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Expression screening of 17q12–21 amplicon reveals GRB7 as an ERBB2-dependent oncogene

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ABSTRACT

Gene amplification is a major genetic alteration in human cancers. Amplicons, amplified genomic regions, are believed to contain "driver" genes responsible for tumorigenesis. However, the significance of co-amplified genes has not been extensively studied. We have established an integrated analysis system of amplicons using retrovirus-mediated gene transfer coupled with a human full-length cDNA set. Applying this system to 17q12–21 amplicon observed in breast cancer, we identified GRB7 as a context-dependent oncogene, which modulates the ERBB2 signaling pathway through enhanced phosphorylation of ERBB2 and Akt. Our work provides an insight into the biological significance of gene amplification in human cancers.

1. Introduction

DNA amplification is a major genetic alteration contributing to oncogenesis [1]. Historically, some proto-oncogenes identified as cellular counterparts of retroviral oncogenes were found to be amplified in human cancers. Thus, it is thought that unidentified proto-oncogenes exist in amplified genomic regions called "amplicons", and amplified proto-oncogenes express large amount of proteins, leading to oncogenesis. We previously constructed gene expression maps of chromosomes in human breast cancer cell lines and extracted six novel amplicons [2]. Nevertheless, it is not easy to identify such proto-oncogenes because amplification events often include multiple genes, and because more information is required, including precise mapping of amplified regions in multiple cancers and deduced function of each gene. A few findings provided the significance of co-amplified genes except specific oncogenes in the amplicon in terms of cancer cell phenotypes. In non-small-cell lung cancer, for instance, co-amplification of *TTF-1* and *NKX2–8* in the 14q13.3 amplicon renders cancer cells resistance to cisplatin [3]. However, the biological significance of co-amplification for oncogenesis has not been validated extensively.

In this study, we focused on the functions of co-amplified genes localized in the 17q12–21 amplicon containing *ERBB2* as a driver gene [4]. The *ERBB2* amplicon is observed in 25% of breast cancers, and also in ovarian, gastric and esophagus cancers [5]. Clinicopathological data indicate that ERBB2 expression is a poor prognostic factor [6]. Furthermore, an active *ErbB2* mutant called *neu* oncogene causes cellular transformation of NIH3T3 cells [7] and breast cancer in trans-

Abbreviations: GRB7, growth factor receptor-bound protein 7; ERBB2, v-erb-b2 erythroblastic leukemia viral oncogene homolog; MMTV, mouse mammary tumor virus; CMV, cytomegalovirus; MAPK, mitogen-activated protein kinase; MEK, MAPK extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; IGF1, insulin-like growth factor-1

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genic mice expressing *neu* oncogene under the control of MMTV promoter [8]. These results support the idea that *ERBB2* functions as a driver gene for oncogenesis when mutated or overexpressed.

To examine the function of co-amplified genes in the *ERBB2* amplicon, we tested these genes for enhancement of ERBB2 cellular transforming activity. For this purpose, we introduced a human wild-type (WT) *ERBB2* expression vector into NIH3T3 cells and established "non-transformed" cells moderately expressing ERBB2 under the CMV promoter. Then, human full-length cDNAs of co-amplified genes were retrovirally introduced into ERBB2-expressing NIH3T3 cells, and the transforming activity was assessed by focus formation assays. Our screening system ensures multiple expression of complete proteins in cells, thereby enabling the examination of combinations of co-amplified genes in the *ERBB2* amplicon.

Here, we show *GRB7* gene, which is located about 10 kb from *ERBB2* locus and is frequently co-amplified with *ERBB2*, caused efficient transformation of NIH3T3 in concert with *ERBB2*; this ERBB2-dependent transforming activity was specific for GRB7 among the GRB7 family proteins. This was consistent with the observation that phosphorylation of ERBB2 was increased when GRB7 was expressed, but not other GRB7 family proteins. Importantly, phosphorylation of Akt at Thr308 and Ser473 was upregulated when both ERBB2 and GRB7 were expressed. We further examined the transforming activity of a series of GRB7 mutants, and showed that a BPS region deletion mutant of GRB7 is a potent activator of ERBB2 and Akt, while the transforming activities of other domain deletion mutants are severely impaired.

Our model of collaborative transformation by ERBB2 and GRB7 proposes that GRB7 is a cytoplasmic activator and adaptor of ERBB2, enhances ERBB2 phosphorylation, and connects ERBB2 to Akt. Our analysis also highlights the biological significance of gene amplification in terms of simultaneous overexpression of a driver gene and "supporter" genes.

