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Clinicopathologic significance of CEP17 copy number gain in breast cancer

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이경열

ABSTRACT

Clinicopathologic significance of CEP17 copy number gain in breast cancer

Kyoungyul Lee
Department of Medicine, Translational medicine Major
The Graduate School
Seoul National University

Background: Increased copy number of chromosome enumeration probe (CEP) targeting centromere 17 is frequently encountered during *HER2* in situ hybridization (ISH) in breast cancer. It is caused by amplification or gain in copy number of the pericentromeric region of chromosome 17. Chromosomal instability (CIN) is defined as a defect that frequently results in the loss or gain of a whole or part of a chromosome during cell division in malignant solid tumors. These similar genetic abnormalities have been reported to be associated with prognosis and treatment response in breast cancer. The aim of this study was to clarify the clinicopathologic implication of CEP17 copy number gain in breast cancer

Methods: We analyzed 945 cases of invasive breast cancers whose *HER2* fluorescence ISH reports were available from 2004 to 2011 at a single institution and evaluated the association of CEP17 copy number gain with clinicopathologic features of tumors and patient survival. To identify the correlation between CEP17 copy number gain and CIN, CIN status was determined by summing copy number gains of four CEPs (CEP1, CEP8, CEP11 and CEP16) on fluorescence in situ hybridization (FISH) using another 463 cases of breast cancer, and was correlated with clinicopathologic features and survival of the patients. In addition, CIN scores using next generation sequencing were calculated to validate the correlation between CEP17 copy number and CIN in 71 cases of breast cancer.

Results: We detected 186 (19.7%) cases of CEP17 copy number gain (CEP17 ≥3.0) among 945 invasive breast cancers. In survival analysis, CEP17 copy number gain was not associated with disease-free survival of the patients in the whole group. Nonetheless, it was found to be an independent adverse prognostic factor in *HER2*-negative group, but not in *HER2*-positive group. In further subgroup analyses, CEP17 copy number gain was revealed as an independent poor prognostic factor in *HER2*-negative and hormone receptor-positive breast cancers, and it was associated with aggressive histologic variables including high T stage, high histologic grade, lymphovascular invasion, P53 overexpression, and high Ki-67 proliferative index. High CIN was associated with adverse clinicopatholgic parameters of breast cancer.

Among them, positive HER2 status, high Ki-67 index and CEP17 copy number gain were found as independent predictors for high CIN. High CIN was associated with poor clinical outcome of the patients in whole group and in luminal/HER2-negative and HER2-positive subtypes as well. However, no predictive value of high CIN was found in response to anthracycline or anthracycline & taxane-based chemotherapy. CEP17 copy number was significantly higher in the high-CIN-score group than in the low-CIN-score group. A positive linear correlation between the mean CEP17 copy number and the CIN score, calculated by NGS, was found.

Conclusion: We found that elevated CEP17 count can serve as a prognostic marker in luminal/*HER2*-negative subtype of invasive breast cancer. High CIN was proved as a poor prognostic factor in breast cancer. CEP17 copy number was confirmed as a useful predictor for CIN in breast cancer, suggesting that CEP17 status need to be evaluated carefully and should be included in HER2 ISH report.

Keywords: CEP17 copy number gain, *HER*2, Breast cancer, Chromosomal instability, Next generation sequencing

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LIST OF ABBREVIATIONS

HER2: human epidermal growth factor receptor 2

IHC: immunohistochemistry

ISH: in situ hybridization

CEP: chromosome enumeration probe

BRCA1: breast cancer 1 gene

TOP2A: DNA topoisomerase II alpha gene

TP53: tumor protein p53 gene

CIN: chromosomal instability

NGS: next generation sequencing

FISH: fluorescence in situ hybridization

TMA: tissue microarray

ER: estrogen receptor

PR: progesterone receptor

ASCO/CAP: American Society of Clinical Oncology/College of American Pathologists

CMF: cyclophosphamide, methotrexate and fluorouracil

TCH: docetaxel, carboplatin and trastuzumab

INTRODUCTION

Human epidermal growth factor receptor 2 (*HER2*) gene amplification is found in about 20% of invasive breast cancers (1). It is an established prognostic marker associated with poor clinical outcome and a robust predictive marker of clinical benefits from *HER2*-targeted agents (2, 3). As *HER2*-targeted therapy has become a standard treatment for *HER2*-positive breast cancers in adjuvant and metastatic settings, determination of *HER2* status is now an important daily practice in pathology.

In breast cancer, *HER2* status is determined by immunohistochemistry (IHC) first and then, in situ hybridization (ISH) is employed to confirm gene amplification in equivocal cases on IHC. *HER2* oncogene maps to the long arm of chromosome 17 (4). While interpreting *HER2* ISH using dual-colored probes, a copy number gain of chromosome enumeration probe (CEP) targeting the centromere 17 is often observed. At first, such finding was attributed to polysomy 17- increased number of chromosome 17, itself. However, recent studies have revealed that increased signals of CEP17 on ISH are not true polysomy 17. Instead, it is the amplification or gain in copy number of the pericentromeric region of chromosome 17 that leads to the increased copy number of CEP17 (5-9).

Besides *HER2* gene, several breast cancer-related genes are located on chromosome 17 such as breast cancer 1 (*BRCA1*), DNA topoisomerase II alpha (*TOP2A*), and tumor protein p53 (*TP53*) (10). Chromosome 17 is one of the smallest human chromosomes, and there exist both various structural and numerical aberrancies as shown in different cancers (11-13). In breast cancers, CEP17 copy number gain has been reported to be associated with adverse clinicopathologic parameters (14-16). However, as for its prognostic significance, there have been conflicting results. While a few earlier studies proved that CEP17 copy number gain correlated with poor prognosis (17, 18), the prognostic significance of CEP17 copy number gain remains unknown in breast cancer (19, 20).

Chromosomal instability (CIN) is defined as a defect that frequently results in the loss or gain of a whole or part of a chromosome during cell division in malignant solid tumors (21). Defects in chromosome cohesion, mitotic checkpoint function, centrosome copy number, kinetochore–microtubule attachment dynamics, and cell-cycle regulation are thought to be the underlying mechanisms of CIN (22). As a hallmark of cancer, CIN contributes to tumorigenesis through the inactivation of tumor suppressor genes (23). CIN-induced genetic changes lead to intratumoral heterogeneity, which allows tumor cells to adapt to unfavorable environments and therapeutic agents (21, 24). Tumors with high CIN are associated with poor prognoses in various cancer types, including breast cancer (25-27). In addition

to its prognostic implications in malignant tumors, CIN may be a promising predictor for treatment response (28). Especially, high CIN has been reported to be associated with sensitivity to anthracycline (29, 30) and resistance to taxane (31, 32).

To clarify the significance of CEP17 copy number gain in breast cancer, we examined the associations between clinicopathologic features of breast cancer and CEP17 copy number gain in a relatively large series of breast cancer. Then, we investigated the prognostic significance of CEP17 copy number gain in breast cancer in the whole group as well as in different subtypes of breast cancer. We also assessed the correlation between the gain in the CEP17 copy number and CIN in breast cancer to determine whether CEP17 copy number gain reflects CIN in a large set of breast cancers. Finally, we analyzed the correlation between CEP17 and CIN scores which were measured by analyzing copy number variations in next generation sequencing (NGS) data in a small subset of breast cancer patients.

MATERIALS AND METHODS

1. Patient population and tissue collection

We selected 1,013 cases of invasive breast cancers diagnosed between June 2004 and December 2011 at Seoul National University Bundang Hospital, whose *HER2* fluorescence ISH (FISH) reports were available as we had used in our previous study (9). *HER2* FISH was performed upon diagnosis of invasive breast cancer, irrespective of HER2 IHC score. Of the 1013 cases, 68 cases were excluded for this study due to recurrent breast cancer, advanced breast cancer with distant metastasis at presentation, incomplete resection, male breast cancer, or bilateral breast cancer. Finally, a total of 945 invasive breast cancers were included in this study. Clinicopathologic information was obtained from the medical records and hematoxylin and eosin-stained sections. The following histopathologic variables were evaluated: histologic subtype, T stage, N stage, Bloom-Richardson histologic grade, and lymphovascular invasion. The baseline characteristics are shown in **Table 1**.

We used two more sets of breast cancer samples in this study for CIN of breast cancer. The second set was a total of 463 invasive breast cancer samples, which were consecutively resected between 2003 and 2008 at Seoul National University Bundang Hospital. It was used for analyses of CIN using

multiple CEP probes and determination of its prognostic and predictive values. The clinicopathologic information was obtained from medical records and hematoxylin-and-eosin-stained sections. The following histopathological variables were recorded: T stage, N stage, histologic subtype (by WHO classification), Bloom-Richardson histological grade and lymphovascular invasion. The baseline clinicopathologic characteristics of second set are shown in **Table 2**. The third set, which was composed of 71 cases of invasive breast cancer surgically resected between 2010 and 2012, was used for correlation of CEP17 copy number with CIN scores based on NGS. A significant proportion (35.2%) of the third set was mucinous carcinoma which had been analyzed for other study (not published). The baseline characteristics are shown in **Table 3**.

Table 1. Baseline characteristics of the first set

Clinicopathologic characteristics	Number of subjects (%)
Age	
< 50 years	251 (54.2)
≥ 50 years	212 (45.8)
Sex	
Male	3 (0.6)
Female	460 (99.4)
Histologic subtype	
Invasive ductal carcinoma, NOS	398 (86.0)
Invasive lobular carcinoma	21 (4.5)
other subtypes	44 (9.5)
pT stage	
pT1	198 (42.8)
pT2	238 (51.4)
pT3	19 (4.1)
pT4	8 (1.7)
Lymph node metastasis	
Absent	251 (54.2)
Present	212 (45.8)
Histologic grade	
I	80 (17.9)
II	152 (34.1)
III	214 (48.0)
Estrogen receptor	
Positive	313 (67.6)
Negative	150 (32.4)
Progesterone receptor	
Positive	270 (58.3)
Negative	193 (41.7)
Hormone receptor	
Positive	323 (69.8)
Negative	141 (30.2)
HER2 status	
Negative	375 (81.0)
Positive	88 (19.0)
p53 overexpression	
Absent	357 (77.1)
Present	106 (22.9)
Ki-67 index	
<20%	270 (58.3)
≥20%	193 (41.7)
Molecular subtype	
Luminal/HER2-negative subtype	283 (61.1)
Luminal/HER2-postive subtype	40 (8.6)
HER2-positive subtype	48 (10.4)
Triple-negative subtype	92 (19.9)
CEP17 copy number gain	
Absent	401 (87.2)
Present	59 (12.8)

Table 2. Baseline characteristics of the second set

Clinicopathologic characteristics	Number of subjects (%)
Age	
< 50 years	251 (54.2)
≥ 50 years	212 (45.8)
Sex	
Male	3 (0.6)
Female	460 (99.4)
Histologic subtype	
Invasive ductal carcinoma, NOS	398 (86.0)
Invasive lobular carcinoma	21 (4.5)
other subtypes	44 (9.5)
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<20%	270 (58.3)
≥20%	193 (41.7)
Molecular subtype	
Luminal/HER2-negative subtype	283 (61.1)
Luminal/HER2-postive subtype	40 (8.6)
HER2-positive subtype	48 (10.4)
Triple-negative subtype	92 (19.9)
CEP17 copy number gain	
Absent	401 (87.2)
Present	59 (12.8)

Table 3. Baseline characteristics of the third set

Clinicopathologic characteristics	Number of subjects (%)
Age	
< 50 years	37 (52.1)
≥ 50 years	34 (47.9)
Sex	
Male	0 (0)
Female	71 (100)
Histologic subtype	
Invasive ductal carcinoma, NOS	44 (62.0)
Mucinous carcinoma	25 (35.2)
Metaplastic carcinoma	2 (2.8)
pT stage	
pT1	35 (49.3)
pT2	28 (39.4)
pT3	8 (11.3)
Lymph node metastasis	
Absent	48 (67.6)
Present	23 (32.4)
Histologic grade	
I	17 (23.9)
II	28 (39.4)
III	26 (36.6)
Estrogen receptor	
Positive	60 (84.5)
Negative	11 (15.5)
Progesterone receptor	
Positive	48 (67.6)
Negative	23 (32.4)
Hormone receptor	
Positive	60 (84.5)
Negative	11 (15.5)
HER2 status	
Negative	61 (85.9)
Positive	10 (14.1)
p53 overexpression	
Absent	57 (80.3)
Present	14 (19.7)
Ki-67 index	
<20%	42 (59.2)
≥20%	29 (40.8)
Breast cancer subtype	
Luminal/HER2-negative subtype	56 (78.9)
Luminal/HER2-postive subtype	4 (5.6)
HER2-positive subtype	5 (7.0)
Triple-negative subtype	6 (8.5)
CEP17 copy number gain	
Absent	54 (76.1)
Present	17 (23.9)

2. Tissue microarray construction

All the slides of each breast cancer from the second set were reviewed to select representative sections. Tissue microarrays (TMAs) of 2mm diameter were constricted from representative formalin-fixed paraffin-embedded blocks (SuperBioChips Laboratories, Seoul, South Korea) for immunohistochemistry and fluorescence in situ hybridization.

