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Impact of Using Median *vs.* Mean in Calculating ERBB2 FISH Results in Breast Cancer

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ABSTRACT

INTRODUCTION

Erb-b2 receptor tyrosine kinase 2 (ERBB2) testing is used to measure the status of ERBB2 gene expression and DNA amplification. Test results have been reported with a discrepancy as high as 20%. The purpose of this study was to improve ERBB2 fluorescence in situ hybridization (FISH) sensitivity by evaluating results generated by median as well as mean calculations.

METHODS

We retrospectively identified breast cancer cases at our institution in which ERBB2 FISH testing was performed in-house from June 2018 to May 2020. FISH results were classified using the 2018 American Society of Clinical Oncology/College of American Pathologists guidelines: groups 1 and 5 are FISH positive and negative, respectively, and groups 2-4 are equivocal requiring additional work-up. FISH counting sheets were collected and regrouped by median ERBB2 copy number counts and median ERBB2/CEP17 ratio and compared with the mean ERBB2 and mean ERBB2/CEP17 ratio. Intra-tumor genetic heterogeneity and CEP17 copy number gain (CEP17 ≥3) were assessed to see if they affect the discrepancy between median and mean groups.

RESULTS

Seventy-two breast cancer cases were collected and evaluated. Eleven cases (11 of 72 [15%]) had discrepant grouping by mean and median calculations. A significant number of discrepancies were found for CEP17 copy number gain ($p = 0.027$) but not for ERBB2 ($p = 0.411$), the ERBB2/CEP17 ratio ($p = 0.445$), FISH results ($p = 0.194$), or genetic heterogeneity ($p = 0.465$). Among the four cases regrouped to median group 1, 2 were from mean group 3 and underwent anti-ERBB2 targeted therapy and 2 were from mean groups 4 and 5 may have benefitted from targeted therapy with more than 30% amplified cells. The

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median may be better to reflect the monosomy subclone within tumor tissues for the case 387 moved from mean group 5 to median group 2. The 6 cases moved from mean group 5 to median group 4 with CEP17 copy number gain may have had a poor prognosis based on other study result.

CONCLUSION

Including the median calculation may increase ERBB2 sensitivity and identification of CEP17 copy number gain. Further clinical studies are necessary to examine the outcome of including median in calculating ERBB2/CEP17 values.

KEYWORDS

ERBB2; CEP17; FISH; Breast cancer, Mean, Median

INTRODUCTION

Erb-b2 receptor tyrosine kinase 2 (ERBB2), also known as human epidermal growth factor receptor 2 (HER2), located on 17q12 [1], is one of the most well-known proto-oncogenes; it encodes an epidermal growth factor receptor with tyrosine kinase activity. Routine ERBB2 testing by fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) has been used as a predictive biomarker for targeted therapy and disease prognosis [2-5], as it is overexpressed in about 20% of breast cancer cases [6]. In 2018, the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) published ERBB2 testing clinical practice guidelines to improve the accuracy of these tests [3]. In dual-probe FISH, 20 cells are normally counted for ERBB2 and chromosome enumeration probe 17 (CEP17) signals and the mean ERBB2/CEP17 is calculated to generate the ERBB2/CEP17 ratio. FISH results can be classified into 5 groups, with groups 1 and 5 being FISH positive and negative, respectively, and groups 2-4 being equivocal requiring additional work-up.

ERBB2 testing has been reported with discordance rates as high as 20% [7,8]; false negative test results were estimated to be about 2%-4% [9,10]. The exact determination of ERBB2 status in equivocal groups is further complicated by ERBB2 heterogeneity and CEP17 copy number gain [11]. ERBB2 heterogeneity describes the subsets of tumor cells with distinct ERBB2 amplification characteristics which contributes to equivocal ERBB2 status [12]. ERBB2 genetic heterogeneity (GH) is a standardized approach to assessing non-uniform ERBB2 amplification; it is defined as the presence of infiltrating tumor cells with a ERBB2/CEP17 ratio of higher than 2.2 in 5%-50% cells [13]. Genetic heterogeneity is found in 11%-45% of breast tumors and leads to discrepant ERBB2 status [11,14]. A previous study estimated that 13% low-grade amplified cells in tumors with regional genotypic variability affected patients' trastuzumab eligibility, indicating a need to increase sensitivity of interpreting cellular ERBB2 variation [14]. CEP17 copy number gain indicates the presence of 3 or more copies of chromosome 17 and is reported in 13%-46% of breast carcinomas [15]. Cases with heterogeneous amplification or co-amplification of the centromeric marker complicate the interpretation of results; consequently, an evidence-based approach is required to make the final determination of ERBB2 status [16].

