ANTICANCER RESEARCH *38*: 367-372 (2018)
doi:10.21873/anticanres.12231

Evaluation of Expression of Human Epidermal Growth Factor Receptor 2 (HER2) in Gastric and Gastroesophageal Junction Adenocarcinoma Using IHC and Dual-ISH

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Abstract. *Background/Aim: Trastuzumab® is used for human epidermal growth factor receptor 2 (HER2)-overexpressing metastatic gastric/gastroesophageal junction (GEJ) adenocarcinoma. Our aim was to compare HER2 expression by immunohistochemistry (IHC) and dual in situ hybridization (DISH) in early-stage vs. late-stage gastric and GEJ tumors. Materials and Methods: Fifty early-stage and 50 late-stage gastric tumors and a similar number of early-stage and late-stage GEJ tumors were studied. HER2 was analyzed by IHC and dual-ISH using tissue microarray. Results: Of 200 selected cases, 168 had satisfactory results. Among the 110 cases with both tests successfully performed, there were only five cases with discrepancy between assays (4.5%). Seven equivocal (2+) cases by IHC were all found to be amplified by dual-ISH. When compared with IHC, dual-ISH identified 12 additional HER2-positive cases (10.9%). Conclusion: The 12.5% overall overexpression/amplification in gastric and GEJ adenocarcinomas is in concordance with previous reports. No correlation was found between tumor stage and HER2 overexpression/amplification. Determination of HER2 in limited tissue samples benefits from combinational IHC and ISH testing.*

Presented as a poster at the CAP Annual Meeting in Nashville, TN, October 4-7, 2015.

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Key Words: HER2, gastric cancer, esophageal cancer, DISH.

Adenocarcinoma of the esophagus, gastroesophageal junction (GEJ) and stomach represents a global healthcare problem (1). In 2017, 28,000 new cancer gastric and 16,940 new esophageal cancer cases are estimated to be diagnosed in the United States and an estimate of 10.960. and 15,690 people, respectively, will die of their disease. In 2014, there were an estimated 95,764 and 45,547 people living with stomach cancer and esophageal cancer in the United States (2). It is considered the third most common gastrointestinal malignancy after colorectal and pancreatic carcinoma in North America and the second worldwide. The prognosis for advanced gastric cancer remains poor and the 5-year survival rate for all stages is about 30% (2, 4). The main underlying causes related to the pathogenesis of gastric and gastro/esophageal cancer include environmental, ethnic and behavioral factors, infectious agents, and specific genetic alterations, among others (5).

Human epidermal growth factor receptor protein 2 (HER2) is a member of the epidermal growth factor receptor (EGFR) family. The *HER2* oncogene encodes for a 185-kDa transmembrane glycoprotein receptor with intracellular tyrosine kinase activity. It is located in the long arm of human chromosome 17, and is involved in a variety of cellular processes that affects tumor cell biology in several ways, including cell proliferation, apoptosis, adhesion, migration, and differentiation (1, 6). *HER2* amplification is seen in several malignancies such as breast and ovarian cancer. HER2 is overexpressed in 10-25% of gastric cancer cases (4). This was established using the standard techniques of immunohistochemistry (IHC) and *in situ* hybridization for HER2 assessment (1). The use of IHC techniques, and *in situ* hybridization assays, including dual-ISH (DISH), fluorescent *in situ* hybridization (FISH), chromogenic *in situ* hybridization (CISH), and silver *in situ* hybridization (SISH), to assess *HER2* overexpression have been researched and

compared in recent years. The use of IHC as the first step in the HER2 testing algorithm has been recommended due to its low cost and high concordance rate with *in situ* hybridization methods (7-9).

The objectives of this retrospective study were: i) to compare HER2 expression in early-stage vs. late-stage gastric and esophageal/GEJ adenocarcinomas, ii) to assess if DISH can be used for testing HER2, iii) to evaluate if co-testing with IHC and ISH is a better alternative to the performance of ISH only when IHC result is 2+ (equivocal).

