Methylation biomarkers for pleomorphic lobular breast cancer - a short report

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Abstract

Background Pleomorphic invasive lobular cancer (pleomorphic ILC) is a rare variant of ILC that is characterized by a classic ILC-like growth pattern combined with an infiltrative ductal cancer (IDC)-like high nuclear atypicality. There is an ongoing discussion whether pleomorphic ILC is a dedifferentiated form of ILC or in origin an IDC with a secondary loss of cohesion. Since gene promoter hypermethylation is an early event in breast carcinogenesis and thus may provide information on tumor progression, we set out to compare the methylation patterns of pleomorphic ILC, classic ILC and IDC. In addition, we aimed at analyzing the methylation status of pleomorphic ILC.

Methods We performed promoter methylation profiling of 24 established and putative tumor suppressor genes by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) analysis in 20 classical ILC, 16 pleomorphic ILC and 20 IDC cases.

Results We found that pleomorphic ILC showed relatively low TP73 and MLH1 methylation levels and relatively high RASSF1A methylation levels compared to classic ILC. Compared to IDC, pleomorphic ILC showed relatively low MLH1 and BRCA1 methylation levels. Hierarchical cluster analysis revealed a similar methylation pattern for pleomorphic ILC and IDC, while the methylation pattern of classic ILC was different.

Conclusion This is the first report to identify TP73, RASSF1A, MLH1 and BRCA1 as possible biomarkers to distinguish pleomorphic ILC from classic ILC and IDC.

Keywords Sporadic breast cancer · Lobular breast cancer · Pleomorphic lobular breast cancer · DNA hypermethylation · MS-MLPA · Epigenetics

1 Introduction

Invasive lobular breast cancer (ILC) is the second most prevalent histological breast cancer type that accounts for 10–15% of all breast cancers [1, 2]. ILC differs from invasive ductal carcinoma (IDC) in biology, histology, clinical presentation and response to therapy (reviewed in [3]). In contrast to ductal tumors, most lobular tumors show loss of E-cadherin expression, which often results from inactivating gene mutation and subsequent loss of heterozygosity or promoter hypermethylation [4]. Indeed, conditional knock-out mouse models have shown that somatic inactivation of E-cadherin leads to ILC development and progression [5, 6]. Among the eight different ILC variants described, classic ILC and pleomorphic ILC are the most common ones [3, 7]. Although the exact frequency of these ILC subtypes has not extensively been documented, approximately 60% of all ILC cases is classic and approximately 13% is pleomorphic (reviewed in [3]). Phenotypically, classic ILC is composed of small regular low grade and dissociated cells with intra-cytoplasmic...
vacuoles and small nuclei that exhibit a highly trabecular infiltrative growth pattern, often distributed in targetoid patterns around uninvolved ducts [8]. Pleomorphic ILC shows a similar growth and invasion pattern, but is composed of high grade polygonal cells with eccentric and highly pleomorphic nuclei. Furthermore, pleomorphic ILC tumors have been reported to be significantly larger than classic ILC tumors, and pleomorphic ILC patients often present with lymph node involvement and a higher rate of metastatic disease compared to classical ILC [9]. Moreover, the overall survival and recurrence rates of pleomorphic ILC patients are worse compared to classic ILC patients [10], indicating that pleomorphic ILC is a more aggressive form of breast cancer than classic ILC.

