The effect of an alternative chromosome 17 probe on fluorescence *in situ* hybridization for the assessment of HER2 amplification in invasive breast cancer

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Abstract. Fluorescent *in situ* hybridization (FISH) is commonly used to determine the ratio of human epidermal growth factor receptor 2 (HER2) to centromere enumeration probe for chromosome 17 (CEP17), which further determines HER2 gene status in breast cancer. However, due to copy number alteration in CEP17, inaccurate diagnoses can occur. The current study was performed to investigate the diagnostic value of an alternative CEP17 reference probe for HER2 status in invasive breast cancer. A higher-order repeat in the centromeric region of chromosome 17 was identified and an alternative probe (SCEP17) was subsequently prepared. Karyotype analysis of peripheral blood was used to detect SCEP17 probe specificity. Using a HER2/CEP17 probe, karyotype analysis revealed two strong green signals at the centromere of chromosome 17 and one weaker signal at the other centromere. However, two strong hybridization signals at the centromere of chromosome 17 were observed when the HER2/SCEP17 probe was used. In the 425 patients with invasive breast cancer, no statistical difference was observed between HER2/SCEP17 and HER2/CEP17 when detecting HER2 gene amplification (P=0.157). However, in terms of copy number, the SCEP17 probe exhibited a reduced number compared with the conventional CEP17 probe (P<0.001). In conclusion, the HER2/SCEP17 probe may lead to increased accuracy HER2 status assessment in invasive breast cancer. However, a further large-scale and prospective clinical trial is required for confirmation of the potential benefits of using the HER2/SCEP17 probe.

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Introduction

Human epidermal growth factor receptor 2 (HER2) is located on chromosome 17 q12-21.32 and has been identified to be an oncogene in breast cancer. HER2 is involved in the modulation of cancer cell proliferation, invasion, metastasis and apoptosis via the PI3K/AKT and RAS/mitogen activated protein kinase pathways (1). In total, ~15-20% of invasive breast cancers exhibit chromosomal HER2 amplification and protein overexpression (2-4). Previous research has demonstrated that HER2-positive breast cancer is associated with more aggressive clinical progression and a poor prognosis (5,6). HER2 status also predicts sensitivity to anthracycline-based chemotherapy regimens as well as resistance to cyclophosphamide-based regimens and tamoxifen-based therapies in estrogen receptor-positive breast cancer (7-9). Furthermore, HER2 is an effective therapeutic target for the humanized monoclonal antibody, trastuzumab and anti-HER2 monoclonal antibodies, pertuzumab and ertumaxomab (10,11). The accurate evaluation of HER2 status is therefore critical for the effective clinical management of breast cancer. Evaluations should be performed using standardized testing techniques to accurately assess HER2 status.

HER2 status is traditionally evaluated using immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) (12). IHC detects the expression of HER2 proteins, whereas FISH detects HER2 gene amplification. FISH is less affected by pre-analytic and analytic variables and is therefore considered to be a more reliable, sensitive and accurate testing procedure (12,13). The most commonly used FISH assay is the US Food and Drug Administration approved dual-probe assay of HER2 and the centromere enumeration probe for chromosome 17 (CEP17), which presents a HER2/CEP17 ratio (12). The HER2/CEP17 ratio has long been regarded as a better

reflection of the HER2 gene status than the mean HER2 copy number because it compensates for the loss of signals by tissue sectioning and adjusts for the natural increase in the number of chromosomes during replication (14,15).

Chromosome 17 has complex structural and numerical aberrations and is one of the smallest human chromosomes (16-18). CEP17 probes are conventionally prepared using a chromosome 17 centromere sequence (the P17H8 sequence of alpha satellite DNA) (19). This sequence is composed of short interspersed nuclear element (SINE) and long interspersed nuclear element (LINE) higher-order repeat structures, which are also present on various chromosomes in the human genome, including chromosome 11 and X (20,21). A CEP17 probe may hybridize with other chromosomal centromeric repeat sequences, producing a variety of detrimental signals that interfere with the evaluation of HER2 gene amplification and aneuploidy of chromosome 17.

