Expression of the ERBB Family of Ligands and Receptors in Gastric Cancer

Sun-ju Byeon^a Hye Seung Lee^c Min-A Kim^a Byung Lan Lee^b Woo Ho Kim^a

Departments of ^aPathology and ^bAnatomy, Seoul National University College of Medicine, and ^cDepartment of Pathology, Seoul National University Bundang Hospital, Seoul, Korea

Keywords

Gastric cancer · ErbB receptors · Ligands · Analysis

Abstract

Objective: Gastric cancer (GC) is the second most common cancer and the third leading cause of cancer-related death in Korea. Alterations in the ERBB (homology to the erythroblastoma viral gene product, v-erbB) receptor family and ERBB-related signaling pathways are frequently observed in GC. However, the roles of the ERBB receptors and their ligands in GC are not well established. **Methods:** We evaluated the expression levels of various ERBB receptor ligands (i.e., heparin-binding epidermal growth factor-like growth factor [HBEGF], transforming growth factor- α [TGFA], amphiregulin [AREG], epiregulin [EREG], epidermal growth factor [EGF], and betacellulin [BTC]) and 3 ERBB family receptors (i.e., epidermal growth factor receptor [EGFR], human EGFR2 [HER2], and ERBB3) in 313 cases of GC using immunohistochemistry, fluorescence in situ hybridization, and mRNA in situ hybridization. **Results:** A high expression of EGFR, HER2, and ERBB3 was observed in 30, 32, and 27 cases, respectively. A high expression of HBEGF, TGFA, AREG, EREG, EGF, and BTC was observed in 91, 97, 151, 74, 26, and 37 cases, respectively. A high expression of TGFA was associated with better

survival, while a high expression of BTC was associated with worse survival. These results were confirmed using Cox proportional hazards analysis. HBEGF, TGFA, AREG, tumor-node-metastasis classification, Lauren's classification, and ERBB3 were significant survival parameters in multivariate analysis.

Conclusion: Among the ERBB family receptors and ligands examined, 3 ligands (i.e., TGFA, HBEGF, and AREG) and ERBB3 had a prognostic impact.

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Introduction

Gastric cancer (GC) is one of the most common cancers in the world, accounting for 8% of the total cases and 10% of the total cancer-related deaths [1]. In Korea, GC is the second most common cancer and the third leading cause of cancer-related death [2]. Several studies have been performed to elucidate gastric carcinogenesis, and alterations in receptor tyrosine kinases have been identified as one of the major factors associated with GC [3, 4]. Among the receptor tyrosine kinases involved in GC, aberrant ERBB (homology to the erythroblastoma viral gene product, v-erbB) receptors are widely known, and extensive research has been performed to investigate their

role in GC [5]. In GC, overexpression or amplification of the epidermal growth factor receptor (EGFR) and ERBB2 (also known as human EGFR2 or HER2) are correlated with a poor prognosis, and several clinical trials targeting EGFR and HER2 have been conducted [6]. However, only HER2-targeted therapy has shown sufficient survival effects in HER2-overexpressed and/or HER2-amplified GC [7]. Small-sized trials have indicated the potential of cetuximab ($p > 0.05$) in EGFR-overexpressed and/or EGFR-amplified GC [8, 9]. The mutation status of the Kirsten Ras (KRAS) protooncogene GTPase is the most predictive marker for EGFR-targeted therapy in colorectal carcinoma [10]. However, GC have a lower frequency of the KRAS mutation compared to colorectal cancers. According to The Cancer Genome Atlas (TCGA) data, 42.5% (90/212) of colorectal cancers have KRAS gene alterations [11], but their proportion in GC is only 4.0–16.0% [12–14]. Various other data sources have also reported a low proportion of EGFR alterations (5–11%) in GC [13, 14]. Due to the low frequency of KRAS mutation and EGFR alteration in GC, some clinical trials did not evaluate the status of KRAS and EGFR in cetuximab-treated GC, and hence these studies did not reveal any efficacy of cetuximab in GC patients [15, 16].

