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ORIGINAL ARTICLE

Comparison of HER2 amplification status among breast cancer subgroups offers new insights in pathways of breast cancer progression

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Abstract Although the prognostic and predictive significance of human epidermal growth factor receptor 2 (HER2) in invasive breast cancer is well established, its role in ductal carcinoma in situ (DCIS) remains unclear. Reports on combined evaluation of both HER2 protein expression and HER2 amplification status in pure DCIS and DCIS adjacent to invasive ductal carcinoma (i.e., admixed DCIS) are scarce. In this study, immunohistochemistry and fluorescence in situ hybridization (FISH) were used to assess HER2 status in 72 cases of pure DCIS, 73 cases of DCIS admixed with invasive ductal carcinoma (IDC), and 60 cases of pure IDC. HER2 copy number-based amplification was present in 49% of pure DCIS, 16% of admixed DCIS, 18% of admixed IDC, and 8% of pure IDC. Amplified pure DCIS with clusters of HER2 signals showed a significantly lower HER2 copy number than amplified admixed DCIS with clusters. Whereas pure DCIS and admixed DCIS presented significant differences, the in situ and invasive component of admixed tumors showed striking similarities regarding mean HER2 and chromosome 17 centromere (CEP17) copy number, grade, and estrogen and progesterone receptor expression. The discrepant prevalence of HER2 amplification among breast cancer subgroups indirectly suggests that HER2 may not play a crucial role in the transition of in situ to invasive breast cancer. The similarities in HER2 amplification status between the in situ and invasive component of admixed tumors hint at a common biological pathway for both components. Our data support the theory that pure DCIS, pure IDC, and admixed lesions have a common progenitor, but can progress as separate lineages.

Keywords Breast cancer progression · HER2 amplification · Clusters · HER2 protein overexpression · Ductal carcinoma in situ (DCIS)

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Abbreviations

CEP	Chromosome enumeration probe
DCIS	Ductal carcinoma in situ
ER	Estrogen receptor
FISH	Fluorescence in situ hybridization
HER2	Human epidermal growth factor receptor 2
IDC	Invasive ductal carcinoma
IHC	Immunohistochemistry
PR	Progesterone receptor

Introduction

The human epidermal growth factor receptor 2 (HER2) is a 185-kDa transmembrane tyrosine kinase receptor which has no known ligand of its own but preferentially dimerizes with

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other members of the HER family, where it subsequently acts as a co-receptor [4]. Although HER2 status is a well-established prognostic and predictive marker in invasive breast cancer [34], its role in ductal carcinoma in situ (DCIS) remains unclear. HER2 positivity is estimated to be present in 14% of invasive breast cancers, with a range of 7–27% as determined by a multicenter observational study [43]. This well-defined range indirectly serves as a quality control for laboratories that perform HER2 testing, since HER2 positivity rates below or above this range are hard to explain by biological factors [43]. Contrary to the narrow range in invasive breast cancer, reports on HER2 status in DCIS mention positivity rates ranging from 8 to 67% [5, 38, 49]. In most studies, immunohistochemistry was applied to investigate HER2 status. Hitherto, only three studies have been published in which HER2 amplification status was examined by fluorescence in situ hybridization (FISH) in a consecutive and unselected series of DCIS with and without invasive component [6, 18, 37]. Although FISH is considered to be a more robust technique than IHC [44], these studies still yielded conflicting results, with reported amplification rates ranging from 22.7 to 50% in pure DCIS, and a range of 23.8 to 30% in admixed DCIS. Unlike the vast majority of studies on DCIS, the authors of these three reports did separately evaluate pure DCIS and DCIS admixed with invasive ductal carcinoma (IDC), which will be further referred to as admixed DCIS and admixed IDC [6, 18, 37].

Besides obscurity concerning average HER2 positivity rates in DCIS, its prognostic significance in DCIS remains undefined as well. Because of ample contradictory evidence, it is currently unclear whether patients with HER2-positive DCIS manifest a worse outcome compared with HER2-negative DCIS. Some authors reported no association between HER2 status and recurrence risk in DCIS [3, 19–21, 36], whereas, others described a significant correlation between HER2 positivity and either increased in situ, invasive, or overall recurrence [13, 16, 35, 40, 52]. Despite these contradictory data, all studies are consistent in reporting that HER2-positive DCIS present more often high nuclear grade and extensive comedonecrosis compared with HER2-negative DCIS [36, 48, 51], which seems to allude to a more malignant phenotype. Further investigations will have to elucidate the exact role of HER2 in recurrence risk stratification of DCIS, as well as its predictive significance. Such a study is currently performed. The efficacy of postoperative trastuzumab in HER2-positive DCIS is assessed in the NSABP B-43 trial, which is now closed to accrual [47]. In this prospective randomized phase III clinical trial, radiotherapy is compared with radiotherapy plus trastuzumab in patients with HER2-positive DCIS treated with breast-conserving surgery [46, 47].

The importance of HER2 status in DCIS is likely to increase. Given the lack of in-depth analyses of HER2 amplification status in DCIS, we decided to perform an extensive comparative study on HER2 status in pure DCIS, the in situ and invasive components of admixed tumors, and pure invasive breast cancer (breast cancer of no special type, further referred to as invasive ductal carcinoma or IDC). To this purpose, both immunohistochemistry (IHC) and FISH were performed on series of consecutive cases. This study enabled us to assess HER2 positivity rates among breast cancer subgroups, as well as investigation of the prevalence of clusters of HER2 signals. Correlations between HER2 status and tumor grade were performed. Similarities between the in situ and invasive components of admixed tumors were investigated. These findings enabled us to ponder the different hypotheses on breast cancer progression pathways [29, 50].

