The frequency and clinical significance of centromere enumeration probe 17 alterations in human epidermal growth factor receptor 2 immunohistochemistry-equivocal invasive breast cancer

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Background and aims: Chromosome 17 alterations affect the assessment of HER2 gene amplification in breast cancer (BC), but its clinical significance remains unclear. This study aimed to identify the prevalence of centromere enumeration probe 17 (CEP17) alterations, and its correlation with response to neoadjuvant therapy (NAT) in BC patients with human epidermal growth factor receptor 2 (HER2) immunohistochemistry-equivocal score.

Methods and results: A large BC cohort (n = 6049) with HER2 immunohistochemistry score 2+ and florescent in-situ hybridisation (FISH) results was included to assess the prevalence of CEP17 alterations. Another cohort (n = 885) with available clinicopathological data was used to evaluate the effect of CEP17 in the setting of NAT. HER2-amplified tumours with monosomy 17 (CEP17 copy number < 1.5 per nucleus), normal 17 (CEP17 1.5–< 3.0) and polysomy 17 (CEP17 ≥ 3.0) were observed in 16, 59 and 25%, respectively, compared with 3, 74 and 23%, respectively, in HER2-non-amplified tumours. There was no significant relationship between CEP17 alterations and pathological complete response (pCR) rate in both HER2-amplified and HER2-non-amplified tumours. The independent predictors of pCR were...
oestrogen (ER) negativity in HER2-amplified tumours [ER negative versus positive; odds ratio (OR) = 11.80; 95% confidence interval (CI) = 1.37–102.00; \( P = 0.02 \)], and histological grade 3 in HER2 non-amplified tumours (3 versus 1, 2; OR = 5.54; 95% CI = 1.61–19.00; \( P = 0.007 \)).

Keywords: alterations, breast cancer, CEP17, chromosome 17, HER2

Introduction

Human epidermal growth factor receptor 2 (HER2) protein overexpression and/or gene amplification occurs in approximately 15% of invasive breast cancer (BC).\(^1,2\) Patients with HER2-positive BC are often treated with a combination of sequential chemotherapy and HER2-targeted therapy in the neoadjuvant and/or adjuvant setting.\(^3\) HER2 status is determined by immunohistochemistry (IHC) and in-situ hybridisation (ISH). The current American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines and United Kingdom (UK) recommendations for HER2 testing recommend a two-tiered system using IHC and ISH testing if required. BC with HER2 IHC score 2+ are considered equivocal and require the assessment of HER2 gene amplification status using dual-probe ISH.\(^4-6\) In the dual-probe ISH assay, HER2 gene copy number is reported relative to the centromere enumeration probe 17 (CEP17) nuclear signal as an internal control, and hence the HER2/CEP17 ratio is influenced not only by average HER2 copy number but also by CEP17 alterations.

Previous studies have shown that the HER2/CEP17 ratio and HER2 gene copy number are predictive for neoadjuvant trastuzumab and chemotherapy response in HER2-positive BC.\(^7-10\) Our previous study demonstrated that the maximum benefit of neoadjuvant anti-HER2 therapy is observed in the subgroup of patients with tumours that are HER2 IHC score 3+, histological grade 3 or HER2 IHC score 2+/HER2-amplified co-existing with oestrogen receptor (ER) negativity in HER2-positive BC patients.\(^11\) Although CEP17 alterations are common, their significance has received less attention in clinical practice.

