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HER2 status in gastric/gastro-oesophageal junctional cancers: should determination of gene amplification by SISH use HER2 copy number or HER2:CEP17 ratio?

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Summary

The aim of this study was to compare HER2 amplification, as determined by the HER2 copy number (CN) and the HER2/CEP17 ratio, with protein expression in gastric and gastro-oesophageal junction (G/GOJ) adenocarcinoma. HER2 immunohistochemistry (IHC) and silver *in situ* hybridisation (SISH) were performed in 185 cases. Modified gastric criteria were used for IHC scoring. HER2 and CEP17 CNs were counted in at least 20 cancer cells and the ratio calculated as per previously defined protocols. These two SISH methods were statistically compared against the different IHC scores. Thirty-four cases showed amplification, by both methods in 29, and either method in five. IHC score was 3+ in 29 cases; 26 showed amplification by both methods, one by ratio only and two were not amplified. IHC score was 2+ in 24 cases; three showed amplification by both methods and two by either. One each of IHC 1+ and 0 showed an increased ratio but not CN. The HER2 CN and ratio for IHC score 3+ compared to scores 2+, 1+ and 0 were significantly different (all $p<0.01$). The CN for IHC 2+ vs IHC 1+ and IHC 0 was significantly different (both $p<0.01$) but the ratio was not ($p=0.5711$ and $p=0.2857$, respectively). The CN and the ratio for scores 1+ and 0 were not significantly different ($p=0.9823$ and $p=0.9910$, respectively). The HER2 CN differentiates between the different IHC scores better than the HER2:CEP17 ratio. Cases that show IHC3+ and high CN may not require calculation of the ratio. Furthermore, consideration should be given to the CN when IHC negative cases appear amplified by the ratio only.

Key words: Amplification, copy number, double probe method, gastric/gastro-esophageal junctional carcinoma, HER2 status, ratio, single probe method.

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INTRODUCTION

HER2 positive status determines the eligibility for HER2 targeted therapy of advanced and metastatic gastric and gastro-oesophageal junction (G/GOJ) adenocarcinomas. The HER2 status can be determined by estimation of protein expression by immunohistochemistry (IHC) and/or assessment of HER2 gene copy number and centromeric probe 17 (CEP17) ratio by *in situ* hybridisation (ISH). ISH can be performed by bright field techniques such as chromogenic ISH (CISH) or silver ISH (SISH) or by dark field methods using fluorescence (FISH). Although there is evidence that bright field methods are superior to FISH in determining the gene amplification for G/GOJ cancers,[1–3] there is no universal acceptance of a method for testing at this point.[4,5–7] The Trastuzumab for Gastric Cancer (ToGA) study defined ISH positive status for G/GOJ cancer as HER2:CEP17 ratio $\geq$2.0 irrespective of the copy number.[4] This criterion has been used for clinical testing and in most studies related to the HER2 status of gastric/GOJ cancer. The majority of studies show a perfect or near perfect correlation between IHC3+ expression and gene amplification.[4,8–10] However, they also report that a proportion of equivocal and negative IHC cases are ISH positive when the ratio is used. IHC is a semi-quantitative test that is subject to a variety of pre analytical and analytical issues involving tissue sampling, scoring criteria and inherent subjectivity of IHC interpretation. In contrast, ISH technique is considered superior, being more quantitative and reproducible. There are specific issues confounding HER2 testing in G/GOJ cancers by either method, such as the type of specimen used (i.e., endoscopic biopsies, resections or metastatic material) and a greater degree of heterogeneity reported in these cancers.[2,8]

Considering the special issues, a modified IHC scoring system was established for HER2 testing of G/GOJ cancers.[9] Superiority of the modified/gastric cancer scoring system has been validated by others subsequently.[4,10] A positive IHC reaction in G/GOJ cancers includes basolateral/lateral membrane staining as opposed to the requirement of complete membrane staining in breast carcinomas. Additionally the cut-off for a positive test is only 10% positive tumour cells for resections and a cluster of five positive tumour cells for endoscopic biopsies.[9,10]

There are two methods to establish gene amplification. One is the single probe method by counting the actual HER2 copy number (CN) per nucleus, the cut-off for amplification being $\geq$6.0. The other is using dual probes to calculate the ratio of HER2 genes to centromere 17 (HER2/CEP17), the cut-off for amplification being $\geq$2.0. The ratio distinguishes increased HER2 gene copy number secondary to extra copies of CEP17 that may occur due to true polysomy or

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co-amplification. For the ToGA study, HER2 positive status was defined by either protein expression that showed a IHC 3+ score, or FISH gene amplification defined as a HER2/CEP17 ratio $\geq$2.0, not a HER2 CN $\geq$6.0. Significantly, subgroup analysis showed that there was no survival benefit with trastuzumab therapy for the HER2 amplified but IHC negative group (score 0 or 1+) which accounted for ~23% of the positive cohort. The exact reason for this has not been established to date. A recent review on the issue of HER2 testing in gastric cancer by an expert panel[2] recommends taking into consideration the gene count, i.e., CN when the ratio suggests borderline amplification (a ratio close to 2.0). Furthermore, the GaTHER study conducted in Australia concluded that the inter-laboratory agreement on CISH/SISH scoring was good/very good when HER2 copy number was used ($\kappa = 0.68$ to 0.86), but was reduced when HER2:CEP17 ratio was used.[1] In spite of the above, there are no studies that have compared the performance of the ratio versus the HER2 copy number across the range of IHC. In our experience with HER2 testing of gastric/GOJ cancers, we have encountered a few cases that just reach the cut-off ratio of 2 whilst the actual HER2 copy number does not exceed 6. Therefore, we embarked on a detailed analysis of the HER2 copy numbers and HER2/CEP17 ratio across the range of IHC scores, also comparing the results against tumour type/grade, and taking into account the potential impact on clinical testing.