Materials and Methods

Patients. The study consisted of a retrospective analysis of 200 patients with a previous diagnosis of gastric or esophageal/GEJ adenocarcinoma, reported between 2000 and 2011. For clinical purposes, the cases were classified as esophageal adenocarcinoma when the tumors were located entirely above the GEJ, and as gastric adenocarcinoma when they were located below the GEJ junction; tumors located across the GEJ were considered GEJ adenocarcinomas. For the purpose of the study, all cases were considered as one group.

This study was performed using an Institutional Review Board-approved protocol (MCC 16754).

Formalin-fixed paraffin embedded tissue (FFPE) samples, clinical records, and pathology reports were retrieved from the electronic medical records and pathology files from the Department of Anatomic Pathology at Moffitt Cancer Center. The tumors were graded as well-, moderately and poorly differentiated. The cases were subsequently divided as early-stage (stage 0-IB) and late-stage (stage IIIA-IV) according to the AJCC Cancer Staging Manual (10). Four main cohorts were selected as follows: 50 cases of early-stage gastric, 50 cases of late-stage gastric, 50 early-stage esophageal (E)/GEJ and 50 late-stage E/GEJ.

Pathological evaluation and tissue microarray (TMA) construction. The hematoxylin and eosin (H&E) slides from each case with a previous diagnosis of gastric and GEJ adenocarcinoma were selected and evaluated by two pathologists (DC and MR) to confirm the diagnosis. The tumor was outlined with a permanent marker and these areas were used to build the gastric and E/GEJ adenocarcinoma cancer TMA. The TMA was composed of 209 (1 mm) cores of tissue representing 50 early-stage gastric, 50 later-stage gastric, 50 early-stage E/GEJ, 50 late-stage E/GEJ and nine controls of breast cancer tissue. The tissue cores were randomly distributed on the TMA and 3 μm sections were obtained from the TMA and subjected to IHC and DISH tests.

IHC was analyzed using the Ventana PATHWAY® system (Ventana Medical Systems, Inc. Tucson, AZ, USA) using the 4B5 rabbit monoclonal antibody to HER2/neu.

For DISH, the Ventana INFORM HER2 Dual ISH DNA Probe Cocktail assay was used. This assay detects the *HER2* gene using a dinitrophenyl-labeled probe and the chromosome 17 centromere using a digoxigenin-labeled probe. The *HER2* gene was visualized as a discrete black signal, using Ventana ultraview silver ISH dinitrophenyl (SISH). The chromosome 17 centromere was visualized as a red signal using the Ventana ultraview Red ISH digoxigenin detection. Using ×40 or ×60 objectives, analysis of ISH was performed on the areas of interest. For each nucleus, we conducted a manual count of the number of *HER2* signals and the number of centromere 17 (CEP17) signals using bright-field microscopy. The ratio of the average number of *HER2* gene copies to the average number of chromosome 17 copies was calculated. Indeterminate results were either due to absence of target cells, no tissue core present, unacceptable nuclear morphology, unacceptable background (SISH 'dust'), or weak/absent staining in targeted cells. Per guidelines (10), a HER2-to- chromosome 17 ratio of less than 2.0 was considered negative, whereas a ratio of 2.0 or more was considered positive. The first and last sections of each series were stained with H&E to check for the conservation of the relevant tissue during testing.

The HER2 expression was evaluated by two pathologists (DC and MR) using the modified Trastuzumab for Gastric Cancer trial scoring criteria established for gastric and gastroesophageal carcinomas (10). Indeterminate and discrepant cases were reviewed to reach a final consensus (Table I).

Results

Clinicopathological characteristics. Our study population comprised of 200 cases. Thirty-two cases (16%) were excluded because the tissue cores either did not contain tumor or they were lost in the deeper sections used for analysis. The remaining 168 cases were analyzed. The majority of patients were males 76.2% (n=128). The age ranged from 32 to 97 years (median=71 years). The tumor size ranged from 0.3 to 24 cm, (median 3.5 cm.). The tumor characteristics are listed in Table II. These data reflect the expected distribution and features of these tumors.