In the current study, a short higher-order repetitive sequence was identified in the centromere of chromosome 17. This was identified using comparative analysis of the (GenBank) database and by the preparation of a highly specific short CEP17 probe (SCEP17) using PCR. Karyotype analysis of peripheral blood was also performed to evaluate HER2/SCEP17 specificity. Furthermore, the impact of SCEP17 on HER2 gene amplification was assessed using FISH in 425 patients with invasive breast cancer.

Materials and methods

Preparation of the HER2 probe. Two bacterial artificial chromosome (BAC) clones RP11-1044P23 and RP11-62N23 were used in the current study (each, Invitrogen; Thermo Fisher Scientific, Inc.). DH10B competent cells (gifted from Huazhong University of Science and Technology, Wuhan, China) were removed from an -80˚C freezer and placed on ice to thaw. Electroporation cuvettes (0.2 cm, Bio-Rad Laboratories, Inc.) were chilled on ice. The BAC clones RP11-1044P23 and RP11-62N23, were added to DH10B competent cells prior to the complete thaw of cells. After electroporphoration in electroporation cuvettes, 1 ml luria-bertani (LB) liquid medium (1% bacto trypton, 0.5% yeast extract, 0.5% NaCl) was added and cells were resuspended and transferred to a centrifuge tube. Cells were shaken for 1 h at 37˚C, at 220 rpm. After centrifugation for 5 min at 4,500 x g and 4˚C, 200 μl supernatant was spread onto selective LB/agar plates which contained 30 μg/ml chloramphenicol, tetracycline or kanamycin. Media were purchased from BD Biosciences and chemicals from Sigma-Aldrich. Clones were subsequently cultured at 37˚C overnight. The DNA of BAC clones was extracted using the E.Z.N.A. Endo-Free Plasmid Maxi kit (Omega Bio-Tek, Inc.) according to the manufacturer's protocol. BAC clones were then labeled using Biotin-nick translation reactions with Cy3-dUTP dye according to the manufacturer's protocol (cat. no. 18160-010; Invitrogen; Thermo Fisher Scientific, Inc.).

Preparation of CEP17 probe. The CEP17 probe was prepared using the P17H8 sequence of alpha satellite DNA on chromosome 17, and the sequence from 5'-3' was presented in Fig. 1.

This sequence was synthesized to a pUc-CEP17 plasmid purchased from Biocin Healthcare. The DNA sequence was labeled using the following primers (forward, 5'-GGAATC TGCAAGTGGATATG-3' and reverse, 5'-CAGAACTACTCT ATGAAAAGC-3'). The PCR was performed in a volume of 20 μl, including 2 μl cDNA template, 10 μl Taq PCR mix (Takara Biotechnology Co., Ltd.), 1 μl forward and reverse primers (10 μm) and 6 μl nuclease-free water. The PCR conditions were as follows: 5 min at 94˚C, followed by 30 cycles of 10 sec at 94˚C, 10 sec at 52˚C or 55˚C and 2 min at 72˚C with a final 5 min extension step at 72˚C. PCR products were labeled using a PCR Labeling kit (Invitrogen; Thermo Fisher Scientific, Inc.) with FITC dye. The HER2/CEP17 probe set was a combination of HER2 probe and CEP17 probe.

Preparation of SCEP17 probe. To develop an alternative chromosome 17 probe, a higher-order repetitive sequence on the centromeric region of chromosome 17 was identified by performing a comparative analysis of the NCBI (Genbank) database (https://www.ncbi.nlm.nih.gov/genbank/). The sequence is as follows: 5'-AAGCATTCTCAGAAACTTCTC TGTGATGTTTGTGTTCAACTCCCAGAGTTTCACATT GCTTTTCATAGAGTAGTTCTGAAACATGCTTTTCGT AGTGTCTACAAGTGGACATTTGGAGCGCTTTCAGGC CTGTGGTGGAAAACGAATTATGGTCACATAAAAACT GGAG-3'. This sequence exhibited a higher specificity due to its high number of repeats in the centromeric region of chromosome 17 and appropriately short length. This DNA sequence was subsequently labeled with primers (forward, 5'-AAGCAT TCTCAGAAACTTCTCTG-3' and reverse, 5'-CTCCAGTTT TTATGTGACCATAA-3'). The preparation process of new probe was the same as CEP17 probe, and was named SCEP17. The HER2/SCEP17 probe set was a combination of HER2 probe and SCEP17 probe.