At the molecular level, classic and pleomorphic ILCs show similarities and differences. Both variants lack expression of basal markers like cytokeratin (CK)5 and CK14, but expresses the luminal epithelial markers CK8 and CK18 [11, 12]. ILCs are usually ‘luminal’ type breast cancers that express the estrogen receptor (ER) gene and genes involved in ER activation, including the progesterone receptor (PR) gene [13, 14]. Cytosolic translocation of p120-catenin due to inactivation of E-cadherin is a hallmark of ILC, whereas classic and pleomorphic ILCs do not over-express the epidermal growth factor receptor (EGFR) gene [1, 15, 16]. While most classic ILCs lack expression of HER2 (ERBB2) [1], up to 81 % of pleomorphic ILCs express HER2 [17]. Moreover, although the somatic TP53 mutation frequency in pleomorphic ILC may be as high as 46 %, this is a rare event in classic ILC (approximately 6 %), suggesting a role for p53 loss in the etiology of pleomorphic ILC [17–19]. These findings are supported by observations in mammary-specific E-cadherin and p53 knock-out mice that develop a mouse variant of pleomorphic ILC [6]. Furthermore, in contrast to classic ILC, pleomorphic ILC often expresses the apocrine differentiation marker gross cystic disease fluid protein 15 (GCDFP15) and the androgen receptor (AR) [17, 20]. The origin of pleomorphic ILC tumors is still under debate and, since results from conditional mouse models have suggested that all lobular cancer types are evolutionary linked (reviewed in [21]), it is currently unclear whether pleomorphic ILC is a dedifferentiated form of classic ILC or whether it evolves from ductal type tumors. The differential diagnosis between these breast cancer subtypes is important because surgery planning of ILC requires preoperative MRI, due to an often more diffuse and multifocal growth pattern of lobular tumors and a higher incidence of contralateral tumors [22].

In cancer, DNA methylation is often disturbed and can act as a driving force during tumor progression [23, 24] (reviewed in [25]). DNA methylation occurs by the enzymatic transfer of a methyl group onto the carbon-5 position of a cytosine (often part of a cytosine phosphate guanosine (CpG) dinucleotide), which can result in gene silencing [26]. Promoter hypermethylation of tumor suppressor genes is considered to be an early event in carcinogenesis since high methylation levels have been found in columnar cell lesions, the earliest recognized breast cancer precursors [27]. Hence, methylation patterns may give insight in tumor progression and, thus, shed light on the precursor origin of pleomorphic ILC tumors. In light of the possible future extrapolation to methylation detection in biopsy, blood, nipple fluid and urine samples, DNA hypermethylation is a promising area in the clinical biomarker field. DNA hypermethylation analyses can be performed on formalin-fixed tissues and, thus, are suited for molecular profiling and the identification of markers that predict therapeutic responsiveness.

Here we have identified promoter methylation patterns in pleomorphic ILC in relation to ILC and IDC to identify pleomorphic ILC biomarkers. Methylation was assessed by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), a highly reproducible technique that only requires small amounts (10 ng) of short DNA fragments and that shows high concordance with other established techniques such as quantitative multiplex methylation-specific PCR [28, 29]. MS-MLPA can be used in samples with mixed populations of cells. As long as 30 % of methylated DNA/tumor DNA is present in the sample, the methylation status will be recognized correctly [30]. We assessed the promoter methylation status of 24 tumor suppressor genes and compared 16 pleomorphic ILC, 20 classic ILC and 20 IDC cases. We found that the methylation patterns of classic ILC and IDC were comparable, and that the classic ILC and IDC profiles were mildly different from pleomorphic ILC. Furthermore, we found that the methylation status of the RASSF1A, TP73, MLH1 and BRCA1 gene promoters can be used as stratification markers to distinguish pleomorphic ILC from classic ILC and IDC.

2 Materials and methods

2.1 Patient material

Patient samples were derived from the archives of the Departments of Pathology at the University Medical Centre Utrecht, the Radboud University Medical Centre, Nijmegen, The Netherlands, the Institute of Pathology, Paderborn, Germany, and the Department of Pathology, Bács-Kiskun County Teaching Hospital, Kecskemé, Hungary. The clinicopathological characteristics of the patient samples are provided in Table 1. Classic and pleomorphic ILC and IDC cases were selected based on examination of haematoxylin and eosin (H&E)-stained slides by at least two pathologists. The use of left-over material was approved by the Tissue Science Committee of the UMC Utrecht [31]. Histological grades were assessed according to the Nottingham modification of the Scarff-Bloom-Richardson grading system [32]. ER and
PR were considered positive when ≥10% of the cells showed positive nuclear staining. HER2 was scored according to the modified DAKO scoring system, where only a score of 3+ was considered positive. The mitotic activity index (MAI) was assessed as reported before [33].