Chromosome 17 (Ch17) alterations include whole chromosome gains or losses, gene copy number anomalies, allelic losses and structural rearrangements.\(^12,13\) Whether the number of CEP17 signals by ISH reflects true polysomy or monosomy of Ch17 has been examined, and recent data have shown that an increased CEP17 copy number is usually related to focal peri-centromeric gains rather than to true polysomy.\(^12,14\) However, most previous studies have interpreted CEP17 copy number by ISH as whole Ch17 alterations.\(^12,15\) CEP17 copy number was reported to affect HER2 status assessment, and is associated with the clinical outcome and tumour grade.\(^15\) However, the definition of CEP17 alterations (monosomy, duplication or polysomy), BC cohorts (early BC, metastatic BC, HER2-positive BC or HER2-amplified/non-amplified BC) and type of chemotherapy and anti-HER2 therapy have varied among studies, leading to conflicting results. Some earlier reports observed that CEP17 copy number gains correlated with poor prognosis, whereas other studies did not demonstrate any prognostic significance.\(^15,16\) Based on the retrospectively assessed clinical trials, the N9831 adjuvant trastuzumab trial suggests a treatment benefit independent of CEP17 alterations in patients with HER2-positive tumours.\(^17\) While the NEAT/ER9601 adjuvant epirubicin trial suggests that CEP17 duplication predicts benefit from anthracyclines in early stage BC.\(^18\)

In clinical practice, ISH testing is performed in IHC HER2 equivocal tumours. As this subgroup of tumours comprises a relatively low proportion of BCs and includes both HER2-positive (HER2-amplified) and HER2-negative (HER2-non-amplified) BCs, a large cohort of HER2 tested BCs is required to analyse the clinical significance of centromere alterations adequately. Herein, we hypothesise that patients with IHC HER2-equivocal BC with different CEP17 alterations show variable responses to therapy. This study included two large cohorts of BCs with IHC HER2-equivocal for which HER2 florescent in-situ hybridisation (FISH) data were available and aimed to identify the prevalence of CEP17 alterations and to assess their clinical impacts in the setting of neoadjuvant treatment (NAT).

Materials and methods

STUDY COHORT

The initial cohort included 6049 BCs with HER2 IHC 2+ and available HER2 FISH data from a single
centre, the University Hospitals Birmingham NHS Foundation Trust, as a large, unselected patient cohort. This cohort was mainly used to assess the prevalence of CEP17 alteration in BC. Detailed clinical and pathological data were available in the second multicentre cohort (n = 885). The majority of patients were treated at Nottingham University Hospitals NHS Trust, Nottingham (n = 395), with additional patients from Addenbrookes Hospital, Cambridge; University Hospitals of Leicester NHS Trust; St Vincent’s University Hospital, Dublin; University Hospital Galway, Galway; Burney Breast Unit, St Helens and Knowsley Teaching Hospital NHS Trust, Liverpool; Guy’s and St Thomas’ NHS Foundation Trust, London; Ninewells Hospital, Dundee; and University of Turin, Turin, Italy.

Patients were considered eligible for anti-HER2 therapies if their tumours showed a HER2/CEP17 ratio ≥ 2.0 regardless of the HER2 gene copy number or if the HER2 gene copy number was ≥ 6.6 Patients were divided into three groups: NAT received including chemotherapy alone; chemotherapy with trastuzumab; and chemotherapy with dual anti-HER2 agents (i.e. trastuzumab with either pertuzumab or lapatinib). The type of chemotherapy included anthracycline and taxane, anthracycline without taxane and non-anthracycline regimens. Pathological complete response (pCR) was defined as no residual invasive carcinoma in both breast and axillary lymph nodes regardless of the presence of residual ductal carcinoma in situ (DCIS) (ypT0/Tis ypN0).19 All data used in the analysis were derived from the original pathology reports.

Statistical analysis was performed using EZR software (Saitama Medical Center Jichi Medical University; http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html), which is a graphical user interface for R (the R Foundation for Statistical Computing, Vienna, Austria, version 2.13.0).22 Associations between CEP17 alterations and clinical-pathological variables or pCR were examined with Fisher’s exact tests or Pearson’s χ² test, as appropriate. A logistic regression model was applied to evaluate the effect of covariates on pCR. If a variable remained at a level of P value ≤ 0.15, it was incorporated into the final multivariable model.23 A P-value < 0.05 was considered statistically significant.