Materials and methods

Patients, tissue samples, and morphological evaluation

The specimens included in this study originated from patients who consecutively underwent mastectomy or lumpectomy at the Ghent University Hospital and who did not receive neoadjuvant therapy. Due to the lower incidence of DCIS compared with invasive breast cancer, the time periods for patient selection were thus chosen to ensure that each group of tumors contained a similar number of cases, to allow subsequent statistical analysis. Cases of pure DCIS originated from 1 January 2007 to 1 January 2011 ($n = 72$). Admixed tumors were diagnosed between 1 April 2011 and 1 January 2012 ($n = 73$), and pure IDC were diagnosed between 1 January 2010 and 1 January 2012 ($n = 60$). Pure IDC was defined as pure invasive breast cancer (no special type, formerly known as invasive ductal carcinoma or IDC), whereas admixed tumors were defined as IDC admixed with a DCIS component of any size. Due to the limited cohort size, the group of admixed tumors was not further subdivided according to the size of the in situ component, as this would hamper the power of subsequent statistical analysis. This study was approved by the ethics committee of Ghent University Hospital.

All resection specimens were received freshly and fixed in 10% neutral-buffered formalin for 6–72 h after being sliced at 5-mm intervals, in line with the ASCO/CAP guidelines [26, 27]. Grading of DCIS and IDC was performed on archived H&E-stained slides as previously described [10, 45].

Immunohistochemistry

Stainings were performed on 4-μm-thick sections mounted on Superfrost plus slides (Menzel-Gläser, Germany) using an automatic immunostainer (Benchmark XT, Ventana Medical Systems, Arizona), according to the manufacturer's instructions. The rabbit monoclonal antibodies 4B5, SP1, and 1E2 (ready-to-use (RTU), Roche, Switzerland) were used for the detection of HER2, estrogen receptor (ER), and progesterone

receptor (PR), respectively. For all antibodies, visualization was achieved with the ultraView Universal DAB Detection kit (Ventana Medical Systems, Arizona). Heat-induced epitope retrieval was performed using Cell Conditioning 1 for ER and PR and Cell Conditioning 2 for 4B5. The HER2 expression was scored according to the 2013 update of the ASCO/CAP guidelines as 0/negative, 1+/negative, 2+/equivocal, or 3+/positive [53]. As we previously described, some lesions presented moderate incomplete membrane staining in <10% of tumor cells, which is not covered by the 2013 ASCO/CAP guidelines [23]. HER2 IHC score was recorded as a missing value for these rare cases. Expression of ER and PR was assessed according to the Allred score as described by Harvey et al. [14]. An Allred score ≥ 3 was considered positive in accordance with the ER and PR IHC ASCO/CAP guidelines [12].

FISH

After mounting of 4-μm-thick sections on Superfrost slides, 7 to 10 μl (dependent on the tissue size) of the HER2/neu and chromosome 17 centromere (CEP17) probe (PathVysion HER-2 DNA Probe Kit, Abbott, Illinois) was added. After denaturation during 5 min at 75 °C in a hot water bath, sections were hybridized overnight at 37 °C in an incubator. Ten microliter DAPI was added, followed by covering with a coverslip. Red (for *HER2*) and green (for CEP17) signals were assessed and non-overlapping signals were counted in 20 nuclei using a fluorescence microscope equipped with appropriate filters (Olympus BX40, Japan). According to the ASCO/CAP guidelines [53], 20 non-overlapping nuclei were counted, considering only intact nuclei and excluding nuclei without signals or signals of only one color. As previously described [22], the presence of clusters was noted. The mean *HER2* and CEP17 copy number was determined by dividing the total number of signals by the number of counted nuclei and the *HER2*/CEP17 ratio was calculated. We did not apply the ASCO/CAP algorithm for reflex HER2 testing in case of equivocal results [11, 54], as we aimed to assess the existence of a gene dosage effect [22]. Therefore, we regarded all IHC and FISH data as separate entities, which enabled us to correlate HER2 protein expression with the mean HER2 copy number. Since true polysomy of chromosome 17 is a very rare event, we disregarded HER2/CEP17 ratio as well [32] and defined amplification as an average HER2 copy number of ≥6.0.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics 21.0 software (Chicago, IL, USA). Continuous data were analyzed with Mann-Whitney or Kruskal-Wallis test and are reported as mean ± standard deviation. Categorical data were analyzed with chi square or Fisher's exact test when appropriate and are reported as *n* (percentage). Correlations between in situ and invasive components of admixed tumors were assessed with Spearman correlation. All mentioned statistical tests were two-sided, and $p < 0.05$ was considered statistically significant. In case of three or more groups, the level of significance was adjusted according to the Bonferroni correction when necessary.

Results

Tumor characteristics among the four subgroups according to amplification status

HER2 amplification status differed significantly among the four tumor groups ($p < 0.001$). As shown in Table 1, this was due to the high HER2 positivity rate of 49% in the pure DCIS group compared to 16% amplified admixed DCIS, 18% amplified admixed IDC, and only 8% amplified pure IDC. The in situ and invasive components of the admixed breast tumors presented quite similar HER2 amplification rates. The few discordant admixed cases will be more thoroughly discussed later on. There were no significant differences regarding grade distribution, ER, and PR expression among the four tumor groups (Table 1).

A strong correlation between amplification status and grade was seen only in pure DCIS and admixed DCIS (Table 2). Amplified DCIS presented more often with high grade. Of note, grading in the in situ lesions was based on nuclear atypia [45], whereas grading of invasive lesions was based on the Nottingham grading system (the Elston-Ellis modification of the Scarff-Bloom-Richardson grading system), which includes not only nuclear atypia, but also mitotic count and glandular differentiation as well [10]. Patient age at diagnosis was not associated with amplification status, regardless the tumor type (Table 2). Obviously, HER2 protein overexpression showed a strong correlation with amplification status in each tumor group ($p < 0.001$; Table 2).

