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ARTICLE

ERBB2 amplification status in 67 salivary duct carcinomas assessed by immunohistochemistry, fluorescence in situ hybridization, and targeted exome sequencing

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Salivary duct carcinoma (SDC) is an aggressive salivary gland malignancy with poor survival. Approximately 30% SDC harbor *HER2* amplification and response to trastuzumab has been reported. However, a systematic approach for HER2 status assessment in this tumor type has not been established. A total of 67 tumor samples were evaluated for HER2 protein overexpression or *ERBB2* gene amplification using at least 2 methods: immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), and/or targeted exome next-generation sequencing (NGS). NGS assessed *ERBB2* copy number fold change (FC) and total copy number (TCN). HER2 status was first determined by IHC/FISH according to the 2018 ASCO/CAP breast cancer guidelines. FISH results, the "gold standard", were compared with the NGS results. All (15/15) IHC positive, 35% (6/17) equivocal, and no (0/19) IHC negative SDC were *HER2* amplified by FISH. HER2 FISH signal/cell showed a good correlation with FC (Spearman correlation: 0.708, R^2: 0.501, $p < 0.0001$) and TCN (Spearman correlation: 0.763, R^2: 0.582, $p < 0.0001$). Receiver operating characteristics curve estimation showed an area under curve (AUC) of 0.975 for *ERBB2* FC. FC cutoff of ≥1.8 corresponded to an accuracy of 95.2% for *ERBB2* amplification (Youden's index: 0.84, sensitivity: 89.47%, specificity: 100%). FC < 1.3 could be reliably classified as *ERBB2* not amplified and FC ≥ 1.3 and <1.8 as equivocal. TCN estimation showed AUC of 0.981. TCN cutoff of >6.0 corresponded to an accuracy of 92% for HER2 amplification (Youden's index: 0.81, sensitivity: 81.2%, specificity: 100%). TCN < 4 could be reliably classified as *ERBB2* not amplified and TCN ≥ 4.0 and ≤6.0 as equivocal. FC and TCN were binarized with respective cutoffs of ≥1.8 and ≥6.0 and the proportion of agreement with FISH were 95% and 92%, respectively. The assessment of *ERBB2* copy number by NGS is accurate and reliable with FC or TCN nearly equivalent to FISH in identifying *HER2* amplified SDC.

Modern Pathology (2022) 35:895–902; https://doi.org/10.1038/s41379-021-00999-0

INTRODUCTION

Salivary duct carcinoma (SDC) is a high-grade apocrine adenocarcinoma most frequently found in the parotid gland and represents 2–5% of malignant salivary gland neoplasms[1,2]. Standard treatment regimen includes surgery, chemoradiation, and androgen receptor (AR) inhibition, but the survival rate remains low and less than 45% patients survive 5 years[1]. The human epidermal growth factor receptor 2 (HER2) protein, a 185-kDa transmembrane tyrosine-kinase receptor encoded by the *HER2 (ERRB2)* gene is overexpressed in a variety of malignancies, including approximately 25–30% SDC[1,3–7]. When activated, the HER2 protein forms heterodimers on the cell surface, which propagates activation of the PI3K and RAS pathways and affects proliferation, survival and angiogenesis[8]. Therapy with the monoclonal antibody trastuzumab, which causes internalization and downregulation of the HER2 protein, has led to dramatic improvements in disease-free survival in breast cancer patients harboring *HER2* amplified tumors[9–11]. Given the pathologic and molecular similarities between SDC and a subset of invasive mammary carcinomas[1,7,12–14], trastuzumab has been identified as a potential treatment option for HER2 positive SDC. A number of case reports and small case series have demonstrated its effectiveness in the treatment of select SDC patients, especially when combined with other chemotherapeutic drugs[15–32]. A more recent series of trastuzumab-treated SDC showed most notable responses in cases with HER2 protein overexpression by IHC[33]. These results support the need for an accurate HER2 scoring system in SDC, which is largely missing at present. The standard methods recommended by the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) for evaluation of HER2 status in breast cancer include detection of the protein overexpression by immunohistochemistry (IHC) and gene amplification by in situ hybridization (ISH)[34]. The emerging next-generation sequencing (NGS) technologies used in clinical cancer medicine to identify molecular therapeutic targets commonly allow for concurrent detection of copy number alterations (CNA) and can be a cost-effective alternative to multiple single-gene tests. The MSK-IMPACT assay is a clinically validated and U.S. Food and Drug Administration (FDA)-cleared targeted exome sequencing assay that detects point mutations,

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Received: 31 August 2021 Revised: 6 December 2021 Accepted: 8 December 2021
Published online: 28 December 2021

insertions or deletions, rearrangements, and CNAs[35]. Using a large set of breast and gastroesophageal carcinomas, MSK-IMPACT was clinically validated to reliably detect *ERBB2* amplification and proved to be highly concordant with the traditional methods, IHC and fluorescence in situ hybridization (FISH)[36]. In this study, we evaluated the HER2 protein expression and *ERBB2* gene amplification status in a large cohort of SDC, aiming to assess the concordance between IHC, FISH, and a targeted exome NGS assay in this cancer type.

