

Molecular differences between ductal carcinoma *in situ* and adjacent invasive breast carcinoma: A multiplex ligation-dependent probe amplification study

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Abstract. Ductal carcinoma *in situ* (DCIS) accounts for approximately 20% of mammographically detected breast cancers. Although DCIS is generally highly curable, some women with DCIS will develop life-threatening invasive breast cancer, but the determinants of progression to infiltrating ductal cancer (IDC) are largely unknown. In the current study, we used multiplex ligation-dependent probe amplification (MLPA), a multiplex PCR-based test, to compare copy numbers of 21 breast cancer related genes between laser-microdissected DCIS and adjacent IDC lesions in 39 patients. Genes included in this study were ESR1, EGFR, FGFR1, ADAM9, IKBKB, PRDM14, MTDH, MYC, CCND1, EMSY, CDH1, TRAF4, CPD, MED1, HER2, CDC6, TOP2A, MAPT, BIRC5, CCNE1 and AURKA.

There were no significant differences in copy number for the 21 genes between DCIS and adjacent IDC. Low/intermediate-grade DCIS showed on average 6 gains/amplifications versus 8 in high-grade DCIS ($p = 0.158$). Furthermore, alterations of AURKA and CCNE1 were exclusively found in high-grade DCIS, and HER2, PRDM14 and EMSY amplification was more frequent in high-grade DCIS than in low/intermediate-grade DCIS. In contrast, the average number of alterations in low/intermediate and high-grade IDC was similar, and although EGFR alterations were exclusively found in high-grade IDC compared to low/intermediate-grade IDC, there were generally fewer differences between low/intermediate-grade and high-grade IDC than between low/intermediate-grade and high-grade DCIS.

In conclusion, there were no significant differences in copy number for 21 breast cancer related genes between DCIS and adjacent IDC, indicating that DCIS is genetically as advanced as its invasive counterpart. However, high-grade DCIS showed more copy number changes than low/intermediate-grade DCIS with specifically involved genes, supporting a model in which different histological grades of DCIS are associated with distinct genomic changes that progress to IDC in different routes. These high-grade DCIS specific genes may be potential targets for treatment and/or predict progression.

Keywords: DCIS, IDC, MLPA, laser microdissection, breast cancer

1. Introduction

The most widely held concept regarding the development of human breast cancer suggests that breast cancer progression is a multistep process that manifests itself as a sequence of pathologically defined

stages with ductal carcinoma *in situ* (DCIS) as final pre-invasive stage before progression to invasive ductal carcinoma (IDC). Pathologically, DCIS is a clonal epithelial proliferation that does not breach the basement membrane. IDC, on the other hand, is an abnormal proliferation of breast epithelial cells that infiltrates through the basement membrane into the surrounding stroma. The proportion of patients diagnosed with DCIS, and with a mixture of DCIS and IDC, is increasing as screening mammography becomes more common. DCIS now accounts for approximately 20% of mammographically detected breast cancers. Some

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women with DCIS will develop life-threatening invasive breast cancer, but the determinants of progression to IDC are largely unknown. Therefore, all women with DCIS are generally treated fairly aggressively with surgery and radiation. Biologic markers that predict recurrence and/or progression to invasive cancer other than traditional grading are thus warranted.

In the most popular model to explain the development of IDC, low-grade DCIS tends to progress to low-grade IDC, and high-grade DCIS tends to progress to high-grade IDC by accumulation of fairly specific chromosomal and gene alterations [5,6,23,24,37]. In the last decades, much progress has been made in understanding the molecular and genetic events that underlie the transition from preinvasive lesions such as DCIS to IDC. The majority of molecular changes that are observed in breast cancer seem to be already evident in the DCIS stage [33,36]. The search for biologic markers that determine the transition from DCIS to IDC and thereby predict the natural course of DCIS is, however, ongoing.

An impediment to our understanding of the biological course of DCIS has been the limited extent of DCIS in clinical specimens. Laser capture microdissection (LCM) has, however, enabled the acquisition of pure populations of target cells and has proven to be popular for use in comparative genomic hybridization (CGH) [2] and loss of heterozygosity (LOH) studies [4]. Still, the amount of DNA that can be isolated in this way is often a limiting factor. In contrast with other high throughput analysis methods, multiplex ligation-dependent probe amplification (MLPA) requires only small quantities of short DNA fragments, which makes it very suitable for analysis of microdissected paraffin embedded material [30]. In previous studies we used MLPA to detect HER2 [28,30] and TOP2A [31] amplification and used its multiplex aspect to determine the frequency of polysomy 17 in breast tumors [32].