Karyotype and FISH analyses. Metaphase and interphase chromosomes were obtained from phytohaemagglutinin stimulated lymphocyte cultures from normal peripheral blood (donated by two healthy volunteers) with standard methods in accordance with previous study and International System for Human Cytogenetic Nomenclature guidelines (22,23). The chromosome localization of HER2/CEP17 and HER2/SCEP17 was investigated using FISH analysis on the metaphase chromosome spreads according to standard protocol (24).

Case selection. The current study was performed according to research protocols approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (Wuhan, China). A cohort of 425 patients diagnosed with invasive breast carcinoma at the Zhongnan Hospital of Wuhan University (Wuhan, China) between September 2012 and September 2015 were included in the current retrospective study. No patients included in the present study received any chemotherapy, radiotherapy or immunotherapy at the time of breast tissue specimen collection. All tissue specimens were fixed with 10% formalin for 12 h at room temperature and embedded in paraffin. Patient data is presented in Table I.

IHC analysis for HER2 status. Sections of 4 μm thickness were cut and placed on acid pretreated poly-L-lysine-coated slides to incubate overnight at 56˚C. IHC analysis was performed using anti-HER2 antibodies (1:1,000; cat. no. ab16901; Abcam)

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1       TGAACATTCC TATTGATAGA GCAGTTTGGA AACACTCTTG TTGTGGAATG TGCAAGTGGA GATTTGGAGC GCTTTGAGGC
81      CTATGGTAGT AAAGGGAATA GCTTCATAGA AAAACTAGAC AGAAGCATTC TCAGAAAATA CTTTGTGATG ATTGAGTTTA
161     ACTCACAGAG CTGAACATTC CTTTGGATGG AGCAGGTTTG AGACACTCTT TTTGTACAAT CTACAAGTGG ATATTTGGAC
241     CTCTCTGAGG ATTTCGTTGG AAACGGGATA ACTGCACCTA ACTAAACGGA AGCATTCTCA GAAACTTCTT GGTGATGTTT
321     GCATTCAAAT CCCAGAGTTG AACCTTCCTT TGATAGTTCA GGTTTGAAAC ACTCTTTTTG TAGGATCTGC AAGTGGATAT
401     TTGGACCACT CTGTGGCCTT CGTTCGAAAC GGGTATATCT TCGCATAAAA TCTAGACAGA AGCCTTCTCA GAAACTTCTC
481     TGTGATGATT GCATTCAACT CACAGAGTTG AACCCTCCTA TGGATAGAGC AGTGTTGAAA CTCTCTTTTT GTGGAATCTG
561     CAAGTGGATA TGTGGACCTC TCCGAAGATG TCTTTGGAAA CGGGAATATC TTCACATAAA AACTAAACAG AAGCATTCTC
641     AGAAACTTCT CTGTGATGTT TGTGTTCAAC TCCCAGAGTT TCACATTGCT TTTCATAGAG TAGTTCTGAA ACATGCTTTT
721     CGTAGTGTCT ACAAGTGGAC ATTTGGAGCG CTTTCAGGCC TGTGGTGGAA AACGAATTAT GGTCACATAA AAACTGGAG
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Figure 1. The DNA sequence of CEP17. CEP17, the centromere enumeration probe for chromosome 17.

with a Elivision super horseradish peroxidase (Mouse/Rabbit) IHC kit (cat. no. KIT-9921, MXB Biotechnologies) and DAB Detection kit (DAB-2031; MXB Biotechnologies) after deparaffinization via two changes of xylene, rehydration in graded ethanol and antigen retrieval in EDTA retrieval buffer 100˚C for 2 min according to the manufacturer's protocol. All slides were counterstained with hematoxylin for 2 min at room temperature. Slides were measured at x200 magnification and scored from 0 to 3+ according to the 2013 American Society of Clinical Oncology/College of American Pathologists guidelines by at least two pathologists in a blinded fashion. A score of 0 or 1+ (no staining or weak, incomplete membrane staining in any proportion of tumor cells) was considered negative, 2+ (complete membrane staining that is non-uniform or weak but circumferential in distribution in ≥10%) was considered equivocal and 3+ (uniform intense membrane staining of >10% tumor cells) was considered positive (12).