2.2 Methylation-specific multiplex ligation-dependent probe amplification

H&E stained sections were used to reveal pre-invasive lesions, necrosis and admixed lymphocytic infiltrates and to guide micro-dissections for DNA extraction. Areas with necrosis, dense lymphocytic infiltrates and pre-invasive lesions were intentionally avoided. All areas selected for micro-dissection had a tumor percentage of at least 70%. Tumor tissue was derived from 4 μm thick sections (5 to 10, formalin-fixed paraffin embedded) and DNA was isolated by overnight incubation in lysis buffer (50 mM Tris–HCl, pH 8.0; 0.5% Tween 20) with proteinase K (10 mg/ml, Roche) at 56°C, followed by boiling for 10 min. After a 5 min centrifugation step (12,000 g), 5 μl supernatant was used for MLPA analysis according to the manufacturer’s instructions, using the ME001-C2 methylation kit (MRC-Holland). The principle of MS-MLPA has been described elsewhere [28] and the PCR and data analysis procedures were performed as reported before [27]. The ME001-C2 MS-MLPA probe mix contains 26 probes, detecting the methylation status of promoter CpG sites of 24 established and putative tumor suppressor genes (Table 2) that are frequently silenced by hypermethylation in tumors, but not in blood-derived DNA of healthy individuals. In addition, we included 15 reference probes. The cumulative methylation index (CMI) was calculated as the sum of the methylation percentage of all genes, as described before [34].

2.3 Statistics

Statistic calculations and ROC curve analyses were performed using IBM SPSS statistics v20.0 (SPSS Inc., Chicago, IL, USA). Two-sided \( p<0.05 \) was considered significant. Absolute methylation levels were used to calculate \( p \)-values upon comparing classic ILC, pleomorphic ILC and IDC samples, using the Student’s t-test or Mann–Whitney U Test, and the Kruskal-Wallis test. Through Bonferroni-Holm correction of all \( p \)-values we excluded false-positives caused by multiple comparisons. Logistic regression analysis was used to reveal the best (combination of) genes able to discriminate pleomorphic ILC from classic ILC and/or IDC. A backward stepwise method was used until the most predictive variables remained. Unsupervised hierarchical clustering (Euclidean metric) using the statistical program R was performed on Z-scores to identify relevant clusters.

3 Results and discussion

Kruskal-Wallis one-way ANOVA analysis was carried out to assess differential methylation patterns in our non-parametric methylation data of the three breast cancer subtypes, i.e.,

| Table 1 Clinicopathological characteristics of breast cancer patients |
|-----------------|-----------------|-----------------|-----------------|
| Feature         | Grouping        | Classic ILC N (%) | Pleomorphic ILC N (%) | IDC N (%) |
| N Range         | 20              | 16              | 20              |
| Histological grade | 52–88           | 43–80           | 44–87           |
| 1               | 8 (40.0)        | 0 (0.0)         | 5 (25.0)        |
| 2               | 6 (30.0)        | 5 (31.3)        | 5 (25.0)        |
| 3               | 5 (25.0)        | 11 (68.8)       | 10 (50.0)       |
| Not available   | 1 (5.0)         | 0 (0.0)         | –               |
| MAI (%) Mean Range | 0–26            | 9–100           | 0–8             |
| 1               | 1 (5.0)         | 1 (6.3)         | –               |
| Lymph node status | Negative\(^a\) | 8 (50.0)        | 7 (35.0)        |
| 2               | 7 (35.0)        | 8 (50.0)        | 13 (65.0)       |
| Receptor status | PR positive     | 10 (50.0)       | 10 (62.5)       | 13 (65.0) |
| Her2 positive   | 0 (0.0)         | 0 (0.0)         | 3 (15.0)        |
| Tumor size (cm) | ≤2.0            | 1 (5.0)         | 4 (25.0)        | 8 (40.0)  |
| >2.0            | 18 (90.0)       | 12 (75.0)       | 12 (60.0)       |
| Not available   | 1 (5.0)         | –               | –               |