IHC and DISH results. The IHC and DISH results are summarized in Table III. HER2 overexpression or amplification was observed in 12.5% of all evaluable adenocarcinomas (21/168 cases) (Figure 1). Among the 110 cases in which both tests were successfully performed, there were discrepant results between IHC and DISH (4.5%) in only five cases (Table IV). These discrepant cases consisted of negative IHC result with amplification by DISH. Seven equivocal (2+) cases by IHC were all amplified by DISH. Thus, *in toto*, when compared with IHC, DISH identified 12 additional HER2-positive cases (10.9%).

When broken-down by stage and site, the positive cases included 11 early-stage cases (8 gastric and 3 E/GEJ) and 10 late stages (3 gastric and 7 E/GEJ) (Table V).

Discussion

Trastuzumab® is a recombinant humanized monoclonal antibody that targets the extracellular domain IV of the HER2 protein. In 2010 after the results of the ToGA trial (11) evaluating Trastuzumab® in metastatic overexpressing (3+ HER2) gastric and GEJ cancers, the US Food and Drug Administration (FDA) approved the drug in combination with cisplatin and capecitabine or 5-fluorouracil for the

Table I. *Criteria used for scoring human epidermal growth factor receptor 2 (HER2) expression by immunohistochemistry in gastric and esophagogastric junction adenocarcinoma in biopsy specimens.*

Score	Reaction	HER2 expression assessment
0	No reactivity or no membranous reactivity in any cancer cell	Negative
1+	Cancer cell cluster with a faint or barely perceptible membranous reactivity, regardless of percentage of positive cancer cells	Negative
2+	Cancer cell cluster with a weak to moderate, complete basolateral or lateral membranous reactivity, regardless of percentage of positive cancer cells	Equivocal
3+	Cancer cell cluster with a strong, complete basolateral or lateral membranous reactivity, regardless of percentage of positive cancer cells	Positive

treatment of patients with HER2-overexpressing advanced stage metastatic gastric or GEJ adenocarcinoma(12). However, metastatic gastric cancer has already a poor prognosis and the use of targeted therapies such as Trastuzumab® may also benefit patients with non-metastatic/ early stage HER2 positive adenocarcinomas (3, 14).

In our study, we found a 12.5% overall overexpression/ amplification in gastric and esophageal/GEJ adenocarcinomas. Compared to IHC, DISH detected a larger number of HER2 positive cases (Table III).

Several reviews reported that 19 (but as low as 4.4%) to 22% (but as high as 53.4%) of gastric and esophageal adenocarcinomas are HER2 (out of 1,962 and 475 patients, respectively) (15). In addition, the overexpression of HER2 in patients with gastric or gastro/esophageal adenocarcinoma is most frequently seen within tumors of intestinal-type, higher disease stage, late onset presentation, and higher metastasis rate (16, 17).

A review of the English literature showed a few studies on the correlation between HER2 status by IHC, FISH and DISH. Kazuki *et al*. (18), found a concordance rate of 100% between FISH and DISH methods in 108 gastric cancer cases and concluded that the use of different techniques did not affect the evaluation of HER2 status. Grin *et al*. (19), also described a high concordance rate of 98% (49/50) between DISH and FISH. They reported on the sole discrepant case that was non-amplified by FISH but showed focal amplification by DISH and attributed the discrepancy to tumor heterogeneity, which was a frequent finding (78% of HER2-positive cases). Cho *et al*. (20) described a 95%, statistically significant ($p<0.000001$) concordance rate between IHC and SISH. Their findings also support the use of other *in situ* techniques in equivocal cases. Our study showed five discrepant cases (4.5%). Discrepant results between IHC and ISH methods can be secondary to fixation protocols, commercial antibodies used, surgical *versus* biopsy specimens, interobserver variability, whole-tissue sections *versus* tissue microarrays, and one of the most important factors mentioned in several studies is tumor heterogeneity (8, 21).

