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ARTICLE

Integrated genomic characterization of ERBB2/HER2 alterations in invasive breast carcinoma: a focus on unusual FISH groups

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Abstract

In patients with invasive breast cancer, fluorescence in situ hybridization (FISH) testing for HER2 typically demonstrates the clear presence or lack of *ERBB2* (*HER2*) amplification (i.e., groups 1 or 5). However, a small subset of patients can present with unusual *HER2* FISH patterns (groups 2–4), resulting in diagnostic confusion. To provide clarity, the 2018 CAP/ASCO HER2 testing guideline recommends additional testing using HER2 immunohistochemistry (IHC) for determining the final HER2 status. Despite this effort, the genomic correlates of unusual *HER2* FISH groups remain poorly understood. Here, we used droplet digital PCR (ddPCR) and targeted next-generation sequencing (NGS) to characterize the genomic features of both usual and unusual *HER2* FISH groups. In this study, 51 clinical samples were selected to represent FISH groups 1–5. Furthermore, group 1 was subdivided into two groups, with groups 1A and 1B corresponding to cases with *HER2* signals/cell ≥6.0 and 4–6, respectively. Overall, our findings revealed a wide range of copy number alterations in *HER2* across the different FISH groups. As expected, groups 1A and 5 showed the clear presence and lack of *HER2* copy number gain, respectively, as measured by ddPCR and NGS. In contrast, group 1B and other uncommon FISH groups (groups 2–4) were characterized by a broader range of *HER2* copy levels with only a few select cases showing high-level gain. Notably, these cases with increased *HER2* copy levels also showed HER2 overexpression by IHC, thus highlighting the correlation between *HER2* copy number and HER2 protein expression. Given the concordance between the genomic and protein results, our findings suggest that HER2 IHC may inform *HER2* copy number status in patients with unusual FISH patterns. Hence, our results support the current recommendation for using IHC to resolve HER2 status in FISH groups 2–4.

Introduction

Amplification of the *ERRB2* (*HER2*) oncogene and overexpression of the HER2 protein occur in 10–20% of patients with invasive breast carcinoma [1–3]. In these patients with HER2-positive tumors, selective treatment with HER2-targeted therapy results in improved disease-free and overall survival [4–7]. Given this benefit, HER2 status represents an important predictive biomarker for HER2-targeted therapy in patients with invasive breast carcinoma.

Accurate assessment of HER2 status is critical for identifying HER2-positive patients who will benefit from HER2-targeted therapy and recognizing HER2-negative patients who may be harmed by inappropriate HER2-based treatments. The current 2018 CAP/ASCO HER2 testing guideline recommends HER2 testing by using either immunohistochemistry (IHC) for HER2 protein overexpression or in situ hybridization (ISH) for *HER2* gene

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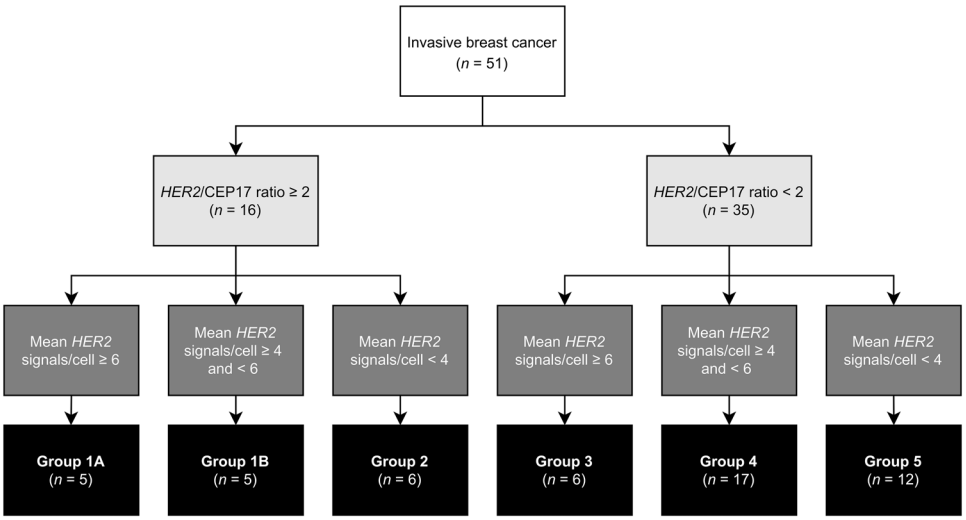

Fig. 1 Classification of *HER2* FISH groups. The distribution of 51 cases into 5 FISH groups based on the various combinations of *HER2* signals/cell and *HER2*/CEP17 ratios.

amplification [5]. In many clinical laboratories, IHC is used as an initial-screening method, and dual probe ISH testing is pursued in select cases with equivocal IHC results. In general, dual probe ISH testing utilizes one probe for the *HER2* gene locus and one centromeric control probe (commonly CEP17), thus resulting in five ISH patterns (groups 1–5) based on various combinations of *HER2* signals/cell and *HER2*/CEP17 ratios [7]. In the majority of patients, ISH testing results in group 1 pattern with clear amplification (*HER2*/CEP17 ≥ 2.0 and *HER2* signals/cell ≥ 4.0) or group 5 pattern with clear lack of amplification (*HER2*/CEP17 < 2.0 and *HER2* signals/cell < 4.0), thus separating the patients into two distinct treatment groups. In contrast, groups 2–4, while less common, remain a significant challenge given their unusual ISH patterns and lack of strong, prospective clinical evidence demonstrating benefit to HER2-targeted therapies [7].