Ethical approval and consent to participate
This study was approved by the Nottingham Research Tissue Bank Access Committee under the IRAS Project ID: 184265. All patients included were consented to participate in research. Data collected were fully anonymised. The study was performed in accordance with the Declaration of Helsinki.

| Table 1. HER2 status and CEP17 alterations |
|-------------------------------------------|
| Term                                      |
| HER2-amplified                            |
| HER2/CEP17 ratio ≥ 2.0 or HER2 CN ≥ 6.0  |
| HER2-non-amplified                        |
| HER2/CEP17 ratio < 2.0 and HER2 CN < 6.0  |
| CEP17 alternations                        |
| CEP17 monosomy (monosomy 17)              |
| CEP17 signal number < 1.5 per nucleus     |
| CEP17 normal (normal 17)                  |
| CEP17 signal number 1.5–3.0 per nucleus   |
| CEP17 polysomy (polysomy 17)              |
| CEP17 signal number ≥ 3.0 per nucleus     |

CEP17, centromere enumeration probe 17; HER2, human epidermal growth factor receptor 2; FISH, fluorescent in situ hybridisation; CN, copy number.
HER2 amplified tumours

Monosomy 17  Normal 17  Polysomy 17

HER2 non-amplified tumours

Figure 1. The examples of human epidermal growth factor receptor 2 (HER2) fluorescent in-situ hybridisation (FISH) result categories focusing on centromere enumeration probe 17 (CEP17) alterations.

Results

The prevalence of CEP17 alterations

Fourteen per cent of tumours with IHC score 2+ were HER2-amplified (HER2-positive) and 86% were non-amplified (HER2-negative). Of the amplified group, 87% of cases were defined based on a HER2/CEP17 ratio of ≥ 2.0, while 13% of cases were defined based on the mean HER2 copy number alone (≥ 6.0). Figure 2 shows the distribution of CEP17 alterations (cut-off of 1.5 CEP17 copy number for monosomy and 3.0 CEP17 copy number for polysomy). HER2-amplified

Figure 2. Scatter plot showing the prevalence of centromere enumeration probe 17 (CEP17) alterations in the initial cohort (n = 6049). A. HER2-amplified tumours (n = 877); B. HER2-non-amplified tumours (n = 5172).
tumours with monosomy, normal and polysomy of CEP17 were observed in 16, 59 and 25% of cases, respectively, and in HER2-non-amplified tumours in 3, 74 and 23%, respectively (P < 0.05).

**THE RELATIONSHIP BETWEEN CEP17 ALTERATIONS AND CLINICOPATHOLOGICAL FEATURES**

In the second cohort (n = 885), 369 cases showed HER2 gene amplification while 516 tumours did not show evidence of HER2 gene amplification. The median CEP17 signal number was 2.1 (range = 1.0–12.6) and HER2 gene copy numbers was 4.1 (range = 1.4–42.4). HER2-amplified tumours with monosomy, normal and polysomy of CEP17 were observed in 20, 62 and 18% of cases, respectively, while HER2-non-amplified tumours with monosomy, normal and polysomy of CEP17 were observed in 7, 66 and 27%, respectively (Table 2). In HER2-non-amplified tumours, polysomy 17 was associated with higher tumour grade (P < 0.001). There was no significant relationship between CEP17 alterations and histological type or hormone receptor status.

**THE RELATIONSHIP BETWEEN CEP17 ALTERATIONS AND pCR**

Full data on NAT were available in 39% of patients (n = 345 of 885), including 165 with HER2-amplified and 180 with HER2-non-amplified tumours. pCR rate was calculated in tumours classified according to HER2 status and CEP17 alterations, taking account of treatment regimens (Table 3). There was no significant relationship between CEP17 alterations and pCR rate in patients with HER2-amplified tumours and HER2-non-amplified tumours. The association between clinicopathological parameters and the attainment of a pCR was examined in both HER2-amplified tumours and HER2-non-amplified tumours by univariate and multivariate stepwise regression models (Table 4). In HER2-amplified tumours, ER negativity was identified as an independent predictor of pCR [ER negative versus positive: odds ratio (OR) = 11.80; 95% confidence interval (CI) = 1.37–102.00; P = 0.02]. In HER2-non-amplified tumours, histological grade 3 was an independent predictor of pCR (3 versus 1, 2; OR = 5.54; 95% CI = 1.61–19.00; P = 0.007).