\(^a\) negative = N0 or N0(i+); \(^b\) positive = ≥N1mi (according to TNM 7th edition, 2010)
classic ILC, pleomorphic ILC and IDC (Fig. 1; patient samples listed in Table 1). Sixteen of the 24 genes tested (listed in Table 2), including \textit{BRCA1}, showed significant differences between the groups. However, after correction for multiple comparisons only the methylation patterns of \textit{TP73} ($p<0.002$), \textit{MLH1}$_{\text{ba}}$ ($p<0.002$) and \textit{RASSF1A}$_{\text{ax}}$ ($p<0.002$) were found to be significantly different between the three breast cancer subtypes (Fig. 2).

A (post hoc) Mann–Whitney test followed by multiple comparisons correction was carried out, using the 16 genes derived from the above Kruskal-Wallis analysis, to specify the differences between classic and pleomorphic ILCs. By doing so, we found that the methylation patterns of \textit{TP73}, \textit{MLH1}$_{\text{y}}$ and \textit{RASSF1A}$_{\text{x}}$ were significantly different between the classic and pleomorphic ILCs. When compared to classic ILCs, pleomorphic ILCs showed less promoter methylation of the \textit{MLH1}$_{\text{y}}$ ($p=0.002$) and \textit{TP73} ($p=0.001$) genes (Fig. 2), while the promoter methylation of the \textit{RASSF1A} gene was found to be higher in pleomorphic ILCs ($p=0.001$). The CMI of the pleomorphic ILCs was not significantly different from that of classic ILCs (353.3 versus 390.0, respectively; $p=0.437$). In logistic regression analyses \textit{TP73} ($p=0.017$) and \textit{RASSF1A} ($p=0.005$) showed a joint independent discriminative value for pleomorphic ILCs versus classic ILCs (area under the curve (AUC) 0.888, CI 0.764-1.000, $p<0.001$), with a combined receiver operating characteristic (ROC) curve-based sensitivity and specificity of 81 and 100 %, respectively.

After correction for multiple comparisons, we found that the methylation levels of both \textit{MLH1}$_{\text{y}}$ ($p=0.001$) and \textit{BRCA1} ($p=0.002$) were significantly lower in pleomorphic ILCs than in IDCs (Fig. 2). The mean CMI of pleomorphic ILCs was not significantly different from that of IDCs (353.3 vs. 392.6, respectively; $p=0.357$), indicating that the overall methylation patterns of these two breast cancer subgroups were similar. Logistic regression analysis showed that only \textit{BRCA1} methylation ($p=0.002$) had an independent

### Table 2

Probes directed against the CpG islands of 24 tumor suppressor genes in the MS-MLPA kit (ME001-C2, MRC-Holland)