In the 2018 ASCO/CAP HER2 testing update, additional testing was recommended for groups 2–4 including concurrent HER2 IHC for final interpretation and repeat counting of ISH signals with equivocal (2+) IHC results [5]. Using these criteria, group 2 or 4 cases with HER2 overexpression (3+) by IHC are considered HER2-positive, and group 3 cases with 3+ and 2+ expression are interpreted as HER2-positive. The use of alternative probes has been discouraged due to the concerns of "false-positive" ISH ratios from heterozygous deletions of frequently used control probe sites [8]. Hence, additional clinical methods to resolve these unusual groups are currently lacking, and their genomic features remain poorly understood. Droplet digital PCR (ddPCR) and targeted next-generation sequencing (NGS) represent alternative clinical methods for copy number analysis that may provide additional insights. Specifically, ddPCR is a useful assay that allows for absolute quantification of small genetic targets [9]. In comparison, NGS provides a comprehensive copy number analysis across broader segments of the genome [10]. Herein, we use both ddPCR and targeted NGS to characterize the genomic landscape of *HER2* alterations in patients with groups 1–5 ISH results by fluorescence in situ hybridization (FISH).

Materials and methods

Clinical samples

This study was approved by the institutional review board at Stanford University. The pathology database from Stanford was searched for invasive breast cancer cases with *HER2* FISH and HER2 IHC results from 2001 to 2016. Fifty-one unique patient samples were selected and classified into six FISH groups: (1) group 1A (*HER2*/CEP17 ratio ≥ 2.0 and mean *HER2* signals/cell ≥ 6.0) ($n = 5$), (2) group 1B (*HER2*/CEP17 ratio ≥ 2.0 and mean *HER2* signals/cell ≥ 4.0 and <6.0) ($n = 5$), (3) group 2 (*HER2*/CEP17 ratio ≥ 2.0 and mean *HER2* signals/cell < 4.0) ($n = 6$), (4) group 3 (*HER2*/CEP17 ratio < 2.0 and mean *HER2* signals/cell ≥ 6.0) ($n = 6$), (5) group 4 (*HER2*/CEP17 ratio < 2.0 and mean *HER2* signals/cell ≥ 4.0 and <6.0) ($n = 17$), and (6) group 5 (*HER2*/CEP17 ratio < 2.0 and mean *HER2* signals/cell < 4.0) ($n = 12$) (Fig. 1).

FISH

HER2 FISH was performed at the Stanford Cytogenetics Laboratory (Stanford, CA) using the Abbott Vysis PathVysion (Des Plaines, IL) FDA-cleared kit per manufacturer's recommendations. The results were scored by at least two experienced cytogenetics personnel. Counts

greater than ten signals/nucleus were recorded as ten signals given the significant crowding of *HER2* signals and loss of signal resolution for accurate quantification.

IHC

HER2 IHC was performed using 4B5 antibody from Ventana (Tucson AZ) as part of the FDA-cleared PATHWAY kit according to manufacturer's instructions on Ventana XT or Ultra instruments at the Stanford Immunohistochemistry Laboratory (Stanford, CA). The results were interpreted according to the 2018 ASCO/CAP HER2 testing guideline.

DNA purification for ddPCR and NGS

Genomic DNA (gDNA) was purified from 1–5 formalin-fixed paraffin-embedded (FFPE) curls (10–20 µm) using ReliaPrep FFPE gDNA Miniprep System (Promega, Sunnyvale, CA), with the following modifications: FFPE curls were incubated for 16 h at 65 °C in lysis buffer containing proteinase K, incubated 1 h at 90 °C, and flash cooled. The entire mixture was transferred to a 0.45 µm cellulose acetate filter (Corning COSTAR, Corning, NY) and centrifugated for 15 min at 4 °C at 16,000 × *g*, after which the filtrate was processed according the manufacturer's guidelines. Purified DNA was used directly for ddPCR assays and sheared prior to input into the TOMA OS-Seq protocol as follows: up to 1 µg of DNA was sheared with a Covaris E210R sonicator (Covaris, Woburn, MA) to a target base pair peak of 550 bp according to the manufacturers' recommendations. The recommended minimum DNA input for our NGS and ddPCR is 10 ng of DNA. To ensure optimal performance, ~200 and 50 ng of input DNA were loaded into our NGS and ddPCR assays, respectively.

ddPCR

ddPCR was used for DNA quantitation, for library quantitation, and for *HER2* copy number calling. Specifically, gDNA samples were quantified at input and after the excision repair step of the library preparation (see below) by ddPCR using the *RPP30* gene as a surrogate for genomic equivalents. *RPP30* is a standard housekeeping gene that is widely used as a reference gene for ddPCR experiments for many cancer types including breast cancer [9, 11, 12]. ddPCR reactions consisted of 11 µl of Droplet PCR Supermix for probes (no dUTP) (cat no 186–3024, BioRad, Hercules, CA), 1.1 µl of *RPP30* assay (HEX-labeled) (Assay ID: dHsaCP2500313; BioRad, Hercules, CA), 2.2 µl DNA, and nuclease free water to a final volume of 22 µl. Next, 20 µl of this reaction mixture was used to generate droplets that were PCR amplified and subsequently analyzed using the QX200™ Droplet Digital™ PCR System according to the manufacturer's recommended guidelines using QuantaSoft v1.7.4.0917 (BioRad, Hercules, CA). Values were converted from copies/µL to ng/µL using 30 ng per 10,000 copies of genome equivalents. Library quantitation by ddPCR is described in the library preparation section below. For copy number assays by ddPCR, the experiments were performed as described for DNA input quantitation above, except that the 1.1 µl of HEX-labeled *RPP30* control assay (listed above) was combined with 1.1 µl of FAM-labeled *HER2* assay for each sample, and the water amount was adjusted accordingly.

Two separate ddPCR assays were used to capture two different exons of the *HER2* tyrosine kinase domain: (1) a commercially available assay for targeting exon 19 (Assay ID: dHsacp1000116; BioRad, Hercules, CA) and (2) a custom assay for targeting exon 21 using specific PCR primers for exon 21 and standard protocols using the QX200™ Droplet Digital™ PCR System (BioRad, Hercules, CA). ddPCR ratios were calculated by dividing the *HER2* values with the *RPP30* value for each sample. Furthermore, ddPCR ratios were transformed into amplification calls based on an algorithm that adjusts for tumor cellularity [13].