Mean ERBB2 expression and mean ERBB2/CEP17 ratios have been traditionally used to calculate the ERBB2 FISH results. However, among the 20 counted cells, the signals may not be normally distributed or may be skewed because of heterogeneous ERBB2 status, a well-known feature of breast cancer [17]. Depending on prevalence, these populations of ERBB2 amplified cells may be sensitive to targeted drugs [12]. Statistically, the median describes the

middle value in skewed distributions in addition to resisting outliers [18]. Consequently, we investigated ERBB2 FISH testing of ERBB2 and CEP17 signals with respect to median and mean signals.

MATERIALS AND METHOD

Case Selection and FISH

This study was approved by the institutional review board at The University of Texas Medical Branch (Galveston, Texas). We performed a retrospective chart review of breast cancer cases that underwent ERBB2 FISH testing from June 2018 to May 2020, after the implementation of the 2018 ASCO/CAP guidelines. Formalin-fixed, paraffin-embedded breast cancer specimens were tested for ERBB2 gene amplification using dual-probe FISH (PathVysion; Abbott Laboratories). Areas of invasive carcinoma for FISH analysis were marked by a breast pathologist on accompanying H&E slides prior to FISH testing. ERBB2 FISH signals were captured by CytoVison software (Leica Biosystems, Inc.) and analyzed manually according to the assay protocol (PathVysion; Abbott Laboratories).

Pathologic tumor data, including grade, AJCC stage, lymphovascular invasion, lymph node invasion, Ki67, estrogen receptor (ER) and progesterone receptor (PR) status, were extracted from pathology reports. Clinical data, including treatment information, were collected from patients' electronic medical records.

Discordant Cases

Discordant mean and median groups were based on the difference between mean groups and median groups. We classified the FISH results into 5 groups according to the 2018 ASCO/CAP guideline mean values [3]. We then classified the FISH results into 5 groups according to the median values. Median group 1 had a median ERBB2 of ≥4 and median ERBB2/CEP17 ratio of ≥2; group 2 had a median ERBB2 of <4 and median ERBB2/CEP17 ratio of ≥2; group 3 had a median ERBB2 of ≥6 and median ERBB2/CEP17 ratio of <2; group 4 had a median ERBB2 of ≥4 and <6 and median ERBB2/CEP17 ratio of <2; and group 5 had a median ERBB2 of <4 and median ERBB2/CEP17 ratio of <2. Median group 1 was positive, and group 5 was negative. Median group 2-4 were equivocal.

Genetic Heterogeneity (GH)

ERBB2 GH was evaluated using the 2009 ASCO/CAP definition of heterogeneity: more than 5% but less than 50% of infiltrating tumor cells with an ERBB2/CEP17 ratio of higher than 2.2 [13].

Amplified Cells

Cells in each case were further divided into 6 categories [19]. A "Classic amplified" cell had a ratio of ≥2 and ERBB2 copy number of ≥6. A "Co-amplified/polysomy" cell was defined as a ratio of <2 and ERBB2 copy number of ≥6. A "Low amplified" cell was defined as a ratio of ≥2 and ERBB2 copy number ≥4 and <6. An "Equivocal" cell had a ratio of <2 and ERBB2 copy number of ≥4 and <6. A "Monosomy" cell was defined as a ratio of ≥2 and ERBB2 copy number of <4. A "Classic non-amplified" cell had a ratio of <2 and ERBB2 copy number of <4. "Classic amplified", "Co-amplified/polysomy" and "Low amplified" cells were all considered as amplified cells.

Statistical Analysis

The chi-square test was used to compare the variables between the mean and median groups. A p value of <0.05 was considered significant.

RESULTS

Clinical and Pathologic Data

Case counting sheets of 72 breast cancer specimens with FISH test results were collected and regrouped by median ERBB2 copy number and the median ERBB2/CEP17 ratio (Table 1). All patients were female and aged 32 years - 92 years, median aged 65 years at the time of July 2020.