Differences in mean HER2 and CEP17 copy number

Taking a closer look at HER2 amplification status, mean HER2 copy number, mean CEP17 copy number, and histopathological features of each group separately, we observed that the mean HER2 copy number correlated with grade only in the amplified pure DCIS lesions (Table 3). Within the group of amplified pure DCIS, high-grade lesions presented with a higher mean HER2 copy number ($p = 0.005$), and accordingly, with a higher mean HER2/CEP17 ratio ($p = 0.048$). This association was not observed in the non-amplified pure DCIS, nor in the other tumor subgroups. No differences in CEP17 copy number were noted among the tumor subgroups (Tables 3, 4, 5, and 6).

Table 1 Comparison of grade/differentiation, ER and PR expression, and amplification status among the four tumor subgroups

	Pure DCIS *n* (%)	In situ component mixed tumors *n* (%)	Invasive component mixed tumors *n* (%)	Pure IDC *n* (%)	*p* value*
Grade/differentiation					0.061
Low/well	2 (3)	10 (14)	13 (18)	3 (5)	
Intermediate/moderate	41 (57)	35 (48)	33 (45)	32 (53)	
High/poor	29 (40)	28 (38)	27 (37)	25 (42)	
ER					0.401
Negative	10 (14)	7 (10)	11 (15)	12 (20)	
Positive	62 (86)	66 (90)	62 (85)	48 (80)	
PR					0.754
Negative	14 (19)	12 (16)	11 (15)	13 (22)	
Positive	58 (81)	61 (84)	62 (85)	47 (78)	
HER2 IHC					**<0.001**
0	2 (3)	13 (19)	23 (32)	11 (19)	
1+	3 (4)	12 (17)	11 (16)	6 (10)	
2+	24 (34)	26 (37)	21 (30)	37 (63)	
3+	41 (59)	19 (27)	16 (23)	5 (8)	
Copy number-based HER2 amplification status					**<0.001**
Non-amplified	37 (51)	61 (84)	60 (82)	55 (92)	
Amplified	35 (49)	12 (16)	13 (18)	5 (8)	

Statistically significant *p*-values are shown in bold

ER estrogen receptor, *DCIS* ductal carcinoma in situ, *IDC* invasive ductal carcinoma, *IHC* immunohistochemistry, *PR* progesterone receptor

*Chi square or Fisher's exact test

The gene dosage effect

The mean HER2 copy number correlated with the HER2 IHC score in the non-amplified lesions of the in situ ($p = 0.005$) and invasive components ($p = 0.001$) of admixed tumors, but not in the other non-amplified tumor subgroups, although there was a tendency towards significance within the non-amplified pure DCIS group. The higher the HER2 IHC score, the higher the

Table 2 Associations between copy number-based HER2 amplification status and pathological and immunohistochemical features for each tumor subgroup

	Non-amplified *n* (%) or mean ± SD	Amplified *n* (%) or mean ± SD	*p* value*	Non-amplified *n* (%) or mean ± SD	Amplified *n* (%) or mean ± SD	*p* value*
	Pure DCIS			In situ component mixed tumor		
Age (years)	54.8 ± 10.7	54.6 ± 10.4	0.748	56.0 ± 11.9	53.4 ± 10.6	0.618
Grade			**<0.001**			**0.002**
Low	2 (5)	0 (0)		10 (16)	0 (0)	
Intermediate	29 (78)	12 (34)		33 (54)	2 (17)	
High	6 (16)	23 (66)		18 (30)	10 (83)	
HER2 IHC			**<0.001**			**<0.001**
0	2 (6)	0 (0)		13 (22)	0 (0)	
1+	3 (8)	0 (0)		12 (21)	0 (0)	
2+	24 (67)	0 (0)		26 (45)	0 (0)	
3+	7 (19)	34 (100)		7 (12)	12 (100)	
	Pure IDC			Invasive component mixed tumor		
Age (years)	60.8 ± 14.1	60.2 ± 14.9	0.809	56.0 ± 12.1	53.2 ± 9.7	0.479
Differentiation			0.728			0.218
Well	3 (6)	0 (0)		13 (22)	0 (0)	
Moderate	30 (55)	2 (40)		26 (43)	7 (54)	
Poor	22 (40)	3 (60)		21 (35)	6 (46)	
HER2 IHC			**<0.001**			**<0.001**
0	11 (20)	0 (0)		23 (40)	0 (0)	
1+	6 (11)	0 (0)		11 (19)	0 (0)	
2+	37 (69)	0 (0)		19 (33)	2 (15)	
3+	0 (0)	5 (100)		5 (8)	11 (85)	

Significant *p* values are shown in bold

*Mann-Whitney *U* test or chi square or Fisher's exact test when appropriate

DCIS ductal carcinoma in situ, *IDC* invasive ductal carcinoma, *IHC* immunohistochemistry, *SD* standard deviation

Table 3 Grade and HER2 immunohistochemical scores in relation to HER2/CEP17 ratio and mean HER2 and CEP17 copy number for pure DCIS, according to copy number-based HER2 amplification status