NGS library preparation

Samples were processed using the TOMA SIGNOME 130-gene library preparation kit according to manufacturer's recommendation (TOMA Biosciences, Holland, MI) as described previously using up to 1 µg of DNA as input to excision repair and 100–200 ng as input to ligation [14]. Excision repair, adapter ligation, target capture, and library expansion were carried out according to the TOMA SIGNOME 130-gene library preparation kit. A series of 100-fold dilutions of the resulting libraries were performed in TE buffer and the 10^{-6} dilutions were quantified via ddPCR using the TOMA ILQ assay, using the following PCR cycling parameters: 95 °C 10 min; 30 s at 94 °C, 30 s at 55 °C, 60 s at 70 °C, 40 cycles; followed by 5 min at 70 °C. This assay measures P7 (labeled by FAM) and P5 (labeled by HEX). Linkage values determined by QuantaSoft v1.7.4.0917 (BioRad, Hercules, CA) identified library molecules with both P5 and P7 adapters and were used to calculate the number of library molecules per µl. Next, 1.0–1.4 billion total library molecules were loaded onto the NextSeq 500 (Illumina, San Diego, CA) according to the manufacturer's recommendations with the following adjustments: libraries from a total of 12 samples per run were pooled, and the volume was adjusted to 20 µl with TE buffer. The pooled libraries were denatured by the addition of 1 µl 0.5 M NaOH, incubated for 5 min at room temperature, followed by the addition of chilled HT1 buffer (1280 µl) and was prepared to be loaded into an Illumina NextSeq 500/550 Mid Output v2 kit (300 cycle) sequencing

cartridge. The TOMA sequencing primers were diluted and used as indicated in the TOMA SIGNOME 130-gene library preparation kit. Libraries were then sequenced as paired-ends (2 × 150 bp).

NGS analysis

We used the TOMA SIGNOME Analysis software (TOMA Biosciences, Foster City, CA) to map reads. Copy number alterations were identified by the SIGNOME Analysis copy number caller by comparing with a normal diploid control sample included in every run, either as full (all exons of a gene) or partial (some exons of a gene). Specifically, copy number gains were called when the absolute value of the log 2 ratio was ≥1.2. Our threshold of 1.2 corresponds to ~3 excess copies of *HER2* in a pure tumor sample. This value was established to avoid false-positive calls using reference materials and validated down to 10 ng of input DNA. Furthermore, we used the Integrated Genome Viewer (IGV) to visually inspect the extent of copy number changes in the *HER2* coding region [15]. Variant calling for single-nucleotide variants (SNVs) and small insertions and deletions (indels) in *HER2* was performed as described previously [14]. All of our samples passed important quality control metrics (e.g., fraction of on-target reads >0.4, fraction of read alignment >0.7, and median unique coverage for regions of interest >200×) for optimal performance.

Data analysis

GraphPad Software version 8.1.1 (San Diego, CA) and R version 3.4.0 (Vienna, Austria) were used for statistical analyses. All tests of significance were two-tailed, and $p < 0.05$ was considered statistically significant.

Results

Patient characteristics

Fifty-one patients with invasive breast carcinoma were included in our cohort (Table 1). The median age was 53 years with a range of 35–91 years. The majority of samples were resection specimens (78%) and invasive ductal carcinomas of no special type (88%). Median invasive tumor cellularity was 60% (range: 10–90%) with median in-situ ductal carcinoma (DCIS) cellularity of 0% (range: 0–40%). Eighty-six percent of cases had positive estrogen receptor expression, and 70% were Stage I–II. A minority of samples (11/51, 21%) were previously treated with systemic therapy, including cytotoxic chemotherapy and/or HER2-targeted therapy. No endocrine therapy was administered prior to sample collection. The majority of the samples (35/51, 69%) were not pretreated with any systemic therapy, and treatment information was not available for five patients (5/51, 10%). Detailed information is provided in (Supplementary Table 1).

Table 1 Patient and sample information.

Characteristics ($n = 51$)	Data (%)
Age, years	
Median (range)	53 (35–91)
Specimen type	
Biopsy	11 (22)
Resection	40 (78)
Location	
Breast	46 (90)
Lymph node	2 (4)
Distant metastasis	3 (6)
Histologic type	
Ductal	45 (88)
Lobular	4 (8)
Mucinous	1 (2)
Metaplastic	1 (2)
Tumor cellularity, %	
Invasive carcinoma, median (range)	60 (10–90)
In situ carcinoma, median (range)	0 (0–40)
Estrogen receptor status*	
Positive	44 (86)
Negative	7 (14)
Clinical stage	
I	20 (39)
II	16 (31)
III	10 (20)
IV	5 (10)

*Estrogen receptor status was considered positive if ⩾ 1% of tumor cell nuclei were immunoreactive and negative otherwise.

HER2 expression is concordant between invasive carcinomas and DCIS

Twenty-five samples had DCIS on the diagnostic H&E slide. Out of these, five samples (5/25, 20%) had no residual DCIS present on the HER2 IHC slide for review. In the remaining 20 samples (20/25, 80%), HER2 expression was analyzed in the DCIS component and compared with the HER2 expression in the invasive component. In nearly all samples (19/20, 95%), the DCIS and invasive cancer showed concordant HER2 IHC status (e.g., positive [3+], equivocal [2+], and negative [0/1+]). One sample from group 4 (case 26) showed a minor discordance in HER2 IHC status, with DCIS showing equivocal (2+) expression and invasive cancer showing negative (1+) expression.