In addition to aberrations in the ERBB receptor tyrosine kinases, their ligands have important roles in cancer genetics and biology. The expression levels of amphiregulin (AREG) and epiregulin (EREG) have been associated with the response to cetuximab in KRAS^{wild} colorectal adenocarcinoma [17, 18]. Meetze et al. [19] reported neuregulin 1 (NRG1) as a predictive biomarker for ERBB3 (also known as HER3)-targeted therapy. Hobor et al. [20] showed that transforming growth factor- α (TGFA) and AREG paracrine networks play important roles in promoting resistance to EGFR blockade in colorectal cancer cell lines. Small-sized studies have shown that low levels of TGFA are associated with a good cetuximab response in GC patients [9, 21]. However, the association between variable ERBB ligands and GC is not well established [22, 23].

In our experience, commercial antibodies are not always appropriate for immunohistochemistry (IHC) of low abundant proteins, although they are considered useful for detection in formalin-fixed and paraffin-embedded tissues. Several research studies have revealed that mRNA expression levels measured using in situ hybridization methods are positively correlated with protein expression in disease-related genes [24]. In this study, we evaluated 6 well-known EGFR ligands (i.e., TGFA, heparin-binding epidermal growth factor-like growth fac-

tor [HBEGF], AREG, EREG, epidermal growth factor [EGF], and betacellulin [BTC]) and 3 ERBB receptors (i.e., EGFR, ERBB2, and ERBB3) and their association with clinicopathological parameters in a GC tissue microarray (TMA).

Materials and Methods

Patient Samples

We studied GC patients who were admitted to the Seoul National University Hospital and underwent surgery from January 2004 to December 2004. Data on patient age, sex, clinical stage, and pathological parameters were retrieved from their medical records. We requested survival information from the Ministry of Security and Public Administration (Korea) according to institutional regulations as previously described [25]. The pathological stages were reclassified according to the 7th International Union against Cancer/American Joint Committee on Cancer. The maximum follow-up period was 60 months. TMA methods were applied to IHC analysis as previously described. Briefly, representative tumor tissues (2-mm diameter) were taken from individual paraffin-embedded gastric tumors (donor blocks) and arranged in new recipient paraffin blocks (TMA blocks) using a trephine apparatus. One core tissue was taken from each GC case, and 8 TMA were made. Sections with a thickness of 4 μ m were cut from each TMA block, deparaffinized, and dehydrated for IHC staining.

IHC, Fluorescence in situ Hybridization, and mRNA in situ Hybridization

Bond-MAX (Leica Microsystems, Wetzlar, Germany) was used for EGFR (1:50 dilution, catalog No. 28-0005; Life Technologies, Carlsbad, CA, USA) and ERBB2 (1:100 dilution, catalog No. A0485; Dako, Glostrup, Denmark) IHC. VENTANA XT BenchMark (Ventana Medical Systems, Tucson, AZ, USA) was used for ERBB3 (1:100 dilution, catalog No. M3710; Spring Bioscience, Pleasanton, CA, USA) IHC. A dual-color PathVysion HER-2 DNA Probe Kit (Abbott Laboratories, Abbott Park, IL, USA) was used for fluorescence in situ hybridization (FISH). Gene amplification was defined when the HER2 gene ratio was more than 2.0. Scoring of ERBB2 IHC and FISH was done as previously described [26]. Membranous staining of EGFR and ERBB3 was evaluated using a proportional score and an intensity score. The proportional score was assigned as: 0 (negative), 1 (<1% of tumor cells), 2 (1–10% of tumor cells), 3 (11–33% of tumor cells), 4 (34–66% of tumor cells), or 5 (>66% of tumor cells). The intensity score was assigned as: 0 (no tumor cell staining), 1 (weak), 2 (moderate), or 3 (strong). The total score was calculated by summing the proportional score and the intensity score. Cases were considered to represent a “high” expression of EGFR and ERBB3 when the total score was greater than 5. mRNA in situ hybridization for TGFA, HBEGF, AREG, EREG, EGF, BTC, and ubiquitin C (positive control) was performed and interpreted manually using an RNAscope[®] 2.0 High-Definition Kit (BROWN; Advanced Cell Diagnostics, Hayward, CA, USA) according to the manufacturer’s protocol. Briefly, the scores were evaluated as: score 0, no staining or 1 dot per cell; score 1, 1–3 dots per cell; score 2, 4–10 dots per cell and no or very few dot clusters; score 3, more than 10 dots per cell; and score 4, 10% positive cells having dot clusters. Cases with a score of 3 or 4 were