	HER2/CEP17 ratio	*p* value*	Mean HER2 copy number	*p* value*	Mean CEP17 copy number	*p* value*
Non-amplified						
Grade		0.532		0.267		0.604
Low	1.18 ± 0.14		2.65 ± 0.14		2.28 ± 0.39	
Intermediate	1.45 ± 0.58		3.73 ± 1.04		2.67 ± 0.63	
High	1.24 ± 0.20		3.18 ± 0.92		2.55 ± 0.50	
HER2 IHC		0.067		0.035		0.981
0	1.15 ± 0.09		3.10 ± 1.06		2.70 ± 0.71	
1+	1.15 ± 0.06		2.95 ± 0.45		2.57 ± 0.50	
2+	1.31 ± 0.30		3.42 ± 0.95		2.63 ± 0.59	
3+	1.97 ± 0.90		4.68 ± 0.86		2.65 ± 0.78	
AMPLIFIED						
Grade		**0.048**		**0.005**		0.444
Low	–		–		–	
Intermediate	6.37 ± 2.16		14.01 ± 5.34		2.28 ± 0.66	
High	8.19 ± 3.21		19.90 ± 5.49		2.47 ± 0.69	
HER2 IHC		–		–		–
0	–		–		–	
1+	–		–		–	
2+	–		–		–	
3+	7.72 ± 2.88		18.22 ± 5.81		2.39 ± 0.68	
HER2 clusters		0.092		0.092		0.457
Absent	2.22[a]		6.35[a]		2.85[a]	
Present	7.72 ± 2.88		18.22 ± 5.81		2.39 ± 0.68	

Values are mean ± SD. Significant *p* values are shown in bold, with adapted significance level $\alpha = 0.01667$ (for 3 groups) or $\alpha = 0.00833$ (for 4 groups) according to Bonferroni correction

DCIS ductal carcinoma in situ, *IDC* invasive ductal carcinoma, *IHC* immunohistochemistry, *SD* standard deviation

*Mann-Whitney test or Kruskal-Wallis test

[a] HER2/CEP17 ratio and HER2 and CEP17 copy numbers are constant due to a single case

mean HER2 copy number. We previously designated this phenomenon the "gene dosage effect" [22]. The gene dosage effect could not be detected in the amplified groups, because the majority of these lesions presents with a 3+ HER2 IHC score (Tables 3, 4, 5, and 6). The mean CEP17 copy number did not correlate with the HER2 IHC score, regardless the amplification status or the tumor type.

Copy numbers and clusters among tumor subgroups

Table 7 illustrates the comparison of mean HER2 and CEP17 copy numbers, and HER2/CEP17 ratio among the four different tumor groups. Among the non-amplified lesions, a higher HER2 and CEP17 copy number was observed in the pure DCIS group ($p < 0.001$ each), whereas there were no significant differences between the three other groups. The mean CEP17 copy number did not differ between the different tumor groups among the amplified lesions. The mean HER2 copy number was significantly lower in amplified pure DCIS with clusters when compared with amplified admixed DCIS with clusters; i.e., amplified pure DCIS presented with smaller clusters of HER2 signals than amplified admixed DCIS (Table 7; Fig. 1).

The presence of clusters of HER2 signals differed somehow among the breast tumor subgroups. Clusters of HER2 signals were noted in 35 of 36 amplified pure DCIS (97%), in 9 of 12 amplified admixed DCIS (75%), in 9 of 14 amplified admixed IDC (64%), and in 4 of 5 amplified pure IDC (80%). Generally, amplified lesions without clusters of HER2 signals presented a mean HER2 copy number close to the cutoff value of 6, i.e., "low-level" amplification (Tables 3, 4, 5, and 6).

Concordance within admixed breast tumors

Both components of non-amplified admixed tumors showed a significant correlation for HER2 copy number, CEP17 copy number, HER2/CEP17 ratio, grade, ER and PR expression, and HER2 IHC score (Table 8). Figure 2 illustrates the proportional correlation between both components of non-amplified admixed tumors regarding CEP17 copy number and HER2 copy number. In amplified admixed lesions, both components displayed a significant correlation for all variables except for the tumor grade, HER2 copy number, and presence of clusters of HER2 signals. Only three admixed cases showed discordant HER2 amplification status between the in situ and invasive components. However, each discordant admixed case presented with a mean HER2 copy number

Table 4 Grade and HER2 immunohistochemical scores in relation to HER2/CEP17 ratio and mean HER2 and CEP17 copy number for the in situ component of admixed tumors, according to copy number-based HER2 amplification status

	HER2/CEP17 ratio	*p* value*	Mean HER2 copy number	*p* value*	Mean CEP17 copy number	*p* value*
Non-amplified						
Grade		0.283		0.165		0.623
Low	1.08 ± 0.11		2.11 ± 0.21		1.96 ± 0.16	
Intermediate	1.18 ± 0.25		2.49 ± 0.73		2.08 ± 0.44	
High	1.23 ± 0.27		2.65 ± 1.12		2.13 ± 0.63	
HER2 IHC		0.186		**0.005**[a]		0.154
0	1.12 ± 0.06		2.07 ± 0.37		1.85 ± 0.34	
1+	1.09 ± 0.13		2.30 ± 0.59		2.10 ± 0.33	
2+	1.22 ± 0.23		2.63 ± 0.89		2.11 ± 0.51	
3+	1.29 ± 0.53		3.11 ± 1.22		2.41 ± 0.68	
Amplified						
Grade		0.519		0.667		0.162
Low	–		–		–	
Intermediate	11.64 ± 1.67		20.78 ± 0.53		1.80 ± 0.21	
High	11.62 ± 9.51		26.53 ± 16.84		2.62 ± 1.06	
HER2 IHC		–		–		–
0	–		–		–	
1+	–		–		–	
2+	–		–		–	
3+	11.63 ± 8.62		25.57 ± 15.40		2.48 ± 1.01	
HER2 clusters		**0.021**		**0.021**		0.266
Absent	4.14 ± 2.48		10.73 ± 7.01		2.60 ± 0.61	
Present	14.12 ± 8.52		30.51 ± 14.37		2.44 ± 1.14	

Values are mean ± SD. Significant *p* values are shown in bold, with adapted significance level $\alpha = 0.01667$ (for 3 groups) or $\alpha = 0.00833$ (for 4 groups) according to Bonferroni correction

DCIS ductal carcinoma in situ, *IDC* invasive ductal carcinoma, *IHC* immunohistochemistry, *SD* standard deviation

*Mann-Whitney test or Kruskal-Wallis test

[a] *p* value <0.00833 due to statistically significant difference between score 0 and score 3+ and between score 0 and score 2+

close to the cutoff value of 6.00. No pronounced discrepancies in mean HER2 copy number were observed. These three discordant cases were omitted from the analyses shown in Table 8.