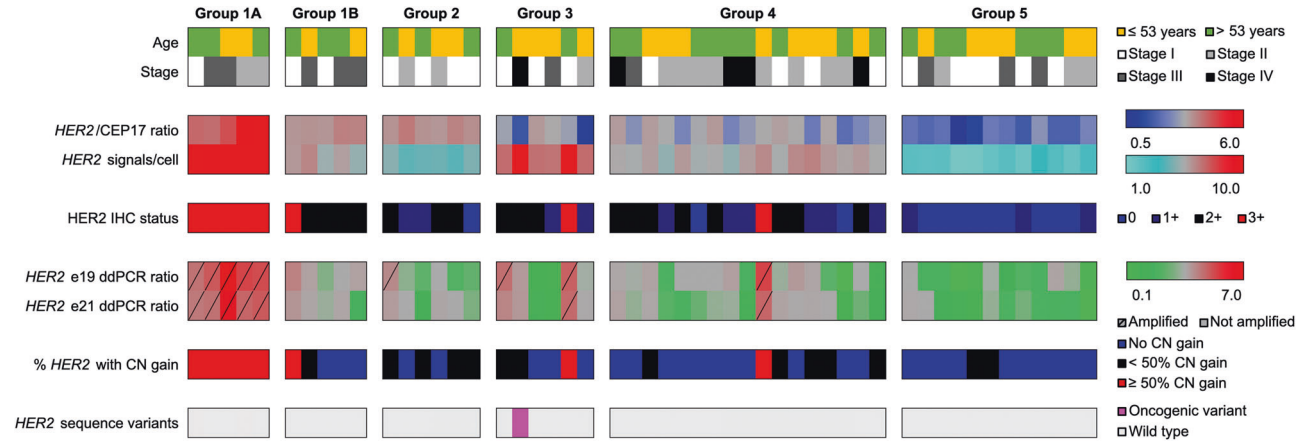

Fig. 2 Summary of clinicopathologic and genomic findings. Visualization of findings from FISH, immunohistochemistry, droplet digital PCR, and next-generation sequencing. IHC immunohistochemistry, e19 exon 19, e21 exon 21, CN copy number, WT wild type.

HER2 FISH groups and HER2 IHC expression in invasive cancers

As expected, all cases from group 1A (5/5, 100%) had 3+ HER2 expression by IHC (Fig. 2). Interestingly, only one case from group 1B (case 6, 1/5, 20%) showed 3+ expression. No cases in group 2 had 3+ expression. One case from group 3 cases (case 21, 1/6, 17%) and one from group 4 (case 32, 1/17, 6%) showed 3+ expression. 3+ expression was not present in group 5.

HER2 copy number ratios between exon 19 and 21 are correlated

We used two different ddPCR assays to determine the normalized copy number ratios for *HER2* exons 19 and 21. These exons were selected given that they encode the tyrosine kinase domain in HER2. The tyrosine kinase domain is critical for HER2 activity and intracellular signal induction and plays an important role in oncogenic transformation and addiction [16–18]. To evaluate for interassay reliability, we compared the copy number ratios between the two exons using Spearman's rank-order correlation. As expected, we found a strong correlation ($\rho = 0.83$, $p < 0.0001$) in the copy number ratios between exons 19 and 21.

HER2 copy number ratios are associated with FISH groups and HER2/CEP17 ratios

To assess the relationship between ddPCR ratios and FISH results, the copy number ratios were compared across the six different FISH groups (Fig. 3a, b). The median copy number ratios of exons 19 and 21 varied significantly across the different FISH groups (Kruskal–Wallis test, $p < 0.01$). Specifically, the median ratios of group 1A were significantly greater than those of group 5 (Dunn's multiple comparison test, $p < 0.001$) for both exons 19 and 21 (Supplementary Tables 2A, 2B). In addition, the median ratio of group 1A was significantly greater than that of group 4 ($p = 0.03$) for exon 21. No other paired comparisons were significantly different.

To further explore the association of *HER2* levels between ddPCR and FISH, Spearman's rank-order correlation was performed using *HER2/RPP30* ratios from ddPCR and *HER2*/CEP17 ratios from FISH (Fig. 4). For both exons 19 and 21, ddPCR copy number ratios were significantly correlated with the *HER2*/CEP17 FISH ratios ($\rho_{e19} = 0.44$, $p = 0.001$ and $\rho_{e21} = 0.46$, $p = 0.0006$, respectively) despite the use of different control probes (e.g., *RPP30* vs. CEP17).

HER2 copy number ratios are associated with IHC expression

In addition to the FISH, we also measured the association between *HER2* copy ratios and HER2 expression by IHC. Notably, the median ddPCR ratios differed significantly across the three IHC scores for both exons 19 and 21 (Kruskal–Wallis test, $p < 0.0001$) (Fig. 3c, d). Specifically, the median ratios of cases with 3+ HER2 expression were significantly greater than those with 2+ (Dunn's multiple comparison test, $p < 0.05$) and no expression (1+/0) ($p < 0.0001$) (Supplementary Tables 2C, 2D). In addition, the median ratios of 2+ cases were significantly greater than those with 1+/0 expression ($p < 0.05$).

Amplification calling using copy number ratios from ddPCR

In this study, we used a previously validated algorithm to infer amplification status from ddPCR ratios [13].