3. Immunohistochemical analyses and scoring

The expression of the estrogen receptor (ER), progesterone receptor (PR), HER2, p53 and Ki-67 was evaluated in representative tumor sections of surgical specimen at the time of diagnosis. In cases with missing data, immunohistochemical staining on representative tissue sections was carried out in a BenchMark XT autostainer (Ventana Medical Systems, Tucson, AZ) using an UltraView detection kit (Ventana Medical Systems). The following antibodies were used: anti-ER (1:100; clone SP1; LabVision, Fremont, CA), anti-PR (1:70; PgR 636; Dako, Carpinteria, CA), anti-HER2 (ready to use; 4B5; Ventana Medical Systems), anti-p53 (1:600; D07; Dako), and anti-Ki-67 (1:250; MIB-1; Dako).

A tumor was regarded as positive for ER or PR if it showed at least 1% positive nuclear staining with the relevant antibody. For HER2, 3+ on immunohistochemistry or the presence of gene amplification on FISH was

considered positive. Nuclear staining in 10% or more of the tumor cells was considered positive for p53. Nuclear staining in 20% or more of the tumor cells was considered to indicate a high Ki-67 proliferation index.

Immunohistochemical expression of the standard biomarkers was used to categorize the tumor samples into breast cancer subtypes. Breast cancer subtypes were categorized according to the criteria : luminal/HER2-negative subtype (ER+ and/or PR+, HER2-, luminal/HER2-positive subtype (ER+ and/or PR+, HER2+), HER2-positive subtype (ER-, PR-, HER2+), and triple-negative subtype (ER-, PR-, HER2-).

4. Fluorescence in situ hybridization

To identify the HER2 status and CEP17 copy number in each case, HER2 FISH was performed on TMAs of the second set and all tissue sections of the second set. FISH targeting CEP1, CEP8, CEP11, and CEP16 was performed on TMAs to assess CIN. These CEP probes around the centromere have been reported to show frequent copy number gains in breast cancer (27, 33, 34).

Briefly, 4 μm deparaffinized tissue sections were incubated in pretreatment solution (Abbott Molecular) at 80 °C for 30 min and then in protease solution (Abbott Molecular) for 20 min at 37 °C. Probes were diluted in tDen-Hyb-2 hybridization buffer (InSitus Biotechnologies, Albuquerque,

NM). The probes and the DNA in the tissue sections were denatured together by incubating them for 5 min at 73 °C in HYBrite™ (Abbott Molecular), and then hybridized for 16 h at 37 °C. Post-hybridization washes were performed according to the protocol supplied. Slides were mounted and viewed with a fluorescence microscope.

5. Definition of HER2 status, CEP17 copy number gain and CIN

We reviewed the FISH results of the first set and recorded mean *HER2* copy number, mean CEP17 copy number, *HER2*/CEP17 ratio, and the number of nuclei counted. In accordance with updated 2013 ASCO/CAP guidelines, *HER2* status was re-evaluated. *HER2* copy number of 6.0 or higher per cell or a *HER2*:CEP17 ratio of 2 or higher was considered amplified. *HER2*/CEP17 ratios <2 and *HER2* copy numbers of 4 to 6 signals per cell were classified as equivocal. *HER2* copy numbers <4 signals per cell and *HER2*/CEP17 ratios <2 were considered non-amplified (35). Of the 945 cases, 212 (22.4%) were *HER2*-amplified, 679 (71.9%) were non-amplified, and 54 (5.7%) were equivocal. In this study, *HER2*-equivocal cases were regarded as *HER2*-negative for statistical analyses.

CEP17 copy number gain was defined with two thresholds in the first set: (1) CEP17 ≥2.6, for possible truncation effect and (2) CEP17 ≥3.0, which is the commonly adopted threshold (Figure 1A). Centromere 17 was

regarded as amplified in cases with an average copy number ≥6.0 (Figure 1B), based on the criteria for *HER2* amplification (35).

The signals for each CEP probe in FISH were counted in at least 20 non-overlapping tumor nuclei. The mean CEP counts per cell for chromosomes 1, 8, 11, 16 and 17 were calculated. CEP copy number gain was defined as a mean CEP count of ≥ 3.0 (Figure 2). A mean CEP count of < 1.6 was defined as CEP copy number loss.

Aneuploidy is a consequence of CIN, and FISH using multiple CEP probes is accepted as an appropriate method to assess the degree of CIN (36). As CEP copy number loss was rarely found only in CEP8 (13 cases, 2.8%), CIN status was determined by summing the copy number gains for CEP1, CEP8, CEP11, and CEP16 in each case. A high-CIN tumor was defined as a tumor with copy number gains in at least three CEPs. Copy number gain in one or two CEPs or no copy number gain was regarded as low-CIN.

Figure 1. Typical fluorescence in situ hybridization images of CEP17 copy number gain and amplification. (A) CEP17 copy number gain without HER2 amplification; (B) CEP17 amplification without HER2 amplification (CEP17, green signal; HER2, red signal).

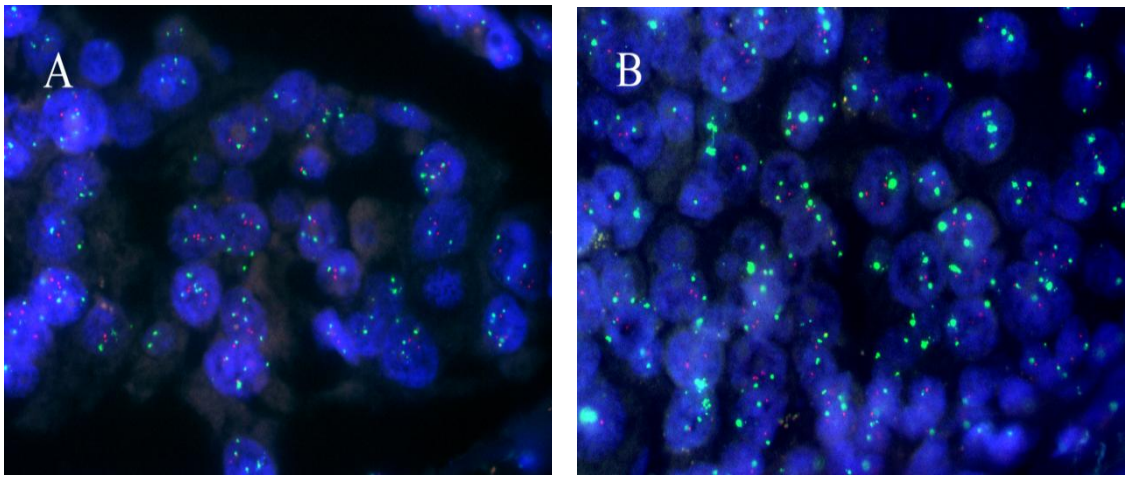
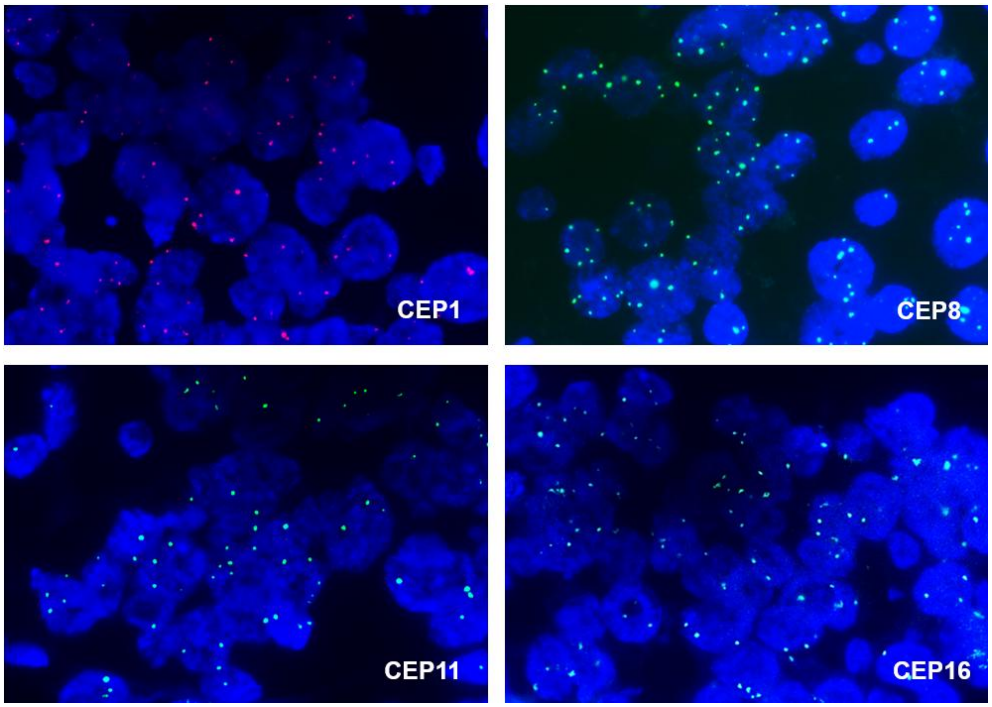


Figure 2. CEP copy number gain detected in fluorescence in situ hybridization. Representative images of CEP1, CEP8, CEP11 and CEP16 copy number gain with an increased number of three or more signals per cell.



6. Determination of CIN score with NGS

Genomic DNA was extracted from 3 μg of the formalin-fixed paraffin-embedded tissues of the third set. DNA library preparation and target enrichment were performed with the SureSelectXT Target Enrichment Kit (Agilent Technologies, Santa Clara, CA). Deep targeted sequencing was performed with a cancer gene panel that included 170 cancer driver genes (**Appendix A1**). Target region bases were sequenced for each sample using the HiSeq 2500 system (Illumina, San Diego, CA), achieving average coverage depth 715× (Macrogen Inc., Seoul, Republic of Korea).

The adapter sequences were eliminated with cuadapt. The reads were aligned to the reference genome (GRCh37/hg19) using Burrows–Wheeler Aligner MEM (BWA-MEM). Poorly mapped reads (mapping quality below 20) and duplicated reads were removed with SAMtools version 1.3.1 and MarkDuplicates (version 2.2.4), respectively. The base quality of the deduplicated reads was recalibrated with GATK BaseRecalibrator. To estimate the degree of CIN, we calculated the Z-score of the normalized number of reads in 2,897 predefined regions in each sample and scored them by counting the number of regions with $|Z| > 3$.