Fig. 1. Immunohistochemical staining of ERBB receptor and mRNA in situ hybridization of ERBB ligands.

considered as “high” mRNA expression [24]. The results of IHC staining of the ERBB receptors and mRNA in situ hybridization of the ERBB ligands are shown in Figure 1.

mRNA Expression of the ERBB Ligands in Other Datasets

To compare the mRNA expression status of the ERBB ligands, we analyzed the RNA sequencing data using the fragments per kilobase of transcript per million mapped reads with upper quartile normalization (FPKM-UQ) method. We downloaded the RNA sequencing data of 407 stomach adenocarcinomas from TCGA via the Genomic Data Commons data portal (<https://gdc-portal.nci.nih.gov/>), and the microarray expression data (GSE62254) of 300 stomach adenocarcinomas from the Asian Cancer Research Group (ACRG) via the Gene Expression Omnibus (<https://www.ncbi.nlm.nih.gov/geo/>) [13, 14]. The TCGA data were analyzed after log (base 2) transformation.

Statistical Analysis

Statistical analyses were conducted using R version 3.2.2. The following statistical analyses were performed: Pearson χ^2 test, Fisher’s exact test, Spearman’s correlation test, Kaplan-Meier survival analysis using the log-rank test, and univariate and multivariate survival analyses using Cox proportional hazards (CoxPH) regression models. Stepwise model selection in multivariate analysis was

performed using Akaike’s information criterion. To test the proportional hazards assumption of Cox, regression was performed using the Grambsch-Therneau test. $p < 0.05$ was considered statistically significant.

Results

Comparison between the ERBB Family of Receptors and Ligands and Clinicopathological Parameters

Out of 313 GC cases, a high expression of EGFR, ERBB2, and ERBB3 was found in 30 (9.6%), 32 (10.2%), and 27 (8.6%) cases, respectively. A high expression of TGFA, HBEGF, AREG, EREG, BTC, and EGF was found in 97 (31.0%), 91 (29.1%), 151 (48.2%), 74 (23.6%), 37 (11.8%), and 26 (8.3%) cases, respectively. The results of the detailed comparison between the expression status and the clinicopathological parameters are summarized in Table 1. The number of patients with stage I, II, III, and IV was 78, 72, 118, and 45, respectively.

Table 1. Comparison between clinicopathological parameters and the expression status of ERBB family receptors and ligands

	EGFR		ERBB2		ERBB3		TGFA		HBEGF		AREG		EREG		BTC		EGF	
	low/high	p value	low/high	p value	low/high	p value	low/high	p value	low/high	p value	low/high	p value	low/high	p value	low/high	p value	low/high	p value
Gender																		
Male	199/24	0.265	198/25	0.364	205/18	0.582	147/76	0.063	153/70	0.155	107/116	0.035	168/55	0.503	197/26	0.889	203/20	0.504
Female	84/6		83/7		81/9		69/21		69/21		55/35		71/19		79/11		84/6	
Age																		
<60 years	142/15	0.985	142/15	0.695	139/18	0.073	112/45	0.372	115/42	0.364	91/66	0.028	124/33	0.273	136/21	0.393	146/11	0.403
≥60 years	141/15		139/17		147/9		104/52		107/49		71/85		115/41		140/16		141/15	
Lauren's class																		
Intestinal	114/16	0.168	111/19	0.031	121/9	0.366	73/57	<0.001	79/51	0.001	55/75	0.005	84/46	<0.001	114/16	0.822	119/11	0.933
Nonintestinal	169/14		170/13		165/18		143/40		143/40		107/76		155/28		162/21		168/15	
TNM																		
I/II	124/13	0.361	134/16	0.804	135/15	0.406	93/57	0.010	96/54	0.010	69/81	0.051	115/35	0.902	138/12	0.045	139/11	0.549
III/IV	159/17		147/16		151/12		123/40		126/37		93/71		124/39		138/25		148/15	
Perineural invasion																		
Absent	124/13	0.960	120/17	0.260	123/14	0.376	80/57	<0.001	85/52	0.002	58/79	0.003	95/42	0.010	122/15	0.673	128/9	0.326
Present	159/17		161/15		163/13		136/70		137/39		104/72		144/32		154/22		159/17	
Lymphatic invasion																		
Absent	97/7	0.226	93/11	0.885	91/13	0.085	66/38	0.134	71/33	0.465	55/49	0.778	84/20	0.195	96/8	0.110	100/4	0.050
Present	186/23		188/21		195/14		150/59		151/58		107/102		155/54		180/29		187/22	
Venous invasion																		
Absent	226/22	0.402	227/21	0.045	223/25	0.084	172/76	0.796	173/75	0.374	127/121	0.705	190/58	0.836	222/26	0.152	230/18	0.189
Present	57/8		54/11		63/2		44/21		49/16		35/30		49/16		54/11		57/8	
EBV																		
Negative	262/29	0.707	261/30	1.000	264/27	0.236	199/92	0.478	204/87	0.332	151/140	0.864	221/70	0.795	258/33	0.310	267/24	0.703
Positive	21/1		20/2		22/0		17/5		18/4		11/11		18/4		18/4		20/2	
MSI status																		
MSS	261/26	0.294	255/32	0.090	262/25	1.000	203/84	0.029	206/81	0.271	151/136	0.314	222/65	0.169	255/32	0.211	266/21	0.052
MSI	22/4		26/0		24/2		13/13		16/10		11/15		17/9		21/5		21/5	