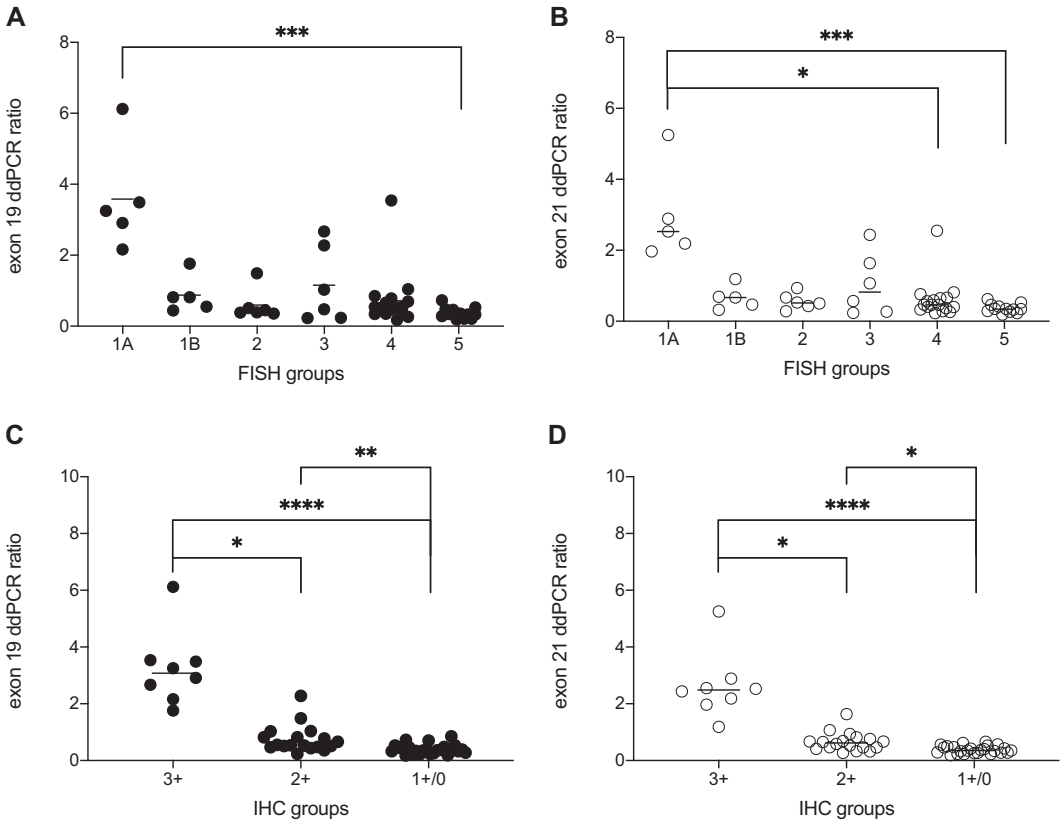

Fig. 3 Distribution of *HER2* copy number ratios in different FISH groups and IHC scores. A: *HER2* exon 19 ddPCR ratios among the 6 FISH groups. B: *HER2* exon 21 ddPCR ratios among the 6 FISH groups. C: *HER2* exon 19 ddPCR ratios among the 3 IHC scores; D: *HER2* exon 21 ddPCR ratios among the 3 IHC scores. *$p < 0.05$; **$p \le 0.01$; ***$p \le 0.001$; ****$p \le 0.0001$.

Importantly, the algorithm adjusts the threshold for amplification calls based on the tumor cellularity of the analyzed sample. For exon 19, nine amplification calls were made using this algorithm, with all cases in group 1A called amplified (Fig. 2). Four cases in groups 2–4 also were called amplified: case 11 in group 2 (1/6, 17%), cases 17 and 21 in group 3 (2/6, 33%), and case 32 in group 4 (1/17, 6%). Of note, two out of the four samples with ddPCR amplification calls, namely case 21 and 32, also had 3+ HER2 expression. Similarly, seven amplification calls were made for exon 21, with all cases in group 1A called amplified. In addition, two samples in the FISH groups 2–4 were called amplified: case 21 in group 3 (1/6, 17%) and case 32 in group 4 (1/17, 6%), both of which had 3+ HER2 expression. No amplification calls were made in group 1B, group 5, or cases with 1+/0 HER2 expression for both exons 19 and 21.

Amplification from ddPCR is concordant with 3+ HER2 expression

Although ddPCR amplification events were observed across the different FISH groups, the amplification calls coincided closely with 3+ HER2 expression. To further explore this relationship, Cohen's kappa statistic was measured by comparing the amplification calls from ddPCR with 3+ HER2 expression by IHC (Table 2). 3+ HER2 expression and amplification from exon 19 showed high numerical concordance (94%) with substantial agreement ($\kappa = 0.79$), whereas 3+ expression and exon 21 amplification showed high numerical concordance (98%) with almost perfect agreement ($\kappa = 0.92$), suggesting a close association between ddPCR-based amplification and protein overexpression.

NGS reveals copy number gains in HER2

In contrast to ddPCR, NGS allows for comprehensive copy number analysis across the entire coding region of *HER2*. Using this approach, we observed a wide spectrum of copy number gain events in our cohort. In 14 (27%) samples, copy number gain events were detected in <50% of *HER2* (minor copy number gain), whereas in eight (16%) samples, copy number gain events were observed in ≥50% of *HER2* (major copy number gain), including three samples (1, 3, and 21) that showed gains in all the exons. Copy number

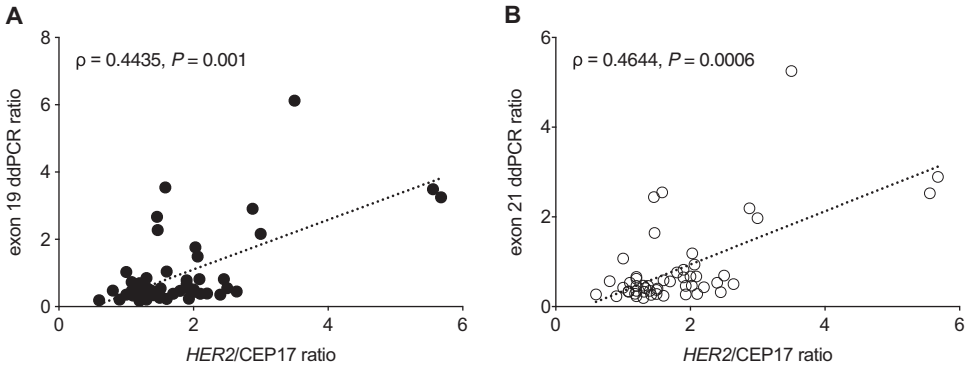

Fig. 4 Correlation between *HER2*/RPP30 ratios from ddPCR and *HER2*/CEP17 ratios from FISH. A: Correlation of *HER2* exon 19 ddPCR ratios with FISH ratios. B: Correlation of *HER2* exon 21 ddPCR ratios with FISH ratios. ρ Spearman's rank correlation coefficient.