In the current study, we applied MLPA to compare copy number changes in 21 breast cancer related genes between laser-microdissected DCIS and adjacent IDC lesions in 39 patients.

2. Materials and methods

2.1. Patient material

From paraffin embedded breast cancer resection specimens of 39 patients, tissue blocks with DCIS

and adjacent IDC were selected by an experienced pathologist (PJvD) based on the H&E stained sections. Grading of DCIS and invasive cancer was done according to usual methods [16,20]. DCIS and IDC lesions were subdivided into low/intermediate-grade DCIS/IDC (DCIS $n = 19$ and IDC $n = 15$) and high-grade DCIS/IDC (DCIS $n = 20$ and IDC $n = 24$). Grading was done independently by two pathologists and discordances were discussed until consensus was reached. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospital [46].

2.2. Laser microdissection

Laser microdissection was performed on 8 μm thick paraffin sections (by comparing with a serial H&E stained slide where IDC and DCIS were marked). For laser microdissection, sections were baked at 56°C for 1 h, deparaffinized in xylene for 10 min and rehydrated through graded alcohols (100%, 85% and 70% for 1 min each). After staining with haematoxylin for 5 s, slides were rinsed in water and dipped in eosin for 5 s. Finally, slides were dehydrated in 100% ethanol for 1 min and air dried. At this point Liquid Cover Glass (PALM AG, Bernried, Germany) was applied by aerosol to improve morphology and to allow larger tissue areas to be laser pressure-catapulted, and sections were air dried for at least 30 min. A PALM microdissection system with UV laser was used to separate between 4 and 20 mm^2 of DCIS or IDC from their surrounding tissue. Subsequently, these areas were catapulted by laser pressure catapulting into a cap of a common microfuge tube moistened with a drop of mineral oil.

2.3. Multiplex ligation-dependent probe amplification (MLPA)

After laser microdissection, DNA was isolated by 1 h incubation in proteinase K (10 mg/ml; Roche, Almere, The Netherlands) at 56°C followed by boiling for 10 min. This DNA solution (50 μl) was, after centrifugation, used in the MLPA analysis according the manufacturers' instructions, using the P078-B1 breast kit (MRC Holland, Amsterdam, The Netherlands). Table 1 shows the contents of this kit and includes chromosomal locations of all probes. All tests were per-

Table 1
Contents of the P078-B1 MLPA kit (MRC Holland, The Netherlands)

Gene	Chr	Mapview position	No. probes	Transcript description	Ref.
<i>ESR1</i>	06q25	06-152.423838 06-152.457215	2	Transcription factor	[21]
<i>EGFR</i>	07p11	07-055.191055 07-055.233957	2	Receptor tyrosine kinase involved in signal transduction	[39]
<i>FGFR1</i>	08p12	08-038.391533 08-038.434092	2	Receptor tyrosine kinase involved in signal transduction	[11,27]
<i>ADAM9</i>	08p11	08-038.998319	1	Metalloproteinase associated with protein metabolism	[11,27]
<i>IKKKB</i>	08p11	08-042.292902 08-042.302676	2	Serine/threonine kinase associated with signal transduction	[11]
<i>PRDM14</i>	08q13	08-071.130073	1	Transcription regulatory protein	[35]
<i>MTDH</i>	08q22	08-098.742504 08-098-788082	2	Metastasis promoting gene involved in chemoresistance	[22]
<i>MYC</i>	08q24	08-128.821796 08-128.822001 08-128.822151	3	Transcription factor involved in apoptosis and cell proliferation	[42]
<i>CCND1</i>	11q13	11-069.167779 11-069.175089	2	Cell cycle control protein involved in signal transduction	[26]
<i>EMSY</i>	11q13	11-075.902087 11-075.926543	2	Transcription regulatory protein	[26]
<i>CDH1</i>	16q22	16-067.328716 16-067.404826	2	Adhesion molecule associated with signal transduction	[12]
<i>TRAF4</i>	17q11	17-024.098403	1	Adaptor molecule involved in signal transduction, cell proliferation and apoptosis	[10]
<i>CPD</i>	17q11	17-025.795018	1	Carboxypeptidase involved in protein metabolism	–
<i>MED1</i>	17q12	17-034.840858	1	Transcription regulatory protein involved in signal transduction	[48]
<i>HER2</i>	17q12	17-035-118101 17-035.127183 17-035-133169 17-035.136344	4	Receptor tyrosine kinase associated with signal transduction	[30]
<i>CDC6</i>	17q21	17-035.699283	1	Cell cycle control protein involved in signal transduction	[1]
<i>TOP2A</i>	17q21	17-035.812698 17-035.816651 17-035.818297	3	DNA topoisomerase protein involved in regulation of the topological status of DNA	[38]
<i>MAPT</i>	17q21	17-041.423085	1	Structural protein involved in cell growth and/or maintenance	–
<i>BIRC5</i>	17q25	17-073.722036 17-073.722396 17-073.724340	3	Adapter molecule involved in signal transduction, cell communication and cell survival	[15]
<i>CCNE1</i>	19q12	19-035.000150 19-035.005214	2	Cell cycle control protein involved in signal transduction	[9,25]
<i>AURKA</i>	20q13	20-054.389980	1	Serine/threonine kinase involved in signal transduction	[18,43]

