-1 *in vitro*, we established neu/Egr-1 WT and KO tumor cell lines derived from breast tumors developed in each mouse. Egr-1 deletion delayed tumor development *in vivo* and decreased the rate of cell growth *in vitro*. These results suggest that Egr-1 plays an oncogenic role in HER2/neu-driven mammary tumorigenesis.

1. Introduction

Early growth response factor-1 (Egr-1) has been associated with multiple cellular processes including growth, differentiation, apoptosis and wound healing [1]. In addition, Egr-1 is known as a direct regulator of multiple tumor suppressors including TGFβ1 [2], PTEN [3], p53 [4], and fibronectin [5]. Egr-1 expression is lower in non-small cell lung carcinoma (NSCLC) [6] and glioblastoma [7], suggesting that Egr-1 expression is often inhibited in tumors. However, Egr-1 serves as an oncogene in prostate cancer [8,9]. The role of Egr-1 in breast cancers is controversial. Overexpression of Egr-1 in human breast tumor cells suppresses transformed cell growth and tumorigenicity [10]. Also, human and mouse breast cancer cell lines and tumors tested had decreased Egr-1 expression compared to their normal counterparts [11]. On the other hand, Egr-1 DNAzyme, which inhibits Egr-1 expression, suppresses MCF-7 human breast cancer growth in nude mice [12].

To clarify the role of Egr-1 in breast tumors *in vivo*, we used mouse models of breast cancer induced by human epidermal growth factor receptor 2 (HER2/neu). HER2/neu receptor kinase is a member of the epidermal growth factor family of receptor tyrosine kinases comprised of EGFR/ErbB-1, HER2/ErbB-2/neu, HER3/ErbB-3, and HER4/ErbB-4 [13]. Neu is the murine homologue of HER2. Dysregulation of ErbB expression is implicated in cancer development [14], and overexpression of HER2 is found in about 20–30% of breast cancers [15]. Mouse mammary tumor virus (MMTV)-neu transgenic mice that overexpress neu protein with a strong viral promoter/enhancer develop focal mammary tumors with a latency period of 7–8 months [16].

This study sought to elucidate the functional role of Egr-1 in tumor initiation of HER2/neu-driven breast cancer. We established three types of bigenic mouse models by crossing MMTV-neu and Egr-1 knockout (−/−) mice: neu/Egr-1 wild type (+/+) (neu/Egr-1 WT), neu/Egr-1 heterozygote (+/−) (neu/Egr-1 het), and neu/Egr-1 knockout (neu/Egr-1 KO). Neu/Egr-1 KO mice were compared with neu/Egr-1 WT or het mice with respect to onset of tumor appearance and number of tumors per mouse. Furthermore, we established neu/Egr-1 WT and KO tumor cell lines derived from each mouse tumor. Neu/Egr-1 KO cells were compared with neu/Egr-1 WT cells for cell proliferation, colony formation, cell cycle progression, cell adhesion, and cell migration. We also found that Egr-1 regulated the activity of Notch and STAT3 and expression of cyclin D1, p27, and vimentin.

2. Materials and methods

2.1. Generation of bigenic mice

MMTV-neu (Fvb/N background) mice were purchased from Jackson Laboratory (http://www.jax.org). Egr-1 knockout (ICR background) mice were kindly provided by Professor H. Song (CHA University, Seoul, Korea). To generate bigenic neu/Egr-1 knockout mice, we first crossed Fvb/N strain MMTV-neu mice with ICR strain Egr-1 heterozygous (+/−) mice that were backcrossed to an Fvb/N background for 10 generations to generate compound heterozygotes. The resulting mice were then intercrossed to obtain neu/Egr-1 wild type (+/+) (neu/Egr-1 WT), neu/Egr-1 heterozygote (+/−) (neu/Egr-1 het), and