Table 2 Concordance of HER2 between IHC and ddPCR.

		HER2 IHC		
		3+	2+, 1+, 0	Total
HER2 exon 19 ddPCR	Amplified	7	2	9
	Not amplified	1	41	42
	Total	8	43	51
	Concordance = 94%, κ = 0.79, SE = 0.12, 95% CI = 0.56–1.0			
HER2 exon 21 ddPCR	Amplified	7	0	7
	Not amplified	1	43	44
	Total	8	43	51
	Concordance = 98%, κ = 0.92, SE = 0.077, 95% CI = 0.77–1.0			

κ Cohen's kappa coefficient, *SE* standard error, *CI* confidence interval.

gain events were not detected in the remaining 29 (57%) samples.

Concordance of HER2 copy number gains from NGS with results from FISH, IHC, and ddPCR

To correlate our findings, we compared our NGS results with those from FISH, IHC, and ddPCR. Notably, major copy number gains were seen in all cases in group 1A (5/5, 100%). In contrast, only one case in group 1B (case 6, 1/5, 20%) with 3+ HER2 expression showed major copy number gain. Major copy number gains were also identified in a case from group 3 (case 21, 1/6, 17%) and one from group 4 (case 32, 1/17, 6%), both of which also showed 3+ expression (Fig. 2). As expected, major copy number gains were concordant with group 1A pattern, showing substantial agreement (κ = 0.74) (Table 3). Notably, there was 100% concordance between 3+ HER2 expression by IHC and major copy number gain by NGS, highlighting the close correlation between the genomic and protein levels. Lastly, major copy number gains were also concordant with ddPCR-based amplification status for exons 19 and 21, showing substantial ($\kappa_{e19} = 0.79$) and almost perfect ($\kappa_{e21} = 0.92$) agreement, respectively.

Table 3 Concordance of HER2 NGS status with IHC and ddPCR.

		HER2 NGS		
		≥50% CN gain	0–50% CN gain	Total
HER2 FISH	1A	5	0	5
	1B, 2, 3, 4, 5	3	43	46
	Total	8	43	51
	Concordance = 94%, κ = 0.74, SE = 0.14, 95% CI = 0.46–1.0			
HER2 IHC	3+	8	0	8
	2+, 1+, 0	0	43	43
	Total	8	43	51
	Concordance = 100%, κ = 1.0, SE = 0, 95% CI = 1.0–1.0			
HER2 exon 19 ddPCR	Amplified	7	2	9
	Not amplified	1	41	42
	Total	8	43	51
	Concordance = 94%, κ = 0.79, SE = 0.12, 95% CI = 0.56–1.0			
HER2 exon 21 ddPCR	Amplified	7	0	7
	Not amplified	1	43	44
	Total	8	43	51
	Concordance = 98%, κ = 0.92, SE = 0.077, 95% CI = 0.77–1.0			

κ Cohen's kappa coefficient, *SE* standard error, *CI* confidence interval.

Groups 1A and 1B show different HER2 copy number levels

In our limited analysis, we observed differences in *HER2* copy number levels between groups 1A and 1B. For example, all cases in group 1A showed amplification by ddPCR and major copy number gain by NGS, whereas only one case in group 1B showed major copy number gain. No ddPCR amplifications were called in group 1B. Given the variable tumor content of our samples, it is possible that the lower *HER2* copy number observed in group 1B may be the result of lower tumor cellularity. To investigate, we compared the tumor cellularity between group 1A and 1B

and detected no significant difference (Mann–Whitney *U* test, $p = 0.3$), suggesting that the variation in *HER2* copy level is likely not an artifact of tumor content. Furthermore, there was no correlation between tumor cellularity and the extent of *HER2* copy number gain ($\rho = 0.54$, $p = 0.1$), exon 19 copy number ratios ($\rho = 0.37$, $p = 0.3$), and exon 21 copy number ratios ($\rho = 0.42$, $p = 0.23$) in our combined group 1 cohort, thus demonstrating no association between tumor cellularity and *HER2* copy levels.

HER2 gain in group 3 is restricted to cases with 3+ HER2 expression

Similar to groups 2 and 4, group 3 cases with 3+ HER2 expression are considered HER2-positive. However, in contrast to other groups, group 3 cases with 2+ HER2 expression are also considered HER2-positive given that the repeat FISH testing results in another group 3 pattern. In our study, repeat FISH testing was not performed, thus precluding an accurate analysis of the guideline recommendations. Nevertheless, in our group 3 cohort, *HER2* gain events were largely restricted to cases with 3+ HER2 expression. Specifically, the only case with both amplification in exons 19 and 21 and major copy number gain was case 21 which had 3+ HER2 expression. There were three cases with 2+ expression, and only one case (case 17) in this subset had amplification in exon 19 without any other copy gain events. The remaining two cases showed no ddPCR-based amplification or major copy number gain.

Sequence variants in HER2

In addition to copy number analysis, we used our sequencing data to detect SNVs and small indels in *HER2*. In total, 11 sequence variants were identified from seven patients with various FISH results (Supplementary Table 3). Furthermore, all sequence variants were missense variants occurring in different coding regions of *HER2*, including three in the receptor L domain, one in the furin-like cysteine rich region, and three in the tyrosine kinase domain (Supplementary Fig. 1) [18, 19]. Notably, three patients showed p.R138Q, which is a known recurrent hotspot variant seen in various cancer types [20]. Using OncoKB, one variant (p.R896C) from patient 18 was classified as an oncogenic alteration (level 3A) that may be sensitive to neratinib, a HER2/EGFR tyrosine kinase inhibitor [21–23]. The remaining variants were classified as variants with likely neutral effect or of unknown significance.