**Discussion**

Focusing on BC patients with a HER2 immuno-histochemistry-equivocal score, we revealed the prevalence of CEP17 alterations and their association with response to NAT. Our results showed that polysomy 17, defined by increased CEP17 copy number by ISH, was observed in 24% of HER2 IHC-equivocal BC and was associated with an increasing HER2 copy number. In addition, we observed that polysomy 17 was little more frequent in HER2-non-amplified tumours than HER2-amplified, as shown in a previous study.24 Although Ch17 alterations have been estimated by counting CEP17 copy number by ISH in most previous studies, the definition of CEP17 alterations and the patient cohorts vary among studies, leading to conflicting results.13,15 Our cut-off for polysomy 17 (mean of ≥ 3 CEP17 signals per nucleus) is a commonly adopted threshold.13,15 Merola et al.25 reported a 46% prevalence of polysomy 17 in HER2 IHC-equivocal BC using a similar definition. Although a higher rate of polysomy 17 in HER2 IHC-equivocal BC was reported by Merola et al.,25 their cohort was smaller than our current series and the clinicopathological parameters were not clearly defined. Moreover, we emphasise that ISH analysis is routinely evaluated in HER2 IHC-equivocal BC, so the clinical significance of CEP17 alterations needs to be analysed in a large cohort of HER2 IHC-equivocal BC patients.

Others have reported monosomy 17 in 4% of HER2-positive cases.17 In our cohort, which comprised HER2 IHC-equivocal BC only, monosomy 17 was present in 16% of HER2-amplified tumours compared to 3% of HER2-non-amplified tumours. These findings indicate that some cases with a low HER2 copy number were classified as HER2-amplified because of low CEP17 when positivity is defined using HER2/CEP17 ratio alone.6 Mathematically, the lower the value of denominator (as in cases of Ch17 monosomy), the higher value of the overall ratio, even with a fixed numerator. Biologically, in cells with normal genetic content (2 N), there is usually one active allele for each gene while the other copy is dormant. In monosomy 17, the single CEP17 signal binding would result in a higher HER2/CEP17 ratio (> = 2.0) even with lower levels of amplification of the active HER2 allele. Although the response rate of these HER2 IHC 2+ cases with monosomy 17 and low levels of HER2 gene amplification classified as HER2 positive based on HER2/CEP17 ratio to anti-HER2 therapy is uncertain, there is no evidence that their response is significantly lower than other HER2 IHC 2+ with HER2 gene copy number > 6.0.11 Emerging new anti-HER2 antibody drug conjugates (ADC) for ‘HER2-low expression groups’ would also help to refine the definition of HER2 positivity for therapeutic purposes.26

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There are limited studies showing the relationship between CEP17 alterations and treatment response in the neoadjuvant setting, but multiple studies have investigated pathological biomarkers predicting response or resistance to NAT. HER2 amplification level determined by FISH was associated with pCR, and the pCR rate was significantly higher in highly amplified tumours.\textsuperscript{27} Hormone receptor status, especially ER negativity, and high histological grade have been shown to predict response to neoadjuvant chemotherapy alone, and combined chemotherapy and anti-HER2 therapy in BC.\textsuperscript{11,28–30} In our current cohort of