Note: For each gene the chromosomal position, mapview distance from *p*-telomere, the number of probes present in the MLPA kit, a description of the transcript protein and if possible a relevant (breast cancer) reference is given.

formed in duplicate on an ABI 9700 PCR machine (Applied Biosystems, Foster City, CA, USA). PCR products were analyzed on an ABI310 capillary sequencer (Applied Biosystems). Gene copy numbers were analyzed using Genescan (Applied Biosystems)

and Coffalyser (version 7.0) software (MRC-Holland). Four negative reference samples (normal breast) were taken along in each MLPA run to normalize MLPA ratios. For genes with more than one probe present in the kit, the mean of all the probe peaks of this gene in

duplicate was calculated. A mean value below 0.7 was defined as loss, a value between 0.7–1.3 was defined as normal, 1.3–2.0 as gain and values >2.0 were defined as (high level) amplification, as established previously [7,14].

2.4. Statistics

Statistics were performed using SPSS statistical software. Copy number ratio differences for the 21 individual genes between DCIS and adjacent IDC cases were compared with paired *t*-test. In addition, we compared the mean copy number ratio of each individual gene between the grouped DCIS cases on the one hand and the IDC cases on the other. Next, MLPA data were dichotomized as non-amplified vs. gain/amplified (cut-off 1.3), and these gene dosage categories were compared between DCIS and IDC by McNemar's test. Furthermore, the mean MLPA copy number ratio including all 21 genes in all 39 patients was compared between DCIS and IDC by *t*-test.

Hereafter, we compared different grades of DCIS and IDC. First, we compared the mean copy number ratios between low/intermediate and high-grade DCIS, as well as between low/intermediate-grade and high-grade IDC by *t*-test. Then, we again dichotomized data and compared the mean number of gains/amplifications between low/intermediate and high-grade DCIS, as well as between low/intermediate-grade and high-grade IDC by *t*-test. *p*-values below 0.05 were con-

sidered significant. Bonferroni correction was used to address the problem of multiple comparisons. Finally, unsupervised hierarchical clustering was applied with R as before [32].

3. Results

Supplementary Table 1 (Suppl. Table 1: <http://www.qub.ac.uk/isco/JCO/>) shows the raw copy number data. Paired comparison of copy number ratios between DCIS and adjacent invasive lesions did not yield significance for any of the tested genes. Overall (unpaired) comparison of copy number ratios between the grouped DCIS and invasive lesions also did not yield significance for any of the tested genes.

Figure 1 shows the frequency of gain/amplification of 21 genes analyzed by MLPA in DCIS and adjacent IDC, using dichotomized data. Although BIRC5 (survivin), TOP2A, CCND1 and MED1 (PPARBP) gain/amplification seemed to be more prevalent in DCIS than IDC, overall, there were no significant differences between both components. Chromosomes 8, 11 and 17 seem to show the most frequent alterations in both breast lesions, the analyzed regions on chromosomes 6, 7, 16, 19 and 20 to a lesser extent. We found three genes showing frequent ($>5\%$) loss: FGFR1, CDH1 and MAPT, with also no significant differences between DCIS and IDC.