EGFR, epidermal growth factor receptor; TGFA, transforming growth factor-α; HBEGF, heparin-binding epidermal growth factor-like growth factor; AREG, amphiregulin; EREG, epiregulin; BTC, betacellulin; EGF, epidermal growth factor; TNM, tumor-node-metastasis.

Table 2. Correlation coefficients between the expression status of ERBB family receptors and ligands

	ERBB2	ERBB3	TGFA	HBEGF	AREG	EREG	BTC	EGF
EGFR	0.069 (0.222)	-0.023 (0.689)	0.040 (0.481)	0.102 (0.071)	0.207 (<0.001)	0.202 (<0.001)	0.015 (0.788)	0.099 (0.081)
ERBB2		0.084 (0.138)	0.230 (<0.001)	0.248 (<0.001)	0.075 (0.185)	0.135 (0.017)	0.007 (0.900)	0.051 (0.366)
ERBB3			-0.009 (0.873)	0.029 (0.611)	0.091 (0.110)	0.097 (0.087)	0.028 (0.616)	-0.010 (0.860)
TGFA				0.545 (<0.001)	0.238 (<0.001)	0.278 (<0.001)	0.033 (0.563)	-0.026 (0.641)
HBEGF					0.269 (<0.001)	0.256 (<0.001)	0.027 (0.633)	0.062 (0.272)
AREG						0.396 (<0.001)	0.161 (0.004)	0.150 (0.008)
EREG							0.192 (0.001)	0.296 (<0.001)
BTC								0.392 (<0.001)

Numbers in parentheses are *p* values. EGFR, epidermal growth factor receptor; TGFA, transforming growth factor-α; HBEGF, heparin-binding epidermal growth factor-like growth factor; AREG, amphiregulin; EREG, epiregulin; BTC, betacellulin; EGF, epidermal growth factor.

A high expression of ERBB2 was more common with an intestinal type histology as previously reported [27]. A high expression of TGFA, HBEGF, AREG, and EREG was also more common with an intestinal type histology ($p < 0.05$). However, a high expression of EGFR or ERBB3 did not show a statistically significant association with the clinicopathological parameters.

Correlation Analysis between ERBB Receptors and Ligands

The results of correlation analyses between the ERBB receptor family and the ERBB ligands are summarized in Table 2. No correlations were observed between the 3 ERBB receptors. AREG and EREG expressions were frequently higher in the high-EGFR cases. High expression levels of TGFA, HBEGF, and EREG were associated with the high-ERBB2 cases. High-ERBB3 GC were not significantly correlated with the expression of the ligands. Considerable positive correlations were observed among 6 ERBB ligands (11 combinations out of 15 possible combinations). These positive correlations among the ERBB ligands were also observed in TCGA (6/15; online suppl. Table 1; see www.karger.com/doi/10.1159/000464250 for all online suppl. material) and ACRG (6/15, online suppl. Table 2) datasets. All 3 datasets (our data, TCGA, and ACRG) showed positive correlations among TGFA, HBEGF, and AREG.

