

ARTICLE



RAI1 alternate probe identifies additional gastroesophageal adenocarcinoma cases as amplified following equivocal *HER2* fluorescence in situ hybridization testing: experience from a national reference laboratory

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The College of American Pathologists/American Society of Clinical Oncology recommends *HER2* testing prior to initiation of targeted therapy for patients with advanced Gastroesophageal adenocarcinoma (GEA), using immunohistochemistry (IHC) followed by fluorescence in situ hybridization (FISH) in cases with an equivocal (score 2+) result on IHC. The FISH results are considered indeterminate if the *HER2/CEP17* ratio is <2.0 with an average CEP17 copy number of ≥3.0 and a *HER2* copy number ≥4.0 and ≤6.0 after counting additional tumor cells. Indeterminate results may be resolved by using an alternative chromosome 17 probe such as *RAI1*. The purpose of this study is to review our experience with *RAI1* alternate probe in *HER2* FISH testing of GEA in a large reference laboratory setting. Esophageal, gastroesophageal, and gastric adenocarcinomas received for *HER2* FISH testing in our lab between 9/2018 and 1/2020 were included. *HER2/CEP17* and *HER2/RAI1* ratios, and the average *HER2*, CEP17, *RAI1* signals per cell were recorded. 328 GEA had *HER2* testing performed in our lab during the study period. 101 (30.8%) were amplified, 169 (51.5%) were non-amplified and 58 (17.7%) were indeterminate. Following *RAI1* testing, 42 (72.4%) of 58 indeterminate cases were reclassified as non-amplified and 16 (27.6%) were reclassified as amplified, increasing the total amplified cases to 117 (35.7%). The correlation between the average CEP17 and *RAI1* copy number for all cases was weak ($R^2 = 0.095$). In summary, using the alternate probe *RAI1* reclassifies 27.6% of original *HER2* FISH indeterminate gastroesophageal carcinomas as amplified, which makes them eligible for targeted therapies.

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INTRODUCTION

Gastroesophageal adenocarcinoma (GEA) is the fifth most frequently diagnosed cancer and the third leading cause of cancer-related death worldwide. *ERBB2* (erb-b2 receptor tyrosine kinase 2 or *HER2*) is a biomarker used to identify eligible patients for specific therapy in the setting of advanced GEA. *HER2* overexpression is seen in 12–30% of GEA with no significant differences between primaries and metastases^{1,2}. Currently the standard of practice is to perform *HER2* testing on all patients with advanced GEA who are candidates for *HER2*-targeted therapy, regardless of histologic subtype, specimen type (biopsy or resection), differentiation and precise tumor site (stomach, gastroesophageal junction, esophagus or metastatic deposit). Patients with positive results on *HER2* testing are offered targeted therapy such as Trastuzumab, which is a monoclonal antibody targeting the *HER2* receptor. Trastuzumab reduces proliferation, suppresses angiogenesis and promotes cell death through antibody dependent cell mediated cytotoxicity³. Improved survival has been documented in the randomized controlled trial ToGA (Trastuzumab for Gastric Cancer) for GEA patients treated with

Trastuzumab alongside with chemotherapeutic agents, further emphasizing the importance of accurate *HER2* testing and reporting⁴.

The College of American Pathologists, American Society for Clinical Pathology, and American Society of Clinical Oncology (CAP/ASCO) have published comprehensive guidelines on *HER2* testing in GEA based on literature review and consensus opinion, which differ from the guidelines established for breast cancer^{5,6}. Notably, *HER2* overexpression in GEA may be more heterogeneous and the staining pattern may be more basolateral rather than circumferential on immunohistochemical stains (IHC). In addition, IHC patterns observed in cancer cell clusters consisting of ≥5 neoplastic cells in biopsies (rather than required 10% of the tumor cells in GEA excisions, or any breast tumors) is considered reactive⁵. IHC is the recommended primary test of choice and no further testing is indicated for negative (scores 0 or 1+) or positive (score 3+) cases for GEA. All tumors with equivocal scores (2+) on initial IHC analysis are reflexed to in situ hybridization for determining the amplification status. Both FISH and bright field ISH are acceptable means to assess for *HER2*

