

Molecular Network Pathway of ERBB In Diffuse-Type Gastric Cancer: Mesenchymal Stem Cells and Epithelial-Mesenchymal Transition

Shihori Tanabe^{1*},
Kazuhiko Aoyagi²,
Hiroshi Yokozaki³ and
Hiroki Sasaki²

- 1 Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, Kawasaki, Japan
- 2 Department of Translational Oncology, FIOC, National Cancer Center Research Institute, Tokyo, Japan
- 3 Department of Pathology, Kobe University of Graduate School of Medicine, Kobe, Japan

Abstract

Epithelial-mesenchymal transition (EMT) is related to malignancy and metastasis in cancer. The molecular networks including tyrosine kinases are altered in gastric cancer (GC). This study aims to reveal the role of ERBBs (erb-b2 receptor tyrosine kinases) in EMT, and generate the molecular network pathway of ERBBs in diffuse-type GC and mesenchymal stem cells (MSCs). The expression of ERBB genes was analyzed in MSCs and diffuse-type GC which has mesenchymal characteristics compared to intestinal-type GC. The signaling and molecular network of ERBB was analyzed using several databases, including cBioPortal for Cancer Genomics, Kyoto Encyclopedia of Genes and Genomes (KEGG), BioGRID and VaProS. ERBB2 and ERBB3 gene expression were up-regulated in diffuse-type GC compared to MSCs. The ERBB3 molecular network includes epidermal growth factor receptor (EGFR), cadherin 1 (CDH1), catenin beta 1 (CTNNB1) and EPH receptor A5 (EPHA5). These results demonstrate the importance of the ERBB network in cancer signaling, and revealed a ERBB3 network pathway model in diffuse-type GC and MSCs, and EMT.

Keywords: Epithelial-mesenchymal transition; Gastric cancer; Mesenchymal stem cell; ERBB; Stem cell

***Corresponding author:**
Shihori Tanabe, PhD

✉ stanabe@nihs.go.jp

Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 3-25-26, Tonomachi, Kawasaki-ku, Kawasaki, 210-9501, Japan.

Tel: +81-44-270-6686
Fax: +81-44-270-6703

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Introduction

An abundance of molecular signaling pathways are linked between stem cells and cancer. As cellular phenotype changes, expression levels of specific genes in these signaling pathways are altered. We have previously investigated CTNNB1 networks in epithelial-mesenchymal transition (EMT), and revealed that the CTNNB1 network includes epidermal growth factor receptor (EGFR) and erb-b2 receptor tyrosine kinase (ERBB2) [1]. The ERBB family includes EGFR (ERBB1 or HER1), ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4) [2]. Previous studies have demonstrated that ERBB family members are important for cancer, and so in this article, we analyzed the ERBB network [3-5]. ERBB2 and ERBB3 were found to be overexpressed in an appendiceal adenocarcinoma in which p53 was inactivated and the Ras/RAF/MEK, Akt/mTOR, Wnt, and NOTCH pathways were activated [6]. Somatic mutations in ERBB3 were identified in a significant number of early-onset sporadic diffuse-type gastric

cancer (GC) [7]. Mutations in extracellular domains of ERBB2 are suggested to be oncogenic in lung cancer [8].

Since previous reports have demonstrated that the ERBB members are involved in cancer and stem cell networks, it is important to elucidate the role of ERBBs in EMT and cancer resistance. To further reveal the mechanism of EMT in cancer and stem cells, we investigated the expression of ERBB genes in mesenchymal stem cells (MSCs) and diffuse-type GC, and generated ERBB network pathway model.