METHODS AND MATERIALS

Case selection

After obtaining Institutional Review Board approval, the Pathology archives of Memorial Sloan Kettering Cancer Center (MSKCC) were searched for cases with the diagnosis of SDC, which were assessable by at least two of the following assays: HER2 IHC, HER2 FISH, and/or NGS. The diagnostic features of SDC included high-grade histology, apocrine cytology with prominent nucleoli, positive immunolabelling for CK7 and AR, and negative S100[7,13,14]. A total of 67 SDC were identified, including primary ($n = 40$) and metastatic ($n = 27$) formalin-fixed paraffin-embedded (FFPE) tumors diagnosed between 2003 and 2020. The SDC included resection ($n = 52$), biopsy ($n = 14$) and cytology ($n = 1$) samples. Cases were tested for HER2 protein overexpression and/or gene amplification by IHC ($n = 63$), FISH ($n = 55$), and NGS ($n = 55$). *HER2* copy number fold change (FC) was evaluated for all cases assessed by NGS and a subset of the NGS profiled cases (n = 44) were also evaluated for the total copy number (TCN). A total of 33 cases were assessed by all four methods. The details of the assays performed are provided in Table 1.

Immunohistochemistry

HER2 IHC was performed on 4um FFPE tissue sections using PATHWAY anti-HER-2/neu (4B5) (Ventana, Tucson, AZ) or HercepTest (Dako, Carpinteria, CA). All cases were reviewed by three board-certified anatomic pathologists including one surgical and molecular (DCF), one molecular and breast (DSR), and one molecular and head and neck pathologist (SD). Cases were evaluated using the 2018 ASCO/CAP developed standard system for breast cancer and scored accordingly as depicted in Fig. 1[34].

Fluorescence in situ Hybridization

Fluorescence in situ hybridization (FISH) to detect *ERBB2* amplification was performed using either the HER2 IQFISH pharmDx (Dako) or PathyVysion HER-2 DNA probe Kit (Vysis, Downers Grove, IL) dual probe kits. A minimum of 50 cancer cells were evaluated in each case by one experienced technologist (TZ) and at least one molecular pathologist (DCF, SD), and the cases were grouped based on the 2018 ASCO/CAP guidelines for breast cancer. (Supplementary Table S1)[34].

Copy number assessment by NGS

A hybrid-capture based targeted exome NGS assay (MSK-IMPACT) was performed on 55 cases as previously described, including 29 cases previously published[7,35,37]. In brief, genomic DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue, and macrodissection was employed in select cases to enrich for tumor content. The tumor content median was 55% (range 15% to 90%). Either non-neoplastic FFPE tissue or matched peripheral blood were used as source for normal control DNA. Sequencing was performed at a minimum sequencing coverage depth of 200X to interrogate a panel of 341 to 468 cancer-related genes for the presence of point mutations, insertions, deletions and rearrangements. Additionally, the standard clinical pipeline of MSK-IMPACT evaluates more than 1000 intergenic and intronic single-nucleotide polymorphisms to aid in the assessment of copy number alterations. The level of gene copy number gain or loss is calculated from the proportion of sequencing reads at one site relative to another and is referred to as "fold change" (FC). The clinically set threshold for gene amplification is a FC value of 2.0 or greater. Fraction and Allele-Specific Copy Number Estimates from Tumor Sequencing (FACETS) is an allele-specific copy-number analysis pipeline and open-source software for NGS data that is optimized for the MSK-IMPACT assay[38]. It utilizes heterozygous sites of both a tumor and a normal sample to measure allelic imbalance between the two samples and has the benefit of detecting copy neutral loss of heterozygosity and ploidy. Additionally, this algorithm provides an integer copy number assessment of a specific gene. This value is referred to as for the "total copy number" (TCN).

Statistical analysis for concordance

The optimal cut-offs for FC and TCN relative to the HER2 FISH score which was used as the "gold standard" were determined using receiver operating characteristics (ROC) curve estimation. Spearman correlation was used for evaluation of correlation between non-parametric variables. Kruskal-Wallis test was used for comparison of non-parametric variables between IHC groups. Mann–Whitney test was used to compare NGS FC and TCN based on binarized FISH results. Nam's score test and McNemar test were used for evaluation of equivalence between assays with an alpha of 0.05.