Clinical outcomes to HER2-targeted therapies in select patients

Sixteen patients in our cohort (16/51, 31%) received therapies containing HER2-targeted agents (Table 4). Nearly

Table 4 Treatment and response to HER2-targeted therapy.

Patient	FISH Group	IHC score	IHC status	e19 ratio	e21 ratio	*HER2* gain by NGS	HER2-targeted therapy	Context of therapy	Response
1	1A	3+	Positive	2.16[a]	1.97[a]	Major	T, H	Adjuvant	NED
2	1A	3+	Positive	2.91[a]	2.19[a]	Major	T, C, H	Neoadjuvant	PR
3	1A	3+	Positive	6.12[a]	5.25[a]	Major	A, C, T, H[b]	No surgery	PR
4	1A	3+	Positive	3.49[a]	2.53[a]	Major	T, C, H, P	Neoadjuvant	CR
5	1A	3+	Positive	3.25[a]	2.89[a]	Major	T, C, H, P	Neoadjuvant	CR
7	1B	2+	Equivocal	0.82	0.67	Minor	A, C, T, H	Neoadjuvant	PR
10	1B	2+	Equivocal	0.82	0.32	None	A, C, T, H	Neoadjuvant	PR
12	2	1+	Negative	0.45	0.50	None	T, C, H, P[b]	Neoadjuvant	PR
13	2	1+	Negative	0.38	0.28	Minor	T, H	Adjuvant	NED
15	2	2+	Equivocal	0.36	0.53	Minor	T, P[b]	Neoadjuvant	Unknown
18	3	2+	Equivocal	1.03	1.07	Minor	A, C, T, H, P[b]	Neoadjuvant	PR
19	3	2+	Equivocal	0.24	0.27	None	H	No surgery	POD
28	4	0	Negative	0.54	0.40	None	A, C, T, H[b]	Neoadjuvant	Unknown
32	4	3+	Positive	3.54[a]	2.55[a]	Major	A, C, T, H	Adjuvant	Unknown
34	4	2+	Equivocal	1.04	0.59	None	A, C, T, H	Adjuvant	Unknown
38	4	2+	Equivocal	0.51	0.41	None	T, H	Neoadjuvant	PR

e19 exon 19, *e21* exon 21, *A* Adriamycin, *C* Cytoxan, *T* Taxol, *H* Herceptin (Trastuzumab), *P* Perjeta (Pertuzumab), *PR* partial response, *CR* complete response, *POD* progression of disease, *NED* no evidence of disease.

[a]Amplification by ddPCR.

[b]Therapy administered prior to sample collection.

half of these patients (7/16, 44%) belonged to FISH groups 1A or 1B, and the remaining patients belonged to FISH groups 2, 3, and 4 (9/16, 56%). All patients except for one (patient 19) received other cytotoxic chemotherapy agents along with the anti-HER2 therapies (e.g., Herceptin [Trastuzumab], Perjeta [Pertuzumab]). Five patients (5/16, 31%) received HER2-based treatments before specimen collection; the remaining 11 patients (11/16, 69%) received HER2-based treatments after specimen collection. Furthermore, ten of these treatments were administered in the neoadjuvant setting (10/16, 63%), four in the adjuvant setting (4/16, 25%) and two treatments were not associated with surgical resection (2/16, 12%). Clinical response data were available for 12 patients (12/16, 75%). For group 1A ($n = 5$), there were two partial responses (2/5, 40%) for a neoadjuvant and nonsurgical therapy, two complete responses (2/5, 40%) for neoadjuvant therapies, and one no evidence for disease (1/5, 20%) for an adjuvant therapy. For group 1B ($n = 2$), there were two partial responses (2/2, 100%) for neoadjuvant therapies. For groups 2, 3, and 4 ($n = 9$), there were three partial responses (3/9, 33%) for neoadjuvant therapies, one no evidence of disease (1/9, 11%) for an adjuvant therapy, and one progression of disease (1/9, 11%) for a nonsurgical therapy. Outcomes data were not available for four patients (4/9, 44%). Overall, our data suggest that the combination of HER2-targeted therapies and cytotoxic chemotherapies results in clinical and pathologic responses across a wide range of FISH groups. Notably, progression of disease was observed with anti-HER2 monotherapy in patient 19 with FISH group 3 and lack of *HER2* amplification by ddPCR and CN gain by NGS. Given the small number of patients and combined therapy with chemotherapeutic agents, it is difficult to selectively correlate the clinical responses of HER2-targeted therapies with *HER2* copy levels from ddPCR and NGS. Additional prospective and retrospective studies using larger cohorts are needed to validate the predictive value of ddPCR and NGS-based *HER2* results.

Discussion

Our genomic findings reveal a wide range of *HER2* copy number levels across the different FISH groups. As expected, cases with group 1A and group 5 patterns had the most striking difference in *HER2* copy number. In contrast, the remaining cases in groups 1B-4 were characterized by a broad range in *HER2* copy levels with only a few select cases showing marked increase in copy ratio by ddPCR and major copy number gain by NGS. Notably, most of these cases with increased *HER2* copies also had concomitant HER2 overexpression by IHC. The tight correlation between the protein and genomic markers suggests that these select cases, despite their unusual FISH results, may be uniformly driven by *HER2* amplification and potentially respond to HER2-targeted therapy.

Given the strong concordance between IHC and genomic results, our findings suggest that HER2 IHC may inform *HER2* copy number status in patients with unusual FISH patterns. Specifically, we show that groups 2–4 with 3+ HER2 overexpression often had marked increases in *HER2* copy levels that were indistinguishable from group 1A, thus providing genomic rationale for classifying these cases as HER2-positive. Hence, our results support the 2018 ASCO/CAP HER2 testing update recommendation for using IHC to resolve HER2 status in FISH groups 2–4 [5]. Although it is reasonable to assume that these patients will benefit from HER2-targeted therapies based on their increased genomic and protein levels, clinical data will likely remain limited given their rarity.