Materials and Methods

Gene expression analysis of MSCs and GC

Gene expression in MSCs (6 early- and 6 late- stage cultures, n=12) and diffuse-type GC (n=5) was analyzed with GeneChip® Human Genome U133 Plus 2.0 microarray (Affymetrix, Santa Clara, California, USA), as previously described [1,9,10]. Briefly, total purified cellular RNA was biotinylated and hybridized to the microarray. The signal intensity for each gene was analyzed and compared between MSCs and diffuse-type GC. The microarray data for MSCs and diffuse-type GC are available to the public in the NCBI Gene Expression Omnibus (GEO) database and are accessible via GEO Series accession number GSE7888 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE7888>) and GSE42252 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE42252>), respectively [1,9,10].

Cell culture

Human MSCs from bone marrow (Lonza, Walkersville, MD, USA) were cultured in MSC growth medium (MSCGM; Lonza #PT-3001; MSC basal medium supplemented with mesenchymal cell growth supplement, L-glutamine and penicillin/streptomycin) at 37°C in a CO₂ (5%) incubator. Cells were passaged according to the manufacturer's protocol, however a slight modification was made to use trypsin-EDTA solution (Lonza #CC-3232). Lot numbers of the human MSC batches were as follows: #4F1127, #4F0312, #5F0138, #4F1560, #4F0591 and #4F0760. Informed consent was obtained for Poietics human MSC systems (Lonza) [10]. Passage numbers of MSC cultures were #4 and #22 for #4F1127, #4 and #28 for #4F0312, #4 and #24 for #5F0138, #5 and #28 for #4F1560, #4 and #28 for #4F0591, and #4 and #28 for #4F0760.

Diffuse-Type GC tissues

Diffuse-type GC tissues were originally provided by the National Cancer Center Hospital after obtaining written informed consent from each patient and approval from the National Cancer Center Institutional Review Board. All cancer specimens were reviewed and classified histopathologically according to the Japanese Classification of Gastric Cancer. Following surgical extraction, tissue specimens were immediately frozen with liquid nitrogen and stored at -80°C until microarray analysis [1,9]. Existing, publicly available data were analyzed in the article.

Analysis with Cbioportal for Cancer Genomics

cBioPortal for Cancer Genomics (<http://www.cbioportal.org>) [11,12] was used to analyze cancer genomics data related to ERBB3. The term "ERBB3" was searched in the cBioPortal for Cancer Genomics, and the cross-cancer alteration summary for ERBB3 was obtained. Studies of stomach adenocarcinoma (TCGA, Nature 2014) were further analyzed for pathway network analysis [13].

Gene Ontology Analysis of ERBB3

Gene Ontology of ERBB3 was analyzed using several databases, including the DAVID (<https://david.ncifcrf.gov/home.jsp>), EMBL-EBI (<http://www.ebi.ac.uk/QuickGO/>), AmiGO 2 (<http://amigo.geneontology.org/amigo/landing>) and the Gene Ontology Consortium (<http://geneontology.org/>).

Pathway Network Analysis of ERBB3 and Related Genes

Pathway network analysis was performed using the databases VaProS (<http://pford.info/vapros/>), KEGG (<http://www.genome.jp/kegg/>), cBioPortal for Cancer Genomics (<http://www.cbioportal.org>), and Cytoscape (<http://www.cytoscape.org/>). Molecular interactions were analyzed using the BioGRID (<http://www.thebiogrid.org>) database [14]. Molecule localization was analyzed using The Human Protein Atlas (<http://www.proteinatlas.org/>) [15] and UniProt (<http://www.uniprot.org/>) [16].

Statistical Analysis

The gene expression data was shown in signal intensity or ratio to the average of MSC expression. The ratio to the mean of signal intensity in MSC samples normalized with GAPDH signal intensity was calculated and averaged. The data were shown as the mean ± SE. Student's t-test was performed in Microsoft Excel (Microsoft, Redmond, WA, USA). p<0.001 (n=12 in MSCs, n=5 in diffuse-type GC) was considered as statistically significant.

