High Frequency of Juxtamembrane Domain *ERBB2* Mutation in Gastric Cancer

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Abstract. *Background/Aim:* *ERBB2* mutation is an emerging therapeutic target in solid tumors; its therapeutic responses depend on the location of mutation. In gastric cancer, the profiles of *ERBB2* mutations and their relationship with human epidermal growth factor receptor 2 (*HER2*) overexpression remain unknown. We aimed to describe the details of *ERBB2* mutations in gastric cancer. *Patients and Methods:* Comprehensive panel sequencing was performed in 234 advanced gastric cancer patients. We investigated hotspots and clinicopathologic features of *ERBB2* mutant gastric cancer in a single institute and evaluated the hotspots of *ERBB2* mutation in a public database. *Results:* Eighteen patients (7.7%) had *ERBB2* mutations. The most frequent mutation was p.Arg678Gln (42.1%), which was located in the juxtamembrane domain and was the most common mutation in public databases (20.5%). All 18 *ERBB2*-mutant patients were negative for *HER2* expression. Co-occurring genetic alterations included *KRAS*, *PIK3CA*, and *ATM* mutations. *Conclusion:* *ERBB2* mutations were not associated with *HER2* overexpression in gastric cancer patients. The most common mutation was located in the juxtamembrane domain of *ERBB2*.

ERBB2, widely known as human epidermal growth factor receptor 2 (*HER2*), is a proto-oncogene on chromosome 17q21 that encodes a transmembrane protein with tyrosine kinase activity and is involved in pathways that lead to cell growth and differentiation (1). *ERBB2* gene amplification occurs in many cancer types and has been identified in 20% of patients with gastric cancer (2). *ERBB2* amplification is an established therapeutic target in breast and gastric cancer (3, 4) and can be easily identified by confirming the *HER2* overexpression using immunohistochemistry (IHC) (5). In contrast to *ERBB2* amplification, the *ERBB2* mutation has long been neglected due to its low incidence and lack of available therapeutic options. However, *ERBB2* mutation has been recently attracting attention as large-scale sequencing efforts reported recurrent *ERBB2* mutations in various solid tumors and *ERBB2* mutations were shown to play an oncogenic role like *ERBB2* amplification (6-8). Moreover, recent clinical studies of new targeted agents for *ERBB2*-mutant cancers have shown promising results in solid tumors, particularly breast cancer and non-small cell lung cancer (9-11).

ERBB2 receptors contain an extracellular domain, a transmembrane domain, an intracellular region that consists of a juxtamembrane domain, a kinase domain (exons 18-21), and a C-terminal tail domain (12). *ERBB2* mutations occur most frequently in the tyrosine kinase domain region, but mutational hotspots vary among cancer types (7). Importantly, previous clinical trials have revealed that the efficacy of treatment varies according to the location of the *ERBB2* mutation (7, 10, 13). For example, patients receiving neratinib (pan-HER tyrosine kinase inhibitor) with *ERBB2* kinase domain point mutations had a response rate of 21.4%, whereas those with exon 20 insertions had a response rate of 7.1% (10). These variant-specific differences in clinical outcomes demonstrate that a detailed understanding of the

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landscape of *ERBB2* mutations is necessary to develop effective therapies.

Given the promising results of target agents for *ERBB2*-mutant tumors in other organs, clinical investigations targeting *ERBB2* mutations in gastric cancer are also expected in the near future. However, in gastric cancer, *ERBB2* mutations have not been specifically investigated, in contrast to breast and lung cancers (14-16). Herein, we aimed to describe the details of *ERBB2* mutations in clinical samples of advanced gastric cancer in a single institute (n=234) and from a public database (n=1,566). Unlike *ERBB2* amplification, *ERBB2* mutations have not been subjected to routine clinical testing; therefore, data on the clinicopathologic findings of *ERBB2*-mutant cancer are limited (6). We further retrospectively investigated the clinicopathologic features associated with the *ERBB2* mutation in gastric cancer, which might help clinicians select the suitable candidates for *ERBB2* mutation screening.