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amplification, and reported as positive when *HER2*/CEP17 ratio is ≥ 2.0 or average *HER2* signal number per cell is >6.0 and negative when *HER2*/CEP17 ratio is <2.0 when average *HER2* signal number per cell is <6.0 and average CEP17 signal number per cell is <3.0 . Cases are deemed indeterminate if *HER2*/CEP17 ratio is <2.0 with an average ≥ 4 to ≤ 6 *HER2* signals per cell, and average CEP17 signal number of ≥ 3.0 even after counting an additional 20 tumor cells. Extra (≥ 3) copies of CEP17 were noted in 4% of gastric cancers which is the result of intrachromosomal segmental duplication overlapping the centromere of chromosome 17 also involving the *HER2* gene⁵. The 2016 CAP/ASCO guidelines, offers additional options for indeterminate test results including selecting a different block, retesting with an alternative chromosome 17 probe (instead of CEP17), among other measures such as using genomics or alternative testing⁵.

Retinoic acid-induced 1 gene (RAI1) has been used as alternative probe to resolve indeterminate FISH results using *HER2* and CEP17 probes^{7,8}. *RAI1* is located on the short arm of chromosome 17 (band position 17p11.2) and has a reported low coamplification rate of about 1%, and therefore serves as an attractive alternative locus for *HER2* FISH testing^{8,9}. However, some studies describe frequent deletion in 17p11.2, which could potentially result in overestimating the frequency of amplified cases^{9,10}. Much of our current understanding on using *RAI1* as a probe in *HER2* testing is derived from studies done in breast cancer, although the *HER2* testing in breast cancer no longer recommend using alternative probes to resolve indeterminate FISH results^{6,11}. Despite that fact, alternative probes such as *RAI1* remain within testing algorithms for GEA samples⁵. In a reference laboratory setting, alternate chromosome 17 probe has been best option for resolving indeterminate *HER2* FISH in GEA cases where presence/absence of another tissue block is unknown. The purpose of this study is to do a retrospective analysis and describe our experience with using *RAI1* as an alternative probe locus in the *HER2* testing algorithm for GEA.

MATERIALS AND METHODS

The study protocol was approved by the University of Utah Institutional Board Review (IRB#77507). A search was conducted using our laboratory information system for samples submitted for *HER2* testing by immunohistochemistry (IHC) with reflex to *HER2* FISH or submitted primarily for *HER2* FISH testing. Cases were analyzed at ARUP Laboratories, which serves as a national reference laboratory. Only primary esophageal, gastroesophageal, and gastric adenocarcinomas as designated on the accompanying test requisition and received between September 2018 and January 2021 were included in our study cohort. Metastatic tumors were excluded from the analysis as tumor origin was considered to be uncertain due to limited information on the pathology reports provided by the outside institutions.

Hematoxylin-eosin-stained slides were reviewed to confirm the presence and extent of tumor tissue from both biopsies and resections. Immunohistochemical stain analysis was carried out with HercepTest (Dako, Carpinteria, California) following ASCO/CAP 2016 guidelines for *HER2* testing of GEA⁵ and scored manually by board certified anatomic pathologists using the Ruschoff/Hofmann method¹². For biopsy specimens the interpretation was negative (score of 0 or 1+) if there was no reactivity or faint barely perceptible membranous reactivity, equivocal (score of 2+) if there were ≥ 5 tumor cell clusters with weak to moderate complete, basolateral, or lateral membranous reactivity irrespective of percentage of stained tumor cells. Biopsies were interpreted as positive (score of 3+) if there were ≥ 5 tumor cell clusters with strong complete, basolateral or lateral membranous reactivity irrespective of percentage of stained tumor cells. *HER2* IHC scoring was similar for the resection specimens, but a threshold of 10% was used in the assessment. Cases with an equivocal (or score of 2+) result on IHC analysis were reflexed to *HER2* analysis by FISH.