| Length | Gene | Chromosome | Mapview | Full name                           |
|--------|------|------------|---------|-------------------------------------|
| 142    | TIMP3| 22q12.3    | 22-031.527795 | TIMP metallopeptidase inhibitor 3   |
| 148    | APC  | 5q22.2     | 05-112.101357 | Adenomatosis Polyposis Coli        |
| 161    | CDKN2A| 9p21.3    | 09-021.985276 | Cyclin-Dependent Kinase Inhibitor 2A |
| 167    | MLH1$_{\text{a}}$| 3p22.2     | 03-037.009621 | MutL Homolog 1                     |
| 184    | ATM  | 11q22.3    | 11-107.599044 | Ataxia Telangiectasia Mutated      |
| 193    | RARB | 3p24.2     | 03-025.445559 | Retinoic Acid Receptor, beta       |
| 211    | CDKN2B| 9p21.3     | 09-021.998808 | Cyclin-Dependent Kinase Inhibitor 2B |
| 220    | HIC1 | 17p13.3    | 17-001.905107 | Hypermethylated In Cancer 1        |
| 238    | CHFR | 12q24.33   | 12-131.974372 | Checkpoint with Forkhead and Ring finger domains |
| 246    | BRCA1| 17q21.31   | 17-038.530811 | Breast Cancer 1                    |
| 265    | CASP8| 2q33.1     | 02-201.830871 | Caspase 8                          |
| 274    | CDKN1B| 12p13.1    | 12-012.761863 | Cyclin-Dependent Kinase Inhibitor 1B |
| 292    | PTEN | 10q23.3    | 10-089.612348 | Phosphatase and Tensin homolog      |
| 301    | BRCA2| 13q12.3    | 13-031.787722 | Breast Cancer 2                    |
| 319    | CD44 | 11p13      | 11-035.117389 | CD44 molecule                      |
| 328    | RASSF1A$_{\text{a}}$| 3p21.31   | 03-050.353328 | Ras Association (RalGDS/AF-6) domain Family member 1 |
| 346    | DAPK1| 9q21.33    | 09-089.303075 | Death-Associated Protein Kinase 1 |
| 353    | VHL  | 3p25.3     | 03-010.158426 | Von Hippel-Lindau tumor suppressor  |
| 373    | ESR1 | 6q25.1     | 06-152.170883 | Estrogen Receptor 1                |
| 382    | RASSF1A$_{\text{b}}$| 3p21.31   | 03-050.353347 | Ras Association (RalGDS/AF-6) domain Family member 1 |
| 400    | TP73 | 1p36.32    | 01-003.558977 | Tumor Protein p73                  |
| 409    | FHIT | 3p14.2     | 03-061.211918 | Fragile Histidine Triad            |
| 427    | CADM1| 11q23.3    | 11-114.880585 | Cell Adhesion Molecule 1           |
| 436    | CDH13| 16q23.3    | 16-081.218219 | Cadherin 13                       |
| 454    | GSTP1| 11q13.2    | 11-067.107774 | Glutathione S-transferase pi 1     |
| 463    | MLH1$_{\text{b}}$| 3p22.2     | 03-037.010000 | MutL Homolog 1                     |

*For these genes two probe sets against different CpG sites (a and b) are present.*
A discriminative value for pleomorphic ILC versus IDC (area under the curve (AUC) 781, CI 0.623-0.939, \( p = 0.004 \)), with a ROC curve-based sensitivity and specificity of 75 and 81\%, respectively.

In order to determine if the absolute MS-MLPA values of our samples clearly defined our three breast cancer subtypes, we performed hierarchical Euclidean cluster analysis on all genes tested (Fig. 3a) and on the four genes that showed significant differences in the Mann–Whitney tests (Fig. 3b). Both cluster analyses revealed some clustering of pleomorphic ILC samples with IDC samples, while classic ILC samples usually formed separate clusters with other IDC samples.

The different advantages and possibilities of DNA methylation analyses for disease stratification and prognostication
have led to a large amount of reports in the literature, also on breast cancer. However, the vast majority of these reports has so far focused on IDC, and we are unaware of any report on DNA methylation in pleomorphic ILC. Interestingly, in two reports the DNA methylation patterns were compared in ILC and IDC, and it was found that they were very similar in these
two breast cancer subtypes [35, 36]. Our results are in agreement with this notion, while pleomorphic ILC appeared to exhibit a distinct methylation pattern with a CMI similar to that of classic ILC and IDC.

**BRCA1, MLH1 and RASSF1A** are established tumor suppressor genes and TP73 is a putative tumor suppressor gene. BRCA1 and MLH1 are involved in DNA repair and their functional loss causes an accumulation of gene defects. Interestingly, we found a significant association between the presence of MLH1_x (p=0.013) and BRCA1 (p=0.013) promoter methylation and a high MAI (>12), and between MLH1_x and BRCA1 promoter methylation (p=0.004) (data not shown). Previously, BRCA1 promoter methylation has been observed in 10–15 % of all sporadic breast cancer patients [37, 38]. Only 4–5 % of the lobular breast tumors carry a deleterious BRCA1 mutation [39], and none of the 11 previously analyzed ILC samples showed BRCA1 hypermethylation [37]. As we found that the MLH1 and BRCA1 methylation levels were lower in pleomorphic ILC compared to IDC, they may not be suitable as therapeutic targets, but they may be used as biomarkers.