Discussion

In this study, we examined the HER2 status both at the gene level and the protein level which enabled an in-depth comparison of four tumor subgroups. We noticed a strong concordance between amplification status and protein expression as quantified by the IHC score, which is in agreement with previously published results [22]. The non-amplified in situ and invasive components of admixed tumors presented a gene dosage effect, in which the IHC score proportionally correlated with the mean HER2 copy number. The amplified lesions in each subgroup did not display the gene dosage effect, as nearly all lesions presented a 3+ HER2 IHC score. The gene dosage effect was not present in non-amplified pure DCIS and non-amplified pure IDC: this might be attributed to the 2013 update of the ASCO/CAP guidelines for HER2 testing, as our previous results were based on the 2007 ASCO/CAP guidelines [22]. The changes in the ASCO/CAP guidelines had a considerable impact on the assessment of HER2-negative (i.e., IHC scores 0 and 1+) breast cancers [53]. Before the 2013 update of ASCO/CAP guidelines, we were able to distinguish a group of score 0 tumors that had a significantly lower HER2 copy number than the group of score 1+ tumors [24]. This apparent difference in mean HER2 copy number was lost upon application of the updated guidelines [23].

Shortly after the discovery of the HER2 receptor and its association with poor clinical outcome in invasive breast cancer, several researchers reported that HER2 protein overexpression was much more common in pure DCIS than in invasive breast cancer [1, 2]. Although it was speculated that HER2 might not play an important role in the transition from in situ to invasive breast cancer, at that time, no satisfactory explanation was found to account for this enigmatic paradox. Provided that DCIS is an obligate progenitor lesion of IDC, one would expect that HER2-positive DCIS are more likely to progress to IDC, which should result in a higher rate of HER2-positive IDC cases than is currently diagnosed.

We observed HER2 copy number-based amplification in 49% of pure DCIS, in 16% of admixed DCIS, in 18% of admixed IDC, and in 8% of pure IDC. These differences in amplification rates between the subgroups are in agreement with the previously reported differences in HER2 overexpression as measured by IHC at the protein level [1, 2]. The

Table 5 Grade and HER2 immunohistochemical scores in relation to HER2/CEP17 ratio and mean HER2 and CEP17 copy number for the invasive component of admixed tumors, according to copy number-based HER2 amplification status

	HER2/ CEP17 ratio	*p* value*	Mean HER2 copy number	*p* value*	Mean CEP17 copy number	*p* value*
Non-amplified						
Differentiation		0.040		0.191		0.710
Well	1.09 ± 0.08		2.24 ± 0.45		2.05 ± 0.32	
Moderate	1.14 ± 0.09		2.48 ± 0.64		2.16 ± 0.50	
Poor	1.27 ± 0.29		2.79 ± 1.01		2.15 ± 0.37	
HER2 IHC		0.018		**0.001**[a]		**0.008**[a]
0	1.13 ± 0.13		2.28 ± 0.44		2.00 ± 0.28	
1+	1.10 ± 0.05		2.13 ± 0.35		1.94 ± 0.28	
2+	1.20 ± 0.13		2.81 ± 0.79		2.33 ± 0.55	
3+	1.51 ± 0.45		3.62 ± 1.40		2.37 ± 0.27	
Amplified						
Differentiation		0.886		0.721		0.351
Well	–		–		–	
Moderate	9.12 ± 3.82		21.96 ± 9.32		2.49 ± 0.80	
Poor	11.51 ± 10.46		22.32 ± 17.31		2.18 ± 0.58	
HER2 IHC		0.076		0.060		0.843
0	–		–		–	
1+	–		–		–	
2+	2.94 ± 0.30		6.20 ± 0.07		2.13 ± 0.25	
3+	11.55 ± 7.27		25.02 ± 11.92		2.38 ± 0.76	
HER2 clusters		**0.021**		**0.013**		0.938
Absent	4.43 ± 3.20		9.13 ± 5.88		2.11 ± 0.18	
Present	12.80 ± 7.32		27.91 ± 10.84		2.45 ± 0.83	

Values are mean ± SD. Significant *p* values are shown in bold, with adapted significance level $\alpha = 0.01667$ (for 3 groups) or $\alpha = 0.00833$ (for 4 groups) according to Bonferroni correction

DCIS ductal carcinoma in situ, *IDC* invasive ductal carcinoma, *IHC* immunohistochemistry, *SD* standard deviation

*Mann-Whitney test or Kruskal-Wallis test

[a] $p < 0.00833$ due to statistically significant differences between scores 0 and 3+, between scores 1+ and 3+, between scores 0 and 2+, and between scores 1+ and 2+

Table 6 Grade and HER2 immunohistochemical scores in relation to HER2/CEP17 ratio and mean HER2 and CEP17 copy number for pure IDC, according to copy number-based HER2 amplification status