7. Statistical analysis

All statistical analyses were performed with the statistical package SPSS version 15.0 (SPSS Inc., Chicago, IL). Pearson's χ^2 test was used to compare categorical variables between groups. A simple regression analysis was used to detect linear correlations between variables. The Mann–Whitney U test was used to compare continuous variables between two groups. A multivariate logistic regression analysis was used to detect independent predictive factors for CIN. The odds ratios and 95% confidence intervals (CIs) were calculated for the significant variables. For the survival analyses, Kaplan–Meier survival curves were generated and compared with the log rank test. A Cox proportional hazards regression model was used for the multivariate analysis with a backward stepwise selection method. Hazard ratios and 95% CIs were calculated for the significant variables. p values < 0.05 were considered statistically significant, and all reported p values are two-sided.

RESULTS

1. CEP17 copy number alteration

In 945 invasive breast cancers, mean CEP17 copy number per nucleus was 2.47 (range, 1.1-14.95). With the definition of CEP17 copy number gain as mean CEP17 ≥2.6, CEP17 copy number gain including amplification was observed in 283 (29.9%) of 945 patients. CEP17 amplification was found in 18 (6.4%) of 283 CEP17 copy number gain cases. Breast cancers with a CEP17 copy number loss (<1.6 CEP17 value) was detected in 64 (6.8%) of 945 patients. When defining CEP17 copy number gain as CEP17 ≥3.0, CEP17 copy number gain were found in 186 (19.7%) among 945 patients.

2. Association of clinicopathologic characteristics with CEP17 copy number gain

We evaluated the correlations between clinicopathologic parameters and CEP17 copy number gain in the total group using different thresholds for CEP17 copy number gain. CEP17 copy number gain displayed positive correlations with various adverse histologic parameters: It was significantly associated with higher histologic grade (p <0.001), lymphovascular invasion (CEP17 ≥2.6, p = 0.029; CEP17 ≥3.0, p = 0.005), negative hormone receptor status (p <0.001), HER2 IHC score of 2 or 3 (p <0.001), *HER2* gene

amplification (p <0.001), p53 overexpression (p <0.001), and high Ki-67 proliferative index (p <0.001) regardless of the threshold for CEP17 copy number gain (Table 4).

However, as these findings may have derived from the close relationship between *HER2* amplification and CEP17 copy number gain, we performed subgroup analyses according to *HER2* status. Table 5 lists the correlations between clinicopathologic parameters and CEP17 copy number gain in 733 *HER2*-negative breast cancers. Similar to the whole group, CEP17 copy number gain was significantly associated with high T stage (CEP17 ≥2.6, p = 0.017; CEP17 ≥3.0, p = 0.006), high histologic grade (p <0.001), HER2 IHC score of 2 or 3 (p <0.001), p53 overexpression (CEP17 ≥2.6, p = 0.018; CEP17 ≥3.0, p <0.001), and high Ki-67 proliferation index (p <0.001), irrespective of the threshold for CEP17 copy number gain. However, in *HER2*-positive breast cancers, only negative hormone receptor status was associated with CEP17 copy number gain by both criteria (CEP17 ≥2.6, p = 0.020; CEP17 ≥3.0, p = 0.048) (Table 6).

Table 4. Clinicopathologic features of tumors with CEP17 copy number gain

Clinicopathologic characteristics	CEP17 Copy number gain (CEP17 ≥2.6)			CEP17 Copy number gain (CEP17 ≥3.0)		
	Absent, N (%)	Present N (%)	*p*-value	Absent N (%)	Present N (%)	*p*- value
Age			**0.006**			0.066
<50	369 (55.7)	130 (45.9)		412 (54.3)	87 (46.8)	
≥50	293 (44.3)	153 (54.1)		347 (45.7)	99 (53.2)	
T stage			**0.029**			0.056
T1	422 (63.7)	159 (56.2)		478 (63.0)	103 (55.4)	
T2-T4	240 (36.3)	124 (43.8)		281 (37.0)	83 (44.6)	
Node metastasis[a]			0.669			0.667
Absent	434 (65.8)	182 (64.3)		497 (65.7)	119 (64.0)	
Present	226 (34.2)	101 (35.7)		260 (34.3)	67 (36.0)	
Histologic grade			**<0.001**			**<0.001**
I & II	441 (66.6)	113 (39.9)		488 (64.3)	66 (35.5)	
III	221 (33.4)	170 (60.1)		271 (35.7)	120 (64.5)	
Lymphovascular invasion			**0.029**			**0.005**
Absent	433 (65.4)	164 (58.0)		496 (65.3)	101 (54.3)	
Present	229 (34.6)	119 (42.0)		263 (34.7)	85 (45.7)	
Hormone receptor			**<0.001**			**<0.001**
Negative	136 (20.5)	94 (33.2)		161 (21.2)	69 (37.1)	
Positive	526 (79.5)	189 (66.8)		598 (78.8)	117 (62.9)	
HER2 IHC score			**<0.001**			**<0.001**
0 & 1	393 (59.4)	80 (28.3)		427 (56.3)	46 (24.7)	
2 & 3	269 (40.6)	203 (71.7)		332 (43.7)	140 (75.3)	
HER2 amplification on FISH			**<0.001**			**<0.001**
Absent	568 (85.8)	165 (58.3)		640 (84.3)	93 (50.0)	
Present	94 (14.2)	118 (41.7)		119 (15.7)	93 (50.0)	
P53 overexpression			**<0.001**			**<0.001**
Absent	532 (80.4)	180 (63.6)		604 (79.6)	108 (58.1)	
Present	130 (19.6)	103 (36.4)		155 (20.4)	78 (41.9)	
Ki-67 proliferation index			**<0.001**			**<0.001**
<20%	486 (73.4)	147 (51.9)		547 (72.1)	86 (46.2)	
≥20%	176 (26.6)	136 (48.1)		212 (27.9)	100 (53.8)	

[a]Node status was not available for 2 patients

Table 5. Clinicopathologic features of tumors with CEP17 copy number gain in *HER2*-negative breast cancers

Clinicopathologic characteristics	CEP17 Copy number gain (CEP17 ≥2.6)			CEP17 Copy number gain (CEP17 ≥3.0)		
	Absent, N (%)	Present N (%)	*p*-value	Absent N (%)	Present N (%)	*p*- value
Age			0.119			0.223
<50	311 (54.8)	79 (47.9)		346 (54.1)	44 (47.3)	
≥50	257 (45.2)	86 (52.1)		294 (45.9)	49 (52.7)	
T stage			**0.017**			**0.006**
T1	368 (64.8)	90 (54.5)		412 (64.4)	46 (49.5)	
T2-T4	200 (35.2)	75 (45.5)		228 (35.6)	47 (50.5)	
Node metastasis[a]			0.870			0.804
Absent	379 (65.4)	109 (66.1)		417 (65.4)	62 (66.7)	
Present	196 (34.6)	56 (33.9)		221 (34.6)	31 (33.3)	
Histologic grade			**<0.001**			**<0.001**
I & II	406 (71.5)	78 (47.3)		446 (69.7)	38 (40.9)	
III	162 (28.5)	87 (52.7)		194 (30.3)	55 (59.1)	
Lymphovascular invasion			0.120			**0.027**
Absent	378 (66.5)	99 (60.0)		426 (66.6)	51 (54.8)	
Present	190 (33.5)	66 (40.0)		214 (33.4)	42 (45.2)	
Hormone receptor			0.403			0.217
Negative	104 (18.3)	35 (21.2)		117 (17.3)	22 (23.7)	
Positive	464 (81.7)	130 (78.8)		523 (81.7)	71 (76.3)	
HER2 IHC score			**<0.001**			**<0.001**
0 & 1	388 (68.3)	75 (45.5)		422 (65.9)	41 (44.1)	
2 & 3[b]	180 (31.7)	90 (54.5)		218 (34.1)	52 (55.9)	
P53 overexpression			**0.018**			**<0.001**
Absent	470 (82.7)	123 (74.5)		531 (83.0)	62 (66.7)	
Present	98 (17.3)	42 (25.5)		109 (17.0)	31 (33.1)	
Ki-67 proliferation index			**<0.001**			**<0.001**
<20%	438 (77.1)	99 (60.0)		488 (76.3)	49 (52.7)	
≥20%	130 (22.9)	66 (40.0)		152 (23.8)	44 (47.3)	

[a]Node status was not available for 2 patients.

[b]Five cases were 3+ on HER2 immunohistochemistry.

Table 6. Clinicopathologic features of tumors with CEP17 copy number gain in *HER2*-positive breast cancers

Clinicopathologic characteristics	CEP17 Copy number gain (CEP17 ≥2.6)			CEP17 Copy number gain (CEP17 ≥3.0)		
	Absent, N (%)	Present N (%)	*p*-value	Absent N (%)	Present N (%)	*p*- value
Age			**0.007**			0.182
<50	58 (61.7)	51 (43.2)		66 (55.5)	43 (46.2)	
≥50	36 (38.3)	67 (56.8)		53 (44.5)	50 (53.8)	
T stage			0.880			0.394
T1	54 (57.4)	69 (58.5)		66 (55.5)	57 (61.3)	
T2-T4	40 (42.6)	49 (41.5)		53 (44.5)	36 (38.7)	
Node metastasis			0.347			0.370
Absent	64 (68.1)	73 (61.9)		80 (67.2)	57 (61.3)	
Present	30 (31.9)	45 (38.1)		39 (32.8)	36 (38.7)	
Histologic grade			0.244			0.426
I & II	35 (37.2)	35 (29.7)		42 (35.3)	28 (30.1)	
III	59 (62.8)	83 (70.3)		77 (64.7)	65 (69.9)	
Lymphovascular invasion			0.617			0.461
Absent	55 (58.5)	65 (55.1)		70 (58.8)	50 (53.8)	
Present	39 (41.5)	53 (44.9)		49 (41.2)	43 (46.2)	
Hormone receptor			**0.020**			**0.048**
Negative	32 (34.0)	59 (50.0)		44 (37.0)	47 (50.5)	
Positive	62 (66.0)	59 (50.0)		75 (63.0)	46 (49.5)	
P53 overexpression			**0.010**			0.084
Absent	62 (66.0)	57 (48.3)		73 (61.3)	46 (49.5)	
Present	32 (34.0)	61 (51.7)		46 (38.7)	47 (50.5)	
Ki-67 proliferation index			0.131			0.155
<20%	48 (51.1)	48 (40.7)		59 (49.6)	37 (39.8)	
≥20%	46 (48.9)	70 (59.3)		60 (50.4)	56 (60.2)	

3. Prognostic significance of CEP17 copy number gain

In Kaplan-Meier survival analyses, CEP17 copy number gain was not associated with disease-free survival in the whole group although it tended to be associated with decreased disease-free survival (CEP17 ≥2.6, $p = 0.194$; CEP17 ≥3.0, $p = 0.097$; Figure 3A & 3B). CEP17 copy number gain had no impact on the overall survival of the patients, either (CEP17 ≥2.6, $p = 0.868$; CEP17 ≥3.0, $p = 0.396$). In subgroup analyses according to *HER2* status, the patients with CEP17 copy number gain had shorter disease-free survival time with a threshold of 3.0 ($p = 0.011$), but not with that of 2.6 ($p = 0.063$) in *HER2*-negative tumors (Figure 3C & 3D). However, in *HER2*-positive breast cancers, CEP17 copy number gain did not have prognostic significance irrespective of the threshold for CEP17 copy number gain (CEP17 ≥2.6, $p = 0.538$; CEP17 ≥3.0, $p = 0.411$). In both subgroups, CEP17 copy number gain was not associated with overall survival of the patients [*HER2*-negative subgroup, $p = 0.880$ (CEP17 ≥2.6), $p = 0.734$ (CEP17 ≥3.0); *HER2*-postive subgroup, $p = 0.795$ (CEP17 ≥2.6), $p = 0.553$ (CEP17 ≥3.0)]. We concluded that the generally accepted 3.0 CEP17 threshold for CEP17 copy number gain was more practical than 2.6 for prognostication of survival, and further analyses were performed with 3.0 as the threshold value.