| Characteristics | Total number (\%) | \textbf{HER2}-amplified | \textbf{HER2}-non-amplified | \textbf{HER2}-amplified | \textbf{HER2}-non-amplified |
|-----------------|-------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| Total number    | 885 (100)         | 75 (20.3)               | 227 (61.5)             | 67 (18.2)               | 36 (7.0)                |
| CEP17 copy number (median [range]) | 2.1 [1.0–12.6] | 1.3 [1.0–1.5] | 1.9 [1.5–2.9] | 4.2 [3.0–6.7] | 1.4 [1.2–1.5] |
| HER2 copy number (median [range]) | 4.1 [1.4–42.4] | 3.0 [2.1–24.4] | 4.6 [3.0–38.4] | 6.8 [1.7–31.2] | 2.0 [1.4–2.8] |
| Age (median [range]) | 59 [23–96] | 54 [31–84] | 56 [26–96] | 59 [36–80] | 54 [26–76] |
| Histology type | No special type | 804 (91.0) | 70 (94.6) | 208 (91.6) | 66 (98.5) | 31 (86.1) | 298 (87.9) | 131 (93.6) | 0.08 |
| Lobular | 50 (5.7) | 3 (4.1) | 13 (5.7) | 1 (1.5) | 1 (2.8) | 26 (7.7) | 6 (4.3) |
| Mixed (no special type and lobular) | 29 (3.3) | 1 (1.3) | 6 (2.7) | 0 | 4 (11.1) | 15 (4.4) | 3 (2.1) |
| Tumour grade | 1 | 82 (9.4) | 5 (6.8) | 13 (5.8) | 2 (3.0) | 0.34 | 9 (25.7) | 42 (12.5) | 11 (7.9) | <0.001 |
| 2 | 577 (65.9) | 54 (74.0) | 142 (63.7) | 45 (67.2) | 19 (54.3) | 240 (71.2) | 77 (55.0) |
| 3 | 216 (24.7) | 14 (19.2) | 68 (30.5) | 20 (29.8) | 7 (20.0) | 55 (16.3) | 52 (37.1) |
| ER | Negative | 121 (14.8) | 8 (13.8) | 36 (18.1) | 8 (17.4) | 0.75 | 7 (19.4) | 47 (13.9) | 15 (10.6) | 0.35 |
| Positive | 698 (85.2) | 50 (86.2) | 163 (81.9) | 38 (82.6) | 29 (80.6) | 292 (86.1) | 126 (89.4) |
| PR | Negative | 209 (27.5) | 17 (32.7) | 55 (32.5) | 11 (24.4) | 0.56 | 9 (26.5) | 87 (26.9) | 30 (21.9) | 0.52 |
| Positive | 551 (72.5) | 35 (67.3) | 114 (67.5) | 34 (75.6) | 25 (73.5) | 236 (73.1) | 107 (78.1) |

CEP17, centromere enumeration probe 17; HER2, human epidermal growth factor receptor 2; ER, oestrogen receptor; PR, progesterone receptor.
Significant P-values are shown in bold type.
HER2 IHC-equivocal BC within both HER2-amplified and HER2-non-amplified tumours, there was no significant relationship between CEP17 alterations and pCR rate. Similar to previous studies, hormone receptor status and histological grade highlighted subgroups showing higher pCR rates. Page et al.\textsuperscript{16} provided evidence that patients with HER2-amplified monosomy 17 in early-stage BC benefited from

### Table 3. Correlation between CEP17 alterations and pCR

| Treatment                               | Total number | Monosomy 17 pCR/non-pCR, no. (%) | Normal 17 pCR/non-pCR, no. (%) | Polysomy 17 pCR/non-pCR, no. (%) | P-value |
|-----------------------------------------|--------------|----------------------------------|--------------------------------|---------------------------------|---------|
| **HER2-amplified tumours**              |              |                                  |                                |                                 |         |
| Chemotherapy and anti-HER2 therapy      | 165          | 5/20 (20.0)                      | 25/98 (20.3)                   | 2/15 (11.8)                     | 0.70    |
| Anti-HER2 therapy given (single)        | 146          | 5/18 (21.7)                      | 22/86 (20.4)                   | 1/14 (6.7)                      | 0.43    |
| Anti-HER2 therapy given (dual)          | 19           | 0/2 (0)                          | 3/12 (20.0)                    | 1/1 (50.0)                      | 0.46    |
| Chemotherapy containing anthracycline  | 110          | 2/18 (10.0)                      | 16/64 (20.0)                   | 1/9 (10.0)                      | 0.47    |
| **HER2-non-amplified tumours**          |              |                                  |                                |                                 |         |
| Chemotherapy                            | 180          | 1/15 (6.3)                       | 13/105 (11.0)                  | 7/39 (15.2)                     | 0.59    |
| Chemotherapy containing anthracycline  | 165          | 0/15 (0)                         | 11/102 (9.7)                   | 5/32 (13.5)                     | 0.33    |

CEP17, centromere enumeration probe 17; pCR, pathological complete response; HER2, human epidermal growth factor receptor 2.