Results

Gene expression of EGFR, ERBB2 and ERBB3 in MSCs and diffuse-type GC

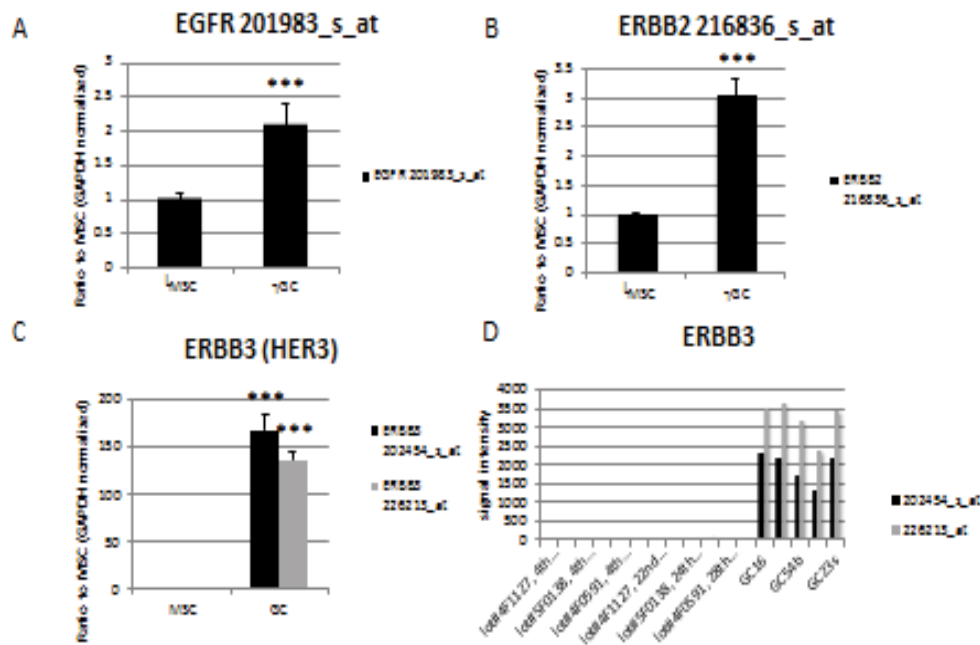
Expression levels of the EGFR, ERBB2 and ERBB3 genes were analyzed in diffuse-type GC and MSCs (**Figure 1A**). ERBB2 gene expression was up-regulated approximately 3-fold in diffuse-type GC compared to MSCs (**Figure 1B**). ERBB3 gene expression was up-regulated by approximately 150-fold in diffuse-type GC compared to MSCs (**Figure 1C**). Signal Intensity of the ERBB3 gene expression in each sample is shown in **Figure 1D**. ERBB4 signal intensity was low.

ERBB3 Interaction Analysis

ERBB3 interactions were analyzed using BioGRID database (**Figure 2**). ERBB3 interactors included growth factor receptor bound protein 2 (GRB2), phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2), C-terminal Src kinase (CSK), SHC adaptor protein 1 (SHC1) and epidermal growth factor (EGF) [2,17].

Network Pathway Analysis of ERBB3

cBioPortal for Cancer Genomics network pathway analysis revealed the ERBB3-associated network and ERBB3 cross-cancer alterations (**Figure 3A and B**). The ERBB3 molecular network was analyzed in stomach adenocarcinoma (TCGA, Nature 2014, tumor samples with sequencing and CNA data 287 samples / 1 gene) [13]. The network contained 51 nodes and included the



***, $p < 0.001$ ($n = 12$ in MSC, $n = 5$ in diffuse-type GC)

Figure 1 (A-D) Gene expression of ERBBs in MSCs and diffuse-type GC.