In *HER2*-negative breast cancers, besides CEP17 copy number gain, age of onset, T stage, lymph node metastasis, histologic grade,

lymphovascular invasion, hormone receptor status, and Ki-67 proliferative index were associated with disease-free survival in univariate analysis (Table 7). In multivariate analysis using the cox proportional hazard model, CEP17 copy number gain was proven an independent prognostic factor (p = 0.039) along with age of onset, T stage, lymph node metastasis, and hormone receptor status (Table 7).

Figure 3. Kaplan-Meier survival curves according to CEP17 copy number gain with different thresholds. (A) Whole patient population using threshold of CEP17 ≥2.6; (B) Whole patient population using threshold of CEP17 ≥3.0; (C) HER2-negative group using threshold of CEP17 ≥2.6; (D) HER2-negative group using threshold of CEP17 ≥3.0

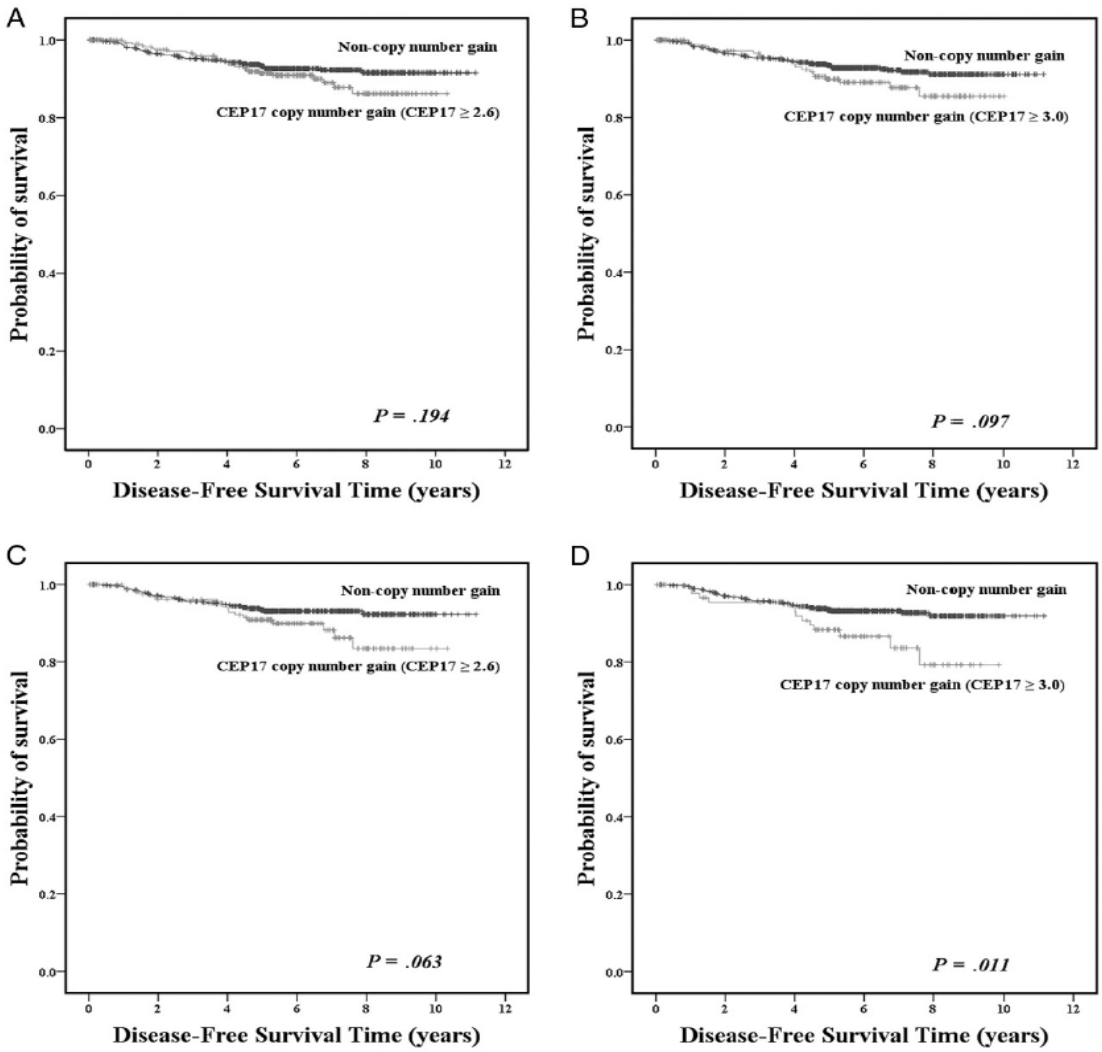


Table 7. Univariate and multivariate analyses of disease-free survival for *HER2*-negative breast cancers

Clinicopathologic variables	Univariate analysis HR (95% CI)[a]	*p-value*	Multivariate analysis HR (95% CI)[a]	*p-value*
CEP17 copy number gain (<3.0 vs ≥3.0)	2.197 (1.179-4.092)	**0.013**	1.941 (1.033-3.650)	**0.039**
Onset age (<50 vs ≥50)	0.570 (0.325-1.002)	0.051	0.563 (0.319-0.993)	**0.047**
T stage (T1 vs T2-4)	2.770 (1.608-4.773)	**<0.001**	1.910 (1.078-3.385)	**0.027**
Lymph node metastasis (absent vs present)	2.556 (1.495-4.368)	**0.001**	2.413 (1.369-4.255)	**0.002**
Histologic grade (1 & 2 vs 3)	1.803 (1.062-3.061)	**0.029**	-	0.923
Lymphovascular invasion (absent vs present)	2.223 (1.307-3.779)	**0.003**	-	0.365
Hormone receptor status (negative vs positive)	0.465 (-0.262-0.824)	**0.009**	0.414 (0.228-0.752)	**0.004**
p53 overexpression (negative vs positive)	1.548 (0.844-2.839)	0.158	-	-
Ki-67 (<20% vs ≥20)	2.125 (1.244-3.632)	**0.006**	-	0.653

[a] Hazard ratio (95% confidence interval) of univariate and multivariate analysis

4. Clinicopathologic significance of CEP17 copy number gain in *HER2*-negative and hormone receptor-positive breast cancers

In further analyses, we sub-classified *HER2*-negative breast cancer into two groups according to hormone receptor status. In Kaplan-Meier survival analyses, patients with CEP17 copy number gain had significantly shorter disease-free survival time than those without in hormone receptor-positive group (p = 0.023; Figure 4A) but not in hormone receptor-negative, that is, triple-negative subgroup (p = 0.331; Figure 4B). In multivariate analyses, CEP17 copy number gain and lymph node metastasis were found to be independent prognostic factors for disease-free survival in *HER2*-negative and hormone receptor-positive group (p = 0.025, p = 0.001, respectively) (Table 8).

Table 9 lists the correlations between clinicopathologic parameters and CEP17 copy number gain in 594 *HER2*-negative and hormone receptor-positive breast cancers. CEP17 copy number gain was significantly associated with most of the poor histologic indicators including high T stage (p = 0.012), high histologic grade (p <0.001), lymphovascular invasion (p = 0.023), p53 overexpression (p <0.001), and high Ki-67 proliferation index (p <0.001). Of note, increased HER2 protein expression (p <0.001) was observed in this group. Through this analysis, we were able to confirm that the established poor prognostic markers had clearly significant associations with the presence

of CEP17 copy number gain in *HER2*-negative and hormone receptor-positive, that is, luminal/*HER2*-negative subtype of breast cancer.

Figure 4. Kaplan-Meier survival curves according to CEP17 copy number gain and hormonal receptor status among HER2-negative breast cancers. (A) HER2-negative, hormone receptor-positive subgroup; (B) HER2-negative and hormone receptor-negative subgroup

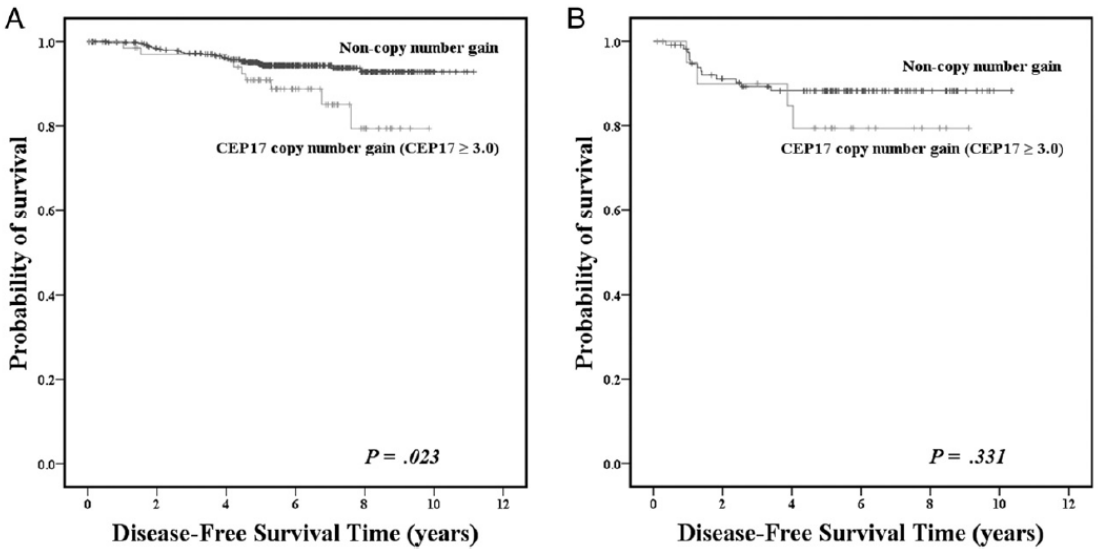


Table 8. Univariate and multivariate analyses of disease-free survival for *HER2*-negative and hormone receptor-positive breast cancers

Clinicopathologic variables	Univariate analysis HR (95% CI)[a]	*p-value*	Multivariate analysis HR (95% CI)[a]	*p-value*
CEP17 copy number gain (<3.0 vs ≥3.0)	2.316 (1.096-4.892)	**0.028**	**2.354 (1.114-4.973)**	**0.025**
Onset age (<50 vs ≥50)	0.659 (0.337-1.290)	0.224	-	-
T stage (T1 vs T2-4)	2.113 (1.117-3.996)	**0.021**	-	0.268
Lymph node metastasis (absent vs present)	3.075 (1.572-6.012)	**0.001**	**3.103 (1.587-6.068)**	**0.001**
Histologic grade (1 & 2 vs 3)	1.761 (0.901-3.442)	0.098	-	0.964
Lymphovascular invasion (absent vs present)	2.050 (1.082-3.887)	**0.028**	-	0.619
p53 overexpression (negative vs positive)	1.510 (0.631-3.612)	0.354	-	-
Ki-67 (<20% vs ≥20)	1.646 (0.755-3.591)	0.210	-	-

[a] Hazard ratio (95% confidence interval) of univariate and multivariate analysis

Table 9. Clinicopathologic features of tumors with CEP17 copy number gain among *HER2*-negative and hormone receptor-positive breast cancers

Clinicopathologic characteristics	CEP17 Copy number gain		*p-value*
	Absent, N (%)	Present, N (%)	
Age			0.683
<50	286 (54.7)	37 (52.1)	
≥50	237 (45.3)	34 (47.9)	
T stage			**0.012**
T1	352 (67.3)	37 (52.1)	
T2-T4	171 (32.7)	34 (47.9)	
Lymph node			0.822
Absent	323 (62.0)	45 (63.4)	
Present	198 (38.0)	26 (36.6)	
Histologic grade			**<0.001**
I & II	426 (81.5)	36 (50.7)	
III	97 (18.5)	35 (49.3)	
Lymphovascular			**0.023**
Absent	338 (64.6)	36 (50.7)	
Present	185 (35.4)	35 (49.3)	
HER2 IHC score			**<0.001**
0 & 1	328 (62.7)	29 (40.8)	
2 & 3	195 (37.3)	42 (59.2)	
P53 overexpression			**<0.001**
Absent	475 (90.8)	52 (73.2)	
Present	48 (9.2)	19 (26.8)	
Ki-67 proliferation			**<0.001**
<20%	463 (88.5)	48 (67.6)	
≥20%	60 (11.5)	23 (32.4)	