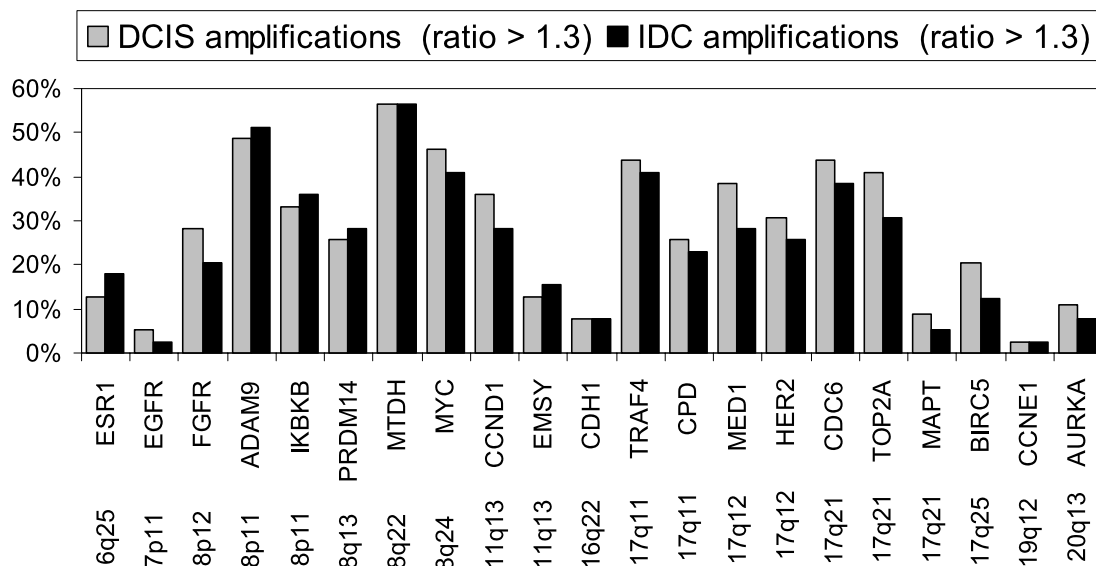


Fig. 1. Amplification frequencies of 21 genes analyzed by multiplex ligation-dependent probe amplification (MLPA) in DCIS and adjacent IDC from 39 breast cancer patients.

Overall, there was no significant copy number difference between DCIS (1.35 ± 0.85) and IDC (1.34 ± 0.88) ($p = 0.604$).

The mean copy number ratio was 1.29 ± 0.71 in low/intermediate-grade and 1.42 ± 0.96 in high-grade DCIS ($p = 0.023$), and 1.30 ± 0.71 in low/intermediate-grade and 1.36 ± 0.97 in high-grade IDC ($p = 0.308$). The mean number of gains/amplifications in the 21 analyzed genes was 6 in low/intermediate-grade DCIS and 8 in high-grade DCIS ($p = 0.158$), and 6 in both low/intermediate-grade and high-grade IDC ($p = 0.903$).

Also, as shown in Table 2, the pattern of alterations differed between low/intermediate-grade and high-grade DCIS, and in a much lesser extent between low/intermediate and high-grade IDC. Alterations of AURKA and CCNE1 were exclusively found in high-grade DCIS, and alterations of EGFR were exclusively found in high-grade IDC. Figure 2 shows that gene copy numbers of HER2 ($p = 0.013$), MYC ($p = 0.009$) and MTDH (not significant) were higher in high-grade DCIS compared to low/intermediate-grade DCIS. Copy numbers of HER2 ($p = 0.037$) were also

significantly higher in high-grade IDC compared to low/intermediate-grade IDC, as were those of TRAF4 ($p = 0.088$), but MAPT ($p = 0.108$) and CCNE1 ($p = 0.070$) gene copy numbers seemed to be lower in high-grade IDC compared to low-grade IDC.

Figure 3 shows the cluster analysis for all 39 DCIS/IDC pairs according to their MLPA profiles based on 21 genes and their grading. Twenty of the 39 pairs of DCIS/IDC (51%) clustered closely together. Although not consistently, most of the low/intermediate-grade DCIS/IDC seemed to belong to a different cluster than high-grade DCIS/IDC.

4. Discussion

This study aimed to investigate and compare copy number changes within DCIS and adjacent IDC by MLPA. Overall, there was no significant copy number difference between DCIS and IDC, and the detected genetic alterations in tumors with synchronous DCIS and invasive ductal cancer showed a high degree of shared changes within the two components.