FISH assay. Tissue sections (4 μm) were mounted on positively charged slides, heated overnight at 56˚C, deparaffinized in xylene, dehydrated in a series of ethanol washes 50, 70, 85 and 100% and air-dried. The slides were placed in 0.2 N HCl (pH 0.24) for 20 min, washed in a 2x saline-sodium citrate (SSC) buffer (Jinpujia Biotechnologies) (pH 7.0) and incubated with 1 N NaSCN solution for 30 min at 80˚C. Subsequently, a protease digestion (100 μg/ml; BBI Life Sciences) was performed at 37˚C for 30 min. The probe mixture was applied to the target tissue and the cover slips were sealed with rubber cement. Denaturation for 5 min at 72˚C following hybridization for 16 h at 42˚C was performed in a hybridizer (CIMO Corporation). After hybridization, the slides were washed in 2xSSC/0.3% NP-40 (Jinpujia Biotechnologies) at 72˚C for 2 min, air-dried prior to counterstaining with 4,6-diamidino-2-phenylindole fluoromount (Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature and covered with a glass coverslip.

FISH results interpretation. FISH samples were analyzed with appropriate filters under a 100x oil immersion objective using an Olympus BX-51 fluorescence microscope (Olympus Corporation, Japan). HER2 and CEP17 signals were assessed by two independent technologists examining 20 non-overlapping nuclei for each tissue. The mean number of HER2 signals, CEP17 signals and the HER2/CEP17 ratio was calculated. HER2 amplification status was classified according to the 2013 ASCO/CAP guidelines (12). The results were graded as: Negative amplification (HER2/CEP17 ratio of <2); Equivocal amplification (HER2/CEP17 ratio <2 with an average HER2 copy number >4 and <6/cell); and positive amplification (HER2/ CEP17 ratio of ≥2 with HER2 ≥4/cell). Diploid CEP17 was defined as an average CEP17 copy number of 1.5-2.6, polysomy CEP17 was defined as a CEP17 copy number >2.6 and monosomy was defined as a CEP17 number <1.5 (25).

Statistical analysis. Statistical analyses were performed with SPSS Statistics version 22 (IBM Corp.). HER2 and CEP17 copy numbers were presented as the mean ± standard deviation. A Wilcoxon signed ranks test was used to compare the difference between two variables. McNemar and McNemar-Bowker tests were used to compare categorical variables. All reported P-values were two-tailed and $P<0.05$ was considered to indicate a statistically significant result. The concordance between different assays was investigated using the kappa (κ) test. Agreement was considered poor when κ coefficient <0.2, fair 0.21-0.40, moderate 0.41-0.60, substantial 0.61-0.80 and almost perfect 0.81-1.00 (26).

Results

Probe specificity in normal human lymphocytes. Karyotype and FISH analyses on lymphocyte metaphase and interphase nuclei from two normal samples confirmed inherent probe hybridization efficiency and specificity. The standard CEP17 probe revealed two bright green spots located at the centromere of chromosome 17 and one weaker signal were identified on the other chromosome centromere (Fig. 2A). Two bright hybridization signals were located at the centromere of chromosome 17, but no marked miscellaneous HER2/SCEP17 signals were observed on the other chromosomes (Fig. 2B). Therefore, karyotype analysis of chromosomal localization verified the hypothesis that SCEP17 exhibits strong specificity.

Patient characteristics. A total of 425 patients with invasive breast cancer were analyzed for HER2 status using IHC and

Table I. Clinical and molecular characteristics of patient tumors.

		Conventional FISH probe (HER2/CEP17)			IHC (HER2)		
	Number	Non-amplified	Equivocal	Amplified	0~1+	2+	3+
Sex							
Male	1	0	0	1	0	1	0
Female	424	300	9	115	253	88	83
Age							
<50	165	119	4	42	100	34	31
≥50	260	181	5	74	153	55	52
Stage							
I~II	360	253	9	98	212	80	68
III~IV	65	47	0	18	41	9	15
ER							
Positive	265	201	4	60	178	51	36
Negative	142	83	3	56	70	25	47
PR							
Positive	211	161	5	45	141	42	28
Negative	196	123	2	71	107	34	55
Ki67							
Positive	304	206	5	93	180	53	71
Negative	81	61	1	19	52	21	8
P120							
Positive	105	77	0	28	74	13	18
Negative	7	3	0	4	4	0	3
E-cadherin							
Positive	82	59	0	23	54	13	15
Negative	27	19	0	8	22	0	5
LNM							
Positive	52	38	1	13	24	18	10
Negative	54	42	0	12	39	6	9