2. Materials and methods

2.1. DNA constructs and antibodies

To construct an ERBB2 expression vector, human ERBB2 (RefSeq: NM_004448) cDNA was inserted in pQCXIN retroviral vector (Clontech, Mountain View, CA). Human full-length cDNAs were obtained from the human proteome expression resource (HuPEX) [9], and cloned into pMXs retroviral vector [10] using the Gateway Cloning system (Life Technologies, Carlsbad, CA) with or without N-terminal FLAG epitope tag. Venus fluorescent protein [11] was used as a control. Primary antibodies were as follows: anti-α-tubulin DM1A (Sigma, St. Louis, MO), anti-FLAG M2 (Sigma), anti-ERBB2 (SV2-61γ, Nichirei Bioscience, Japan), anti-HER2/ErbB2 (#2242, Cell Signaling Technology (CST), Danvers, MA), anti-Phospho-HER2/ErbB2 (Tyr877) (CST#2241), anti-Phospho-HER2/ErbB2 (Tyr1221/1222) (CST#2243), anti-Phospho-HER2/ErbB2 (Tyr1248) (CST#2247), anti-Erk1/2 (CST#4695), anti-Phospho-Erk1/2 (#4370), anti-Phospho-MEK1/2 (CST#9154), anti-Akt (pan) (#4691), anti-Phospho-Akt (Thr308) (CST#2965), anti-Phospho-Akt (Ser473) (CST#4060), and anti-Phosphotyrosine 4G10 (Millipore, Billerica, MA). Secondary antibodies for western blotting were purchased from GE Healthcare (Piscataway, NJ).

2.2. Cell culture

NIH3T3 cells were obtained from RIKEN Cell Bank (Tsukuba, Japan) and cultured in DMEM supplemented with 5% heat-inactivated calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO_2. 3T3-ERBB2 cells were established by retroviral infection of pQCXIN-ERBB2 and selection with 1 mg/ml G418. Plat-E packaging cells were obtained from T. Kitamura (Institute of Medical Science, University of Tokyo), and cultured

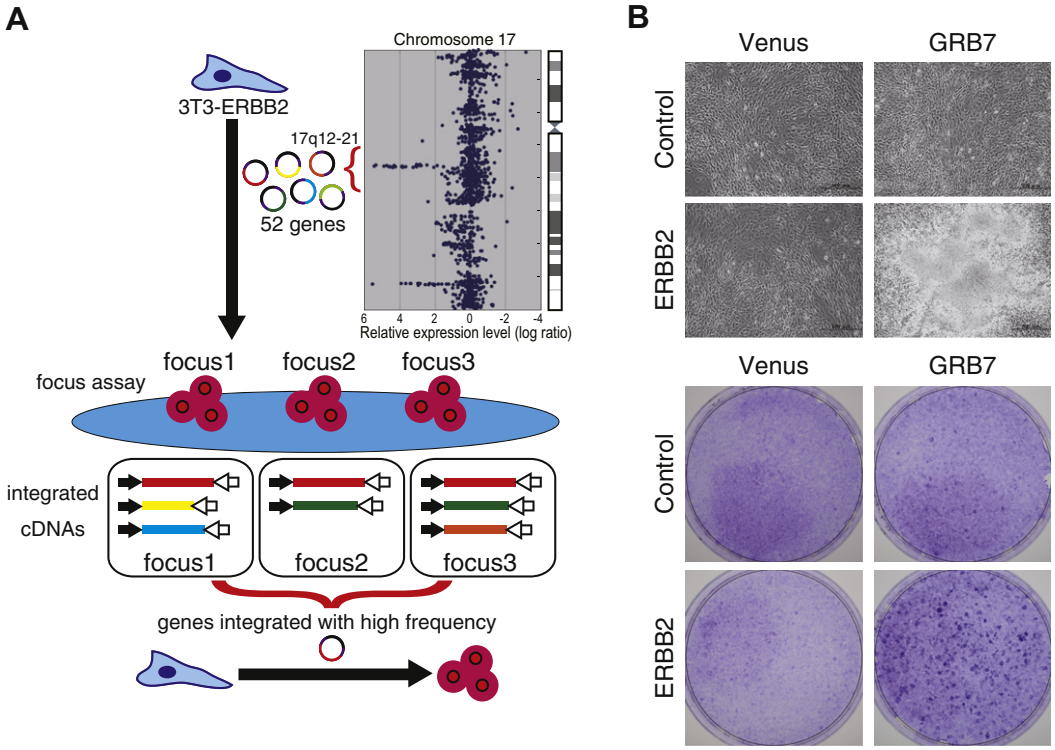

Fig. 1. ERBB2-dependent transforming activity of GRB7. (A) A scheme of oncogene-screening of ERBB2 amplicon. (B) Focus formation assays with ERBB2 and GRB7. 3T3-ERBB2 were infected with GRB7, and cultured for 17 days (upper). Scale bar, 500 μm. Cells were fixed and stained with crystal violet (lower). Venus fluorescent protein was used as a control. Volume of virus stock used in this experiment was 125 μl.