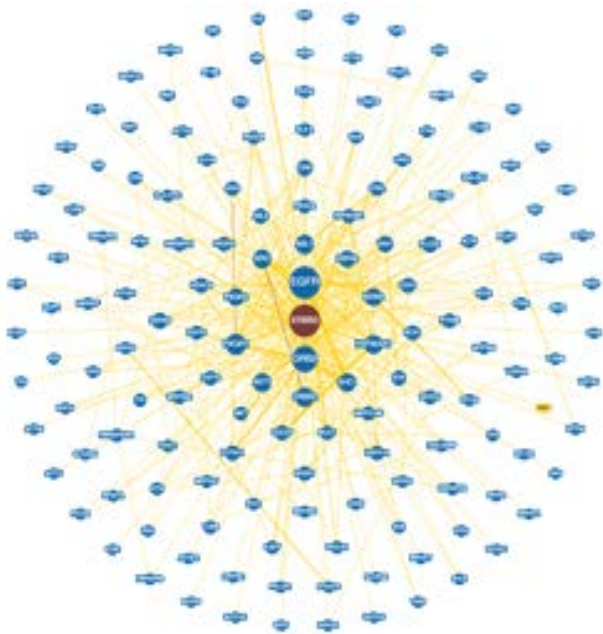


Figure 2 Interactors of ERBB3 (BioGRID).

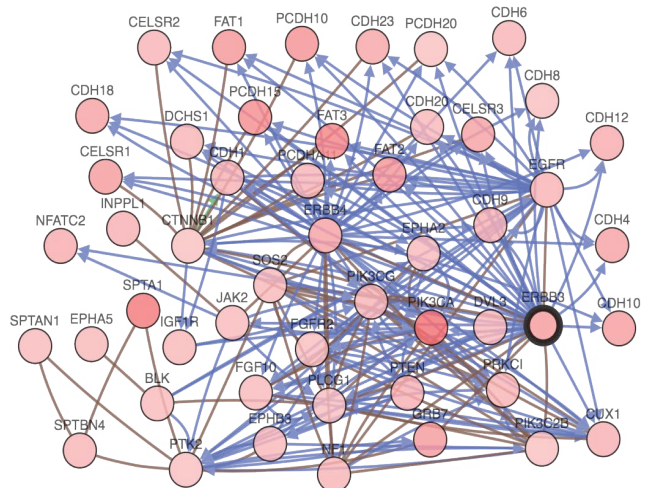


Figure 3a ERBB3 network generated with cBioPortal for Cancer Genomics (Stomach Adenocarcinoma, TCGA, Nature 2014, Tumor Samples with sequencing and CNA data (287 samples) / 1 Gene).

1 query gene ERBB3 as well as the 50 most frequently altered neighboring genes (out of a total of 420) in cBioPortal (Table 1). The network shown in Figure 4 includes the 1 originally queried core gene and the 50 neighboring genes. Analysis of molecular interactions in VaProS demonstrated that ERBB3 interacts with neuregulin 1 (NRG1) and SH2B adaptor protein 3 (SH2B3).

Model Network of ERBB3

The model ERBB3 interaction network is shown in Figure 4. Nodes are colored based on gene expression alteration in diffuse-type GC compared to MSCs. Pink nodes indicate up-regulated (UR) genes and blue nodes indicate down-regulated (DR) genes with $FC > 3$, p value < 0.01 . UR genes with $2 < FC < 3$, p value < 0.01 are highlighted with orange.