Patients and Methods

Patients. Targeted sequencing was performed in patients with advanced gastric cancer (n=234) as a requirement at the Department of Pathology, Samsung Medical Center (Seoul, Korea) between January 2019 and April 2021. The tissue type was surgical resection in 82 (35.0%) patients and biopsy in 152 (65.0%) patients. Formalin-fixed paraffin-embedded (FFPE) tissue samples were used for targeted sequencing. Patients' clinicopathological information, including age, sex, stage according to the American Joint Committee on Cancer (AJCC) 8th edition (17), pathologic diagnosis, and Lauren classification (18), were evaluated by reviewing the electronic medical records. Data for HER2, programmed death-ligand 1 (PD-L1) and mismatch repair protein expression, as well as for Epstein-Barr virus (EBV), and microsatellite instability (MSI) analysis were also retrieved from the medical records; the protocols (IHC and *in situ* hybridization; ISH) for these assessments were described in a previous study (19, 20). This study was reviewed and approved by the Institutional Review Board of the Samsung Medical Center (2020-06-045-002).

DNA/RNA extraction. Genomic DNA and RNA were extracted from FFPE tissue sections and purified using the QIAGEN AllPrep DNA/RNA FFPE Kit (QIAGEN, Venlo, the Netherlands). Manual microdissection was performed for surgical samples and available biopsy samples. The DNA concentrations were measured using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). A Genomic DNA ScreenTape (Agilent Technologies, Santa Clara, CA, USA) on an Agilent 2200 TapeStation system (Agilent Technologies) was used to determine the DNA integrity number, measure of the size of the DNA fragments, and DNA quality.

Library preparation, sequencing, and data analysis. Forty nanograms of DNA were quantified using the Qubit dsDNA HS Assay Kit on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and then sheared using a Covaris E220 Focused-ultrasonicator (Woburn, MA, USA) and an 8 microTUBE-50 Strip AFA Fiber V2, according to the manufacturer's instructions. For DNA library

preparation and enrichment, the TruSight™ Oncology 500 Kit (Illumina Inc., San Diego, CA, USA) was used according to the manufacturer's instructions. Post-enriched libraries were quantified, pooled, and sequenced using a NextSeq 500 system (Illumina Inc.). The sequencing data were analyzed using the TruSight™ Oncology 500 Local App version 1.3.0.39 (Illumina), a comprehensive tumor profiling assay designed to identify known tumor biomarkers, including small variants, splice variants, and fusions. The reads were aligned to the reference genome (GRCh37/hg19) using a Burrows-Wheeler Aligner-MEM (21). Poorly mapped reads with a mapping quality below 20 were removed using SAMtools version 1.3.1 (22). Somatic mutations, including single nucleotide variants and small insertions and deletions, were detected by PISCES and Psara (23). The rest of the pipeline were as follows: CRAFT for copy number variation, TmbRaider for tumor mutation burden, Hubble for MSI, STAR for RNA alignment, and Manta for fusion calling (24). Outputs of data exported from the TSO 500 pipeline (24) were annotated using the Ensembl Variant Effect Predictor Annotation Engine, with information from databases, such as the Single Nucleotide Polymorphism (dbSNP), Genome Aggregation (gnomAD; which includes genome and exome data), 1000 Genomes, ClinVar, COSMIC, RefSeq, and Ensembl databases. The processed genomic alterations were categorized into a four-tier system based on the American Society of Clinical Oncology and College of American Pathologists guidelines (25), annotated with proper references. The following criteria were used to filter the insignificant variants and possible germline variants: (i) variants with <5% allele frequency and <100× read depth at the variant; (ii) variants previously reported to be benign or likely benign in the ClinVar archive (26); and (iii) variants with a frequency greater than 1% in gnomAD (27) were excluded.

Statistical analysis. Statistical analyses were performed using IBM SPSS for Windows version 27 (IBM Corp., Armonk, NY, USA) and R software (version 3.6.3; The R Foundation for Statistical Computing, Vienna, Austria). Pearson's chi-square, Fisher's exact, or Cochran-Armitage trend tests were used to analyze the relationship between *ERBB2* mutation status and clinicopathological parameters. A *p*-value of <0.05 was considered significant.