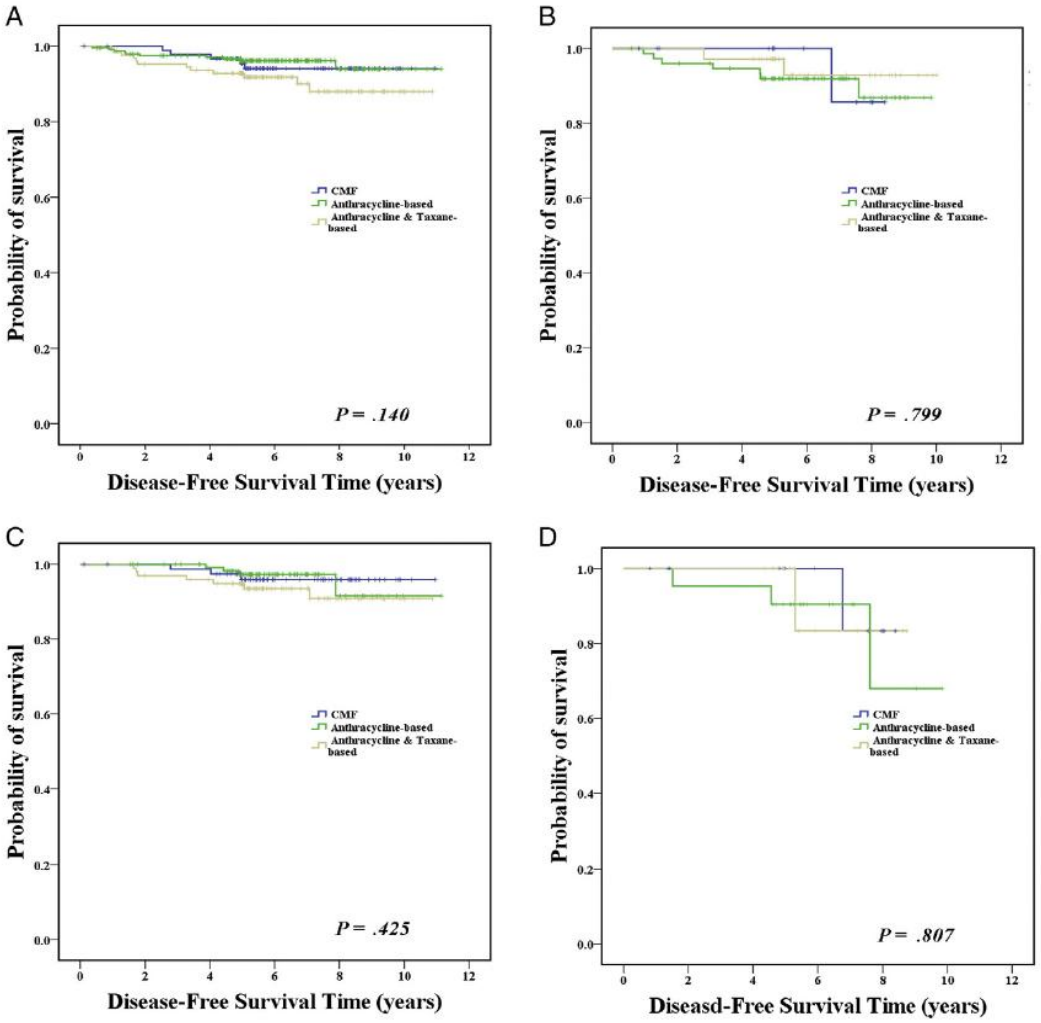
[a]Evaluation for lymph node was not available in a few cases (2 patients)

5. Predictive significance of CEP17 copy number gain according to chemotherapeutic regimens

Of the 945 patients in the first set, 606 patients received adjuvant chemotherapy: 108 patients received CMF (cyclophosphamide, methotrexate and fluorouracil) chemotherapy, 327 received anthracycline-based chemotherapy (with trastuzumab in 43), 163 received combined anthracycline and taxane-based chemotherapy (with trastuzumab in 23), and the remaining 8 received TCH (docetaxel, carboplatin and trastuzumab) chemotherapy. As it has been reported that CEP17 copy number gain is associated with responsiveness to anthracycline-based chemotherapy, we analyzed the prognostic value of CEP17 copy alteration according to different chemotherapeutic regimens in patients receiving adjuvant chemotherapy. However, disease-free survival was not significantly different between subgroups receiving different chemotherapeutic regimens in tumors with CEP17 copy number <3.0 (p = 0.140; Figure 5A) or in those with CEP17 copy number ≥ 3.0 (p = 0.799; Figure 5B). Even after adjusting by tumor stage, there were no significant survival differences between subgroups treated by different chemotherapeutic regimens in tumors with CEP17 copy number <3.0 or in those with CEP17 copy number ≥ 3.0. Finally, in *HER2*-negative, hormone receptor-positive subgroup, there was no difference in disease-free survival according to chemotherapeutic regimen in tumors with

CEP17 copy number <3.0 (p = 0.425; Figure 5C) or in those with CEP17 copy number ≥3.0 (p = 0.807; Figure 5D).

Figure 5. Kaplan-Meier survival curves according to chemotherapeutic regimens. (A) Whole group with CEP17 copy number <3.0 ; (B) Whole group with CEP17 copy number ≥ 3.0; (C) *HER2*-negative, hormone receptor-positive tumors with CEP17 copy number <3.0; (D) *HER2*-negative, hormone receptor-positive subgroup with CEP17 copy number ≥ 3.0

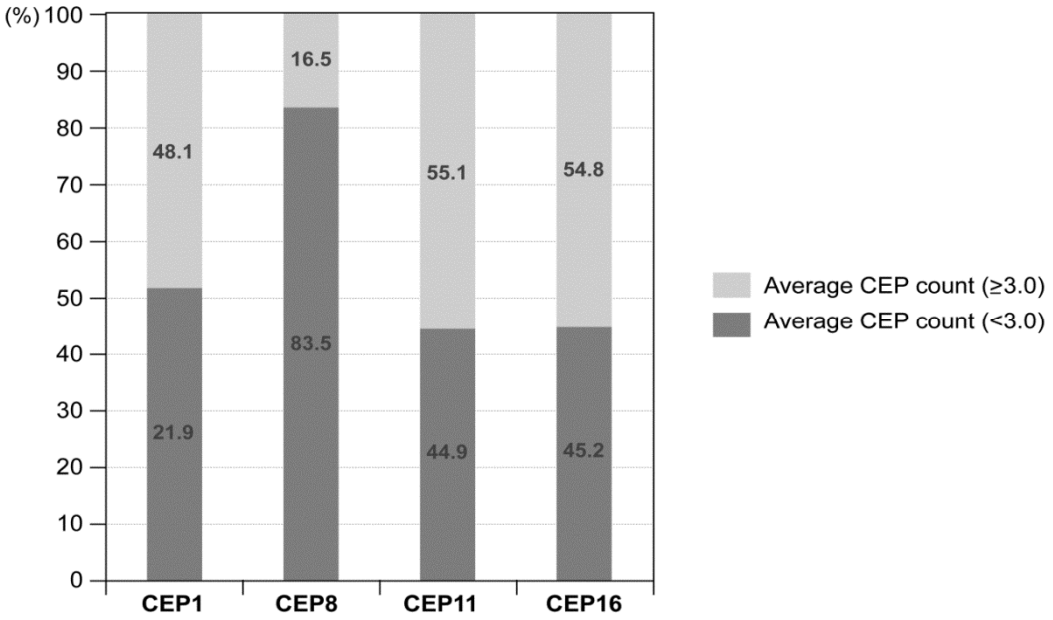


6. CEP copy number gain and CIN

Of the 463 cases in the second set, 88 (19.0%) were HER2-amplified and 375 (81.0%) were non-amplified. CEP17 status was evaluated in 460 cases and copy number gain was detected in 59 cases (12.8%). CEP17 copy number loss (mean CEP17 count <1.6) was found in 3 cases (0.7%). CEP1, CEP8, CEP11, and CEP16 FISH analyses were completed in 443 (95.7%), 462 (99.8%), 448 (96.8%), and 451 (97.4%) cases, respectively. According to the criteria for CEP copy number gain (mean CEP count ≥ 3.0), copy number gains for CEP1, CEP8, CEP11, and CEP16 were noted in 213 (48.1%), 76 (16.5%), 247 (55.1%), and 247 (54.8%) cases, respectively (**Figure 6**).

To assess the degree of CIN, we summed the CEP copy number gains for chromosomes 1, 8, 11, and 16 in each breast cancer. One hundred thirty-two cases (28.5%) showed copy number gain for one CEP, 123 (26.6%) for two CEPs, 97 (21.0%) for three CEPs and 29 (6.3%) for all four CEPs. No gains in four CEPs were found in 82 (17.7%) cases. As mentioned above, 126 (27.2%) breast cancers showing gains in three or more CEP copy numbers were classified as the high-CIN group. The remaining 337 (72.8%) cancers were classified as the low-CIN group.

Figure 6. CEP copy number gain in fluorescence in situ hybridization and their frequencies in breast cancer. A bar chart showing frequencies of CEP1, CEP8, CEP11, and CEP16 copy number gain in breast cancer.



7. Association of CIN with clinicopathologic parameters including CEP17 copy number gain

High CIN correlated with well-known poor prognostic parameters, including the high T stage ($p = 0.007$), lymph node metastasis ($p = 0.010$), high histological grade ($p < 0.001$), lymphovascular invasion ($p = 0.010$), negative hormone receptor status ($p = 0.024$), positive HER2 status ($p < 0.001$), p53 overexpression ($p = 0.001$), and high Ki-67 index ($p < 0.001$). In addition to these acknowledged clinicopathologic factors, the CEP17 copy number gain was clearly associated with high CIN. The proportion of CEP17 copy number gain was significantly higher in high-CIN tumors than in low-CIN tumors (27.8% vs. 7.2%; $p < 0.001$) (**Table 10**).

In order to identify independent predictive factors for CIN, a multivariate logistic regression analysis was performed. Positive HER2 status ($p = 0.021$), high Ki-67 index ($p = 0.027$), and CEP17 copy number gain ($p < 0.001$) were found as independent predictors of high CIN. The odd ratios for positive HER2 status, high Ki-67 index, and CEP17 copy number gain were 1.930 (95% CI 1.105–3.372), 2.007 (95% CI 1.082–3.724), and 3.760 (95% CI 2.026–6.679), respectively (**Table 11**). This analysis demonstrated that CEP17 copy number gain is an independent strong predictor for high CIN.