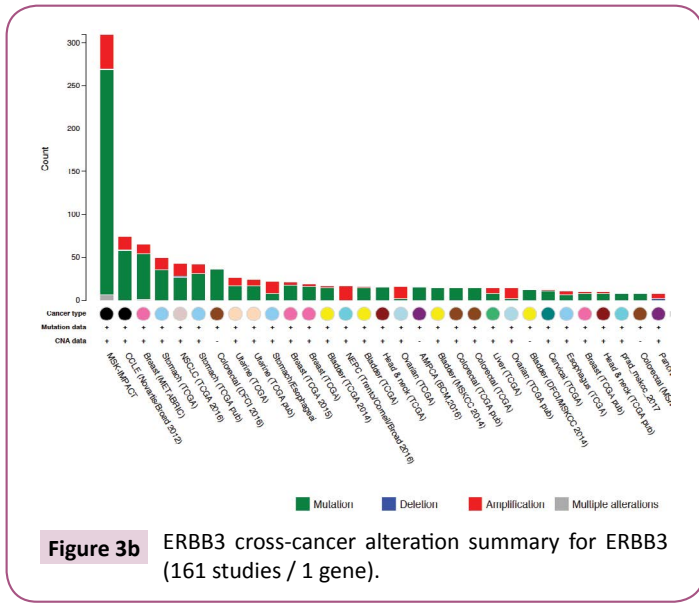


Table 1 Molecules in ERBB3 network.

Gene Symbol	Gene Title	Entrez Gene	Probe Set ID
BLK	BLK proto-oncogene, Src family tyrosine kinase	640	206255_at
CDH1	cadherin 1	999	201131_s_at
CDH10	cadherin 10	1008	220115_s_at
CDH12	cadherin 12	1010	207149_at
CDH18	cadherin 18	1016	206280_at
CDH20	cadherin 20	28316	210913_at
CDH23	cadherin related 23	100653137 / 64072	224527_at
CDH4	cadherin 4	1002	239485_at
CDH6	cadherin 6	1004	205532_s_at
CDH8	cadherin 8	1006	217574_at
CDH9	cadherin 9	1007	207729_at
CELSR1	cadherin EGF LAG seven-pass G-type receptor 1	9620	41660_at
CELSR2	cadherin EGF LAG seven-pass G-type receptor 2	1952	36499_at
CELSR3	cadherin EGF LAG seven-pass G-type receptor 3	1951	40020_at
CTNNB1	catenin beta 1	1499	223679_at
CUX1	cut like homeobox 1	1523	225935_at
DCHS1	dachsous cadherin-related 1	8642	222101_s_at
DVL3	dishevelled segment polarity protein 3	1857	201908_at
EGFR	epidermal growth factor receptor	1956	201983_s_at
EPHA2	EPH receptor A2	1969	203499_at
EPHA5	EPH receptor A5	2044	237939_at
EPHB3	EPH receptor B3	2049	204600_at
ERBB3	erb-b2 receptor tyrosine kinase 3	2065	202454_s_at
ERBB4	erb-b2 receptor tyrosine kinase 4	2066	214053_at
FAT1	FAT atypical cadherin 1	2195	201579_at

FAT2	FAT atypical cadherin 2	2196	208153_s_at
FAT3	FAT atypical cadherin 3	120114	236029_at
FGF10	fibroblast growth factor 10	2255	231762_at
FGFR2	fibroblast growth factor receptor 2	2263	208228_s_at
GRB7	growth factor receptor bound protein 7	2886	210761_s_at
IGF1R	insulin like growth factor 1 receptor	3480	225330_at
INPPL1	inositol polyphosphate phosphatase like 1	3636	201598_s_at
JAK2	Janus kinase 2	3717	205841_at
NF1	neurofibromin 1	4763	212676_at
NFATC2	nuclear factor of activated T-cells 2	4773	228442_at
PCDH10	protocadherin 10	57575	228635_at
PCDH15	protocadherin related 15	65217	1553344_at
PCDH20	protocadherin 20	64881	232054_at
PCDHA11	protocadherin alpha 11	56134 / 56135 / 56136 / 56137 / 56138 / 56139 / 56140 / 56141 / 56142 / 56143 / 56144 / 56145 / 56146 / 56147 / 9752	210674_s_at
PIK3C2B	phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 beta	5287	204484_at
PIK3CA	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha	5290	204369_at
PIK3CG	phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma	5294	206369_s_at
PLCG1	phospholipase C gamma 1	5335	202789_at
PRKCI	protein kinase C iota	5584	209678_s_at
PTEN	phosphatase and tensin homolog	5728	233254_x_at
PTK2	protein tyrosine kinase 2	5747	1559529_at
SOS2	SOS Ras/Rho guanine nucleotide exchange factor 2	6655	212870_at
SPTA1	spectrin alpha, erythrocytic 1	6708	206937_at
SPTAN1	spectrin alpha, non-erythrocytic 1	6709	208611_s_at
SPTBN4	spectrin beta, non-erythrocytic 4	57731	224551_s_at
TNK2	tyrosine kinase non receptor 2	10188	203839_s_at