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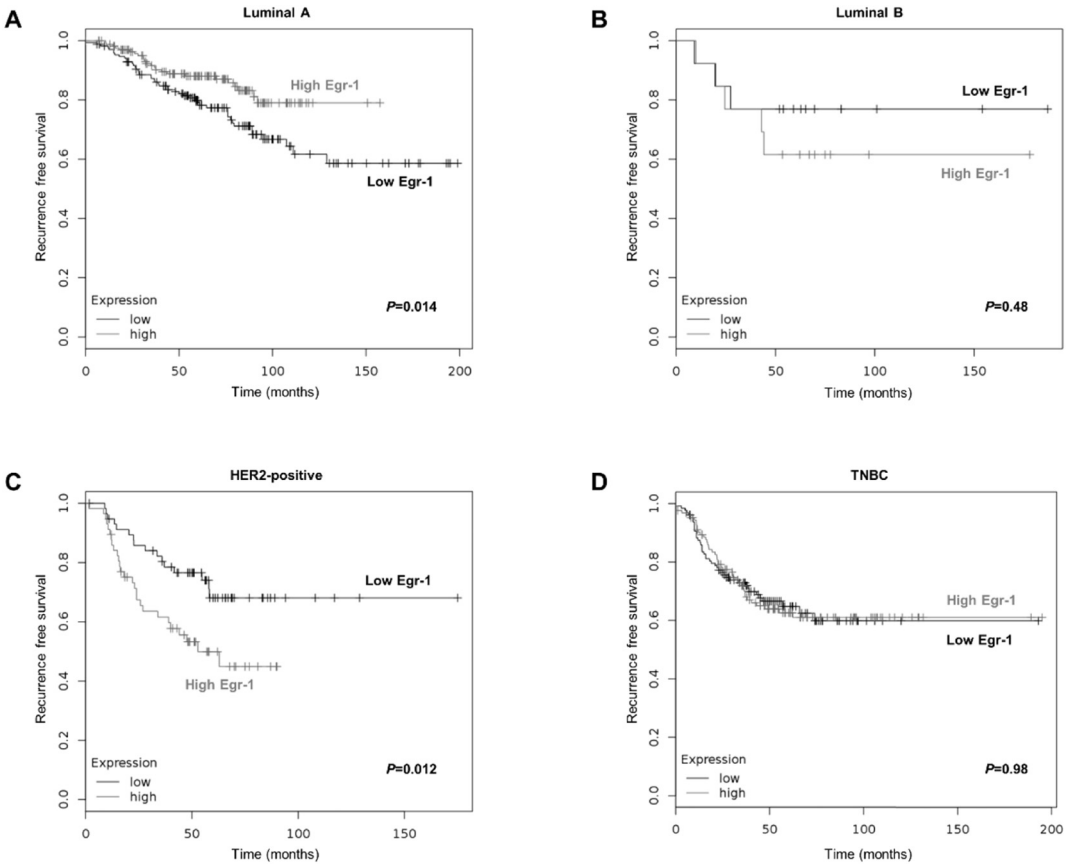

Fig. 1. *Correlation between Egr-1 expression and breast cancer patient survival.*
Kaplan-Meier (KM) survival curves were stratified by Egr-1 expression in luminal A (A), luminal B (B), HER2-positive (C) or triple-negative (D) breast cancer patients. Data were obtained from the Kaplan-Meier Plotter Breast Cancer database (http://kmplot.com/analysis/index.php?p=service&cancer=breast). Shown above figures are *p* values from log-rank tests comparing KM curves.

neu/Egr-1 knockout (−/−) (neu/Egr-1 KO) mice (Fig. 2A). The mice were maintained under standard light (12 h)/dark (12 h) conditions and at a constant temperature in animal facilities (Hanyang University). This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Center for Laboratory Animal Sciences, the Medical Research Coordinating Center, and the HYU industry-University Cooperation Foundation.

2.2. Preparation and culture of mouse tumor cells

Partial mammary tumors were chopped in Trypsin/EDTA (Gibco, MT, USA). The homogenates were incubated for 30 min at 37 °C, and the cells were centrifuged at 1000 rpm for 5 min. The pellets were washed three times with PBS and filtered through cell strainers. Subsequently, the cells were washed with PBS and resuspended in Dulbecco's modified Eagle's medium (Corning, NY, USA) containing 10% fetal bovine serum (FBS) (Youngin Frontier, Seoul, Korea), 100 U/ml penicillin (Corning), and 100 mg/ml streptomycin (Corning). All cell lines were incubated at 37 °C in 5% CO_2 in a humidified atmosphere.

2.3. Antibodies

Antibodies against Egr-1, p-STAT3 (Tyr705), and p-STAT3 (Ser727) were from Cell Signaling Technology (Beverly, MA, USA). Antibodies against Egr-1, Notch1, STAT3, p-ERK1/2 (Tyr204), ERK1, cyclin D1, p27, ZEB1, SNAI-1, E-cadherin, and actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against activated Notch1 (NICD1) and vimentin were from Abcam (Cambridge, UK). Anti-HER2 antibody was from NeoMarkers (Fremont, CA, USA).

2.4. Immunohistochemistry

Mouse mammary gland and tumor tissues were fixed in Bouin's solution (Sigma, St. Louis, MO, USA). Tissues were dehydrated, washed in 70% ethanol, and embedded in paraffin. Five-μm-thick paraffin sections were mounted on poly-L-lysine-coated slides and incubated in a dry oven at 60 °C for 1 h followed by deparaffinization in xylene. The slides were rehydrated by two sequential incubations in 100%, 90%, 80%, and 70% ethanol for 5 min each. The slides were then incubated with primary antibodies in PBS with 5% FBS in a humidified chamber overnight followed by incubation for 30 min with a biotin-conjugated secondary antibody (Vector Laboratories, Burlingame, CA, USA). After washing in PBS, ABC reagent (ABC peroxidase standard staining kit; Thermo, Waltham, MA, USA) was applied to the sections and incubated for 30 min. After washing in PBS, a color reaction was performed with 3, 30-diaminobenzidine (DAB; Vector Laboratories), and the slides were washed with PBS. After counter-staining with hematoxylin (Sigma) and clearing with a graded ethanol series and xylene, the sections were mounted with Canada balsam (Sigma).

2.5. Western blots

Tumor tissues and cells were washed once with PBS and lysed with lysis buffer (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% Triton X-100, 0.1% SDS, 20 mM NaF, 1 mM Na_3VO_4, 1× protease inhibitor; Roche, Basel, Switzerland). Equal amounts of protein in cell lysates were determined by standardization with the BCA Protein Assay Kit (Thermo). Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Whatman, Dassel, Germany).