Furthermore, our findings raise important clinical and biological questions regarding the current HER2 classification system. Although both groups 1A and 1B are considered HER2-positive, we observed notable differences in HER2 levels by ddPCR, NGS, and IHC. Specifically, group 1A consistently showed (i) amplification in exons 19 and 21, (ii) major copy number gain, and (iii) 3+ HER2 overexpression while only one case from group 1B had a major *HER2* copy number gain and a 3+ HER2 overexpression. Other studies have also reported a lower frequency of 3+ HER2 overexpression in group 1B (~10%) compared with group 1A (~70%), suggesting potential differences in HER2 pathway signaling between these two HER2-positive groups [24, 25]. Despite this, patients in group 1B are considered eligible for HER2-targeted therapy given that they show clinical benefit to HER2 blockade [5, 7, 26–28]. However, it remains unclear if group 1B patients derive the same level of benefit as group 1A patients. To investigate this further, subclassification of group 1 into group 1A and group 1B may be useful to capture any potential differences in their response to HER2-targeted therapies. Similarly, we noticed differences in *HER2* copy levels in group 3 cases with 3+ and 2+ HER2 expression despite the fact that both can be considered HER2-positive by the current guideline. For example, in our small cohort analysis, group 3 cases with 2+ expression did not show consistent gains in *HER2* copy number levels. In fact, the only case with both ddPCR-based amplification and major copy number gain by NGS was a sample with 3+ HER2 expression. Further analysis using a larger cohort is needed to validate this observation between 2+ and 3+ IHC cases in group 3 and to explore its potential effect on sensitivity to HER2-targeted therapies.

Currently, there are no standardized guidelines for reporting *HER2* copy number status using ddPCR and NGS. In this study, we explored various strategies for translating the genomic *HER2* results into binary scores (i.e., amplified

vs. not amplified) in order to provide therapeutically relevant information. For ddPCR, we employed a previously reported algorithm that denotes amplification based on the copy number ratio and tumor purity of the tested specimen [13]. For NGS, we primarily used the percentage of *HER2* exons with copy number gains as a marker for HER2 status. Given the concordance of major copy number gains (gains in ≥50% of *HER2*) with HER2 overexpression, our findings suggest that the 50% cutoff may represent an optimal threshold value for calling HER2 positivity using an NGS-based analysis. The significance of minor copy number gains (gains in <50% of *HER2*) remains unclear given that *HER2* amplification often involves amplicons spanning multiple genes in 17q12 [29–31]. Furthermore, it is likely that minor copy number gains are related to technical artifact due to variability in sequencing coverage and may be best interpreted as HER2-negative. Future studies should validate the diagnostic and predictive performance of our 50% threshold using independent datasets.

There are a few limitations to our study. Importantly, we used full-sections scrolls from FFPE blocks for our genomic analysis and did not selectively enrich for the invasive tumor component. Lack of tumor enrichment invariably results in genomic contamination from nontumor cells, which are usually *HER2* nonamplified, and DCIS, which can be *HER2* amplified. To minimize the effect of nontumor cells, we used an algorithm for ddPCR that adjusts amplification thresholds based on tumor cellularity of a given sample [13]. For our NGS-based copy number assessment, tumor fraction or other purity metrics were not included in the analysis. Rather, copy number changes were measured at the overall sample mixture level as compared with a homogenous diploid control sample. Specifically, our algorithm called copy gains when the log 2 ratio of a segment was greater than or equal to 1.2, which corresponds to ~3 excess copies of *HER2* in a pure tumor sample. Given that our samples represent a mixture of tumor and nontumor cells, our threshold corresponds to greater than 3 excess copies of *HER2* for our samples. Hence, our NGS-based copy gain calls may be biased for high-level copy gains and may not reliably call low-level *HER2* copy gains especially in samples with low tumor cellularity. However, low purity samples represent a minority of cases in our cohort (e.g., 3 cases [6%] with <20% tumor cellularity). In addition, high-level copy gains may be more biologically potent and clinically relevant compared with low-level copy gains. Furthermore, all samples had 200 ng of input DNA for our NGS analysis, thus exceeding the minimum DNA input requirement of 10 ng for our NGS assay. To reduce potential contributions from DCIS, we selected cases with minimal in situ components and only included samples where the invasive tumor cellularity was greater than that of DCIS. Notably, there was broad concordance of HER2 IHC scores between DCIS and invasive tumor in our samples, thus suggesting that the DCIS component would not have significantly biased the results of our ddPCR and NGS. Lastly, we observed that the ddPCR ratios for many of our nonamplified samples were less than 1.0. This pattern is likely due to DNA degradation and fragmentation from formalin fixation and other causes. If the fragmentation were more severe in *HER2* than *RPP30*, it is possible that the signals in *HER2* may be reduced due to more frequent breaks within the region targeted for amplification. This negative trend raises the possibility of false-negative results and may be biased against low-level *HER2* copy gains. However, the negative trend would have not interfered with detecting high-level amplification events, which may be more biologically functional and clinically relevant.

In summary, we examined the genomic landscape of *HER2* alterations in patients with group 1–5 patterns. Our findings provide genomic rationale for using IHC to resolve unusual FISH results. The actionability of genomic findings from ddPCR and NGS requires further study and prospective clinical validation.

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Compliance with ethical standards

Conflict of interest S-RY: Received consulting fees from Invitae. YB: Received salary from TOMA Biosciences. FMDeLaV: Received salary and stock from TOMA Biosciences. MB: None. CJK: Received consulting fees from TOMA Biosciences. AV: Received salary from TOMA Biosciences. GJ: Received salary from TOMA Biosciences. KA: None.

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