ER/PR ≥10% defined as positive; ER/PR <10% defined as negative; Ki67 ≥14% defined as positive; Ki67 <10% defined as negative; E-cadherin/P120 ≥25% defined as positive; E-cadherin/P120 <25% defined as negative. HER2, human epidermal growth factor receptor 2; CEP17, the centromere enumeration probe for chromosome 17; ER, estrogen receptor; PR, progesterone receptor; LNM, lymph node metastasis; FISH, fluorescence *in situ* hybridization; IHC, immunohistochemistry.

two FISH methods. Table I summarizes the characteristics of patients included in the current study. The age at diagnosis ranged from 23-94 years, with a median age of 53.6. Among them, one case was male. He suffered from invasive ductal carcinoma (Fig. 3A), with ER 3+ (Fig. 3B), PR- (Fig. 3C), Ki67 20%+ (Fig. 3D) and HER2 2+ (according to IHC; Fig. 3E), which was amplified by FISH (Fig. 3F).

HER2 protein expression by IHC and gene amplification by FISH. The association between FISH and IHC results are presented in Table II. Of the 425 patient specimens, 253 had an IHC score of 0/1+, 89 were 2+ and 83 were 3+. A total of 80 patients with IHC 3+ were also FISH amplified, while 3 patients were FISH equivocal. The concordance rate was 96.39%. Of the patients with an IHC score of 0/1+, 237 were FISH non-amplified, 1 was FISH equivocal and 15 were FISH amplified, with a 93.68% concordance rate. While in the group of IHC 2+, 63 were FISH non-amplified, 5 were FISH equivocal and 21 were FISH amplified.

Assessment of the distribution of invasive breast cancer specimens by HER2/CEP17 and HER2/SCEP17 probes of FISH assays. Table III presents the comparisons of HER2 and CEP17 copy numbers between HER2/CEP17 and HER2/SCEP17 probes. The mean copy number of HER2 and CEP17 detected by the two probes were 4.055±3.904 vs. 4.032±3.665 and 2.243±0.728 vs. 1.959±0.332, respectively. A significant difference was observed between the two probes in the detection of CEP17 copy numbers ($P<0.001$).

The results of HER2 amplification using HER2/CEP17 and HER2/SCEP17 probes are presented in Table IV. By using the HER2/CEP17 probe, it was determined that there were

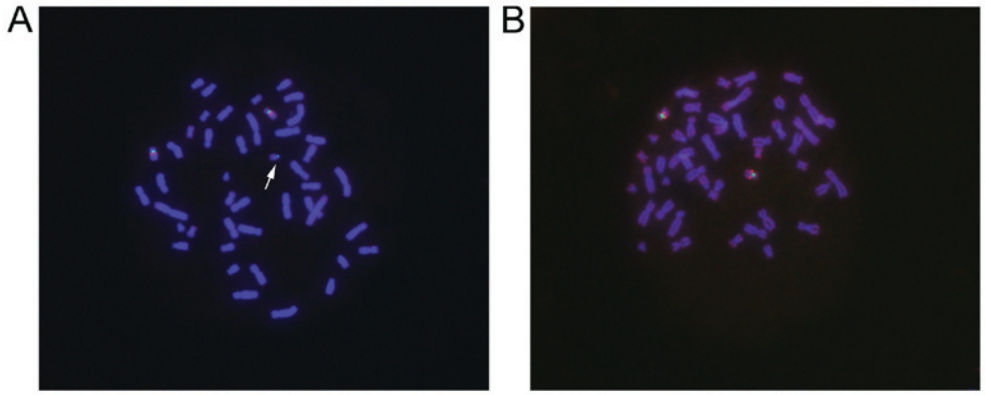


Figure 2. Karyotype analysis of the (A) conventional HER2/centromere enumeration probe for the chromosome 17 probe and (B) HER2/short centromere enumeration probe for chromosome 17 probe. The arrow indicated that there was one weaker green signal on another chromosome (outside 17 chromosome). HER2, human epidermal growth factor receptor 2.