RESULTS

Comparison of IHC and FISH

Of the 63 cases with IHC results, 21 (33.3%) were positive (score 3+), 22 cases (34.9%) were negative (score 0 or 1+) and 20 cases (31.7%) were equivocal (score 2+; Supplementary Fig. 1). Among 55 cases with both IHC and FISH results, all cases with negative HER2 IHC (scores 0 or 1+, $n = 19$) were negative for *HER2* amplification by FISH. All cases with positive HER2 IHC (3+ score, $n = 15$) showed *HER2* amplification by FISH. Equivocal IHC (2+ score) was detected in 17 cases and 6 (35%) showed *HER2* amplification by FISH. Overall, 24 (43.6%) cases were *HER2* amplified and 31 (56.4%) were not amplified by FISH. The respective median HER2/CEP17 ratio and median HER2 signals/cell were 5.2 (interquartile range [IQR], 3.6–7.2) and 10.2 (IQR, 6.4–14.7) for amplified, and 1.2 (IQR, 1.1–1.5) and 2.5 (IQR, 2.2–3.0) for not amplified cases. Two cases with a HER2/CEP17 ratio <2 and the average HER2 signals/cell >4.0 and <6.0 were both considered not amplified because they showed negative IHC (scores 0 and 1+, respectively). The HER2/CEP17 ratio and HER2 signal/cell were significantly different between the IHC groups (Kruskal–Wallis $P: < 0.0001$ for both). The respective median HER2/CEP17 ratio and median HER2 signal/cell were as follows: 1.1 (IQR, 1.0–1.44) and 2.3 (IQR, 2.2–3.0) for IHC negative, 2.8 (IQR, 2.5–4.0) and 6.4 (IQR, 6.2–8.9) for IHC equivocal/FISH amplified cases, 1.3 (IQR, 1.2–1.6) and 2.7 (IQR, 2.5–3.0) for IHC equivocal/FISH not amplified cases, and 5.8 (IQR, 4.2–6.1) and 12.1 (IQR, 10.0–15.6) for IHC positive cases (Fig. 2).

Comparison of NGS and FISH

Out of 42 cases with both FISH and NGS FC results, including 37 with TCN results, 19 (45.2%) cases were amplified, and 23 (54.8%) cases were not amplified by FISH. As determined by the standard clinical pipeline for MSK-IMPACT, the median FC for FISH amplified and FISH not amplified cases were 4.3 (IQR, 2.9–9.2) and −1.0 (IQR, −1.1–1.3; Mann–Whitney $p < 0.0001$), respectively. HER2 signal/cell, as determined by FISH, showed good correlation with FC (Spearman correlation: 0.708, R^2: 0.501, $p < 0.0001$) (Fig. 3A). Of the 37 cases with TCN data, the respective median TCN for FISH amplified ($n = 16$) and FISH not amplified cases ($n = 21$) were 22 (IQR, 10–24) and 1 (IQR, 1–2; Mann–Whitney $p < 0.0001$). HER2 signal/cell, as determined by FISH, showed good correlation with TCN (Spearman correlation: 0.763, R^2: 0.582, $p < 0.0001$; Fig. 3B). ROC curve estimation analysis (Fig. 3C, Supplementary Table S2) using FISH results as the "gold standard" showed an area under curve (AUC) of 0.975 (95% confidence interval [CI], 0.887–0.995) for *ERBB2* copy number FC. A FC cutoff of ≥1.8 corresponded to an accuracy of 95.2% for *ERBB2* amplification status (Youden's index: 0.84, sensitivity: 89.47%, specificity: 100%). At a FC cutoff of ≥1.3 the sensitivity would be 100%, i.e., any case with a FC of <1.3 can be reliably classified as *ERBB2* not amplified. FC of ≥1.3 and <1.8 should be considered as equivocal; two FISH amplified cases and five FISH not amplified cases were in this range.

Table 1. HER2 assessment in SDC.

Case	IHC	MSK-IMPACT		FISH		
	Score	FC	TCN	Result	HER2/CEP17 ratio	HER2 signal/cell
01	3	7	31	Amplified	6.06	13.19
02	3	10.2	24	Amplified	12.17	25.6
03	3	4.7	NA	NA	NA	NA
04	3	NA	NA	Amplified	4.3	10.14
05	0	1.5	NA	Not amplified	1.4	NA
06	2	NA	NA	Not amplified	1.12	2.24
07	2	2.9	NA	Amplified	5.66	14.65
08	3	NA	NA	Amplified	4.23	9.9
09	2	NA	NA	Not amplified	0.99	2.78
10	2	1.5	5	Not amplified	1.2	2.9
11	2	1.73	NA	Not amplified	1.59	2.65
12	NA	1.4	4	Not amplified	1.3	2.5
13	2	NA	NA	Amplified	2.27	5.22
14	1	NA	NA	Not amplified	1.02	2.07
15	0	NA	NA	Not amplified	0.99	2.26
16	3	10.2	NA	Amplified	9.89	25.48
17	3	4	NA	NA	NA	NA
18	0	−1.01	1	Not amplified	0.99	2.26
19	0	−1.1	1	Not amplified	1.53	2.47
20	3	2.9	13	Amplified	9.77	19.55
21	1	−1	1	Not amplified	1.13	2.1
22	0	−1.1	1	Not amplified	1.12	2.6
23	0	−1.3	1	Not amplified	1.13	2.34
24	NA	2.6	7	Amplified	5.32	9.96
25	3	8.3	24	Amplified	6.4	12.33
26	2	1	2	Not amplified	1.57	3.71
27	0	−1	1	Not amplified	0.77	1.8
28	3	1.4	4	Amplified	2.4	6.1
29	0	1	2	NA	NA	NA
30	2	1.02	2	NA	NA	NA
31	2	−1	2	Not amplified	1.3	2.39
32	0	1	2	Not amplified	1.66	3.16
33	1	1.2	1	Not amplified	1.56	3.23
34	3	4.3	NA	Amplified	9.88	16.26
35	2	4	22	Amplified	4.38	9.73
36	0	−1.1	1	Not amplified	1.11	2
37	2	1.8	4	Amplified	2.99	6.44
38	2	3.1	11	Amplified	2.5	6.3
39	NA	10.2	18	Amplified	9.9	5
40	0	−1	2	Not amplified	1.21	3.06
41	0	−1.1	1	Not amplified	1.48	3.02
42	3	4.1	22	Amplified	5.13	12.12
43	NA	22.8	29	Amplified	9.7	18.2
44	3	4.6	26	Amplified	5.79	10.22
45	3	5.5	24	Amplified	6.1	14.9
46	1	−1.1	2	Not amplified	1.66	4.29
47	0	NA	NA	Not amplified	1.14	2.2
48	3	8.1	36	NA	NA	NA
49	2	1.5	6	Not amplified	1.59	3.6
50	3	14.2	43	Amplified	4.08	10.6