Table II. *Clinicopathological characteristics of study cases (n=168).*

Characteristic	Value
Median age (range), years	71 (32-97)
Gender, n	
Male	128
Female	40
Median tumor size (range), cm	3.5 (0.3-24)
Tumor type, n	
Well-differentiated	10
Moderately differentiated	73
Poorly differentiated	85
Tumor type and stage, n	
Early-stage gastric	44
Late-stage gastric	39
Early-stage E/GEJ	42
Late-stage E/GEJ	43

E/GEJ: Esophageal/gastroesophageal junction.

Moelans *et al*. (22) in their study of 199 cases found a concordance rate (92%) in gastric cancer between IHC and CISH, with the additional finding that CISH showed less heterogeneity than IHC. In their study, the cases were divided in two groups; early onset and late onset. Their results showed that none of the patients in the early onset group (<45 years) showed overexpression by IHC and only 2% were amplified by CISH. The late onset group showed an overexpression in 7% of the cases by IHC and 8% amplification by CISH. They concluded that a significant association between HER2 overexpression and age ($p=0.008$) exists. Similarly, in our study we found that only two patients under 45 years of age demonstrated HER2 amplification by DISH, one patient within the early stage gastric category and the other within the late stage esophageal/GEJ subgroup. In these cases, HER2 by IHC showed negative and equivocal results respectively.

Accurate HER2 assessment in gastric and esophageal/GEJ adenocarcinomas is crucial for patient selection for HER2-

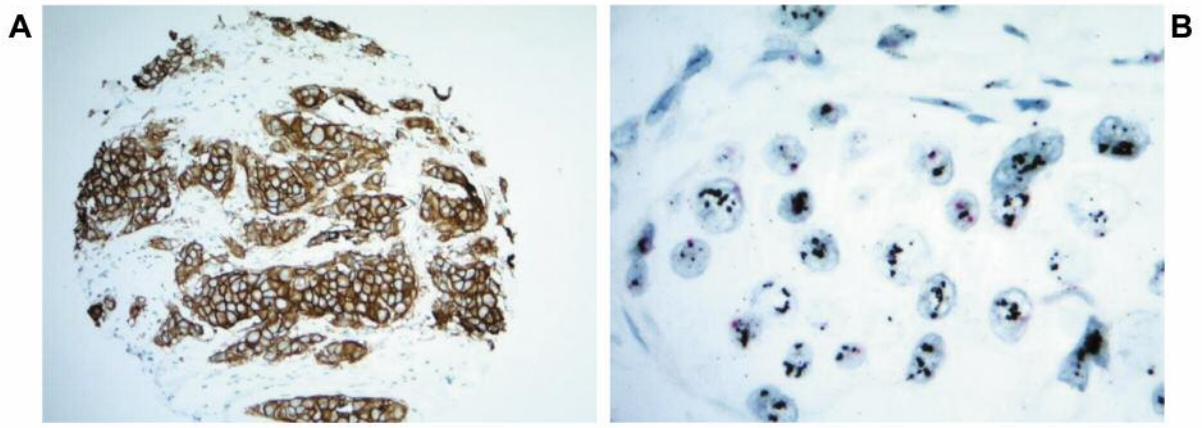

Figure 1. *Biopsy specimen of a case of invasive adenocarcinoma of the gastroesophageal junction with overexpression/amplification of human epidermal growth factor receptor 2 (HER2). A: Immunohistochemical staining showing strong membranous immunoreactivity (3+) with HER2 antibody. B: Dual in situ hybridization result displaying an increased number of HER2 signals (black). Original magnification, ×63.*

Table III. *Results of all 200 cases tested in this study using dual in situ hybridization (DISH) and immunohistochemistry (IHC) tests for human epidermal growth factor receptor 2 (HER2).*