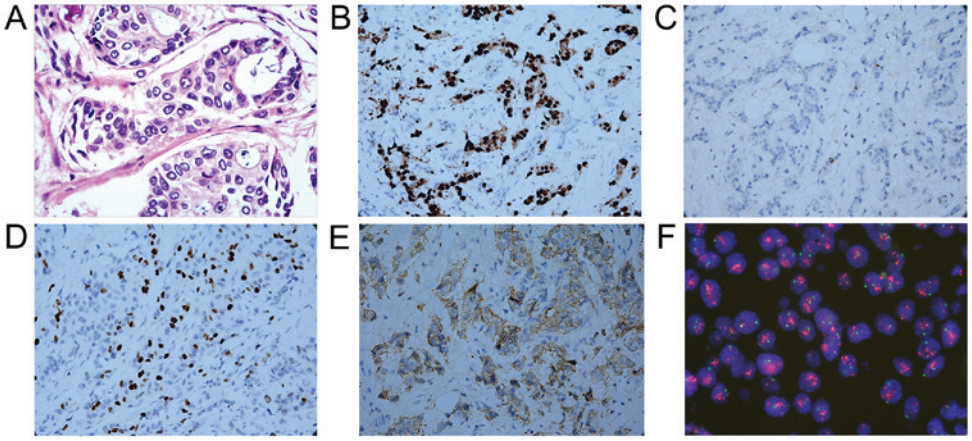


Figure 3. Molecular images of a male patient. (A) hematoxylin and eosin staining, (B) estrogen receptor 3+, (C) progesterone receptor-, (D) Ki67 20%+ and (E) HER2 2+ results were determined by immunohistochemistry. (F) HER2 was amplified by fluorescent *in situ* hybridization. HER2, human epidermal growth factor receptor 2.

Table II. Results of IHC and standard FISH in 425 patients with invasive breast cancer.

	IHC			
FISH (HER2/CEP17)	0-1+	2+	3+	Total
Non-amplified	237	63	0	300
Equivocal	1	5	3	9
Amplified	15	21	80	116
Total	253	89	83	425

IHC, immunohistochemistry; FISH, fluorescence *in situ* hybridization; HER2, human epidermal growth factor receptor 2; CEP17, the centromere enumeration probe for chromosome 17.

116 amplified, 9 equivocal and 300 non-amplified specimens. The HER2/SCEP17 probe revealed HER2 amplification in 118 cases, equivocal in 7 cases and non-amplified in 300 cases. No significant difference was observed between the two probes (McNemar-Bowker test; P=0.157). The agreement between HER2/SCEP17 probe and the HER2/CEP17 probe was 99.5%, with a κ coefficient of 0.989. However, the HER2/SCEP17 probe reclassified two cases from equivocal to amplified. One case was ER -, PR 1+, Ki67 40%+, HER2 2+ in molecular characteristics and the other was ER 3+, PR 2+, Ki67 25%+, HER2 2+. Representative micrographs of one case is presented in Fig. 4. The number of green spots of conventional HER2/CEP17 probe was more than that of HER2/SCEP 17.

The CEP17 probe detected CEP17 polysomy in 100 cases, diploidy in 317 cases and monosomy in 8 cases. The SCEP17 probe detected CEP17 polysomy in 45 cases, diploid in 332 cases and monosomy in 48 cases. A fair agreement existed between CEP17 and SCEP17 probes: 74.8% (318/425); κ coefficient, 0.355 (Table V). A significant difference was revealed between the two probes when analysed using a McNemar statistical test (P<0.001).

Discussion

IHC is used in numerous laboratories for primary HER2 testing, whereas FISH is used for tumor specimens with an IHC score of 2+ (27). In the current study, HER2 status was evaluated for protein expression and gene amplification using IHC and

Table III. Comparison of HER2 and CEP17 copy numbers between HER2/CEP17 and HER2/SCEP17 probes.