Table 1. continued

Case	IHC	MSK-IMPACT		FISH		
	Score	FC	TCN	Result	HER2/CEP17 ratio	HER2 signal/cell
51	2	−1.1	1	Not amplified	1.33	2.51
52	3	NA	NA	Amplified	3.76	7.67
53	2	1.3	5	Amplified	2.53	6.22
54	0	NA	NA	Not amplified	1.2	4.84
55	2	NA	NA	Not amplified	1.36	3.06
56	2	NA	NA	Not amplified	1.72	2.75
57	0	−1.6	1	Not amplified	0.58	1.24
58	3	NA	NA	Amplified	2.03	5.48
59	1	−1.1	1	NA	NA	NA
60	3	4.2	16	NA	NA	NA
61	1	−1.1	1	Not amplified	1.07	2.08
62	2	1.4	2	Not amplified	1.31	2.52
63	2	−1.1	2	NA	NA	NA
64	0	−1.2	1	NA	NA	NA
65	2	1.6	NA	NA	NA	NA
66	3	2	NA	NA	NA	NA
67	3	4.7	NA	NA	NA	NA

FC, fold change; TCN, total copy number; NA, not available.

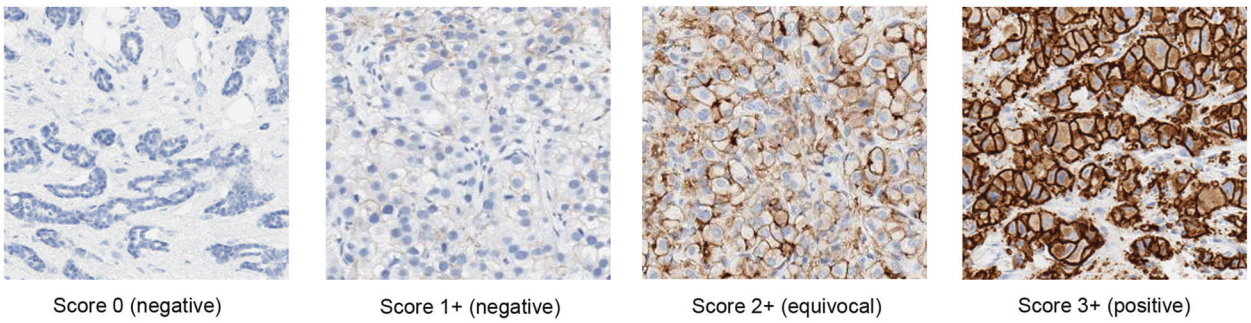

Fig. 1 Visual presentation of the 2018 ASCO/CAP developed scoring system for breast cancer pertaining to IHC in SDC. Score 0 (negative) shows no staining or incomplete/faint membrane staining in ≤10% of invasive tumor cells. Score 1+ (negative) shows incomplete/faint membrane staining in >10% of invasive tumor cells. Score 2+ (equivocal) shows weak/moderate complete membrane staining in >10% of invasive tumor cells. Score 3+ shows complete, intense membrane staining in >10% of invasive tumor cells. Abbreviations: IHC, immunohistochemistry.