METHODS

Cases included in the study were: endoscopic biopsies ($n = 146$, 80.7%), selected blocks of resections ($n = 30$, 16.6%) and metastatic deposits ($n = 9$, 2.7%) of G/GOJ adenocarcinomas with an adequate amount of tumour. HER2 status was assessed by IHC and SISH. IHC scoring (0–3+) was performed using 'modified gastric' criteria. HER2 and CEP17 CNs were counted in at least in 20 cancer cells and the HER2:CEP17 ratio calculated as per standard protocol.[1,4,9] These two methods were statistically compared across the range of IHC scores, as well as the tumour grade and type.

Immunohistochemistry

HER2 IHC was performed on formalin fixed, paraffin embedded tissue using the Ventana XT automated stainer (Ventana, USA), with polyclonal CERB2 antibody (Dako, Denmark) applied at 1:4000 dilution. The resection and/or biopsy specimens were scored for HER2 overexpression according to criteria by Hofman *et al.*[9]

Silver *in situ* hybridisation (SISH)

HER2 amplification was performed on formalin fixed, paraffin embedded tissue using the automated Ventana INFORM HER2 Genomic probe platform. HER2 and CEP17 copy numbers were counted and the CN was averaged per cell. In each case HER2/CEP17 ratio was assessed. The cut-off for amplification by the copy number was $\geq$6 and by the ratio was $\geq$2 as per established guidelines.[1,4,9]

Statistical analysis

Skewed variables were logarithmically transformed where appropriate. Data among groups were compared using general linear models (SAS Proc GLM; SAS Institute, USA). All pair-wise comparisons and their corresponding p values for the test of no difference are reported. Tukey–Kramer test was applied to account for multiple comparisons for a given variable across the four IHC scores. The statistical significance was set at the 5% level.

RESULTS

There were 185 G/GOJ carcinomas in which IHC and HER2 and CEP17 SISH had been performed.

IHC scores and SISH results of CN and ratio

A total of 34 cases demonstrated an increase in either CN or ratio ($n = 5$, CN only in 1 and ratio only in 4) or both ($n = 29$). Table 1 shows the comparison of the HER2 CN, HER2/CEP17 ratio and IHC score. Table 2 shows the raw figures of HER2 and CEP17 signals, ratio and the respective IHC score in discordant cases. Of 29 IHC 3+ cases, 26 showed amplification by both ratio and CN, one case by ratio only, and two cases were not amplified. Of the 24 IHC 2+ cases, three were amplified by both CN and ratio, with two additional cases amplified by either CN or ratio respectively, and 19 cases were not amplified. One each of IHC 1+ and 0 showed increased ratio but not CN. Mean CN for IHC 3+ cases was markedly elevated above 6 (18.92), while all other scores showed a mean copy number that was <6. Fig. 1 shows a concordant case (IHC 3+, copy number $\geq$6 and ratio $\geq$2) and a discordant case (IHC 1, copy number <6 but ratio $\geq$2).

Statistical comparison of ratio and copy numbers across IHC scores

Table 3 shows the comparisons of the ratio and the CN across IHC scores and their corresponding p values. The HER2 CN and ratio for IHC score 3+ versus scores 2+, 1+ and 0 were significantly different from each other (all $p < 0.01$). The CN for IHC 2+ versus IHC 1+ and IHC 0 was significantly different from each other (both $p < 0.01$) but the ratio was not ($p = 0.5711$ and $p = 0.2857$, respectively). The CN and the ratio for scores 1 and 0 were not significantly different ($p = 0.9823$ and $p = 0.9910$, respectively).

HER2 status by definitions used across the world

Using current ToGA (IHC 3+ and/or any ISH positivity by a HER2/CEP17 ratio of $\geq$2.0), European Medicines Agency [(EMEA); all IHC 3+ and IHC 2 and ISH positive by ratio of $\geq$2.0] and Belgian (all SISH positive by ratio of $\geq$2.0 irrespective of IHC) criteria, 36 (19%), 34 (18%) and 33 (17%) out of 185 cases would be positive, respectively. If the copy number was used, positivity would reduce to 34 (18%), 31 (17%), and 30 (16%), respectively. By using recently

Table 1 Comparison of IHC score, HER2 CN and HER2/CEP17 ratio

IHC	SISH+ (ratio)	SISH+ (CN)	SISH– (ratio)	SISH– (CN)	Total	Mean CN	Mean ratio
0	1	0	98	99	99	2.48	1.15
1+	1	0	32	33	33	2.57	1.26
2+	4	4	20	20	24	4.14	1.94
3+	27	26	2	3	29	18.92	9.57
Total	33	30	152	155	185		

CN, copy number; IHC, immunohistochemistry; SISH, silver *in situ* hybridisation.