Survival Analysis according to ERBB Family Receptors and Ligands

The expression status of the 3 ERBB receptors did not show statistical significance in the Kaplan-Meier analysis (Fig. 2). Cases with a high TGFA expression showed better survival ($p = 0.047$), while cases with a high BTC expression showed poor survival ($p = 0.017$). These 2 parameters also showed significant impacts in univariate CoxPH analysis (Table 3). The other ERBB ligands did not show statistical significance in univariate analysis. Tumor-node-metastasis (TNM) classification and TGFA, HBEGF, and AREG expression status were statically significant parameters in multivariate CoxPH analysis using all parameters. Nine clinicopathological parameters remained after the model selection algorithm. The expression statuses of HBEGF and AREG were prognostic factors of a poor prognosis, while the expression statuses of ERBB3 and TGFA were prognostic factors of a good prognosis. A subgroup survival analysis was performed in stage II/III patients and EGFR-positive patients, respectively (online suppl. Table 3 and 4). In multivariate CoxPH analysis, TNM stage, age, MSI status, and AREG

expression showed statistical significances in stage II/III patients. After model selection, these 4 parameters also showed statistical significance. Only TNM stage showed statistical significance in EGFR-positive patients in the multivariate CoxPH analysis. After model selection, TNM stage, Lauren's classification, ERBB2 expression, and EREG expression showed statistical significance.

Discussion

ERBB-related signaling pathways are one of the most widely and deeply studied fields in connection with cancer- and non-cancer-related signal transduction [28]. The ERBB signaling pathway comprises 4 receptors and 13 ligands. Interactions between the ERBB receptors and their ligands show remarkable complexity due to various factors such as structural alterations, homodimeric and heterodimeric ERBB receptor activation, the ligand-independent activity of ERBB2, the absence of a kinase domain in ERBB3, and variable degrees of ligand-receptor interactions. Therefore, it is not easy to design a study that reflects the complexity of the ERBB signaling pathway. Among the 4 ERBB receptors, our study did not evaluate ERBB4 expression status. TCGA and ACRG data showed quite low levels of ERBB4 mRNA expression (online suppl. Fig. 1) and we identified no membranous ERBB4 expression in GC tissues (online suppl. Fig. 2), in accordance with He et al. [29].

After multivariate analysis and model selection, a high ERBB3 expression status was associated with good survival. In contrast, previous studies reported a poor survival in GC with a high ERBB3 expression [30, 31]. Since the cytoplasmic ERBB3 expression was regarded as a positive expression in those reports, a consensus guideline for ERBB3 expression is needed to resolve the contradictory results [32].

Among the 6 ERBB ligands, TGFA, HBEGF, and AREG had statistical impacts on patient survival. Unlike previous studies in colorectal carcinoma and cetuximab-treated GC patients [21], a high TGFA mRNA expression was associated with a good prognosis in our GC cases. Although our patients did not receive anti-EGFR or anti-ERBB2 targeted therapy, our study revealed different impacts of high TGFA expression in conventional chemotherapy. Further research is required to clarify the relationship between TGFA expression level, chemotherapy regimens, and survival. High levels of HBEGF and AREG mRNA were revealed as statistically significant parameters in model-selected multivariate analysis. The expres-

Fig. 2. Survival plots by ERBB family receptor and ligand. **a** EGFR; $p = 0.556$. **b** ERBB2; $p = 0.618$. **c** ERBB3; $p = 0.080$. **d** TGFA; $p = 0.047$. **e** HBEGF; $p = 0.395$. **f** AREG; $p = 0.830$. **g** EREG; $p = 0.261$. **h** BTC; $p = 0.017$. **i** EGF; $p = 0.168$. Solid lines represent a high expression. Dashed lines represent a low expression.