Overall, the concordance rate between the mean group and median group analysis was 85% (61 of 72, Table 1). Sixteen cases remained in group 1, even after median regrouping. Both cases in mean group 3 were regrouped to median group 1. There were 3 concordance cases in group 4 and 42 in group 5.

Group	Number of specimens, mean	Number of specimens, median	Number of concordance cases (%)
1	16	20	16/72 (22%)
2	0	1	0 (0)
3	2	0	0 (0)
4	4	9	3/72 (4%)
5	50	42	42/72 (58%)
Total	**72**	**72**	**61/72 (85%)**

Table 1: Concordant cases for ERBB2 FISH mean and median groups in the 72 cases.

Comparison of Mean and Median FISH Values

We regrouped all cases by median ERBB2 copy number and median ERBB2/CEP17 ratios on the basis of the 2018 ASCO/CAP threshold. The results were summarized by the mean and median, as shown in Table 2. The mean ERBB2 copy number was ≥6 in 10 (14%) cases, ≥4 and <6 in 12 (17%), and <4 in 50 (69%). The median ERBB2 was ≥6 in 11 (15%) cases, ≥4 and <6 in 18 (25%), and <4 in 43 (60%). These differences were not significantly different (p = 0.411). Ten cases were CEP17 copy number gain by the mean and 22 by the median (p = 0.027). The mean and median ERBB2/CEP17 ratio and FISH results did not significantly differ (p = 0.445 and p = 0.194, respectively).

FISH result	Mean, n (%)	Median, n (%)	P value
ERBB2			
≥6	10 (14)	11 (15)	0.411
≥4 & <6	12 (17)	18 (25)	
<4	50 (69)	43 (60)	
CEP17			
≥3	10 (14)	22 (31)	0.027
<3	62 (86)	50 (69)	
ERBB2/CEP17 ratio			
≥2	16 (22)	21 (29)	0.445
<2	56 (78)	51 (71)	
FISH group			
1	16 (22)	20 (28)	0.194
2	0	1 (1)	
3	2 (3)	0	
4	4 (6)	9 (13)	
5	50 (69)	42 (58)	

Table 2: Mean and median FISH results of the 72 cases.

Discrepancy Cases by Mean and Median

Using the median, 11 cases, all invasive ductal carcinoma, (15%) changed to a lower group: 4 to group 1, 1 to group 2, and 6 to group 4 (Table 3). The clinical and pathologic findings are presented in Table 4. Group 1 is defined as FISH positive and had indications for targeted ERBB2 therapy. In case 372 (regrouped from mean group 3 to median group 1), the mean ERBB2 copy number was 6.45, and the median was 6. The mean CEP17 copy number was 3.85, and the median was 3. The mean ratio was 1.68, and the median ratio was 2. In case 376, the mean and median ERBB2 copy number were 6.08 and 6, respectively. The mean CEP17 copy number was 3.18, and the median was 3. The case was reclassified from mean group 3 to median group 1. The mean ratio was 1.91, which was much closer to the threshold of 2. In case 429, the mean ERBB2 and median ERBB2 copy number were 4.08 and 4,

respectively. However, the mean CEP17 was 2.8 (higher than the median of 2), which led to a mean ratio of 1.46 and median ratio of 2. In case 440, the mean ERBB2 copy number was 3.7 and the median was 4. The mean CEP17 was 2.45, and median was 2. The higher median ERBB2 and lower median CEP17 signals resulted in a higher median ratio of 2, which matched group 1. Case 387 was moved to median group 2, with a median ratio of 2, a higher median ERBB2, and a lower median CEP17. Cases 388, 404, 405, 407, 447, and 454 were reclassified as group 4 because they had 4 copies of ERBB2.

Case	Mean				Median			
	ERBB2	*CEP17*	Ratio	Group	*ERBB2*	*CEP17*	Ratio	Group
372	6.45	3.85	1.68	3	6	3	2	1
376	6.08	3.18	1.91	3	6	3	2	1
429	4.08	2.8	1.46	4	4	2	2	1
440	3.70	2.45	1.51	5	4	2	2	1
387	1.70	1.4	1.21	5	2	1	2	2
388	3.8	2.8	1.36	5	4	3	1.33	4
404	3.75	3.05	1.23	5	4	3	1.33	4
405	3.40	2.85	1.19	5	4	2.5	1.6	4
407	3.60	3.1	1.16	5	4	3	1.33	4
447	3.70	2.7	1.37	5	4	3	1.33	4
454	3.70	2.95	1.25	5	4	3	1.33	4

Table 3: Discrepancy cases between means and medians.