Results

Patient characteristics. All patients were Korean individuals, and the mean age was 58.2 years (range=26-86). On histopathological examination, 177 (75.6%) patients were diagnosed with poorly differentiated carcinoma, while 57 (24.4%) were diagnosed with well or moderately differentiated carcinoma. Except for 24 patients, who were not available for pathologic staging, majority (80.3%) of the patients had stage IV disease according to the AJCC 8th staging system at the time of sequencing. There were 21 (9.0%) patients and 1 patient (0.4%) who had stages III and II disease, respectively. None of the patients had stage I disease.

***ERBB2* mutation in our cohort.** Among the 234 gastric cancer patients, *ERBB2* mutations were detected in 18 (7.7%) patients. In one patient, two different types of *ERBB2* mutations were detected; a total of 19 *ERBB2* mutations

Figure 1. Distribution of *ERBB2* mutations in gastric cancer. (A) The locations of *ERBB2* mutations in our sequencing cohort (n=234). (B) The locations of *ERBB2* mutations in public databases (n=1,566).

were detected in this population. The distribution of *ERBB2* mutations is shown in Figure 1A. The most frequently detected mutation was p.Arg678Gln (R678Q; c.2033G>A) (n=8, 42.1%), which is located in the juxtamembrane domain of *ERBB2*. Seven mutations (36.8%) were detected in the active region of the tyrosine kinase domain. These mutations were: V777L (c.2329G>T), V777M (c.2329G>A), V842I (c.2524G>A), T862A (c.2584A>G), L869R (c.2606T>G), and p.Met774dup (c.2320_2322dup), all of which were confined to exons 20 and 21. The remaining 4 (21.1%) mutations occurred in the extracellular domain, and the detected mutations were D277H (c.829G>C), D277Y (c.829G>T), and S310F (c.929C>T). All but one mutation have been previously reported as oncogenic or likely oncogenic according to the public database (ONCOKB; <https://www.oncokb.org>; May 27, 2021). One *ERBB2* duplication mutation, p.Met774dup (c.2320_2322dup), in exon 20 of the kinase domain, has not been reported previously. The variant allele frequency of the detected *ERBB2* mutations ranged from 1.6% to 40%.

***ERBB2* mutation in public databases.** cBioPortal was utilized to analyze *ERBB2* mutations in gastric cancer reported in public databases (28, 29). Combining the reports of 9 different studies (MSKCC 2020; TMUCIH, PNAS 2015;

OncoSG, 2018; Pfizer and UHK, Nat Genet 2014; TCGA, Firehose Legacy; TCGA, Nature 2014; TCGA, PanCancer Atlas; U Tokyo, Nat Genet 2014; and UHK Nat Genet 2011) with exclusion of overlapping cases, the frequency of *ERBB2* mutation was 4.9% (78 of 1,566). The frequency of *ERBB2* mutation varied among studies, ranging from 3.5 to 3.6%. The distribution of the *ERBB2* mutations is shown in Figure 1B. The most common mutation was also R678Q (20.5%) in the juxtamembrane domain, followed by S310F/Y (19.2%) in the extracellular domain, and V842I (7.7%) in the tyrosine kinase domain.

Clinicopathological characteristics of ERBB2-mutant tumors in our cohort. The clinicopathological characteristics of patients with *ERBB2* mutation status in our cohort are summarized in Table I. Among the 18 patients with *ERBB2* mutation, half were men, and the mean age was 55.3 years (range=38-82). On histologic examination, 15 (83.3%) patients were diagnosed with poorly differentiated carcinoma. According to the Lauren classification (18), 14 (77.8%) patients had the diffuse type, while 4 (22.2%) had the intestinal type of carcinoma. Except for 2 patients with missing information on pathologic stage, 2 (12.5%) patients had stage III and 14 (87.5%) had stage IV disease at the time of sequencing. HER2 IHC was performed in all *ERBB2*-

Table I. Patient characteristics.