HER2 FISH analysis was performed using the US Food and Drug Administration (FDA)-approved, dual-probe *HER2* IQFISH (Dako, Carpinteria, California). The FDA-approved probe set was scored according to the ASCO/CAP 2016 guidelines for interpreting GEA *HER2* FISH assays. In

summary a GEA was reported as non-amplified/negative if the *HER2*/CEP17 ratio was <2.0 , *HER2* copy number per cell ≤ 6.0 and average CEP17 copy number per cell <3.0 , amplified/positive if *HER2*/CEP17 ratio ≥ 2.0 , or *HER2* copy number per cell >6.0 regardless of the *HER2*/CEP17 ratio, or indeterminate if *HER2*/CEP17 ratio is <2.0 with an average CEP17 copy number per cell ≥ 3.0 and *HER2* copy number per cell ≥ 4.0 and ≤ 6.0 . At least 20 cells were counted in each case and reviewed by both a technician and a board-certified pathologist trained in anatomic pathology. A minimum of an additional 20 cells were counted if the result was close to the cutoff, if there was significant cell to cell variability in the number of signals, or if the results were indeterminate. The indeterminate cases were retested with *HER2* and *RAI1* probes (both from Agilent Technologies, Santa Clara, California) and interpreted as non-amplified if the *HER2*/*RAI1* ratio was <2.0 and the average number of *RAI1* per cell was 1.5 or greater, or as amplified if *HER2*/*RAI1* ratio was 2.0 or more. *HER2* status was considered unresolved or indeterminate with a suggestion for additional/alternative testing for cases with a deleted *RAI1* (defined as a *RAI1* copy number per cell of <1.5). Concurrent copy number gains in *HER2*, the centromere, and *RAI1* were assumed to be unlikely representation of true *HER2* amplification, and therefore there were no established cutoffs for *RAI1* gains as these cases were more likely to represent polysomy rather than co-amplification. Our laboratory participates in CAP's GEA *HER2* IHC, and *HER2* FISH proficiency testing. All *HER2* immunohistochemical tests have on slide, and FISH tests have batch positive controls.

The results of previous *HER2* immunohistochemical testing (if performed in our laboratory) and FISH data with the FDA approved probe set and the alternate probe set were retrieved from electronic files. The average copy number per cell of *HER2*, CEP17, *HER2*, and *RAI1* from each probe set and their corresponding ratios were recorded.

RESULTS

There were 330 gastric, gastroesophageal and esophageal adenocarcinoma specimens identified that met the search criteria. Two of 330 cases did not have sufficient tissue on FISH slides and were excluded from further analysis. Only one case (0.03%) was received at our laboratory for immunohistochemical testing followed by reflex FISH testing if equivocal (2+). We have performed *HER2* IHC testing on two additional cases. The initial testing with the *HER2* FISH FDA approved probe set showed that slightly over half of all included cases were classified as non-amplified or negative ($n = 169$) and about 30% of the cases were classified as amplified or positive ($n = 101$) using the ASCO/CAP 2016 reporting criteria (Table 1). The indeterminate category retested with the *RAI1* alternate probe, constituted approximately 18% of all studied cases ($n = 58$) (Table 1). These cases have shown a *HER2*/CEP17 ratio of <2 with an average CEP17 copy number of ≥ 3.0 and *HER2* copy number per cell ≥ 4.0 and ≤ 6.0 . Sixteen indeterminate cases (out of 58, or 27.6%) were reclassified as amplified after retesting with the *RAI1* probe, while 42 cases (out of 58, or 72.4%) were reclassified as non-amplified after retesting with the *RAI1* probe (Fig. 1). The results are summarized in Table 1. Of note we did not identify a case with an unresolved *HER2* result after retesting with the *RAI1* alternate probe in our

Table 1. *HER2* fluorescence in situ hybridization testing results of GEA with the CEP17 and *RAI1* alternate probes.