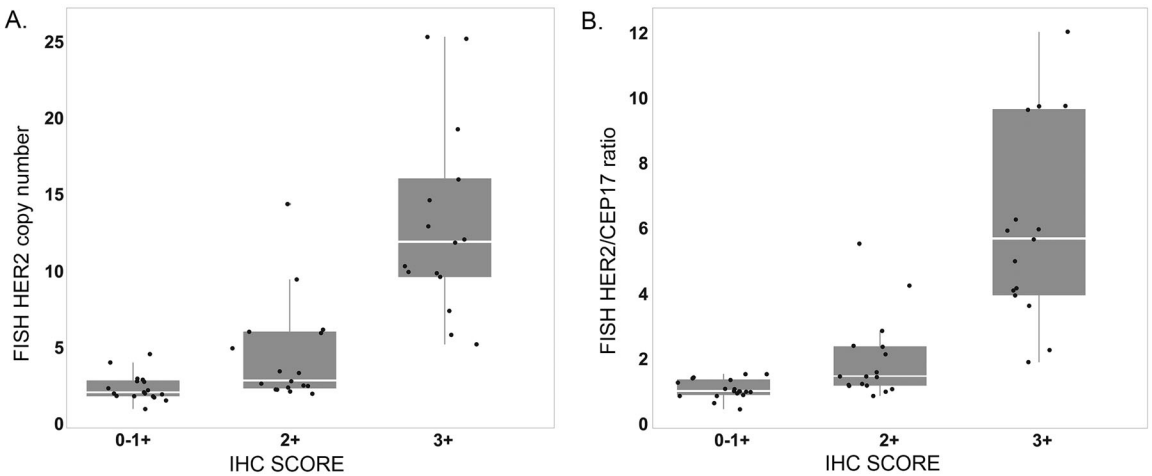

Fig. 2 Comparison of IHC and FISH. Box and whisker plot of IHC score status to FISH results comparing the HER2 copy number as estimated by FISH between IHC groups (**A**) and HER2/CEP17 ratio with IHC groups (**B**). Abbreviations: IHC, immunohistochemistry; FISH, fluorescence in situ hybridization.