### Table 4. Univariate and multivariate logistic regression model for pCR in both HER2-amplified and HER2-non-amplified tumours

| Parameters                  | Risk/reference               | Univariate analysis | Multivariate analysis |
|-----------------------------|------------------------------|---------------------|-----------------------|
|                             |                              | pCR/non-pCR, no. (%)| pCR/non-pCR, no. (%)  | OR          | 95% CI        | P-value |
|                             |                              |                     |                       | OR          | 95% CI        | P-value |
| **HER2-amplified tumour**   |                              |                     |                       |             |              |         |
| CEP17                       | Mono, poly/normal            | 7/35 (16.7)         | 25/98 (20.3)          | 0.78        | 0.31–1.97     | 0.61    |
| ER                          | Negative/positive            | 14/22 (38.9)        | 18/111 (14.0)         | 3.92        | 1.70–9.04     | \textbf{0.001} |
| PR                          | Negative/positive            | 14/36 (28.0)        | 13/65 (16.7)          | 1.94        | 0.83–4.58     | 0.13    |
| Histological grade 3/1, 2   |                              | 22/54 (28.9)        | 10/74 (11.9)          | 3.01        | 1.32–6.88     | \textbf{0.009} |
| **HER2-non-amplified tumour**|                              | 8/54 (12.9)         | 13/105 (11.0)         | 1.20        | 0.47–3.06     | 0.71    |
| CEP17                       | Mono, poly/normal            | 10/31 (24.4)        | 11/128 (7.9)          | 3.75        | 1.46–9.63     | \textbf{0.006} |
| PR                          | Negative/positive            | 12/48 (20.0)        | 6/92 (6.1)            | 3.83        | 1.35–10.80    | \textbf{0.01} |
| Histological grade 3/1, 2   |                              | 16/54 (22.9)        | 4/103 (3.7)           | 7.63        | 2.43–24.00    | \textbf{0.001} |

CEP17, centromere enumeration probe 17; CI, confidence interval; ER, oestrogen receptor; HER2, human epidermal growth factor receptor 2; OR, odds ratio; pCR, pathological complete response; PR, progesterone receptor.

Significant $P$-values are shown in bold type.
trastuzumab, supporting that pCR is not related to CEP17 alterations.

The NEAT/ER9601 adjuvant epirubicin trial\(^\text{18}\) showed CEP17 duplication, determined as CEP17 signal number > 1.86, predicted benefit from anthracyclines in early BC, and Tibau et al.\(^\text{21}\) reported that CEP17 duplication predicted pCR to primary anthracycline-based chemotherapy in BC. However, our cohort showed no difference in the rate of pCR among the different CEP17 in patients who received anthracycline-based NAT.

The strengths of the study include the large sample size of the cohort, including more uncommon FISH patterns (ASCO/CAP FISH group 2, 3 and 4), reliable well-validated FISH assessment in regional testing centres and the inclusion of a large single-centre cohort consisting of unselected patients. However, our study has some limitations. This was a retrospective non-randomised study and our samples were collected from multiple institutions, which may have some selection bias effect. Also, we have not technically determined if the cases in our series have true monosomy or loss of a portion of the chromosome and true polysomy or gain of a portion of the chromosome.

In conclusion, our findings suggest that the clinicopathological significance of CEP17 alterations is not as strong as that of the HER2/CEP17 ratio and HER2 gene copy number. The hormonal receptor status and tumour histological grade are more useful to identify patients who will benefit from NAT in HER2 IHC-equivocal BC.

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Conflicts of interest
The authors have no conflicts of interest to declare.

Data availability statement
The authors confirm the data that has been used is available on reasonable request.

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