Variables	ERBB2 mutation		Total	p-Value
	ERBB2-mutant	ERBB2-wild		
Age, years				
<60	13 (72.2%)	113 (52.3%)	126 (53.8%)	0.104
≥60	5 (27.8%)	103 (47.7%)	108 (46.2%)	
Gender				
Male	9 (50.0%)	82 (38.0%)	91 (38.9%)	0.314
Female	9 (50.0%)	134 (62.0%)	143 (61.1%)	
Histologic type				
W/D, M/D	3 (16.7%)	54 (25.0%)	57 (24.4%)	0.429
P/D	15 (83.3%)	162 (75.0%)	177 (75.6%)	
Lauren classification				
Intestinal	4 (22.2%)	63 (29.2%)	67 (28.6%)	0.398*
Diffuse	14 (77.8%)	125 (57.9%)	139 (59.4%)	
Mixed	0 (0.0%)	23 (10.6%)	23 (9.8%)	
Indeterminate	0 (0.0%)	5 (2.3%)	5 (2.1%)	
Stage				
I	0 (0.0%)	0 (0.0%)	0 (0.0%)	0.692*
II	0 (0.0%)	1 (0.5%)	1 (0.5%)	
III	2 (12.5%)	19 (9.8%)	21 (10.0%)	
IV	14 (87.5%)	174 (89.7%)	188 (89.5%)	
HER2 immunohistochemistry				
Negative	18 (100.0%)	179 (89.1%)	197 (90.0%)	0.228*
Positive	0 (0.0%)	22 (10.9%)	22 (10.0%)	
EBV				
Negative	15 (93.8%)	150 (98.0%)	165 (97.6%)	0.331*
Positive	1 (6.3%)	3 (2.0%)	4 (2.4%)	
MSI				
MSS	17 (94.4%)	212 (98.1%)	229 (97.9%)	0.332*
MSI-high	1 (5.6%)	4 (1.9%)	5 (2.1%)	
Tumor mutation burden				
Low	17 (94.4%)	192 (88.9%)	209 (89.3%)	0.702*
High	1 (5.6%)	24 (11.1%)	25 (10.7%)	
PD-L1				
Negative (CPS=0)	3 (23.1%)	38 (29.2%)	41 (28.7%)	0.758*
Positive (CPS ≥1)	10 (76.9%)	92 (70.8%)	102 (71.3%)	

W/D, Well differentiated; M/D, moderately differentiated; P/D, poorly differentiated; EBV, Epstein-barr virus; MSI, microsatellite instability; MSS, microsatellite stable; PD-L1, programmed cell death ligand-1; CPS, combined positive score. *by Fisher's exact test.

mutant tumors, and all were HER2 negative. EBV ISH was performed in 16 patients, and only one (6.3%) patient was EBV positive. PD-L1 IHC was performed in 13 out of 18 patients, and 10 (76.9%) patients were positive with combined positive scores of 1 to 70.

Next, we compared *ERBB2*-mutant tumors and *ERBB2* wild-type tumors to identify the clinicopathological features associated with *ERBB2* mutation in gastric cancer (Table I). However, there was no factor significantly associated with *ERBB2* mutation, possibly due to the small number of *ERBB2*-mutant cases.

Co-occurring alterations of ERBB2-mutant tumors in our cohort. Table II summarizes the coexisting genetic alterations

(pathogenic/likely pathogenic only) detected by targeted sequencing of 18 *ERBB2*-mutant tumors. Coexisting single nucleotide variants were detected in 10 (55.6%) patients. Copy number alteration was detected in one (5.6%) patient, and no fusion was detected. No additional significant alterations were detected other than the *ERBB2* mutation in 7 (38.8%) patients. The most common single nucleotide variants were *ATM* mutation (n=3, 16.7%), *KRAS* mutation (n=3, 16.7%), and *PIK3CA* mutation (n=2, 11.1%). Three *ATM* mutations were previously reported as pathogenic germline variants (NM_000051.3 [*ATM*]:c.103C>T [p.Arg35Ter], c.8977C>T [p.Arg2993Ter], and c.8432del [p.Lys2811fs]). Considering the high variant allele frequency in patients 8 and 10, the possibility of a germline variant is present. *BRCA2*, *SF3B1*,

Table II. Co-occurring alterations detected in *ERBB2* mutated cases.