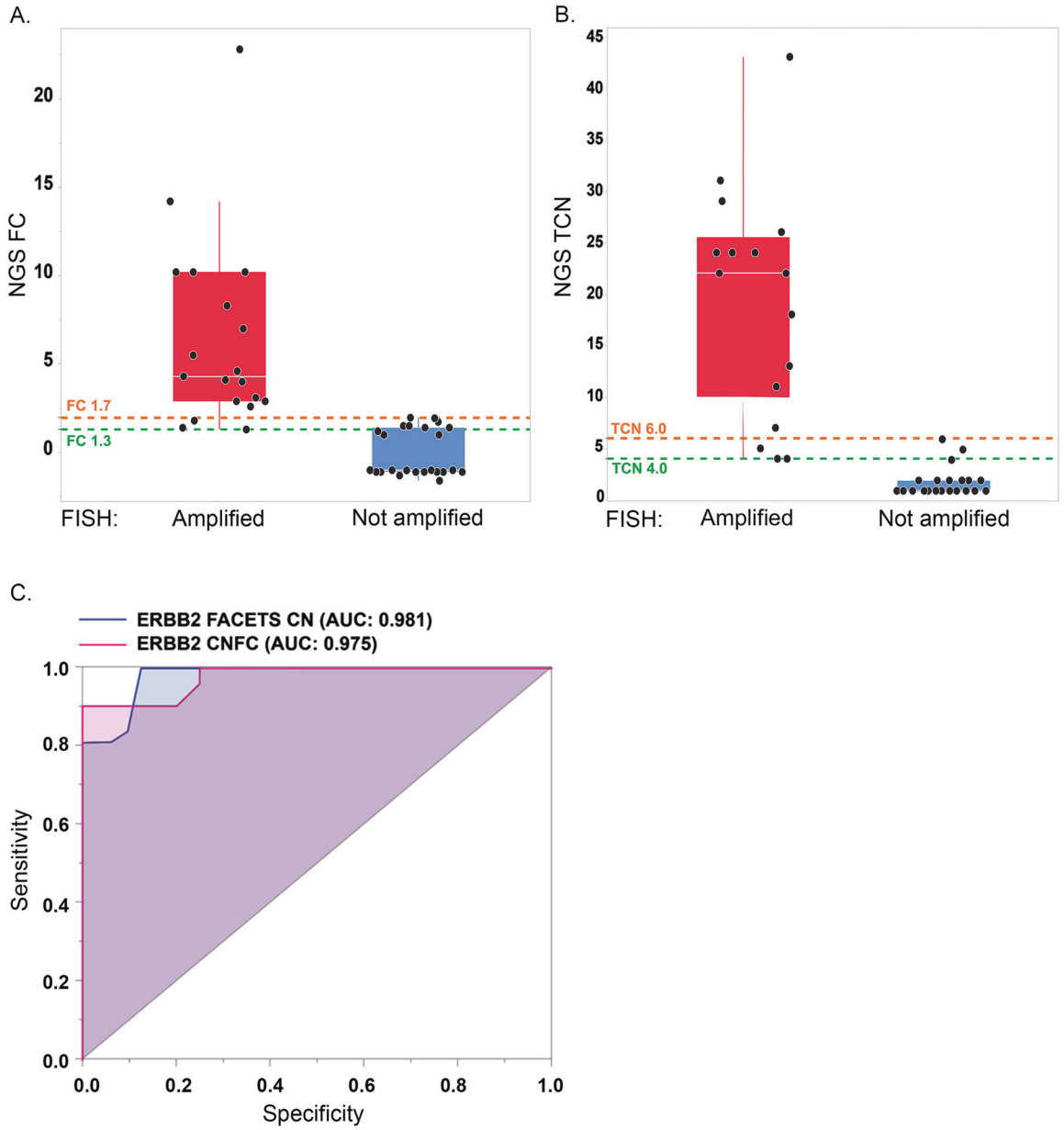

Fig. 3 Box and whisker plot of binarized FISH amplification status to NGS results. A comparison to FC values shows FC of 1.3–1.7 to be equivocal for amplification (**A**). A comparison to TCN values shows TCN of 4–6 are equivocal for amplification (**B**). A ROC curve estimation imputing FISH as the "gold standard" shows both NGS FC and total TCN are reliable predictors of FISH amplification status (**C**). Abbreviations: FISH, fluorescence in situ hybridization; NGS, next-generation sequencing; FC, fold change; TCN, total copy number; ROC, receiver operating characteristics.

TCN estimation showed AUC of 0.981 (95% CI, 0.907–0.996). The estimated TCN cutoff of >6.0 corresponded to an accuracy of 92% for *ERBB2* amplification status (Youden's index: 0.81, sensitivity: 81.2%, specificity: 100%). With the TCN cutoff of ≥4.0, the sensitivity would be 100%, i.e., any case with a TCN of <4.0 can be reliably classified as *ERBB2* not amplified. TCN of ≥4.0 and ≤6.0 should be considered equivocal; three FISH amplified and three FISH not amplified cases were in this range.

TCN and FC showed an excellent correlation (Spearman correlation: 0.94, R2: 0.712, *P*: <0.0001). TCN and FC performed similarly in the assessment of *ERBB2* amplification status; one FISH not amplified case was negative by TCN 2 but equivocal by FC 1.4; one FISH amplified case was positive by FC 1.8 and equivocal by TCN 4; all other cases were concordant. To compare the performance of NGS with FISH, the FC and TCN were binarized with cutoffs of ≥1.8 and >6.0, respectively. After binarization, an equivalence comparison of NGS to FISH was made; the proportion of agreement for FC and FISH was 0.95 (Nam score: −0.05, McNemar *P*: 0.15) and the proportion of agreement for TCN and FISH was 0.92 (Nam score: −0.08, McNemar *P*: 0.08), demonstrating that the accuracy of NGS assessment of *ERBB2* copy number status using either FC or TCN is nearly equivalent to FISH.

Comparison of NGS and IHC

Among 50 cases with both IHC and NGS results available, 18 cases (36%) were IHC negative (score 0 or 1+), 15 cases (30%) were IHC equivocal (score 2+) and 17 (34%) cases were IHC positive (score 3+). Out of 17 IHC positive cases, 16 (94.1%) were positive and

one (5.9%) was equivocal by FC (NGS). Out of 15 cases with IHC equivocal cases, 4 (26.7%) were amplified, 6 (40%) were not amplified, and 5 (33.3%) were equivocal by FC. Out of 5 FC equivocal cases with available FISH results, 1 case was *ERBB2* amplified. FC and TCN were significantly different between the IHC groups (Kruskal–Wallis, $p < 0.0001$ for both). The respective median FC and median TCN were −1.1 (IQR, −1.1 – −1.0) and 1 (IQR, 1–1) for negative IHC, 2.9 (IQR, 1.8–3.1) and 8 (IQR, 5–14) for equivocal IHC/FISH amplified cases, 1.4 (IQR, 0.0–1.5) and 2 (IQR, 1–3) for equivocal IHC/FISH not amplified cases, and 4.7 (IQR, 4.1–8.1) and 24 (IQR, 19–29) for positive IHC (Fig. 4).

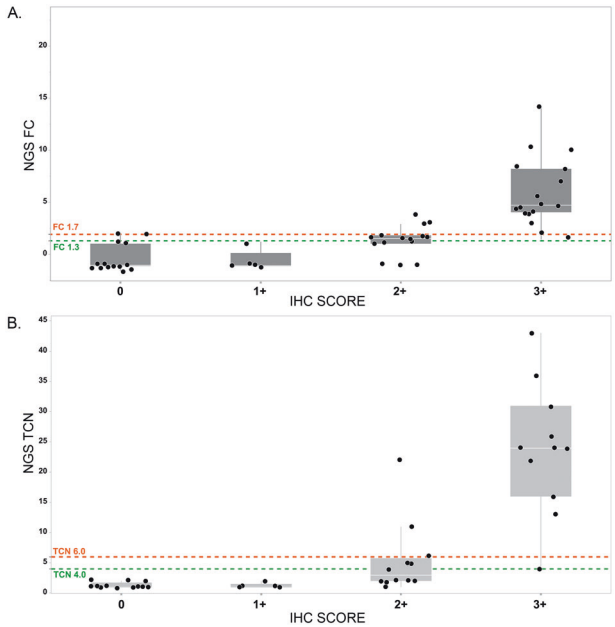

Fig. 4 Box and whisker plot of IHC score status to NGS results. A comparison to FC values shows FC of 1.3–1.7 to be equivocal for amplification (**A**). A comparison to TCN values shows TCN of 4–6 are equivocal for amplification (**B**). Abbreviations: NGS, next-generation sequencing; FC, fold change; TCN, total copy number.