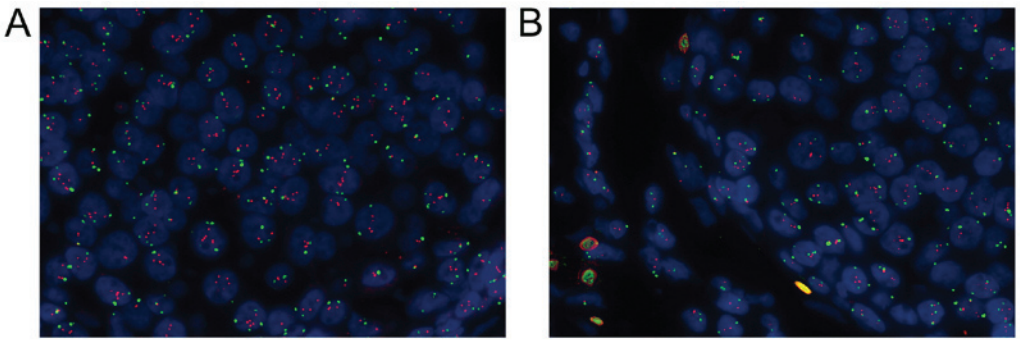
Variables	HER2/CEP17 (M±SD)	HER2/SCEP17 (M±SD)	P-value
HER2 copy number	4.055±3.904	4.032±3.665	0.632
CEP17 copy number	2.243±0.728	1.959±0.332	<0.001

A Wilcoxon signed ranks test was used to calculate P-values. HER2, human epidermal growth factor receptor 2; CEP17, the centromere enumeration probe for chromosome 17; SCEP17, short centromere enumeration probe for chromosome 17; M±SD, mean ± standard deviation.

Table IV. HER2 status according to FISH analyses in 425 invasive breast cancer with HER2/CEP17 and HER2/SCEP17 probes.

	HER2/CEP17			
HER2/SCEP17	Non-amplified	Equivocal	Amplified	Total
Non-amplified	300	0	0	300
Equivocal	0	7	0	7
Amplified	0	2	116	118
Total	300	9	116	425

Overall agreement, 99.5%; κ coefficient, 0.989; McNemar-Bowker test, P=0.157. HER2, human epidermal growth factor receptor 2; CEP17, the centromere enumeration probe for chromosome 17; SCEP17, the short centromere enumeration probe for chromosome 17.



sure 4. Representative micrographs presenting one case reclassified from equivocal to amplified, indicating the (A) conventional HER2 centromere enumeration probe for chromosome 17 probe and (B) the HER2/short centromere enumeration probe for chromosome 17 probe. HER2, human epidermal growth factor receptor 2.

FISH, respectively. The results indicated that patients with an IHC score of 0/1+ were considered as FISH non-amplified and those with an IHC score of 3+ were considered as amplified. However, an IHC score of 2+ was not equivalent to being FISH equivocal and should therefore be re-tested by FISH, as a high proportion of patients with this score may exhibit discordant results. As a methodological comparison assay, the results in the present study are supported by the results obtained from previous studies (28,29).

The most frequently used FISH test for assessment of HER2 gene status in breast cancer is a dual-probe assay, one probe is used for the HER2 gene and the other is used for chromosome 17 (CEP17). The results are reported as a ratio of HER2 signals to CEP17 signals (12). The CEP17 probe hybridizes to a site near the centromere of chromosome 17. The use of this reference probe is intended to compensate the loss of signals by tissue sectioning and to adjust the natural increase in the number of chromosomes during replication (14,15). The CEP17 probe is prepared using the P17H8 sequence of alpha satellite DNA, which is composed of higher-order repeat structures of SINE and LINE, which are also present at various human chromosomes, including chromosome 11 and X (20,21). These phenomena indicate that the CEP17 probe may hybridize with centromeric repetitive sequences of other chromosomes and result in signals that may skew the HER2/CEP17 ratio and therefore affect the assessment of HER2 status. The possibility of this and the impact on the clinical assessment of HER2 status has not, to the best of our knowledge, been assessed previously. The current study identified a higher-order repetitive sequence on the centromeric region of chromosome 17 by comparative analysis of the NCBI (Genbank) database and prepared an alternative CEP17 probe based on this fragment, which was named SCEP17. Karyotype analysis revealed that the SCEP17 probe had increased specificity.

Table V. CEP17 signals according to FISH analysis in 425 patients with invasive breast cancer using HER2/CEP17 and HER2/SCEP17 probes.