Patient no.	<i>ERBB2</i> mutation	Co-occurring single nucleotide variant				Copy number alteration	
		Gene	c.DNA	Amino acid	VAF	Gene	Copy number
2	p.Met774dup	<i>PIK3CA</i>	c.1624G>A	p.Glu542Lys	4.30%		
3	p.Arg678Gln	<i>PIK3CA</i>	c.1624G>A	p.Glu542Lys	13.40%		
8	p.Val842Ile	<i>ATM</i>	c.103C>T	p.Arg35Ter	39.00%		
9						<i>FGFR2</i>	32.8
10	p.Arg678Gln	<i>ATM</i>	c.8977C>T	p.Arg2993Ter	45.90%		
11	p.Arg678Gln	<i>BRCA2</i>	c.7667del	p.Asn2556MetfsTer92	4.20%		
		<i>SF3B1</i>	c.2098A>G	p.Lys700Glu	4.00%		
12	p.Thr862Ala	<i>KRAS</i>	c.38G>A	p.Gly13Asp	1.50%		
13	p.Val777Met	<i>KRAS</i>	c.35G>A	p.Gly12Asp	4.30%		
		<i>ATM</i>	c.8432del	p.Lys2811SerfsTer46	13.80%		
		<i>PTEN</i>	c.801del	p.Lys267ArgfsTer9	4.10%		
		<i>PTEN</i>	c.389C>T	p.Arg130Ter	3.40%		
14	p.Arg678Gln	<i>MAP2K1</i>	c.167A>C	p.Gln56Pro	9.10%		
15	p.Asp277Tyr	<i>KRAS</i>	c.35G>A	p.Gly12Asp	20.60%		
16	p.Arg678Gln	<i>KDM6A</i>	c.2125C>T	p.Gln709Ter	30.00%		

VAF, Variant allele frequency.

MAP2K1, and *KDM6A* mutations were detected in one patient. A coexisting *FGFR2* amplification (estimated copy number: 32.8) was detected in patient 9. No co-occurring *ERBB2* amplification was identified in other patients.

The tumor mutation burden was categorized as high in only one (5.6%) patient, and this patient showed a MSI-high status. The detected *ERBB2* variant was V777M (c.2329G>A).

Discussion

The evidence supporting HER2-directed drugs in *ERBB2*-mutant cancers is promising and is rapidly growing in various solid tumors. Whereas *ERBB2* amplification is a well-established target in the treatment of advanced gastric cancers, *ERBB2* mutations have not been specifically investigated in gastric cancer. Several preclinical and clinical studies have shown that *ERBB2* mutation hotspots vary across cancer types, which is closely associated with drug sensitivity (6, 7, 10). In the present study, we investigated the frequency, mutation details, and clinicopathological features of *ERBB2* mutations in gastric cancer.

The frequency of *ERBB2* mutation was 7.7% (18 of 234 patients), which is slightly higher than the reported frequency in gastric cancer (~4.5%) (7). The most common *ERBB2* mutation was R678Q in the juxtamembrane domain, followed by mutations in the tyrosine kinase and extracellular domains. R678Q was also the most common mutation in gastric cancer as per public databases. This contrasts with other tumor types where the majority of *ERBB2* mutations are located within the kinase and extracellular domains (7). Unlike other oncogenes, such as *BRAF* or *KRAS*, *ERBB2* has no single predominant

mutant allele, and the distribution of mutations varies by tumor type (10). Across all cancer types, the most common location is the tyrosine kinase domain, and the most common single mutation is S310F/Y, which is detected in the extracellular domain (7). Among other tumors with positive response to anti-HER2 therapy, the most common *ERBB2* mutation in non-small cell lung cancer and breast cancer was located in the tyrosine kinase domains of exons 20 and 19, respectively (7). The treatment effect of anti-HER2 agents has been mostly reported in tumors with *ERBB2* tyrosine kinase domain mutations (10).