Comparison of IHC, FC, and TCN and the proposed HER2 testing algorithm in SDC

Based on 33 cases tested by all four methods, the proportion of equivocal cases defined by IHC, FC, and TCN were 10 (30.3%), 5 (15.2%), and 5 (15.2%), respectively. FC and TCN performed similarly in predicting the HER2 status and either method was superior to IHC alone (Fig. 5). A summary of HER2 testing recommendations is depicted in Fig. 6. In brief, a second method would be needed for the equivocal categories including IHC 2+, FC ≥ 1.3 or <1.8, or TCN ≥ 4 or <7.

DISCUSSION

HER2 positivity is a well-established biomarker for a variety of cancer types due to the vastly improved overall survival rates with anti-HER2 targeted therapy. A significant subset of SDC cases are HER2 positive and preliminary data have demonstrated the efficacy of anti-HER2 targeted therapy, such as trastuzumab, in these patients[15–32]. Assembling a relatively large cohort of SDC, we evaluated four testing methods to identify HER2 positive cases, analyzed their concordance, and utilized those results to compile a consistent HER2 scoring system for SDC effective for IHC, FISH, and NGS.

The IHC and FISH results were interpreted according to the 2018 ASCO/CAP guidelines for HER2 testing in breast cancer and this conventional testing approach might be sufficient in most clinical settings. However, the evolvement of genomic medicine and the increasing clinical need for comprehensive molecular testing mandate the development of tissue-efficient alternatives to single-gene testing methods. NGS has proven to be useful in detecting the *ERBB2* amplification in other cancer types including breast, endometrial, gastroesophageal, and colorectal carcinomas[36,39,40], and this approach could also be applied in SDC.

Based on the ROC curve estimation analysis of our SDC data, we found that the optimal threshold for *ERBB2* amplification in SDC would be FC ≥ 1.8, which is lower than the standard cut off for gene amplification by MSK-IMPACT assay, FC ≥ 2[35]. In addition, among the cases in the equivocal range, FC 1.3–1.7, we identified two HER2 positive cases. This observation is consistent with the prior study on *ERBB2* amplification in breast cancer published by our group, where the optimal sensitivity (95%) and specificity

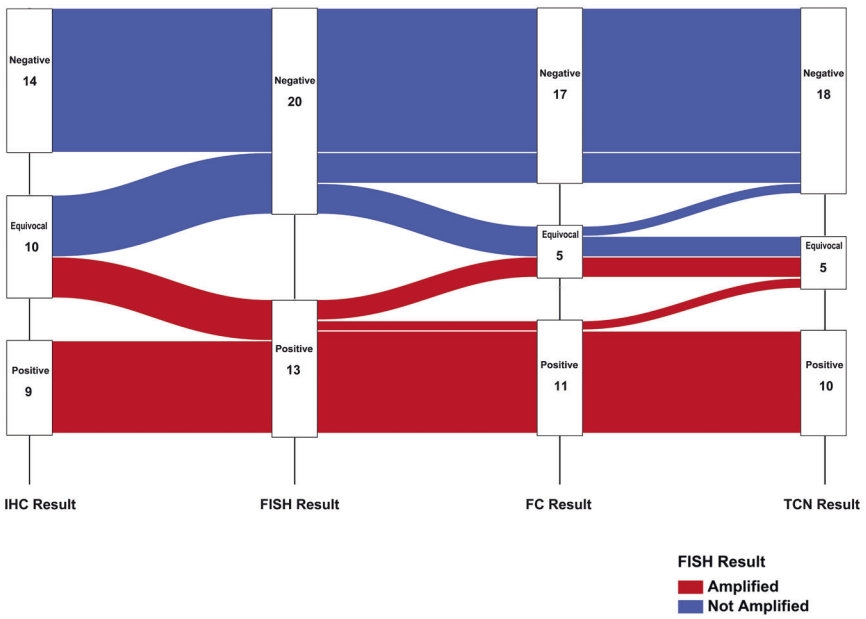

Fig. 5 Parallel coordinates plot showing the results of HER2 testing using IHC, FISH, and NGS assays in 33 cases where all assays were performed. Abbreviations: IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; NGS, next-generation sequencing.