Table 10. Correlations between chromosomal instability status and clinicopathologic characteristics

Clinicopathologic characteristics	Chromosomal instability		*p* value
	Low	High	
Age			0.266
<50 years	188 (55.8)	63 (50.0)	
≥50 years	149 (44.2)	63 (50.0)	
T stage			0.007
T1	157 (46.6)	41 (32.5)	
T2-4	180 (53.4)	85 (67.5)	
Lymph node metastasis			0.010
Absent	195 (57.9)	56 (44.4)	
Present	142 (42.1)	70 (55.6)	
Histologic grade			<0.001
I & II	185 (57.3)	47 (38.2)	
III	138 (42.7)	76 (61.8)	
Lymphovascular invasion			0.010
Absent	203 (60.2)	59 (46.8)	
Present	134 (39.8)	67 (53.2)	
Hormone receptor			0.024
Positive	245 (72.7)	78 (61.9)	
Negative	92 (27.3)	48 (38.1)	
HER2 status			<0.001
Negative	289 (85.8)	86 (68.3)	
Positive	48 (14.2)	40 (31.7)	
p53 overexpression			0.001
Absent	273 (81.0)	83 (66.4)	
Present	64 (19.0)	42 (33.6)	
Ki-67 index			<0.001
<20%	217 (64.4)	53 (42.1)	
≥20%	120 (35.6)	73 (57.9)	
CEP17 copy number gain			<0.001
Absent	310 (92.8)	91 (72.2)	
Present	24 (7.2)	35 (27.8)	

Table 11. Multivariate logistic regression analysis for predictors of high chromosomal instability

Variables	Odds ratio (95% CI)	*p* value
T stage (T1 vs. T2-4)	1.567 (0.969-2.535)	0.067
Lymph node metastasis (Absent vs. Present)	1.604 (0.965-2.668)	0.068
Histologic grade (I & II vs. III)	1.269 (0.683-2.357)	0.451
Lymphovascular invasion (Absent vs. Present)	1.190 (0.717-1.976)	0.501
Hormone receptor (Negative vs. Positive)	1.475 (0.811-2.681)	0.203
HER2 status (Negative vs. Positive)	1.930 (1.105-3.372)	0.021
p53 overexpression (Absent vs. Present)	1.548 (0.875-2.738)	0.134
Ki-67 index (<20% vs. ≥20%)	2.007 (1.082-3.724)	0.027
CEP17 copy number gain (Absent vs. Present)	3.760 (2.026-6.979)	<0.001

CI, confidence interval

8. Prognostic significance of CIN in breast cancer

In the next step, we assessed the prognostic significance of CIN in breast cancer. In the Kaplan–Meier survival analysis, the sum of the CEP copy number gains was significantly associated with disease-free survival and the clinical outcome of the patients became poorer as the sum of CEP copy number gains increased ($p = 0.008$; **Figure 7**). When dividing into high-CIN and low-CIN groups, the high-CIN group showed significantly shorter disease-free survival compared to low CIN group ($p = 0.002$; **Figure 8A**). In subgroup analyses by hormone receptor status, high CIN was associated with shortened disease-free survival time both in hormone receptor-positive and hormone receptor-negative subgroups ($p = 0.049$, $p = 0.035$, respectively; **Figure 8B & 8C**). With regards to breast cancer subtype, high CIN was associated with poor disease-free survival in luminal/HER2-negative and HER2-postive subtypes ($p = 0.038$, $p = 0.032$, respectively; **Figure 9A & 9B**). CIN status was not associated with survival of the patients in luminal/HER2-positive and triple-negative subtypes ($p = 0.555$, $p = 0.447$, respectively; **Figure 9C & 9D**).

Besides high CIN ($p = 0.002$), high T stage ($p = 0.012$), lymph-node metastasis ($p < 0.001$) and lymphovascular invasion ($p < 0.001$) were associated with poor disease-free survival of the patients in univariate analyses (**Table 12**). Negative hormone receptor status tended to be associated

with poor clinical outcome of the patients (p = 0.053). In multivariate analyses, lymph-node metastasis (hazard ratio, 2.528; 95% CI, 1.318-4.850; p = 0.005), lymphovascular invasion (hazard ratio, 2.037; 95% CI, 1.099-3.775; p = 0.024), negative hormone receptor status (hazard ratio, 2.002; 95% CI, 1.169-3.430; p = 0.011) and high CIN (hazard ratio, 1.813; 95% CI, 1.067-3.080; p = 0.028) were revealed as independent poor prognostic factors (**Table 12**).

Figure 7. Kaplan–Meier survival analysis according to the sum of the CEP copy number gains. Disease-free survival of the patients became poorer as the sum of CEP copy number gains increase. Survival difference is most distinct between sum of CEP copy number gain of 2 and 3.

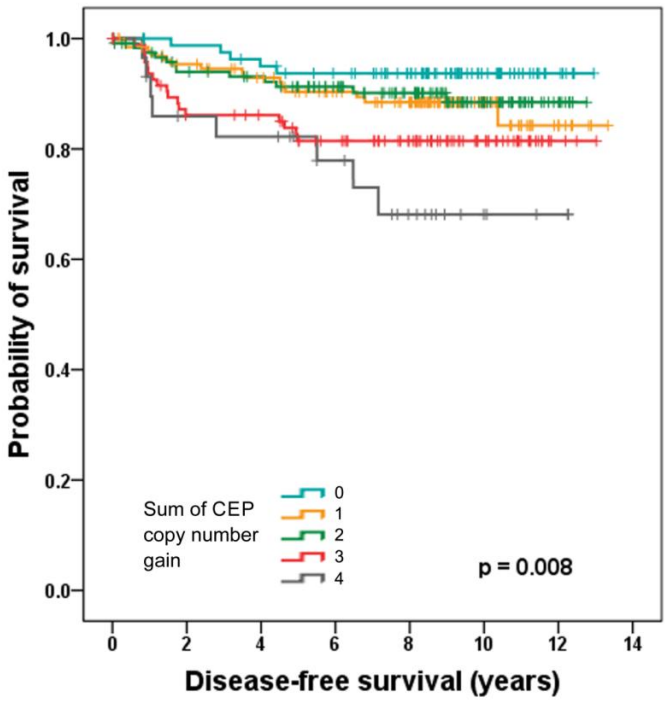


Figure 8. Kaplan-Meier survival analyses according to chromosomal instability status. High chromosomal instability (CIN) is a significant adverse prognostic factor in the whole group (A), in hormone receptor–positive tumors (B), and in the hormone receptor-negative tumors (C)

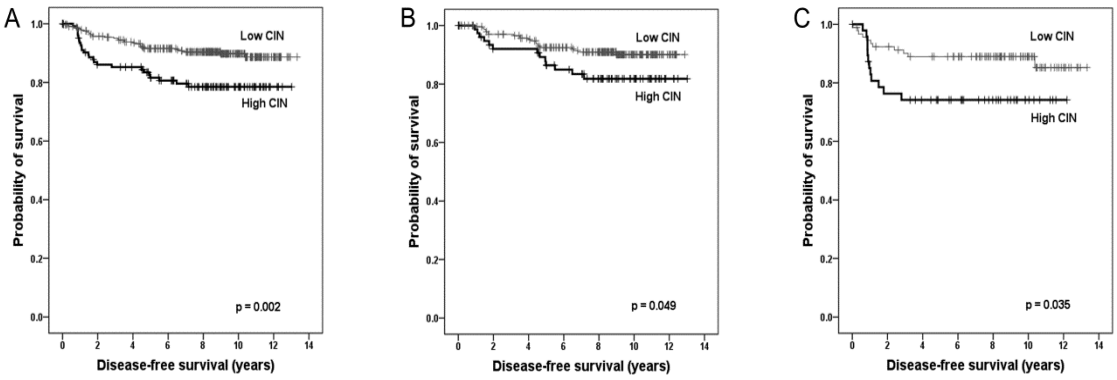


Figure 9. Kaplan-Meier survival analyses based on chromosomal instability status in breast cancer subtypes. Survival analyses in breast cancer subtypes shows that high chromosomal instability (CIN) is a significant poor prognostic factor in the luminal/HER2-negative (A) and HER2-positive subtypes (C), but it is not proven to be a prognostic factor in the luminal/HER2-positive (B) and triple-negative subtypes (D).

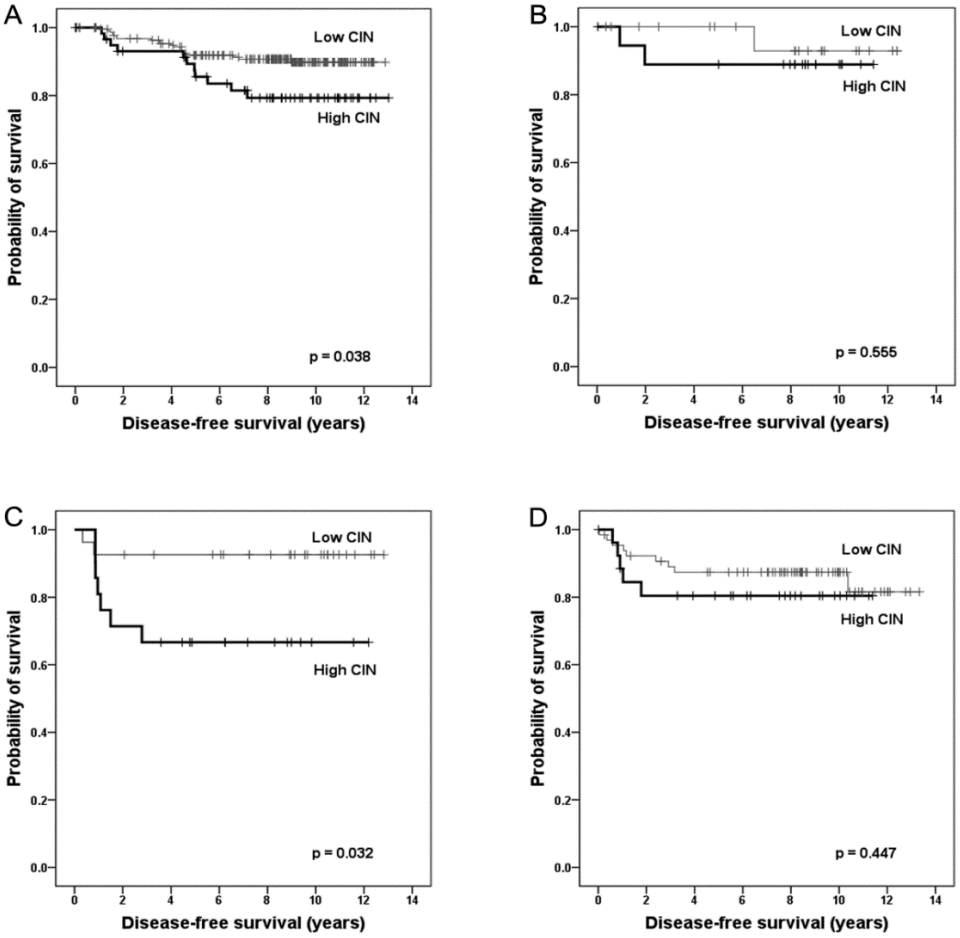


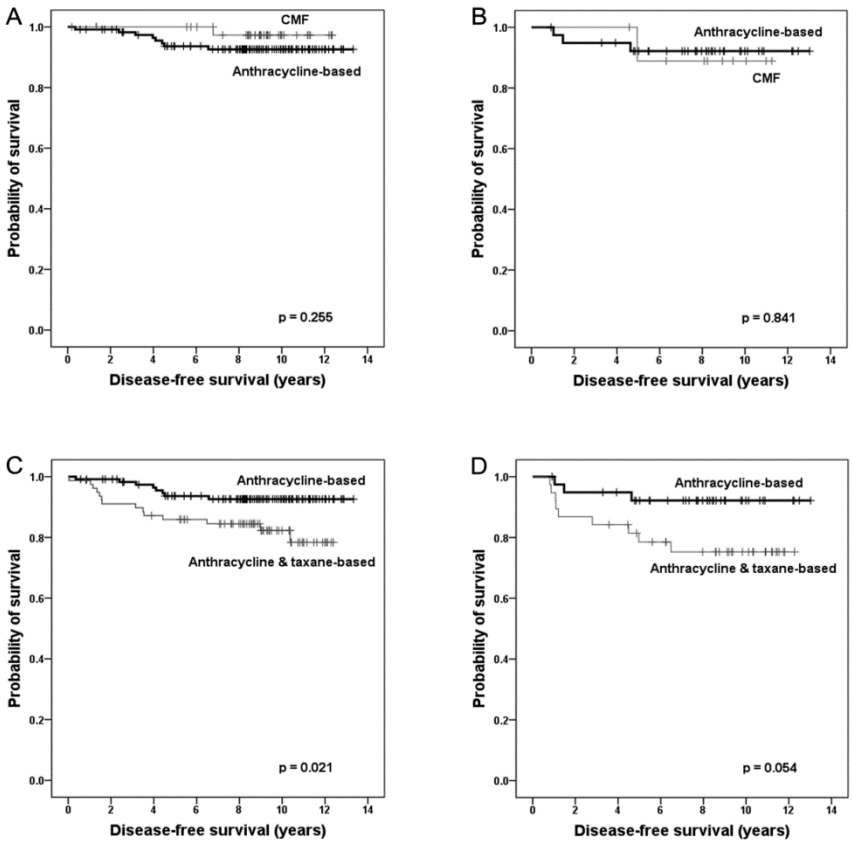
Table 12. Univariate and multivariate analyses of disease-free survival in the whole group