	HER2/ CEP17 ratio	*p* value*	Mean HER2 copy number	*p* value*	Mean CEP17 copy number	*p* value*
Non-amplified						
Differentiation		0.276		0.065		0.093
Well	1.02 ± 0.11		1.80 ± 0.22		1.77 ± 0.06	
Moderate	1.14 ± 0.13		2.52 ± 0.87		2.18 ± 0.53	
Poor	1.23 ± 0.36		2.63 ± 0.73		2.25 ± 0.47	
HER2 IHC		0.702		0.801		0.731
0	1.12 ± 0.11		2.26 ± 0.34		2.04 ± 0.25	
1+	1.34 ± 0.45		2.66 ± 1.12		2.19 ± 0.53	
2+	1.16 ± 0.23		2.58 ± 0.86		2.21 ± 0.53	
3+	–		–		–	
Amplified						
Differentiation		0.564		0.564		0.564
Well	–		–		–	
Moderate	8.66 ± 2.16		23.70 ± 6.79		2.73 ± 0.11	
Poor	11.32 ± 4.70		27.37 ± 6.44		2.73 ± 1.29	
HER2 IHC		–		–		–
0	–		–		–	
1+	–		–		–	
2+	–		–		–	
3+	10.26 ± 3.79		25.90 ± 6.02		2.73 ± 0.91	
HER2 clusters		–		–		–
Absent	15.80[a]		28.50[a]		2.20[a]	
Present	8.87 ± 2.52		23.88 ± 4.58		2.86 ± 0.99	

Values are mean ± SD. Significant *p* values are shown in bold, with adapted significance level $\alpha = 0.01667$ (for 3 groups) or $\alpha = 0.00833$ (for 4 groups) according to Bonferroni correction

*Mann-Whitney test or Kruskal-Wallis test

[a] HER2/CEP17 ratio and HER2 and CEP17 copy numbers are constant due to a single case

DCIS ductal carcinoma in situ, *IDC* invasive ductal carcinoma, *IHC* immunohistochemistry, *SD* standard deviation

Table 7 Comparison of mean HER2 and CEP17 copy numbers, and HER2/CEP17 ratio among the four tumor subgroups, according to copy number-based HER2 amplification status

	Pure DCIS mean ± SD	In situ component mixed tumors mean ± SD	Invasive component mixed tumors mean ± SD	Pure IDC mean ± SD	*p* value*
Non-amplified lesions					
HER2/CEP17 ratio	1.40 ± 0.52	1.18 ± 0.24	1.19 ± 0.19	1.17 ± 0.25	**0.008**[a,c]
HER2 copy number	3.59 ± 1.03	2.48 ± 0.82	2.54 ± 0.78	2.53 ± 0.80	**<0.001**[a,b,c]
CEP17 copy number	2.63 ± 0.60	2.08 ± 0.47	2.13 ± 0.42	2.19 ± 0.50	**<0.001**[a,b,c]
Amplified lesions with or without clusters					
HER2/CEP17 ratio	7.56 ± 2.99	11.63 ± 8.62	10.22 ± 7.38	10.26 ± 3.79	0.315
HER2 copy number	17.89 ± 6.06	25.57 ± 15.40	22.13 ± 12.97	25.90 ± 6.02	0.160
CEP17 copy number	2.41 ± 0.68	2.48 ± 1.00	2.34 ± 0.70	2.73 ± 0.91	0.809
Amplified lesions with clusters					
HER2/CEP17 ratio	7.59 ± 2.94	14.12 ± 8.52	12.80 ± 7.32	8.87 ± 2.52	0.019
HER2 copy number	17.87 ± 6.09	30.51 ± 14.27	27.91 ± 10.84	23.88 ± 4.58	**0.004**[a]
CEP17 copy number	2.38 ± 0.68	2.44 ± 1.14	2.45 ± 0.83	2.86 ± 0.99	0.751

Significant *p* values are shown in bold, with adapted significance level $\alpha = 0.00833$ (for 4 groups) according to Bonferroni correction

CEP17 chromosome 17 centromere, *DCIS* ductal carcinoma in situ, *IDC* invasive ductal carcinoma, *HER2* human epidermal growth factor receptor 2, *SD* standard deviation

*Kruskal-Wallis test

[a] $p < 0.00833$ due to significant difference between pure DCIS and in situ component of admixed tumors

[b] $p < 0.00833$ due to significant difference between pure DCIS and invasive of component admixed tumors

[c] $p < 0.00833$ due to significant difference between pure DCIS and pure IDC

assumption that HER2 is an overt adverse prognostic marker in DCIS is clearly not reflected in the number of HER2-positive admixed IDC. Several studies showed that HER2 positivity was not associated with increased recurrence risk in pure DCIS [3, 19–21, 36]. Moreover, HER2 overexpression in pure DCIS diagnosed by needle biopsy was not associated with an increased risk for upstaging towards invasive carcinoma in the subsequent resection specimen [8].

In agreement with previous studies [1, 2, 6, 18, 37], we observed a high concordance between the in situ and invasive components of admixed tumors regarding amplification status, tumor grade, ER, PR, and HER2 expression. Overall, there were no significant differences in mean HER2 and mean CEP17 copy number within admixed lesions. The presence of clusters of HER2 signals differed slightly among the breast tumor subgroups, although these data might be influenced because of the limited number of amplified lesions in each tumor subgroup. It would be interesting to assess the prevalence and size of clusters of HER2 signals in larger breast cancer cohorts. There were only three admixed lesions with discordant amplification status, and all of these showed a mean HER2 copy number close to the cutoff value of 6.00 (i.e., low-level amplification).