Primary localization information was based on the Cell Atlas in the Human Protein Atlas where available. Secondary localization information was based on subcellular location information from the UniProt database. Gene ontology (GO) of molecules in the ERBB3 model network was related to Wnt receptor signaling

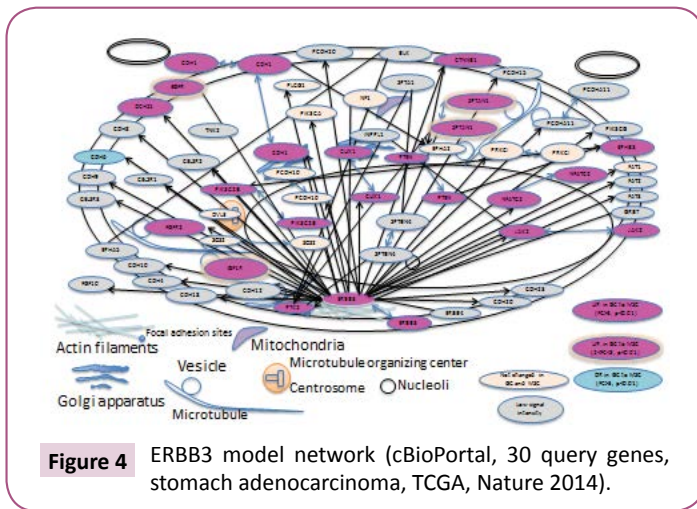


Figure 4 ERBB3 model network (cBioPortal, 30 query genes, stomach adenocarcinoma, TCGA, Nature 2014).

pathway, cell adhesion, cell proliferation, cell differentiation and apoptosis.

KEGG pathway and Gene Ontology of ERBB3

According to KEGG pathway analysis, ERBB3 is involved with EGFR tyrosine kinase inhibitor resistance, the ErbB signaling pathway, the calcium signaling pathway, endocytosis, proteoglycans in cancer, and microRNAs in cancer. The GO of ERBB3 analyzed in AmiGO 2 includes the ERBB3 signaling pathway, the ERBB4-ERBB3 complex, the ERBB2-ERBB3 signaling pathway, and regulation of the ERBB3 signaling pathway (<http://amigo.geneontology.org/amigo/search/ontology?q=ERBB3>). GO biological process of ERBB3 included transmembrane receptor protein tyrosine kinase signaling pathway and regulation of cell proliferation.

DAVID analysis of ERBB3 network molecules

Molecules of ERBB3 network were analyzed in Functional Annotation Tool of DAVID Bioinformatics Resources 6.8, NIAID/NIH (<https://david.ncicrf.gov/home.jsp>). The Gene Ontology and Online Mendelian Inheritance in Man (OMIM) disease for the molecules of ERBB3 network were analyzed. Molecules in ERBB3 network are involved in cell adhesion or mitogen-activated protein kinase (MAPK) cascade.

Enrichment Analysis of ERBB3

The ERBB3 enrichment analysis using cBioPortal for Cancer Genomics revealed that the mRNA expression levels of DLG associated protein 3 (DLGAP3) and RNA polymerase II subunit J2 (POLR2J2) were altered in ERBB3-altered cases and ERBB3-unaltered cases in stomach adenocarcinoma (TCGA, Nature 2014) [13].