Reclassification of Cases in Group 3

Mean group 3 only had 2 cases (2 of 72 [3%]), cases 372 and 376, which were composed of a surgical excision specimen from the left breast and a lymph node metastasis at the right axilla in a patient with recurrent breast cancer. Both cases were scored as IHC 2+ (Table 4) and diagnosis were ERBB2 positive based on the updated 2018 guideline. The addition of the median (group 1 was positive) was in accordance with the clinical diagnosis. Seventy percent of the tumor cells in case 372 were amplified (grade 3). The case was ER-/PR-, ERBB2+, and Ki67 80%; its biological surrogate molecular subtype was HER2 positive. This patient underwent trastuzumab treatment. Case 376 contained 72.5% amplified cells and had lymph node invasion and Ki67 80%; this patient underwent targeted therapy. The signal frequency distribution of the 2 cases is plotted in histograms (Figure 1).

Case	Grade	AJCC stage	Lymphovascular invasion	Lymph node invasion	ERBB2 IHC	ER/PR	Ki67	*ERBB2* clinical status	*ERBB2* GH	Cell amplification	Anti-ERBB2 therapy
372	3	IIA	No	No	2+	-/-	80%	Amp	Present	70%	Herceptin
376	2	IIB	No	Yes	2+	98%/98%	80%	Amp	Present	72.5%	Kadcyla
429	3	IIB	Yes	Yes	2+	90%/80%	40%	No amp	Present	37.5%	None
440	1	IA	No	No	2+	99%/100%	5%	No amp	Absent	30%	None
387	2	IB	No	No	2+	95%/90%	20%	No amp	Absent	0%	None
388	3	IIIB	No	Yes	2+	-/-	95%	No amp	Present	20%	None
404	1	IA	Yes	No	2+	90%/70%	45%	No amp	Present	10%	None
405	1	IA	No	No	2+	-/-	5%	No amp	Absent	20%	None
407	2	IB	No	No	2+	99%/99%	40%	No amp	Absent	5%	None
447	1	IA	No	No	2+	100%/80%	30%	No amp	Present	10%	None
454	3	IA	No	No	2+	95%/30%	30%	No amp	Absent	20%	None

Table 4: Clinicopathologic factors for the 11 cases with discrepancy median and mean groups.

Reclassification of Group 4 Cases

Four (6%) of the 72 cases were in mean group 4. All cases were IHC 2+. After reevaluation by the median, 3 (75%) remained in group 4 and 1 (25%) moved to median group 1. Case 429 converted to ERBB2 positive. Case 429 was grade 3 and stage IIB, with ER 90%/PR 80% and Ki67 40%, 37.5% amplified cells, and ERBB2 GH; the case presented with lymphovascular invasion and lymph node invasion (Table 4).

Reclassification of group 5 cases

Fifty (50 of 72 [69%]) cases were in mean group 5. Among them, 8 (8 of 50 [16%]) changed groups: 1 to median group 1, 1 to group 2, and 6 to group 4. All 8 cases were IHC 2+.

Based on the 2018 guidelines, case 440 was categorized as ERBB2 negative but reclassified as positive median group 1. Case 440 was grade 1 and stage IA with Ki67 5% and strongly ER and PR positive (99% and 100%, respectively).