Variables	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*p* value	Hazard ratio (95% CI)	*p* value
Onset age (<50 years vs. ≥50 years)	0.908 (0.538-1.533)	0.719	-	-
T stage (T1 vs. T2-4)	2.137 (1.185-3.855)	0.012	1.358 (0.740-2.494)	0.323
Lymph node metastasis (Absent vs. Present)	3.402 (1.886-6.135)	<0.001	2.528 (1.318-4.850)	0.005
Histologic grade (I & II vs. III)	1.309 (0.770-2.226)	0.320	-	-
Lymphovascular invasion (Absent vs. Present)	2.904 (1.661-5.077)	<0.001	2.037 (1.099-3.775)	0.024
Hormone receptor (Positive vs. Negative)	1.687 (0.994-2.863)	0.053	2.002 (1.169-3.430)	0.011
HER2 status (Negative vs. Positive)	1.202 (0.636-2.272)	0.571	-	-
p53 overexpression (Absent vs. Present)	1.128 (0.616-2.065)	0.696	-	-
Ki-67 index (<20% vs ≥20%)	1.411 (0.840-2.373)	0.193	-	-
CEP17 copy number gain	1.546 (0.781-3.062)	0.211	-	-
CIN (Low vs. High)	2.270 (1.345-3.831)	0.002	1.813 (1.067-3.080)	0.028

9. Association of CIN with treatment response

Of the 463 patients, 36 (7.8%) patients received neoadjuvant chemotherapy, and 329 (71.1%) received adjuvant chemotherapy. Of the 329 patients treated by adjuvant chemotherapy, 158 (47.6%) received anthracycline-based chemotherapy, 117 received anthracycline & taxane-based chemotherapy, and 54 (16.3%) received CMF chemotherapy. To assess the predictive value of the CIN status on anthracycline or tanxane response, difference in disease-free survival according to different chemotherapeutic regimens was investigated among the patients who received adjuvant chemotherapy. However, disease-free survival did not differ between the patients treated with anthracycline-based chemotherapy and those treated with CMF chemotherapy in either the low-CIN or high-CIN group ($p = 0.255$, $p = 0.841$, respectively; **Figure 10A & 10B**). Moreover, clinical outcome was worse in patients treated with anthracycline & taxane-based chemotherapy than in those treated with anthracycline-based chemotherapy in low-CIN group and tended to be poor in high-CIN groups ($p = 0.021$, $p = 0.054$, respectively; **Figure 10C & 10D**)

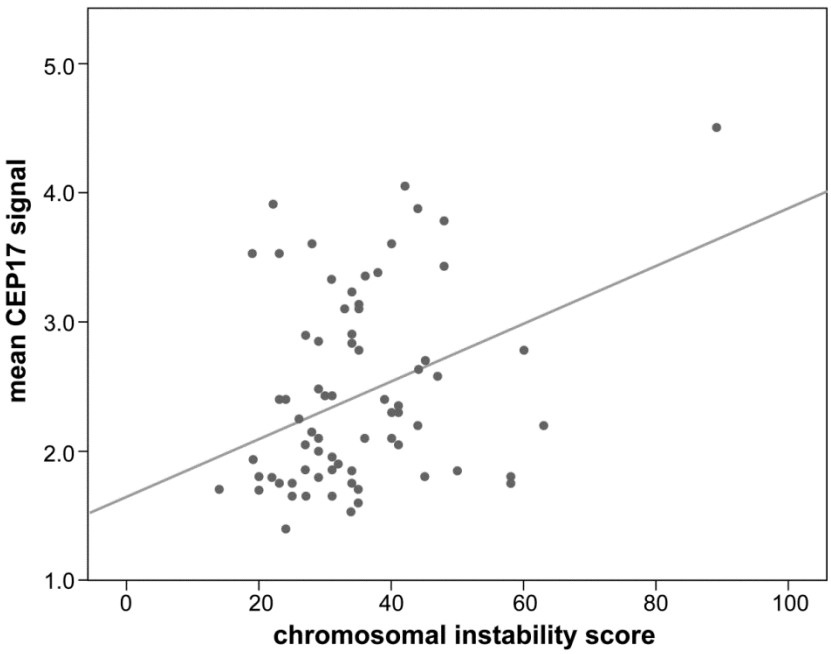
Figure 10. Kaplan–Meier survival analyses of disease-free survival according to chemotherapy regimens. Three are no differences in disease-free survivals between the patients treated with anthracycline-based chemotherapy and those treated with CMF chemotherapy in either the low-chromosomal instability (CIN) (A) or high-CIN group (B). Disease-free survival is poorer in patients treated with anthracycline & taxane-based chemotherapy than in those treated with anthracycline-based chemotherapy in low-CIN group (C), and tends to be poor in high CIN groups (D)



10. Correlation between the CIN score and the CEP17 copy number gain

Using a Z-score of the NGS data in the third set, the CIN scores were calculated from 14 to 89. The mean CEP17 copy number ranged from 1.15 to 4.5. To assess the association of CIN score with CEP17 copy number, tumors with CIN scores above the upper quartile were categorized as the high-CIN group and the remaining were categorized as the low-CIN group. The mean CEP17 copy number was higher in the high-CIN group than in the low-CIN group (2.87 ± 0.94 vs. 2.31 ± 0.65; $p = 0.028$). A simple regression analysis between the CIN score and the mean CEP17 signal was also used to confirm their correlation and a significant positive correlation ($\rho = 0.353$; $p = 0.003$) was found between the CIN score and the mean CEP17 copy number (**Figure 11**).

Figure 11. Correlation between the chromosomal instability score using NGS data and mean CEP17 copy number in the second set. A scatter dot plot shows a positive correlation ($\rho = 0.353$; $p = 0.003$).



DISCUSSION

Elevated CEP17 count is a genetic alteration frequently found in invasive breast cancers during ISH examination using dual-colored probes. One review article reported that the frequencies of CEP17 copy number gain ranged from 3% to 46% depending on the definition and specific subject of research (37). In our study, the proportion of CEP17 copy number gain including 18 CEP17 amplification cases was 29.9% (CEP17 ≥2.6) and 19.7% (CEP17 ≥3.0). CEP17 copy number gain was significantly associated with poor pathological parameters including high histologic grade, negative hormone receptor status, *HER2* amplification, p53 overexpression, and high Ki-67 proliferative index in the whole group, irrespective of the threshold. Similarly, in *HER2*-negative subgroup, CEP17 copy number gain showed an association with aggressive histologic variables including high T stage, high histologic grade, lymphovascular invasion, P53 overexpression, and high Ki-67 proliferative index. The association between CEP17 copy number gain and poor prognostic indicator is consistent with the results from previous studies on CEP17 alterations in breast cancers (14-16).

Breast cancer is a heterogeneous group harboring different molecular subtypes. We can readily classify breast cancers into three major molecular subgroups (*HER2*-positive, luminal and triple-negative subtypes) based on IHC results of ER, PR and *HER2* status (38, 39). These molecular subgroups

show distinct biologic behaviors with different prognoses. Moreover, standard treatment of breast cancer is significantly influenced by molecular subtypes. Although the correlation between CEP17 copy number gain and poor histologic parameters has been recognized, the prognostic significance of CEP17 alteration has not been clarified in breast cancer, especially within the different molecular subgroups. In this study, we evaluated the influence of CEP17 copy number gain on both disease-free and overall survival in each molecular subgroup. We found that CEP17 copy number gain was significantly associated with decreased disease-free survival only in luminal/*HER2*-negative subgroup. CEP17 copy number alteration did not affect disease-free survival in triple-negative and *HER2*-positive subgroups. A recent study has reported results similar to ours by showing that patients with CEP17 gain tumors in luminal B subgroup had intermediate survival between *HER2*-negative and *HER2*-positive breast cancer patients (40). The mechanism by which CEP17 copy number gain influences survival in luminal/*HER2*-negative type breast cancer patients is unknown. A possibility is that because luminal/*HER2*-negative subtype is known to have relatively fewer molecular alterations in contrast with triple-negative or *HER2*-positive subtypes (41), CEP17 copy number gain may reflect genetic instability, which in turn, may be related to poor clinical outcome. Subsequent correlation analysis in luminal/*HER2*-negative subtype revealed that CEP17 copy number gain was associated with most of the poor prognostic indicators of breast

cancer except for lymph node metastasis. From such results, we can infer that CEP17 copy number gain is a definite adverse prognostic factor in luminal/*HER2*-negatve subtype, and thus, CEP17 status may be utilized as an indicator for aggressive treatment and close follow-up.

In the present study, a substantial proportion of breast cancers with elevated CEP17 copy number showed increased HER2 protein expression. In *HER2*-negative breast cancers, HER2 IHC scores of 2 or 3 were observed in 55.9% (41/93) of cases with CEP17 copy number gain (CEP17 ≥3.0) while they were only found in 34.1% (218/640) of cases without CEP17 copy number gain. This effect of CEP17 copy number gain on increased HER2 protein expression raised the question whether *HER2*-targeted therapy would be beneficial in those cases. However, in one previous study, CEP17 copy number gain was associated with increased HER2 protein expression, and mRNA expression level was similar to that of *HER2*-negative tumors (42). Downey et al. reported that CEP17 copy number gain without *HER2* gene amplification was not associated with favorable response to additional *HER2*-targeted treatment in metastatic breast cancers (43). One study reported that trastuzumab benefit was not related to CEP17 gene copy number gain in an adjuvant setting (44). Considering that amplification or copy number gain of the pericentromeric region is the true cause of increased CEP17 copy number, *HER2*-directed therapy may not affect patients showing elevated CEP17 count without *HER2* gene amplification.

Previous studies have reported that CEP17 copy number gain was a strong predictor of response to anthracycline-based therapy. Bartlett et al. showed that CEP17 duplication (>1.86 signals per cell) was related to significant improvement in relapse-free and overall survival with anthracycline use in multivariate analyses (45). Tibau et al. showed that CEP17 copy number gain predicted pathologic complete response to primary anthracycline-based chemotherapy in 140 patients who had operable or locally advanced breast cancer (46). In this study, we also investigated the predictive value of CEP17 copy number gain in response to various chemotherapeutic regimens but did not demonstrate the association between CEP17 copy number gain and responsiveness to anthracycline-based chemotherapy. Although most patients received CMF, anthracycline-based or combined anthracycline- and taxane-based chemotherapy, the number of cases with CEP17 copy number gain and the number of events in each subgroup were quite small. Furthermore, the chemotherapeutic cycle was various and the chemotherapeutic agents were not exactly the same even though they belonged to the same class. In order to evaluate the predictive value of elevated CEP17 count further, more studies with well-designed clinical trials are required.