Discussion

In this study, we investigated the ERBB signaling role in EMT and cancer. Gene expression of ERBB3, as well as ERBB2, was up-regulated in diffuse-type GC compared to MSCs. ERBB3 network pathway model generated in this study included the EMT-

related molecules such as CTNNB1 and CDH1. ERBB3 plays an important role in the PI3K/AKT pathway and is thus a target for the treatment of solid tumors [18]. Cell survival can be inhibited by a mouse monoclonal antibody against ERBB3 [18]. Expression of ERBB3 is correlated with intestinal stem cell markers such as LGR5, EPHB2, CD44 in colorectal cancer, although ERBB3 expression is observed in the differentiated cell population at the top of the crypts [19]. Furthermore, ERBB3 is targeted by miR-205-5p, a tumor suppressor in breast cancer [20]. Knockdown of ERBB3 suppressed migration and invasion of bladder cancer cells [21]. miR-148a-3p targets both ERBB3 and AKT2 and may repress ERBB3- and AKT-mediated proliferation and EMT [21]. ERBB3 is characterized as a marker for circulating tumor cells exhibiting EMT phenotype and therapeutic resistance [22]. The activation of EGF/EGFR-ErbB2 and NRG1/ErbB2-ErbB3 autocrine signaling loops drives triple negative breast cancer exhibiting the chemoresistance [23]. It seems that ErbB3 receptor is important for perineural migration to the bone marrow [24]. The previous immunohistopathology investigation showed that the ERBB2 and ERBB3 proteins were overexpressed in GC [25]. The previous finding indicating the correlation with ERBB3 overexpression and overall survival in GC is consistent with the present discussion in which ERBB3 up-regulation may be related to malignancy or the EMT network in GC [25]. A report suggests that ERBB2 and ERBB3 are down-regulated by DNA-damaging agent such as cisplatin via ERK and p38 signaling pathway [26]. It is also demonstrated that ERBB3 is involved in resistance to EGFR or ERBB2 targeted therapy for cancer [27]. Furthermore, the study of nanoparticles targeting ERBB2 and microRNA21 suggests the importance of ERBB signaling in cancer [28]. These insights indicate the significance of regulation of ERBB2 and ERBB3 signaling in cancer.

The down-regulation of ERBB3 following EMT leads to a reduction in serum-independent proliferation and an enhancement of PI3K-dependent proliferation [29]. Molecules related to intercellular adhesion and metastatic processes (such as PTEN) and endocytosis (such as ERBB3) are involved in liver metastasis [30]. In ERBB2-positive cancers, expression of EGFR, ERBB3 and CDKN1B are demonstrated to predict and distinguish the PI3K and MAPK signaling axes [31]. The ERBBs are inhibited by N-myc downstream regulated gene 1 (NDRG1) [32]. Since ERBBs are involved in oncogenic pathways, such as the PI3K, Akt and STAT3 pathways, further investigation of targeted molecules in the ERBB network is needed. Considering that the cancer cells migrating and metastasizing exhibit EMT-like phenotype, the investigation of ERBB3 signaling in bone-marrow derived MSCs showing mesenchymal phenotype and diffuse-type GC that exhibits more EMT-like phenotype compared to intestinal-type GC may lead to the novel discoveries for the overcome of the therapeutic resistance. Although the current finding showing ERBB3 gene up-regulation in diffuse-type GC compared to MSCs needs to be further examined in terms of the discussion whether the ERBB3 plays crucial role in EMT or not, it is suggested that the ERBB3 signaling is activated in gastric cancer. To further investigate the role of ERBB3 signaling in EMT and CSCs, the analysis in diffuse-type GC and intestinal-type GC would be needed in future.

In conclusion, ERBB3 gene expression was up-regulated in diffuse-type GC compared to MSCs, and ERBB3 network pathway model in EMT was generated. The model of the ERBB3-related molecular network included the EMT-associated molecules such as CTNNB1 and CDH1.

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