sion levels of these 2 ligands were associated with predictive markers for cetuximab-based chemotherapy in colorectal cancers [17]. However, these parameters did not show statistical significance in univariate analysis. We believe that multicollinearity (positive correlation among the expression of ligands) is partly responsible for this phenomenon, and additional studies will be required to identify the multicollinearity. BTC showed a poor

overall survival in our GC cases. However, it was not proven as a statistically significant parameter in multivariate analysis. In a survival analysis limited to stage II/III patients, only a high level of AREG mRNA showed statistical significance. However, TGFA and HBEGF were also significant factors in further model selection. Survival analysis performed in EGFR-positive patients showed different results. However, since the number of

Table 3. Results of the Cox proportional hazards analysis

	Univariate	<i>p</i> value	Multivariate			
			all parameters	<i>p</i> value	model selection	<i>p</i> value
TNM (I/II vs. III/IV)	7.36 (4.69–11.57)	<0.001 ^a	8.38 (5.19–13.51)	<0.001 ^a	8.58 (5.36–13.72)	<0.001 ^a
Sex (male vs. female)	1.39 (0.97–1.98)	0.075	1.33 (0.89–1.98)	0.162	1.33 (0.90–1.98)	0.151
Age (<60 vs. ≥60 years)	1.10 (0.78–1.5)	0.593	1.28 (0.89–1.85)	0.175		
Lauren's classification (intestinal vs. nonintestinal)	1.58 (1.11–2.26)	0.012 ^a	1.41 (0.93–2.13)	0.104 ^a	1.33 (0.90–1.96)	0.158 ^a
EBV (negative vs. positive)	1.16 (0.63–2.15)	0.633	1.74 (0.88–3.43)	0.110	1.72 (0.89–3.33)	0.109
MSI (non-MSI vs. MSI)	0.46 (0.20–1.05)	0.065	0.45 (0.19–1.07)	0.072	0.48 (0.21–1.12)	0.091
EGFR	1.18 (0.68–2.06)	0.556	1.01 (0.57–1.80)	0.971		
ERBB2	1.15 (0.67–1.96)	0.618	1.28 (0.71–2.31)	0.404		
ERBB3	0.51 (0.24–1.10)	0.085	0.48 (0.22–1.05)	0.068	0.45 (0.20–0.98)	0.044
TGFA	0.68 (0.46–1.00)	0.048	0.56 (0.34–0.94)	0.028	0.59 (0.36–0.97)	0.038
HBEGF	0.85 (0.58–1.24)	0.395	1.70 (1.02–2.81)	0.042	1.78 (1.09–2.91)	0.021
AREG	1.04 (0.74–1.46)	0.830	1.57 (1.04–2.37)	0.032	1.71 (1.17–2.50)	0.005
EREG	1.25 (0.85–1.82)	0.262	1.24 (0.77–1.98)	0.372		
BTC	1.72 (1.10–2.70)	0.018	1.19 (0.69–2.04)	0.525 ^a		
EGF	1.47 (0.85–2.56)	0.171	1.04 (0.53–2.04)	0.911		
All parameters				<0.001 ^a		<0.001 ^a

EGFR, epidermal growth factor receptor; TGFA, transforming growth factor- α ; HBEGF, heparin-binding epidermal growth factor-like growth factor; AREG, amphiregulin; EREG, epiregulin; BTC, betacellulin; EGF, epidermal growth factor. ^a Violation of the proportional hazard assumption (defined as *p* value from the Grambsch and Therneau test less than 0.05).

patients used in the analysis is small, further studies are needed.

Our study design has some limitations. Although we normalized the mRNA expression level in each case using ubiquitin C, formalin-fixed and paraffin-embedded samples are known to be vulnerable to mRNA deterioration. Therefore, the high versus low expression in this study is a subjective classification. The model-selection algorithm using Akaike's information criterion may not be suitable for clarifying the relationship between the ERBB receptor family and the various ERBB ligands. We excluded ERBB4 and related ligands (e.g., NRG1–4) from this study. Among NRG1–4, NRG1 and NRG2 were also known as ligands of ERBB3. However, the NRG1-related signal pathway was not properly activated when only ERBB3

was present without ERBB4 [33]. For NRG-related studies, the development of antibodies that can assess ERBB4 membranous expression should be prioritized. Despite these limitations, we found prognostic implications of ERBB ligands and receptors in GC.

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Disclosure Statement

The authors declare no conflict of interest.

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