INITIAL TESTING	Number (%)
Amplified	101 (30.8)
Nonamplified	169 (51.5)
Indeterminate	58 (17.7)
Amplified after <i>RAI1</i>	16 (27.6)
Nonamplified after <i>RAI1</i>	42 (72.4)
FINAL RESULTS (After <i>RAI1</i> retesting)	
Amplified	117 (35.7)
Nonamplified	211 (64.3)
TOTAL	328 (100)

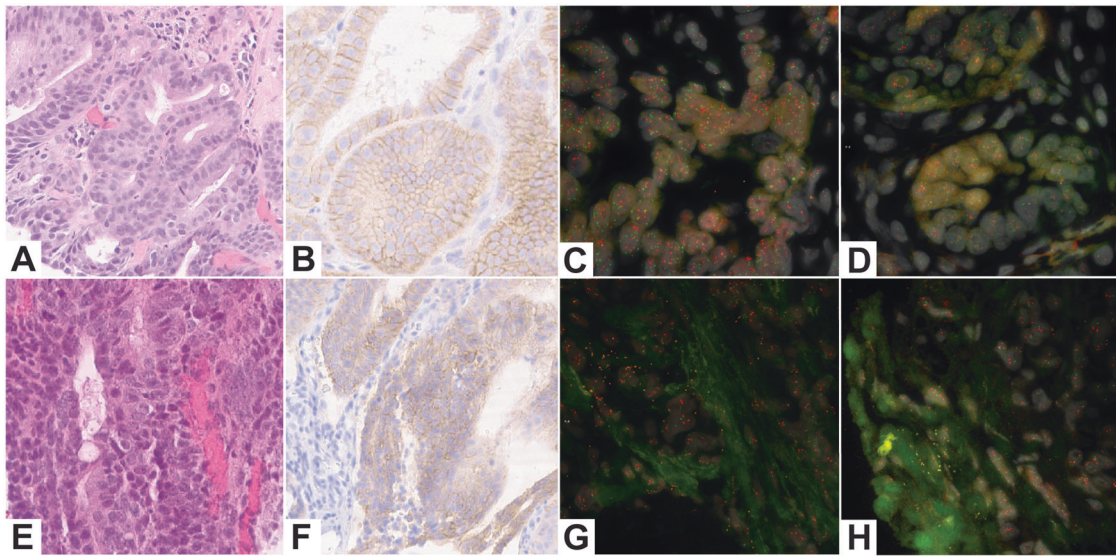


Fig. 1 Gastroesophageal adenocarcinoma (GEA) with equivocal results with US Food and Drug Administration–approved HER2 immunohistochemistry and fluorescence in situ hybridization (FISH) testing that was retested with an alternate *RAI1* probe. **A through D** Example of GEA with equivocal HER2 immunohistochemistry (IHC) and *HER2* FISH results that was reclassified as amplified by the alternate *RAI1* probe with *HER2/RAI1* ratio of 2.5. **E through H** Example of GEA with equivocal HER2 IHC and *HER2* FISH results that had a nonamplified *HER2/RAI1* ratio (original magnification, $\times 40$ [**A, B, E, and F**]; original magnification, $\times 100$ [**C, D, G, and H**]).

Table 2. Features of equivocal cases by *HER2* fluorescence in situ hybridization retested with the *RAI1* alternate probe.