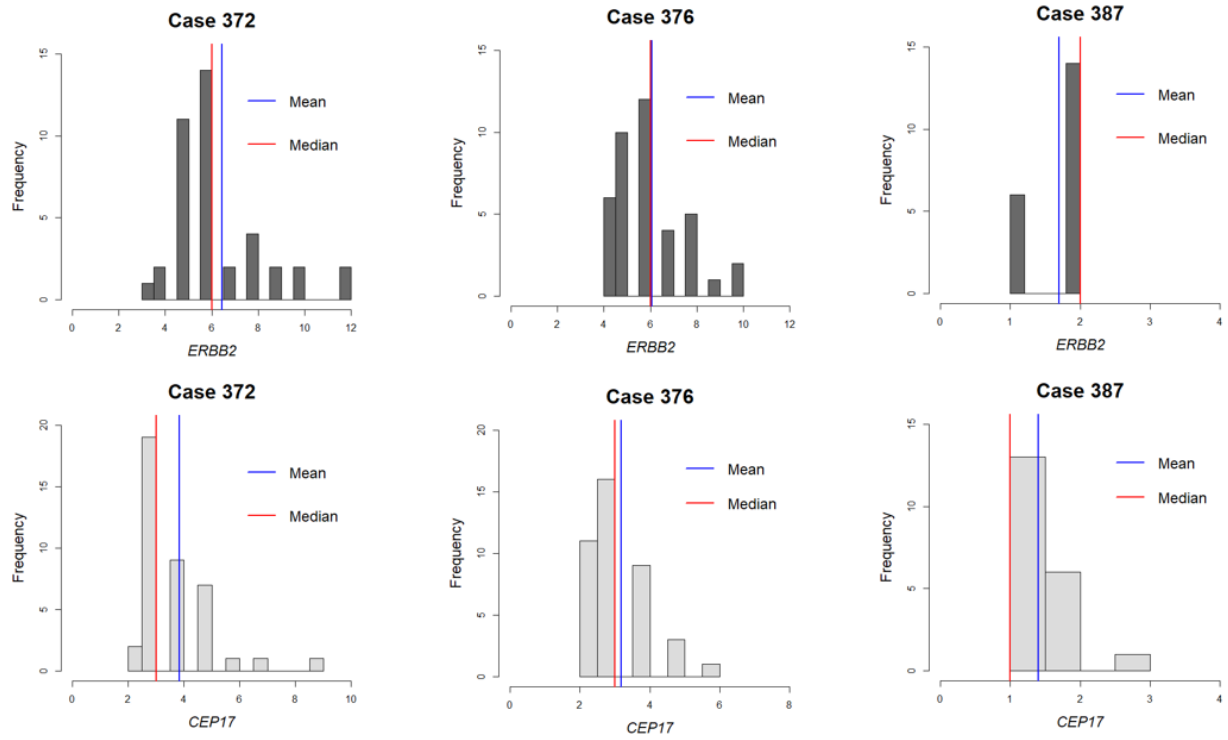

Figure 1: Signal histograms of cases 372, 376, and 387. The signal distributions were varied. The ERBB2 distribution of 40 cells of case 372 ranged from 3% to 12. 65% (26 of 40) cells had ERBB2 signal ≥6. 95% (38 of 40) cells had CEP17 ≥3. The largest subset of ERBB2 copy number was 6% in 35% (14 of 40) cells, which was equal to median ERBB2. The largest subset of CEP17 was 3% in 47.5% (19 of 40) cells, which was equal to median CEP17. The mean line (6.45) in ERBB2 was higher than the median line (6) because 12 cells had ERBB2 signal >6 and as high as 12. The mean line (3.85) in CEP17 was higher than the median line (3) because 47.5% (19 of 40) cells had CEP17 >3 and as high as 9. Compared to the distance between the mean and median ERBB2, the mean and median CEP17 distance was larger. The lower median CEP17 resulted in a ratio of 2 and moving to median group 3. The mean ERBB2 (6.08) was almost equal to the median (6) of case 376, but the median CEP17 (3) was lower than the mean (3.18). The CEP17 distribution in case 376 was 27.5% (11 of 40) cells with 2, 40% (16 of 40) cells with 3, and 32.5% (13 of 40) cells with more than 3 which caused mean CEP17 larger than 3. The median ratio (2) was higher than the mean ratio (1.91) and regrouped to median group 1. All 40 cells in case 376 had ERBB2 signal ≥4, which was finally defined as ERBB2 amplified. Group 1 might have been more appropriate for case 376 than group 3. 70% (14 of 20) cells in case 387 had 2 copies of ERBB2 and 65% (13 of 20) cells had 1 copy of CEP17 which were equal to median values. Also, the largest subclones (9 of 20 cells, 45%) was cells with 2 copies ERBB2 and 1 copy CEP17 which was reflected by median. Blue lines indicate mean values and red lines indicate median values.

There was no lymphovascular invasion, lymph node invasion, or GH present, and it included 30% amplified cells (Table 4). Case 387 was changed to group 2 because of median ERBB2 copy number was 2, median CEP17 was 1 and median ratio was 2 (Table 2). However, the dominate subclone of case 387 was 2 copies of ERBB2 and 1 copy of CEP17, which contained in 45% (9 of 20) cells and could be reflected by median. The copy number distributions of ERBB2 and CEP17 were shown in Figure 1.