To test the reproducibility of MS-MLPA, we have previously taken along 10 primary breast tumor samples in duplicate in at least 8 separate MS-MLPA runs (unpublished data). By doing so, we found that the TP73, MLH1_y, RASSF1A_x and BRCA1 probes have an average standard deviation of 0.01, 0.01, 0.05 and 0.02 per sample, respectively. Based on these findings, we anticipate that the between-group differences observed for TP73 and RASSF1A are reproducible and reliable. The differences between groups observed for MLH1_y and BRCA1 are, however, less pronounced and may be the result of technical variability. We, therefore, recommend validating these findings by an independent highly sensitive and quantitative technique.

TP73 is subject to alternative splicing, and the use of an alternative promoter results in different p73 isoforms that exhibit contrasting effects on tumor development [40]. Although TP73 promoter methylation has been correlated with a poor survival of breast cancer patients [41], this methylation also impairs binding of the transcriptional repressor ZEB1, which may result in an increase in TP73 expression [42]. Unfortunately, studies reporting TP73 methylation levels in normal breast tissue are scarce and not combined with protein or RNA expression analyses [27, 43], and TP73 methylation studies in ILC have not been reported yet. As we found TP73 promoter methylation to be relatively low in pleomorphic ILC compared to classic ILC, it may serve as a biomarker, whereas it is considered less suited as a target for therapy. Further studies are needed to determine the effect of TP73 methylation on protein expression and to determine the functional consequences in pleomorphic ILC.

**RASSF1A** promoter methylation was found to be higher in pleomorphic ILC than in classic ILC. Although uncommon, RASSF1A polymorphisms and deletions have been encountered and RASSF1A promoter hypermethylation has been found to frequently occur in different tumor types [44]. About 70–85 % of ILC and IDC cases show RASSF1A hypermethylation [35, 45]. Also, hypermethylation of RASSF1A in pre-operative serum of breast cancer patients has been found to serve as an independent prognostic marker correlated with a poor overall survival [46]. Since RASSF1A hypermethylation is rarely observed in normal breast tissues, it is considered to be an early event in breast cancer development [46, 47] and, as such, it may serve as a promising breast cancer biomarker. RASSF1 is a member of the RASSF family of genes (RASSF1–8), and gives rise to 8 different isoforms due to alternative splicing and alternative promoter usage [48]. Next to the RASSF proteins, RAF and phosphatidylinositol 3-kinase (PI3K) are also known as RAS effectors, i.e., proteins that specifically bind the GTP-bound form of RAS. In contrast to RAF and PI3K, which control proliferation and survival, the RASSF genes are known to act as tumor suppressors [48]. RASSF1A null-mice show an increased incidence of spontaneous tumor formation, a decreased survival rate and an increased susceptibility for mutagens (reviewed in [48]). In addition, it has been found that exogenous expressions of RASSF1A in different tumor cell lines reduces their viability, proliferation and invasion [48]. These findings, combined with our data showing increased RASSF1A promoter methylation in pleomorphic ILC, renders RASSF1A into an interesting and functional biomarker for lobular breast cancer.

In conclusion, our data indicate that the promoter methylation signature of the TP73, MLH1, RASSF1A and BRCA1 genes may serve as a biomarker to distinguish pleomorphic ILC from classic ILC and IDC. Since pleomorphic ILC is considered to be an aggressive breast cancer variant, and since pre-operative MRI is favorable for ILC patients but not for IDC patients, pleomorphic ILC biomarkers may be useful for treatment design in cases where a pathological distinction between ILC and IDC is questionable. Future research is needed to confirm our findings in an independent patient group and to evaluate the potential of the respective methylation markers as therapeutic targets.

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**Author contributions** The experiments were conceived and designed by CBM, EJV, PJD and PWBD. EJV performed the experiments. EJV, CE and PJD performed the pathological analyses and scoring of human tumor samples. EJV and CBM analyzed the methylation data. EJV, CBM, PJD and PWBD wrote the paper.

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