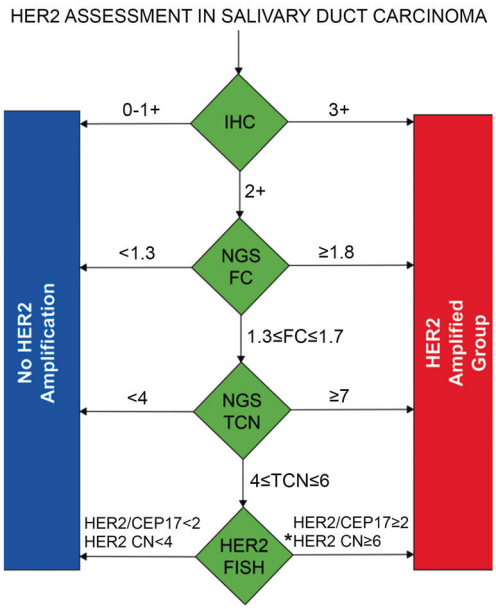

Fig. 6 A comprehensive scoring system for HER2 status in SDC utilizing IHC, FISH, and NGS. The order of reflex testing is interchangeable and can be based on the availability of HER2 testing assay. *Further studies are needed for validation. Abbreviations: SDC, salivary duct carcinoma; IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; NGS, next-generation sequencing; FC, fold change; TCN, total copy number; CN, copy number.

(100%) could also be achieved below FC 2 i.e., an amplified case could show FC ≥ 1.5 but <2. These were typically cases with relatively low tumor content in the tested sample or those showing intratumoral heterogeneity in HER2 expression[36].

Although 15.2% of cases would fall into the equivocal FC range and require alternate testing (i.e., FISH), a proportion of these cases is lower than equivocal cases tested by HER2 IHC supporting the superiority of NGS to IHC in identifying the truly HER2 positive and negative cases. Next, we utilized TCN data generated by FACETS to assess *ERBB2* amplification. Despite FACETS' current lack of widespread clinical use and undefined amplification thresholds, its inclusion in this study represented an opportunity to expand the methods available for identifying HER2 positive cases. In a prior study using MSK-IMPACT for evaluation of *ERBB2* amplification status (prior to use of FACETS) TCN 6 was suggested as the cut off for HER2 amplification[35]. However, in our data set this cut off was associated with a slightly lower accuracy rate (89%) and we found that TCN 7 can reliably detect HER2 positive and TCN < 4 identifies HER2 negative cases.

The scoring schemes utilizing IHC, FISH, and NGS were in complete concordance when analyzed. Although there were equivocal cases for IHC and NGS that were only definitive using FISH, there were no contradictions in returned results and most cases could be definitively scored using any of the three systems/four methods. The order of testing is interchangeable and equivocal results from any assay would require a further testing by an alternate method. In terms of NGS evaluation of *ERBB2* amplification status, the performance of FC and TCN is similar and either method can be used for this purpose.

This study has several strengths. First, given the rarity of SDC in the general population, the large cohort of samples included in this study provides a greater insight into the reliability of testing methods. Second, by comparing the two most commonly applied methods for determining HER2 status in SDC with two emerging NGS methods and finding them concordant, this study demonstrates that NGS can be an effective stand-alone method for this purpose. This is beneficial because NGS, in addition to providing the *ERBB2* amplifications status, also provides information on other therapeutic and prognostic biomarkers. Third, our study examines an investigational bioinformatics algorithm (FACETS) method for determining the *ERBB2* gene amplification and suggests amplification thresholds for future clinical use. This study also has several limitations. A limited number of cases were tested by all four testing modalities and larger number of cases and/or additional datasets would be needed for further validation of the suggested cut-off values. Furthermore, the outcome data relative to the *ERBB2* status for the cases utilized in this study are not available. In order to determine the effectiveness of the suggested scoring systems, the outcome of anti-HER2 treatments must be compared to the ranges identified as HER2 positive. Although this study does provide the framework to apply the existing methods in a consistent manner, it does not make a determination that the ranges within each scoring system are accurate predictors of drug response.

In summary, we examined the utility of four methods to detect HER2 positive SDC cases. We found that in addition to conventional IHC and FISH, NGS can be used as a single method for *ERBB2* amplification detection in the majority of cases. While these data may help identify SDC patients more likely to respond to anti-HER2 therapy, clinical studies focused on the response to targeted therapy in SDC patients are needed for the ultimate clinical validation of the proposed HER2 testing scoring schemes.

DATA AVAILABILITY

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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ACKNOWLEDGEMENTS

We gratefully acknowledge the members of the MSKCC Molecular Diagnostics Service of the Department of Pathology. This work has been supported by the Cancer Center Support Grant of the National Institutes of Health/National Cancer Institute under award number P30CA008748.

AUTHOR CONTRIBUTIONS

D.C.F., A.M.B., and S.D. performed study concept and design; D.C.F., A.M.B., A.L.H., and S.D. were responsible for acquisition of data; D.C.F., A.M.B., A.S.M., T.Z., D.S.R., and S.D. interpreted and analyzed the data; A.M.B. performed statistical analyses; T.Z. provided technical support; D.C.F., A.M.B., and S.D. wrote the manuscript; D.C.F., A.M.B., A.S.M., A.L.H., M.E.A., D.S.R., and S.D. performed review and revision of the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS

No competing financial interests exist for all contributory authors. Research reported in this publication was supported by the Memorial Sloan Kettering Cancer Center Support Grant of the National Institutes of Health/National Cancer Institute under award number P30CA008748. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41379-021-00999-0.

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