	IHC					DISH				IHC-negative DISH-amplified, n
Diagnosis	Positive	Negative	Equivocal	Insufficient	Total	Amplified	Not amplified	Insufficient	Total	
Early-stage gastric, n	4	38	2	6	50	8	25	17	50	2
Late-stage gastric, n	2	37	0	11	50	3	15	32	50	1
Early-stage E/GEJ, n	1	40	1	8	50	3	33	14	50	1
Late-stage E/GEJ, n	2	37	4	7	50	7	16	27	50	1
Total, n (%)	9 (4.5%)	152 (76%)	7 (3.5%)	32 (16%)		21 (10.5%)	89 (44.5%)	90 (45%)		5 (2.5%)

E/GEJ: Esophageal/gastroesophageal junction.

targeted therapy. Therefore, laboratories must ensure that appropriate validation and quality measures are in place. The College of American Pathologists (CAP) requires the implementation of quality control analysis by creating internal revalidation of the IHC and ISH assays used for detection of overexpression/amplification of HER2 in gastric and esophageal/GEJ adenocarcinoma. The goal is to achieve 90 to 95% concordance rate between IHC and ISH, either positive or negative scores (21). Treatment guidelines establish that only patients with a positive result by IHC or ISH can be considered for trastuzumab therapy. Our findings of 5 cases amplified by DISH and negative by IHC raise the discussion regarding current testing guidelines and particularly when to reflex to ISH testing. Our 5 discrepant patients could have been eligible and possibly benefit from trastuzumab therapy if DISH or other ISH assay had been performed during the preliminary assessment of HER2. Further studies should be conducted in order to evaluate the grade of HER2 overexpression/amplification discrepancy between biopsy and surgical specimens. In addition, we found that determination of HER2 using a single testing method in limited samples may result in a significant false-negative or insufficient rate. Based on this, for accurate patient selection, we suggest the combination of IHC and ISH (co-testing).

Limitations of our study included the significant number of cases with indeterminate/insufficient results by one or both testing methodologies. In addition, the size of the sample tested did not allow for adequate evaluation of tumor heterogeneity, especially in cases with discrepancy between IHC and DISH.

In summary, our results of 12.5% overall HER2 overexpression/amplification in gastric and esophageal/GEJ adenocarcinomas are within the range of concordance with the previously published literature. When combining both anatomic sites, no difference in HER2 overexpression was found between early and late-stage tumors. Similarly to HER2-positive breast carcinoma where HER2-targeted-

Table IV. *Results of the 110 cases in which enough tissue was available for interpretation of both dual in situ hybridization (DISH) and immunohistochemistry (IHC) for human epidermal growth factor receptor 2 (HER2).*

Results: DISH/IHC	Number of cases
Not-amplified/0	89
Not-amplified/1+	0
Not-amplified/2+	0
Not-amplified/3+	0
Amplified/0	5*
Amplified/1+	0
Amplified/2+	7
Amplified/3+	9
Total	110

*Cases with discrepancy.

Table V. *Human epidermal growth factor receptor 2 (HER2)-positive cases using one or both immunohistochemistry and dual in situ hybridization methodologies.*

Diagnosis	HER2-positive cases, n
Early-stage gastric	8
Late-stage gastric	3
Early-stage E/GEJ	3
Late-stage E/GEJ	7

E/GEJ: Esophageal/gastroesophageal junction.

therapy is being also used in early-stage cancer, it is possible that early-stage gastric and esophageal/GEJ neoplasms, may be eligible for anti-HER2 therapy. However, further studies with larger number of cases are necessary to elucidate the pattern of expression in early *versus* late stage tumors as well as clinical trials to evaluate the benefits of anti-HER2 therapy in early-stage gastric and esophageal/GEJ adenocarcinomas.

Conflicts of Interest

The Authors declare no conflict of interest in regard to this study.

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Received October 16, 2017
Revised November 13, 2017
Accepted November 16, 2017