Cell Amplification Patterns in 11 Cases

We analyzed cell amplification in all 11 cases and mapped cells of each case into 6 categories [19] (Table 5). In the total amplified row, 4 cases in group 1 contained more than 30% amplified cells, which was higher than the other groups. Cases 372 and 376 had more than 70% amplified cells. Cases 429 had 52.5% cells with ERBB2 ≥4, and case

440 had 70% cells with ERBB2 ≥4 and <6. Monosomy 17 was found in 45% cells in case 387. In cases 388, 404, 405, 407, 447, and 454, the amplified cell percentage was less than 20%. More than 75% of cells were equivocal and classic non-amplified.

Pattern	*ERBB2*/*CEP17* Ratio	*ERBB2*	372	376	429	440	387	388	404	405	407	447	454
Classic amplified (%)	≥2	≥6	40	40	10	0	0	0	0	0	0	0	0
Co-amplified/polysomy (%)	<2	≥6	25	20	10	0	0	5	0	5	0	0	5
Low amplified (%)	≥2	≥4 & <6	5	12.5	17.5	30	0	15	10	15	5	10	15
Equivocal (%)	<2	≥4 & <6	27.5	27.5	15	40	0	35	60	35	55	55	35
Monosomy (%)	≥2	<4	0	0	2.5	0	45	5	5	5	0	10	0
Classic non-amplified (%)	<2	<4	2.5	0	45	30	55	40	25	40	40	25	45
Total amplified (%)			70	72.5	37.5	30	0	20	10	20	5	10	20
Group changes			3 to 1	3 to 1	4 to 1	5 to 1	5 to 2	5 to 4	5 to 4	5 to 4	5 to 4	5 to 4	5 to 4

Table 5: ERBB2 and CEP17 FISH signal patterns in 11 discrepancy cases, analyzed cell by cell.

Discrepancy Cases with Genetic Heterogeneity

We determined the GH in the mean groups [13]. Thirty-two cases presented with GH and 40 cases did not. The results were not statistically significant (p = 0.465). 31% (5 of 16) of group 1 cases presented with GH. 44% of the group 5 cases (22/50) had GH. Three of the 4 cases in group 4 presented with GH, including the discrepancy case 429. Both discrepancy cases in group 3 had GH (Table 4). GH was not the cause of group discrepancy, but the frequency was higher in discrepancy group cases than in same group cases; although this was not significant (6 of 11 *vs.* 26 of 61; p = 0.465).

CEP17	Mean			Median		
	Same group	Discrepancy group	P value	Same group	Discrepancy group	P value
≥3	6	4	0.06	15	7	0.026
<3	55	7		46	4	

Table 6: Significance of CEP17 copy number in case with same groups (n = 11) and discrepancy groups (n = 61).

Discrepancy Cases with CEP17 Copy Number Gain

We compared the mean and median CEP17 in all cases (Table 2); both were significantly different (p = 0.027). More CEP17 copy number gain cases were found for the median. The chi-square test was used to determine whether CEP17 copy number gain resulted in different mean and median groupings (Table 6). By the mean, 4 discrepancy cases were CEP17 copy number gain and 7 were not. This was not statistically significant (p = 0.06), although it was close. However, CEP17 copy number gain was significant in median discrepancy cases (p = 0.026). Three (27%) discrepancy cases were redefined as median CEP17 copy number gain. Three cases (cases 388, 447, and 454) with different mean and median CEP17 values were all from group 5 (Table 3), which might have been caused by equivocal cells. Nine of 61 (15%) same group cases were reclassified as median CEP17 copy number gain.

DISCUSSION

Among the 72 cases, 15% had a discrepant rate between the mean and median for FISH groups. Although we only analyzed a small number of cases, our results indicated that including median to mean calculations may have the potential to increase sensitivity of ERBB2 heterogeneity interpretation.

In mean group 1, no discrepancies were identified. Sixteen cases in mean group 1 were in concordance with the median group. There was a slightly higher ERBB2-positive rate using the median by regrouping 4 cases, although this was not statistically significant.

The ERBB2 status in mean group 2 is uncommon, less than 1% [3]. No cases were included in the mean group, but 1 (case 387) was classified as median group 2. Case 387 contained GH and had 45% monosomy 17 cells, which was

concordant with group 2 features. Nonetheless, clinical trials have shown that trastuzumab therapy has no significant effect in patients in group 2, suggesting that recategorization base on the median for this case would have resulted in no change in indication for ERBB2 targeted therapy [20].