These findings suggest that the invasive component is either derived from the associated in situ component or that both components have a common progenitor and evolve in parallel. This hypothesis is further corroborated by gene expression studies, which did not find significant differences in gene expression profiles between the epithelial compartments of in situ and invasive components of admixed lesions [7, 25, 31]. However, the stromal compartments of admixed in situ and invasive components do show a significant number of differentially expressed genes, which suggests that the tumor microenvironment plays a rather important role in the transition from in situ to invasive breast cancer [7, 31, 42]. Studies using array comparative genomic hybridization (aCGH) demonstrated that the in situ and invasive components of admixed IDC are genomically similar, whereas pure DCIS displayed distinct genomic alterations compared with admixed DCIS [17]. Hernandez et al. confirmed the extensive similarities of the genomic profiles of synchronous admixed DCIS and IDC, but they postulated that a specific repertoire of genetic anomalies drives the selection of non-modal clones in some cases of admixed breast cancer [15]. A recent meta-analysis demonstrated that pure DCIS and admixed DCIS show significant differences in the distribution and number of somatic copy number aberrations, whereas admixed DCIS and admixed IDC do not show significant differences [41].

We observed a similar phenomenon by studying the mean HER2 copy number. Amplified pure DCIS presented a

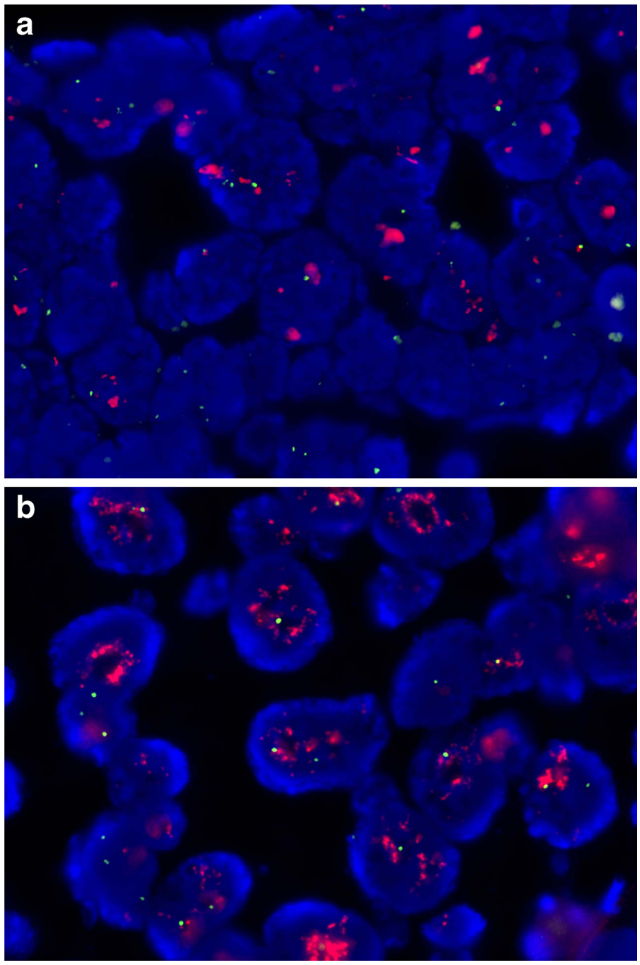

Fig. 1 Photomicrographs of dual-probe FISH analysis (×1000 original magnification). *Red signals* represent a copy of the HER2 gene. The HER2 signals are arranged in clusters (**a**, **b**). *Green signals* signify the location of the CEP17 probe. Pure DCIS lesions present with significantly smaller clusters (**a**) than the in situ components of admixed breast tumors (**b**)

Table 8 Correlation of HER2/CEP17 ratio and mean HER2 and CEP17 copy numbers between the in situ and invasive component of admixed tumors[a]

	Spearman's rho	*p* value*
Both components non-amplified (n = 59)		
HER2/CEP17 ratio	0.372	**0.004**
HER2 copy number	0.493	**<0.001**
CEP17 copy number	0.321	**0.013**
Grade/differentiation	0.622	**<0.001**
ER allred score	0.546	**<0.001**
PR allred score	0.666	**<0.001**
HER2 IHC	0.469	**<0.001**
Both components amplified (n = 11)		
HER2/CEP17 ratio	0.927	**<0.001**
HER2 copy number	0.573	0.066
CEP17 copy number	0.712	**0.014**
Clusters of HER2 signals	0.389	0.237
Grade/differentiation	0.356	0.282
ER allred score	0.820	**0.002**
PR allred score	0.922	**<0.001**
HER2 IHC	_[b]	_[b]

Significant *p* values are shown in bold

*Spearman correlation coefficient

[a] Three lesions with discordant amplification status of both components were omitted from these analyses

[b] Spearman's correlation coefficient cannot be computed because all lesions have 3+ IHC

significantly lower mean HER2 copy number when compared with amplified admixed DCIS. The lower HER2 copy number was reflected in smaller clusters of HER2 signals. We believe that admixed DCIS and the majority of pure DCIS lesions are distinct entities. A subpopulation within the group of pure DCIS lesions will have the potential to evolve towards invasive cancer, and it will remain a challenge for future scientific studies to discern adequate prognostic markers, enabling us to identify these lesions with a propensity for invasive growth. During this search, the role of the tumor microenvironment should not be overlooked [52].

The high prevalence of HER2 positivity in the pure DCIS group, which triples the prevalence of HER2 positivity in admixed DCIS, suggests that HER2 does not play a crucial role in the transition towards invasive carcinoma. We hypothesize that HER2 functions as a driver of proliferation: this clarifies its high prevalence within pure DCIS, which is an intraductal clonal expansion of aberrant epithelial cells. The strong association between HER2 positivity and the presence of comedonecrosis in pure DCIS is probably due to the fast growth rate of the lesion, which usurps tumor oxygenation and causes necrosis of tumor cells [51].