Table 2

Frequencies of alterations (gain, amplification and loss) in low/intermediate-grade and high-grade DCIS and adjacent IDC present in 39 breast tumor samples

	DCIS		IDC	
	Low/intermediate-grade	High-grade	Low/intermediate-grade	High-grade
ESR1	16	15	13	25
EGFR	5	10	0	4
FGFR1	32	55	27	38
ADAM9	58	45	67	50
IKBKB	37	40	47	38
PRDM14	11	40	33	29
MTDH	53	65	47	83
MYC	42	65	47	63
CCND1	53	40	27	42
EMSY	11	20	33	33
CDH1	21	15	7	25
TRAF4	42	65	33	58
CPD	21	40	53	33
MED1	42	50	40	29
HER2	21	65	33	33
CDC6	42	55	47	42
TOP2A	47	45	47	29
MAPT	16	35	7	21
BIRC5	26	30	13	13
CCNE1	0	15	7	4
AURKA	0	25	7	8

Notes: DCIS – ductal carcinoma *in situ*; IDC – invasive ductal carcinoma.

in DCIS and adjacent IDC until now. A previous study has investigated copy number alterations of all genes in this study in a large group of IDC using the same MLPA kit and showed similar copy number alteration frequencies in literature [29].

The number as well as the pattern of alterations differed between low/intermediate and high-grade DCIS, supporting a model in which different histological grades of DCIS are associated with distinct genomic changes [13]. These genes showing alterations may be potential targets for treatment and/or markers of prognosis. HER2 showed more frequent gain/amplification in high-grade DCIS (65%) than in low/intermediate (21%), which is consistent with previous studies showing more frequent gain of 17q and higher HER2 expression levels in high-grade DCIS [13,23,47]. Poorly differentiated DCIS has also been associated with frequent loss of 8p, which is consistent with our data since FGFR1 on 8p11 showed no loss in low/intermediate-grade DCIS compared to 25% loss in high-grade DCIS. MYC amplifications were found in IDC as well as in DCIS without any significant differences in frequency between both components. This is inconsistent with a FISH study that could not demonstrate MYC amplifications in DCIS adjacent to FISH-amplified IDC [41]. In this study, the MLPA ratio was higher in IDC compared to DCIS in 56% of cases, but in most cases (16/22) this did not lead to a different amplification status (gain vs. normal, no differences were found in the 13% high level amplifications) between both lesions. Furthermore, MYC amplifications seem to be more prevalent in high-grade DCIS lesions (65%) than in low/intermediate-grade lesions (42%), which is consistent with previous studies [8].

Some alterations (CCNE1 19q12 and AURKA 20q13) were exclusively found in high-grade DCIS. However, at this moment, no single gene has been identified that differentiates between different types of DCIS. Perhaps the indicated genes could be candidates. Given the small size of the present study group, these data have to be confirmed in larger studies.

Table 2 and statistical analysis show that, overall, the differences between low/intermediate and high-grade DCIS seemed to be bigger than between low/intermediate and high-grade IDC. For example, whereas HER2, EMSY and PRDM14 gains/amplifications were as frequent in low/intermediate-grade IDC as in high-grade IDC, the amplification of these genes seemed to be less frequent in low/intermediate-grade DCIS than in high-grade DCIS. These differences may be prognostically important, or may turn out to be important variables in the choice of therapy.

Cluster analysis showed a trend towards clustering by grade, but several low/intermediate-grade DCIS/IDC clustered together with high-grade DCIS/IDC, indicating that low-grade DCIS can be genetically as advanced as high-grade DCIS. Twenty of the 39 DCIS-IDC pairs clustered closely together, indicating a similar genetic profile of both components. This implies that, at least for the studied genes, DCIS is genetically as advanced as its invasive counterpart. Nevertheless, although no significant differences were observed between DCIS and IDC, not all DCIS/IDC pairs clustered together. It is possible that a larger gene set spread over more different chromosomal locations could better discriminate the samples. For example, Hannemann et al. identified a gene expression classifier of 35 genes which differed between DCIS and invasive breast cancer [19]. This same group also identified a 43-gene classifier to separate well- and poorly-differentiated DCIS samples. Unfortunately, the genes identified in that study did not overlap with the genes investigated using the MLPA P078-B1 kit in our study, except for ESR1. This gene was identified by Hannemann et al. as a discriminator between well- and poorly-differentiated DCIS. However, this gene did not show significant differences in our study (15% and 16% alterations). This could be explained by the fact that Hannemann et al. looked at the expression of ESR1 in contrast to the copy number. Several studies have indicated that there is no absolute relationship between ESR1 gain or amplification and mRNA and protein expression in breast cancer [34,40].

In conclusion, this study showed that MLPA is suited to simultaneously detect amplification or loss of many potential prognostic or predictive genes in breast cancer. We found no significant differences between DCIS and adjacent IDC, but the number as well as the pattern of alterations differed between high- and low/intermediate-grade DCIS. Overall, the differences between low/intermediate and high-grade DCIS seemed to be bigger than between low/intermediate and high-grade IDC.

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