Only 2 cases (372, 376) were in mean group 3, both of which were re-categorized to median group 1 utilizing median grouping. Cases 372 and 376 contained 40% classic amplified cells, 30% co- or low-amplified cells, and 27.5% equivocal cells; these complex subclones resulted in their being classified into different groups. Both cases had a CEP17 copy number gain and ERBB2 copy number <10. Previous studies have reported that group 3 cases are heterogeneous and have more co-amplification of ERBB2 and the centromere rather than true polysomy for the entire chromosome 17 [11,21], which results in a mean ratio of <2. Based on this, the possible causes for discordance of cases 372 and 376 were ERBB2 heterogeneity and CEP17 copy number gain.

Four cases were in mean group 4. They were all IHC 2+ and clinically defined as having no amplification. One case, case 429 changed to median group 1, indicating that this patient may have benefitted from targeted therapy. Moreover, 37.5% of cells in this case had ERBB2 amplification. One study of ERBB2 targeted therapy responses in ERBB2 equivocal tumors, patients with <30% amplified cells did not benefit from targeted therapy [22]. Therefore, case 429 may have received benefit from ERBB2 targeted therapy in addition to hormone therapy.

Two of 6 cases (cases 404 and 407) moved from mean group 5 to median group 4, with a mean CEP17 of >3, and 5 cases (cases 388, 404, 407, 447 and 454) with a median CEP17 of 3. Studies have reported that ERBB2-negative tumors in group 4 are biologically different from those in group 5, which may be partly explained by a CEP17 copy number gain caused by chromosomal instability [23,24]. Cases 404, 407, 447, and 454 were CEP17 copy number gain and ERBB2 negative/hormone receptor positive, biologically close to group 4 and might have poor survival as CEP17 copy number gain is a poor prognostic factor in ERBB2-negative cancers, especially in ERBB2-negative/hormone receptor-positive breast cancers [25]. Most of the cases in groups 4 and 5 were ERBB2 negative; therefore, the cases that moved from mean group 5 to median group 4 were not treated with targeted therapy. Case 440 was regrouped to median group 1 with a borderline ERBB2 positive. This case was grade 1 and hormone receptor positive, with no GH or CEP17 copy number gain; thus, its biological features were similar to those of ERBB2-negative group 5 cases. However, it contained 30% amplified cells and may have benefitted from targeted therapy.

Interestingly, we report that discrepancy cases did not have significant association with GH compared to same group cases. One possible explanation is that approximately 70% of all cases were in group 5 (42 of 61 in the same group and 8 of 11 in the discrepancy group), and these cases share similar biologic features.

The complex abnormalities of chromosome 17 can lead to inaccurate ERBB2 gene copy number assessment in routine diagnostic practice [26]. We report a significant number of CEP17 copy number gain cases was found in the median discrepancy groups ($p = 0.026$) but not in the mean groups ($p = 0.06$). CEP17 copy number gain was found in 10 of 72 mean cases and 22 of 72 median cases, suggesting heterogeneity of CEP17 loci gain in 12 cases. These cases typically had ERBB2/CEP17 ratios <2 because of the co-occurrence of CEP17 gains and relatively low mean ERBB2, as had been reported by others [19,27]. Despite this, we report an upstaging of median discrepancy cases with respect to ERBB2 group status, secondary to heterogeneity of subclones confirmed by ERBB2 and

CEP17. Further, this resulted in a slight increase in the ERBB2-positive interpretation rate because of the reclassification of 4 cases to group 1. The median was concordant with ERBB2 gene status in cases 372 and 376 - both patients underwent targeted therapy. Cases 429 and 440 may have benefitted from targeted therapy since they contained more than 30% amplified cells. The median may have better reflected the monosomy subclone within tumor tissues in case 387. The 6 cases that were moved from mean group 5 to median group 4 with CEP17 copy number gain may have had a poor prognosis. In summary, including the median calculation, there would be no changes in current therapies for case 372 and 376. However, median may increase benefit to risk cases (like cases 429 and 440) since they contained more than 30% amplified cells. The median also helps for the identification of CEP17 copy number gain cases.

Our investigation is limited by lack of clinical validation. Further studies into prognostic implications and outcomes are necessary to determine the clinical significance of utilizing median calculations in ERBB2 FISH interpretation.

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