Several in vitro findings corroborate the theory that HER2 acts as a driver of proliferation instead of being an instigator of invasion. Muthuswamy et al. described that HER2 homodimer activation caused cell proliferation without invasion in an in vitro model of mammary acini cultured in three-dimensional basement membrane gels [33]. Leung and Brugge performed an elegant experiment, in which HER2 expression was induced in a single cell within an organotypic mammary acinus model [28]. This in vitro HER2 overexpression leads to translocation of the activated single cell towards the luminal epithelial layer, with luminal outgrowth resembling pure DCIS in humans [28]. Lu et al. reported that overexpression of HER2 in the MCF10A mammary epithelial cell line resulted in clonal expansion only when grown in three-dimensional matrigel, whereas co-expression of both HER2 and 14-3-3ζ was required to cause invasion in the surrounding matrix [30]. Pradeep et al. proposed a two-hit model for invasive growth, based on in vitro experiments with HER2

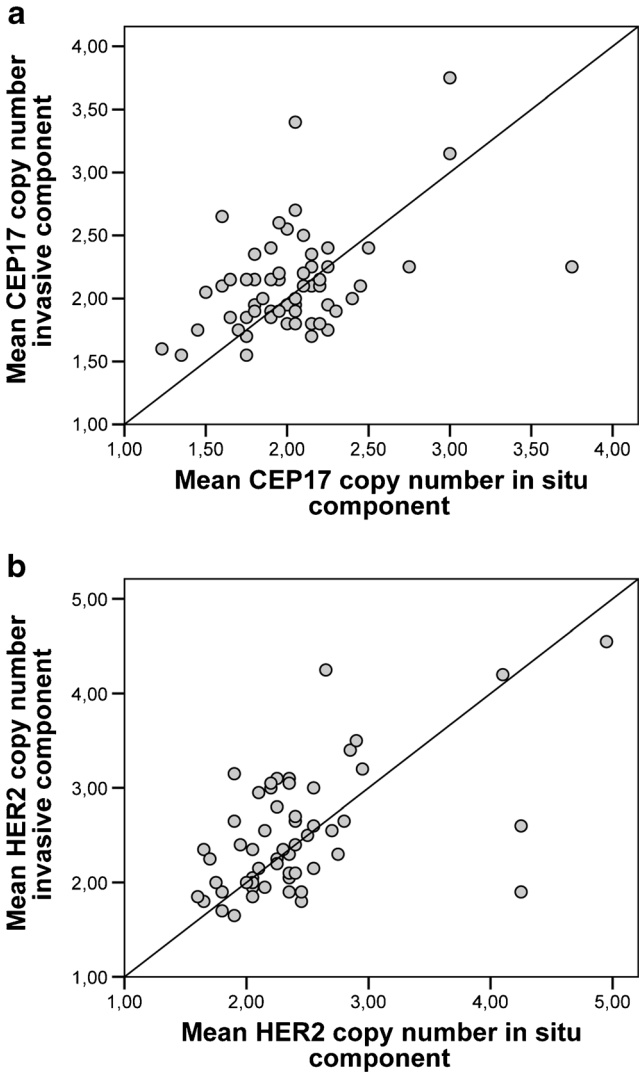

Fig. 2 Scatterplots illustrating the proportional correlation between mean CEP17 copy number (**a**) and mean HER2 copy number (**b**) between the in situ component (*x-axis*) and the invasive component (*y-axis*) of non-amplified admixed breast tumors. Strong concordance was noted for both mean HER2 and mean CEP17 copy number between the in situ and the invasive component of non-amplified admixed tumors

overexpressing cells [39]. In this model, HER2 overexpression (first hit) in MCF10A cells resulted in the formation of filled spheroids when grown in matrigel, but addition of EGF (second hit) was required to induce invasive growth [39]. Again, this in vitro model suggests a crucial role for the tumor microenvironment in evoking invasion.

Based on our own observations and the work of others, we speculate that DCIS is not an obligatory precursor of IDC, and vice versa, that some IDC do not originate from DCIS. The group of pure IDC presented a very low HER2 positivity rate of 8%, which suggests that this tumor subgroup is biologically distinct from IDC admixed with DCIS. Future research should focus on identification of drivers of cancer cell migration and invasion in this distinct subgroup, as these might signify new therapeutic targets. Although the pure IDC group in this study contained only three triple-negative breast cancers (5% of pure IDC), it seems likely that the pure IDC subgroup is enriched in triple-negative tumors, as Doebar et al. reported that triple-negative IDC has the lowest prevalence of associated admixed DCIS compared with other molecular subtypes [9].

Taken together, all these findings made us ponder the pathways of breast cancer progression pathways as proposed by Sontag and Axelrod [50]. By means of mathematical modeling, in which simulated and observed co-occurrence of different grades of IDC and DCIS are studied, they propose several pathways of breast cancer progression. In the parallel pathway, DCIS and IDC diverge from a common progenitor and divergence between in situ and invasive tumors can occur at the same time or at different times within a patient [50]. In contrast with this parallel concept, the branched pathway allows transition from in situ to invasive cancer [50]. A combination of the parallel and branched pathway might explain the observed differences in HER2 status among breast cancer subgroups.

Based on our observations, we think that the time is right to abandon the idea that all IDC have an in situ precursor. Additionally, some DCIS will never develop the propensity for invasive growth, whereas others will evolve into frankly invasive breast cancers. The identification of these 'wolves in sheep's clothing' remains a challenge for future research.

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Authors' contributions KL, MVB, HD, and LL designed the study, analyzed and interpreted the data, and drafted the manuscript. RVDB, VC, and HD acquired patient data. LVDM and SG coordinated and ensured the quality of all analyses. All authors contributed to, read, and approved the final manuscript.

Compliance with ethical standards This study has been approved by the ethics committee of Ghent University Hospital (Ghent, Belgium). The approval carries file number 2013/987 and the Belgian registration number B670201319010.

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