<i>HER2</i> Status Determined by <i>RAI1</i> Probe, No. (%)	<i>RAI1</i> Probe, Range (Average)			CEP17 Probe, Range (Average)		
	Average <i>HER2:RAI1</i> Ratio	Average <i>HER2</i> Copy No.	Average <i>RAI1</i> Copy No.	Average <i>HER2:CEP17</i> Ratio	Average <i>HER2</i> Copy No.	Average CEP17 Copy No.
All cases, ^a 58 (100)	0.8–2.8 (1.65)	2.5–4.4 (4.22)	1.5–6.0 (2.74)	1.0–1.9 (1.44)	3.7–5.9 (4.69)	2.1–5.0 (3.29)
Amplified, 16 (27.6)	2.0–2.8 (2.29)	3.4–6.2 (4.61)	1.5–2.6 (2.03)	1.2–1.9 (1.55)	4.0–5.9 (4.78)	2.1–3.7 (3.13)
Nonamplified, 42 (72.4)	0.8–1.9 (1.41)	2.5–5.5 (4.07)	1.7–6.0 (3.01)	1.0–1.8 (1.40)	3.7–5.9 (4.65)	3.0–5.0 (3.35)

^aEquivocal cases by *HER2* fluorescence in situ hybridization with CEP17.

cohort. Sixteen (27.6%) of the originally indeterminate cases that were classified as amplified after re-testing with alternate *RAI1* probe had an average *HER2/RAI1* ratio of 2.29 (Table 2). The correlation between average CEP17 and *RAI1* copy numbers for these cases was poor (Fig. 2).

DISCUSSION

In our cohort, 58 out of 328 (17.7%) GEA cases required retesting with *RAI1* alternate probe set during the study period in order to resolve indeterminate *HER2* FISH result after the FDA approved original probe set. To the best of our knowledge, this is the largest described cohort of GEA tested with an *RAI1* alternate probe reported in literature. We found that using a *RAI1* probe set reclassifies over 27% of original *HER2* FISH indeterminate gastroesophageal cancer cases as amplified or positive making them potentially eligible for targeted therapy.

The most recent ASCO/CAP guidelines for GEA permit using alternative FISH probes to resolve indeterminate results on *HER2* FISH testing⁵. However, the ASCO/CAP guideline does not comment on the interpretation of the reporting of alternate probes such as *RAI1*. Other recommended options to resolve an indeterminate result on FISH include consulting another scorer regarding selection of tumor areas for scoring or using a different tumor block. In our laboratory both a technician and pathologist review all cases for concordance, which has been an integral part

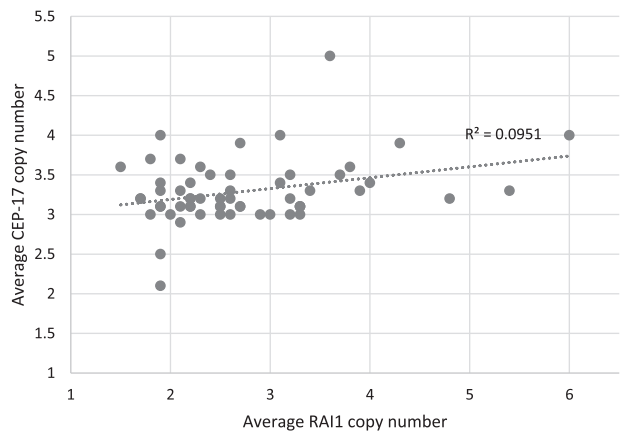


Fig. 2 The correlation between average CEP17 and *RAI1* copy numbers for all cases. The correlation between CEP17 and the alternate *RAI1* probe was poor ($R^2 = 0.095$) among gastroesophageal adenocarcinomas with original indeterminate *HER2* FISH testing result.

of our testing algorithm. Testing a different tumor block is not feasible in the reference laboratory setting because usually only one block is submitted for testing. Also, tumor is frequently present in only one block, particularly in biopsies, so no alternative

blocks are usually available for analysis. Using an alternative probe remains the most feasible option to resolve indeterminate *HER2* FISH cases in reference laboratory setting, unless genomic analysis becomes more readily available as a reflex test option in the future^{13,14}.