The juxtamembrane domain is a relatively short region in the intracellular domain, containing 39 amino acids, whose function is receptor dimerization and stability (12). Juxtamembrane domain mutations are relatively rare, accounting for up to 7.7% of the mutations in reported cases of all cancer types (12). R678Q was most common in transmembrane/juxtamembrane domain mutations and accounted for 3.5%-5.0% of all *ERBB2* mutations (12). To the best of our knowledge, no clinical study has investigated the use of an anti-HER2 therapy for *ERBB2* R678Q-mutant tumors. However, preclinical studies have revealed that R678Q enhances HER2 activity by stabilizing an activating conformation and confers sensitivity to anti-HER2 therapy (12). *In vitro* studies of breast cancer showed that the R678Q mutation is an activating mutation that is sensitive to targeted treatment with trastuzumab, lapatinib, afatinib, and neratinib (10, 12). Other than R678Q, the mutations were dispersed throughout the tyrosine kinase domain and extracellular domains, nearly all of which were previously detected in public databases (<http://www.cbioportal.org>; May 27, 2021). One duplication mutation in exon 20, p.Met774dup (c.2320_2322dup), has not been previously reported. This

mutation was detected close to the common exon 20 insertion, Y772dupYVMA (7).

Next, we evaluated the clinicopathological features of *ERBB2*-mutant gastric cancer in our cohort. Consistent with the findings of previous studies (6, 30), all *ERBB2* mutated cases were negative for HER2 IHC. *ERBB2* amplification and mutation are mostly mutually exclusive, and *ERBB2*-mutant tumors do not overexpress HER2 protein like *ERBB2*-amplified tumors (6). In other tumors, *ERBB2* mutations are reported to be enriched in certain histological types, such as high-grade lobular breast carcinomas (31). In the present study, we failed to identify the clinicopathological features associated with *ERBB2* mutation in gastric cancer, possibly due to the small number of cases. However, it appears that *ERBB2*-mutant gastric cancers are mainly detected in tumors with poor differentiation, negative HER2 IHC results, negative EBV ISH results, and microsatellite stable status, considering the known proportion of those biomarkers in gastric cancer.

The impact of *ERBB2* mutations is likely to be affected by coexisting mutations (6). Previously, *ERBB2* mutations were reported to display significant co-occurrence with *ERBB3*, *RAF1*, *PIK3CA*, and *PIK3R2* alterations but mutually exclusive with *KRAS* alterations in other solid tumors (30). However, in our study, coexisting *KRAS* mutations were detected in three patients. Notably, pathogenic *ATM* variants were detected in these three *ERBB2*-mutant patients. Future preclinical and clinical studies investigating the role of coexisting mutations in *ERBB2*-mutant gastric cancer are warranted.

The present study has the following limitations: the number of patients was relatively small and only the Asian population was assessed. Hence, further studies in a large number of gastric cancer patients are warranted to examine the association between *ERBB2* mutations and clinicopathological factors.

In conclusion, comprehensive panel sequencing detected *ERBB2* mutations in patients with gastric cancer. The most commonly detected variant was R678Q in the juxtamembrane domain in our cohort and public databases. All *ERBB2*-mutated tumors were negative for HER2 expression, which confirmed that it is not possible to detect *ERBB2* mutation by HER2 IHC. Our study might provide useful information for the development of anti-HER2 therapy for *ERBB2*-mutant gastric cancers.

Conflicts of Interest

No potential conflicts of interest exist.

Authors' Contributions

S. A. and K-M. K. designed and supervised the study. S. P. evaluated the data, performed the clinicopathologic analysis, and wrote the manuscript. D. G. K., S. K., and H. K. assisted with the data analysis and writing of the manuscript. All authors have read and approved the final manuscript.

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