	HER2/CEP17			
HER2/SCEP17	<1.5	1.5~2.6	>2.6	Total
<1.5	5	41	2	48
1.5~2.6	3	272	57	332
>2.6	0	4	41	45
Total	8	317	100	425

Overall agreement, 74.8%; κ coefficient, 0.355; McNemar-Bowker test, P<0.001. HER2, human epidermal growth factor receptor 2; SCEP17, the short centromere enumeration probe for chromosome 17; CEP17, the centromere enumeration probe for chromosome 17.

In the 425 cases of invasive breast cancer, 2 patients were reclassified from equivocal to amplified when using the HER2/SCEP17 probe. No statistical difference was exhibited in the detection of HER2 status between the two probes (P=0.157), with a concordance rate of 99.5% and a κ coefficient of 0.989. However, in terms of the average CEP17 signal copy number, the SCEP17 probe exhibited a reduced copy number when compared with the conventional CEP17 probe. A significant difference was also exhibited between the two probes when assessing the ploidy of chromosome 17 (P<0.001). The results of the present study demonstrated that the conventional CEP17 probe could lead to a discordant interpretation of HER2 amplification in invasive breast cancer, but the SCEP17 probe exhibited increased specificity. Use of the SCEP17 probe may therefore lead to a more accurate diagnoses and may guide the appropriate clinical treatment of patients with invasive breast cancer.

An increased number of CEP17 signals are observed in breast cancer and the term 'polysomy 17' is widely used in literature (30). However, pangenomic studies which use comparative genomic hybridization and multiplex ligation-dependent probe amplification demonstrate that true polysomy 17 in breast cancer is rare (31-33). The current ASCO/CAP guidelines for breast cancer (12) and gastroesophageal adenocarcinoma (34) recommend an alternative chromosome 17 probe to reassess the status of patients with equivocal FISH results. Recently, laboratories have focused on an additional set of probes for genes from chromosome 17 including those for the retinoic acid receptor alpha (RARA), Smith-Magenis syndrome (SMS) and the tumor protein P53 (TP53) (35-37). Tse *et al* (35) demonstrated that, by using alternative chromosome 17 reference gene probes including SMS, RARA and TP53, 43.9% of non-amplified breast cancers with CEP17 signals ≥2.6 were scored as amplified and 92.9% of equivocal cases were reclassified as amplified. Similar results were observed by Jiang *et al* in 2015 (36) and Sneige *et al* in 2017 (37). In addition, a study with an alternative chromosome 17 probe D17S122 (located on the short arm of chromosome 17), reclassified ~50% of equivocal cases as amplified (38). However the routine application of alternative reference genes in clinical practice is limited due to frequent heterozygous deletions and additional costs (36,37). The current study utilized alternative reference probes and a conventional CEP17 probe sequence, which was composed of higher-order repeat structures of SINE and LINE. The studies by Tse *et al* (35), Jang *et al* (36), Sneige *et al* (31) and Donaldson *et al* (38) all contained the premise that copy number alteration in α-satellite DNA at the centromeric hybridization site for CEP17 could affect the assessment of HER2 status. The new probe used in the current study was located on the centromere of chromosome 17. However, the other alternative combined or single control probes were located on the short or long arm of chromosome 17.

The sample size used in the current study was relatively small. Resources were additionally focused on studying patients with invasive breast cancer. Other breast cancer subtypes should be included to improve research in future study. The two reclassified patients did not receive HER2-targeted therapy due to the retrospective nature of the current study. Furthermore, the potential benefits of HER2-targeted therapy, assisted by the use of SCEP17 probe are yet to be clinically established.

In summary, the results of the current study indicated that the alternative chromosome 17 probe of SCEP17 exhibited a higher specificity than the CEP17 probe. These results imply a more accurate assessment of HER2 status in invasive breast cancer. However, a multicenter large-scale and prospective clinical trial is required to confirm the clinical benefits of this promising diagnostic tool.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZX, MY and WF designed the study. ZX, WF, PX, BH and QC performed most of the experiments. PX, PW and ZZ analyzed the data. PX and BH wrote the draft of the paper. MY and ZX reviewed and revised the manuscript. All the authors approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Zhongnan Hospital of Wuhan University (Wuhan, China). Because of the

retrospective nature of the study, patient written consent was exempted.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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