Using a unique measure of CIN with sum of CEP copy number gains, we showed that high CIN correlated significantly with aggressive clinicopathologic parameters, including high T stage, lymph-node metastasis,

high histological grade, lymphovascular invasion, negative hormone receptor status, positive HER2 status, p53 overexpression, and high Ki-67 index. The association between high CIN and aggressive clinicopathologic features of breast cancer is in line with the results from a previous study (47). More importantly, high CIN correlated strongly with CEP17 copy number gain. In multivariate logistic regression analysis, the CEP17 copy number gain was revealed as an independent predictor of high CIN with odd ratio of 3.760 (95% CI 2.026-6.979), which indicates an independent strong association between the CEP17 copy number gain and high CIN. To overcome the limitations in assessment of CIN by FISH, we calculated the CIN scores from NGS data in a small subset of breast cancers, using methods similar to those described previously (48, 49). We observed a higher mean CEP17 copy number in the high-CIN-score group than in the low-CIN-score group. We also identified a positive linear correlation between the mean CEP17 copy number and the CIN score. Consistent with this observation, a previous study reported an association between CEP17 copy number and CIN which was assessed using four CEPs (30). Based on these findings, we suggest that an increase in CEP17 copy number is a practical predictor of CIN in breast cancer.

In this study, we showed that the sum of CEP copy number gains correlated strongly with the prognoses of breast cancer patients. In an additional analysis of the dichotomized CIN status, the high-CIN group showed clearly poorer clinical outcomes than the low-CIN group. This result

is consistent with previous studies showing relationship between CIN and clinical outcome of the patients with breast cancer (25-27), although the methods for CIN measurement were different. While we determined CIN status using interphase-FISH with centromere probes, one study employed 'functional aneuploidy profile' from gene expression data (25), and other two studies used single nucleotide polymorphisms array for assessment of CIN (26, 27). In subgroup analysis, high CIN was revealed as a poor prognostic indicator in patients with the luminal/HER2-negative subtype. In the present study, although CEP17 copy number gain was not associated with clinical outcome of the patients in this subtype ($p = 0.114$; data not shown), probably due to small sample size, our data of first set have demonstrated that CEP17 copy number gain is a poor prognostic factor only in the luminal/HER2-negative subtype of breast cancer. This finding also supports that CEP17 copy number gain and CIN are closely related. Previous studies also have shown that CIN is associated with clinical outcome in luminal subtype of breast cancers (26, 27).

Our study also showed that high CIN is associated with a poor prognosis in the HER2-positive subtype of breast cancer. Smid et al. (27) also showed that CIN-score was significantly associated with prognosis in HER2-postive subtype. The reason why high CIN is associated with poor prognosis in HER2-postive subtype is not clear, but in the present study high CIN status correlated with lymph node metastasis and lymphovascular invasion in this

subtype ($p = 0.005$, $p = 0.011$, respectively; data not shown). Further studies would be needed to confirm the prognostic significance of CIN and its mechanism of action in HER2-postive breast cancer.

In contrast, we observed that CIN was not a relevant prognostic factor in triple-negative subtype. Triple-negative breast cancer is characterized by complex-pattern genomes and thus high CIN status (27, 50). High CIN generally leads to intratumoral heterogeneity, which allows tumor cells to avoid the immune system at the genetic level and leads to tumor progression (24). However, extremely high CIN which is found in a subset of triple-negative breast cancer can reduce tumor viability through activation of immune surveillance. A previous study showed that extreme CIN was associated with a better prognosis in ER-negative breast cancer patients (51). Triple-negative breast cancer is heterogeneous group of disease, and thus CIN would be quite variable, although within high level. Therefore, simple dichotomization of CIN into low or high CN groups would not provide prognostic information in triple-negative breast cancer patient.

Although results have been conflicting, several studies have reported that CIN can predict the responsiveness of breast cancer patients to specific chemotherapeutic agents (28-32). Those studies have shown that high CIN is associated with a favorable anthracycline response and taxane resistance. Since considerable patients received anthracycline-based chemotherapy,

anthracycline & taxane-based chemotherapy or CMF chemotherapy in this second cohort, the association between anthracycline or taxane responsiveness and CIN status was analyzed. However, in comparison with CMF chemotherapy, no predictive value of high CIN in response to anthracycline-based chemotherapy was found. Similarly, the association of high CIN with taxane resistance was not demonstrated in this study.

There were some limitations to this study. First, although the assessment of CIN status using CEP probes is accepted as an appropriate method, the limited number of CEP probes used in this study may have affected the accuracy of the CIN measurements. However, we selected chromosomes that are known to show frequent copy number gains in breast cancer to evaluate CIN. Second, we calculated the CIN scores with targeted sequencing data confined to 170 genes, which may also influence on the accuracy of CIN measurement. Finally, as a retrospective study, the patients were treated with various chemotherapeutic agents even within same classes of anthracycline or anthracycline & taxane-based chemotherapy. To validate our findings, studies with large numbers of samples in evenly treated patients are required.

CONCLUSION

We identified the association between CEP17 copy number gain and poor prognostic parameters. We also discovered the value of elevated CEP17 count as a prognostic marker in luminal/*HER2*-negative subtype of invasive breast cancer. Second, the degree of CIN was revealed as an independent prognostic factor for patients with breast cancer in a whole group, and high CIN was a meaningful prognostic indicator in several molecular subtypes of breast cancer. In particular, this study clearly demonstrated a strong positive correlation between the CEP17 copy number and CIN in breast cancer. As a tumor's CEP17 status is readily accessible with routine HER2 ISH testing, the CEP17 copy number gain can be used as a useful predictor of high CIN. In addition to the HER2 status, CEP17 status should be evaluated carefully and be reported in HER2 ISH report.

APPENDIX

A1. List of 170 cancer-related genes in targeted sequencing

ABL1	BCL2	CDKN1B	ERBB3	FLCN	JAK3	MEN1	NOTCH3	PPARG	SMAD4
ABL2	BRAF	CDKN2A	ERBB4	FLT1	KDR	MET	NOTCH4	PTCH1	SMARCA4
AKT1	BRCA1	CDKN2B	ERCC2	FLT3	KIT	MITF	NPM1	PTEN	SMARCB1
AKT2	BRCA2	CDKN2C	ERG	FLT4	KMT2A	MLH1	NRAS	RAB35	SMO
AKT3	BRD2	CEBPA	ERRFI1	FOXL2	KRAS	MPL	NTRK1	RAD50	SRC
ALK	BRD3	CHEK2	ESR1	GNA11	MAP2K1	MSH2	NTRK2	RAF1	STK11
APC	BRD4	CREBBP	ETV1	GNAQ	MAP2K2	MSH6	NTRK3	RARA	SYK
AR	CBFB	CRKL	ETV4	GNAS	MAP2K4	MTOR	NUTM1	RB1	TET2
ARAF	CCND1	CSF1R	ETV5	HDAC9	MAP3K1	MYC	PDGFB	RET	TMPRSS2
ASXL1	CCND2	CTNNB1	ETV6	HGF	MAP3K4	MYCN	PDGFRA	RHEB	TOP2A
ATM	CCND3	DDR1	EWSR1	HRAS	MAPK1	MYD88	PDGFRB	RICTOR	TP53
ATR	CCNE1	DDR2	EZH2	IDH1	MAPK3	NF1	PIK3CA	RNF43	TSC1
AURKA	CDH1	DNMT3A	FBXW7	IDH2	MAPK8	NF2	PIK3CB	ROS1	TSC2
AURKB	CDK12	DOT1L	FGFR1	IGF1R	MCL1	NFKBIA	PIK3CD	RSPO1	VHL
AURKC	CDK4	EGFR	FGFR2	IGF2	MDM2	NKX2-1	PIK3R1	RSPO2	WT1
AXL	CDK6	EPHA3	FGFR3	JAK1	MDM4	NOTCH1	PIK3R2	RUNX1	XPO1
BAP1	CDKN1A	ERBB2	FGFR4	JAK2	MED12	NOTCH2	POLE	SMAD2	ZNRF3

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국문 초록

서론: 유방암에서 *HER2* 제자리부합법 검사 과정 중 17 번 염색체 동원체 부위를 대상으로 한 chromosome enumeration probe (CEP)증가가 때때로 관찰된다. 이와 같은 현상은 17 번 염색체 동원체 주변 부위의 국소적 복제수 증가 혹은 증폭에 기인한 것으로 알려져 있다. 염색체 불안정성은 악성 고형성 종양에서 비정상 세포 분열로 인한 염색체 전체 혹은 일부의 손실 또는 증가를 초래하는 결함으로 정의된다. 이러한 유사한 두가지 유전자 이상은 유방암에서 예후 및 치료 반응과 관련이 있다고 알려져 있다. 이번 연구의 목적은 CEP17 복제수 증가의 임상병리학적 의의를 확인하는데 있다.

방법: 분당서울대학교병원에서 2004 년부터 2011 년까지 HER2 유전자의 형광제자리부합법 결과가 존재하는 945 례의 침윤성 유방암을 이용하여 CEP17 복제수 증가의 임상병리학적 소견 및 예후와의 연관성을 분석하였다. CEP17 복제수 증가와 염색체 불안정성과의 관계를 확인하기 위하여 다른 463 례의 유방암을 이용하여 CEP1, CEP8, CEP11 및 CEP16 의 형광제자리부합법 검사를 실시하여 염색체 불안정성의 정도를 측정하였다. 도출된 유방암 염색체 불안정성의 결과와 임상병리학적 소견 및 예후와의

관련성을 분석하였다. 마지막으로 71 례의 침윤성 유방암을 이용하여 차세대염기서열분석법을 적용하여 염색체 불안정성 점수를 도출하고 CEP17 복제수 증가와의 관련성을 분석하였다.

결과: 945 례의 침윤성 유방암 중 185 건에서 (19.7%) CEP17 복제수 증가가 (CEP17 ≥3.0) 확인되었다. 전체 유방암 집단 및 *HER2* 양성 그룹에서 CEP17 복제수 증가는 환자의 예후와 관련이 없었으나 *HER2* 음성 그룹에서 독립적인 나쁜 예후 인자로 밝혀졌다. 추가적인 분석에서 *HER2* 음성 및 호르몬 수용체 양성 그룹에서 CEP17 복제수 증가는 높은 병기, 나쁜 조직학적 등급, 림프관 침윤, P53 과발현 및 높은 Ki-67 지수 등의 조직학적 변인과 상관성이 있었고 독립적인 나쁜 예후 인자로 나타났다. 463 례의 두번째 유방암 코호트를 이용한 염색체 불안정성 측정에서 높은 염색체 불안정성이 나쁜 예후와 관련된 임상병리학적 인자와 상관성을 보였다. *HER2* 양성, 높은 Ki-67 지수 및 CEP17 복제수 증가가 염색체 불안정성의 독립적인 예측 인자로 확인되었다. 전체 유방암 그룹, *HER2* 양성 그룹과 호르몬을 발현하는 *HER2* 음성 그룹에서 높은 염색체 불안정성은 나쁜 예후와 관련성이 있었다. 반면에 염색체 불안정성과 anthracycline 및 taxane 제제의 화학요법에 대한 반응과의 관련성은 관찰되지

않았다. CEP17 복제수 증가는 높은 염색체 불안정성을 보이는 그룹에서 통계적으로 유의미하게 자주 관찰되었다. 71 례의 유방암을 이용하여 차세대염기서열분석법을 통해 각각의 유방암의 염색체 불안정성 지수를 도출하였고 염색체 불안정성 지수와 CEP17 복제수 평균값 사이에 선형 비례 관계를 확인하였다.

결론: 호르몬 수용체를 발현하는 *HER2* 음성 유방암 그룹에서 CEP17 복제수 증가는 독립적인 예후 인자임을 확인할 수 있었다. 높은 염색체 불안정성은 유방암에서 독립적인 예후 인자임을 확인하였다. 유방암에서 CEP17 복제수 증가는 유방암의 염색체 불안정성에 대한 유용한 예측 인자임을 확인할 수 있었다. 이러한 결과를 통해 *HER2* 제자리부합법 검사 결과에 *HER2* 뿐 아니라 CEP17 복제수에 대한 평가도 포함될 필요가 있다고 생각된다.

주요어: CEP17 복제수 증가, *HER*2, 유방암, 염색체 불안정성, 차세대염기서열분석법

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