In the past several loci have been evaluated as alternate probe sites for resolving equivocal *HER2* FISH results for breast cancer, including *RARA* (retinoic acid receptor alpha)—17q21.2, *RAI1* (retinoid acid-induced 1, previously known as *SMS* for Smith-Magenis syndrome)—17p11.2, and *TP53*—17p13.1⁸. The indeterminate *HER2* FISH cases have reported an upgrade rate of up to 40% when using *RAI1* in breast cancer with 3.1% still unclassifiable due to deletion of *RAI1* in a similar, reference laboratory setting¹¹. The deletion of 17p11.2 and chromosome 17 polysomy likely occurs at a different rate in breast and GEAs^{9,10,14}. The deletions in the *RAI1* locus are a potential cause of overestimation of amplified cases in breast cancers because the deletion of a control probe would result in ratios in the amplified range, even in cases with low (<4) *HER2* copy numbers. Notably, we have not identified any cases with deleted *RAI1* probe in our cohort of GEAs. Thus, the deletion in the 17p11.2 region may be less common in GEAs as compared to breast cancers. The poor correlation between CEP17 and *RAI1* in cases is linked to the extent and mechanism of copy number changes occurring in given tumors. While true polysomy is unusual in breast carcinoma, the co-amplification of CEP17 and *HER2* are usually due to gene amplification encompassing the centromeric or pericentromeric region^{8,15}. In contrast, in gastric carcinomas, the mechanism is intrachromosomal segmental duplication of the *HER2* and centromeric region¹⁶. Furthermore, while evidence remains limited, studies in breast carcinoma show lack of evidence of adverse outcomes in patients with amplified *HER2* status based on an alternate probe testing compared to equivocal or negative cases using the CEP17 probes¹⁰.

Although some patients with GEA show durable response to targeted therapy, most either show primary or acquired resistance. This may be due to tumor heterogeneity and/or previously undocumented discrepancy between primary tumors and metastatic carcinomas^{1,17}. More recent studies have shown that the level of *HER2* amplification correlates with response to trastuzumab in patients with metastatic GEA cancers having ratios ≥ 3.0 or ≥ 4.0 ^{1,18}. The clinical response to targeted therapy in GEA based on a positive result obtained from *RAI1* alternate probe use is currently not known. However, all 16 cases in this cohort that were re-classified as amplified following alternate probe testing had *HER2/RAI1* ratio <3.0 (range:2.0–2.8; average; 2.29).

This study was conducted in a large reference laboratory setting, which precluded access to clinical charts in the majority of tested samples. Therefore, prior *HER2* testing at the referring institution could not be confirmed in our cohort. Although only one case had an immunohistochemical test followed by reflex FISH testing request ordered, two additional cases had IHC done by our lab. However, it is assumed that the remaining cases had prior *HER2* IHC testing performed by the referring institutions following the current *HER2* testing guidelines⁵. Segmental duplication of chromosome 17 with 3 or more copies of CEP17 were noted in 4.1% of gastric cancers in the ToGA trial^{4,5}. The high proportion (17.7%) of indeterminate cases in this study is likely due to referral bias.

Our reference laboratory serves many out of state clients and smaller hospitals, and thus is subject to referral bias. Overall, it is uncertain if cases are representative of all GEA in the general population. We identified that 30.8% and 35.7% of GEA are amplified following testing with FDA approved probe set and additional testing with an alternate probe in indeterminate cases respectively, which falls within the range of published rates of positivity in GEA, reported as 4.4–53.4% with a mean of 17.9%^{5,19}.

In summary, our study showed that *RAI1* alternate probe reclassifies more than a quarter of GEA with indeterminate *HER2*

FISH result as amplified making them eligible for targeted therapy. However, it is largely unknown whether this subgroup of patients will benefit from such therapies.

DATA AVAILABILITY

All data analyzed in this study are included in this paper.

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AUTHOR CONTRIBUTIONS

The study was conceptualized and written by J.J. and H.E.G. N.U. provided technical and material support. J.J., D.S., N.U., D.S. reviewed and edited the final paper.

COMPETING INTERESTS

The authors declare no competing interests.

ETHICAL APPROVAL

The study was approved by the University of Utah Institutional Review Board.

ADDITIONAL INFORMATION

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