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Clonal profiling of mixed lobular and ductal carcinoma revealed by multiplex ligation-dependent probe amplification and fluorescence *in situ* hybridization.

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Running title: Mixed lobular and ductal carcinoma

## Abstract

A needle biopsy of a mass in the right breast of a 36-year-old woman revealed invasive ductal carcinoma (IDC), and approximately 20% of cancer cells showed unequivocal membranous staining with the HercepTest<sup>TM</sup>. After systemic therapy with trastuzumab and paclitaxel followed by FEC (fluorouracil + epirubicin + cyclophosphamide), a right mastectomy was performed. By histological and immunohistochemical examinations, the resected tumor consisted mainly of E-cadherin-negative invasive lobular carcinoma (ILC), and the rest was ERBB2-positive IDC; thus, the diagnosis was mixed ductal and lobular carcinoma. Multiplex ligation-dependent probe amplification and fluorescence *in situ* hybridization (FISH) analyses revealed that ILC and IDC shared high-level amplification of *CCND1* in homogeneously staining regions (HSR) and that IDC had an additional HSR-type amplicon of *ERBB2*. These findings strongly indicate that IDC and ILC had a common precursor cell with *CCND1* amplification. Review of the biopsy specimen with FISH showed IDC with gene amplifications of *CCND1* and *ERBB2* as a minor component, IDC without amplification of *CCND1* or *ERBB2* as a major component, and a minute portion of ILC with *CCND1* amplification. We speculate that chemotherapy and trastuzumab caused a marked reduction in IDC; however, ILC with *CCND1* amplification was resistant to chemotherapy and grew.

Key words: breast cancer, ductal carcinoma, lobular carcinoma, MLPA, FISH

## Introduction

Invasive ductal and lobular breast carcinomas (IDC and ILC, respectively) are the most common histological types of invasive carcinoma of the breast. Molecular profiling studies, clinical and follow-up data, and common metastatic patterns suggest that these histological types of breast cancer have genetic and biologic differences.<sup>1, 2</sup> Tumors with mixed ductal and lobular morphology, referred to as mixed ductal and lobular carcinoma (MDLC), are relatively rare and account for 3%-8% of invasive breast cancers; the clinical and biological significance of MDLC is unknown, as few studies have focused on these tumors.<sup>3, 4</sup>

ILC often have inactivating mutations or methylation silence of the E-cadherin gene (*CDH1*) mapping to chromosome 16q22.1 in combination with loss of the wild-type *CDH1* allele.<sup>5</sup> Thus, most ILC cases (85%) are completely E-cadherin-negative, while most IDC cases have no reduction or only a heterogeneous reduction in E-cadherin expression.<sup>2</sup> Therefore, the negative immunostaining of E-cadherin is the critical diagnostic aid in the differential diagnosis of ILC.

The *CCND1* gene located on 11q13 is a cell-cycle regulatory gene that has been implicated in breast cancer progression.<sup>6</sup> *CCND1* amplification was associated with higher tumor grade, and patients with highly amplified *CCND1* had a significantly worse prognostic score than the low amplification group.<sup>7</sup> Although *CCND1* is amplified in 5%-20% of primary breast cancers,<sup>8</sup> the product of *CCND1*, cyclinD1, is overexpressed at the mRNA and protein level in over 50% of breast cancers without a straightforward association with the gene amplification.<sup>7</sup>

Gene amplification of *ERBB2* (also called *HER2*), which has been the target of molecular therapy, occurs in about 15% of breast cancers. In ILC, *ERBB2* amplification is very rare as compared to in IDC,<sup>7</sup> and the reported frequency of the *CCND1* amplification ranges greatly from 0% to 27% of cases.<sup>7, 9</sup>

Multiplex ligation-dependent probe amplification (MLPA) is a new, high-resolution method for detecting numerous copy number variations in genomic sequences in a single reaction requiring only small amounts of DNA extracted from formalin-fixed paraffin-embedded (FFPE) tissues.<sup>10, 11</sup> A detection kit for 21 genes frequently amplified and deleted in breast cancers, including *ERBB2*, *CCND1* and *CDH1*, is commercially available. Here, we present a case of MDLC, in which the clonal profile could be well described by IHC, MLPA and fluorescence in situ hybridization (FISH).

### **Clinical summary**

A 36-year-old woman presented with a palpable mass in her right breast in September

2007. Physical examinations revealed a tumor measuring approximately 7 cm in diameter in the upper medial quadrant of the right breast. A needle biopsy of the lesion revealed invasive ductal carcinoma. Immunohistochemically, estrogen receptor (ER) (ii) approximately 60% and PgR) were positive of the tumor nuclei, respectively. The HercepTest™ was also positive (score 3), as approximately 20% of the cancer cells showed unequivocal staining of the cytoplasmic membrane. Systemic examination revealed multiple metastases in the lymph nodes, liver, and bones. After six cycles of paclitaxel (80 mg/m<sup>2</sup>) and trastuzumab (4 mg/kg loading dose, followed by 2 mg/kg once per week) as a chemotherapy for the luminal B-like cancer, followed by four cycles of fluorouracil epirubicin (75 mg/m<sup>2</sup>) and cyclophosphamide (FEC)<sup>12</sup> as systemic therapy, a right mastectomy and axillary lymph node dissection were performed for the purpose of disease control in spring 2008. The post-operative course was uneventful, but in October 2009 the patient died of brain metastasis. An autopsy was not performed.

#### Surgical specimens

An ill-defined tumor measuring approximately 8×7 cm was found. Histologically, the tumor consisted of cuboidal cells forming solid nests or tubules and smaller round cells growing discohesively (Fig. 1A). By immunohistochemistry (IHC), the former cells were E-cadherin positive and diagnosed as IDC, but the latter cells were negative for E-cadherin and considered to be ILC. Our diagnosis of the tumor was MDLC. (Fig. 1B) Metastatic cancer was found in 11 lymph nodes, and the histology was lobular carcinoma in seven nodes, ductal carcinoma in one node and MDLC in three nodes.

The biopsy specimen was reviewed and reclassified as MDLC because a minute portion of ILC was found. Neither ductal carcinoma *in situ* nor lobular carcinoma *in situ* was found in the biopsy specimen or resected tumor.

#### IHC

Antibodies against ER (clone 6F11, Novocastra, Newcastle upon Tyne, UK; working dilution of 1:50), PgR (clone PgR636, DAKO A/S, Glostrup, Denmark, ready-to-use), the internal domain of human ERBB2 (polyclonal, Nichirei, Tokyo, Japan; working dilution of 1:100), E-cadherin (mouse monoclonal, NCH-38, DAKO; ready-to-use), human cyclin D1 (rabbit monoclonal, SP 4, Nichirei, ready-to-use) and Ki-67 antigen (mouse monoclonal, clone MIB-1, DAKO; working dilution of 1:100) were used. Antibodies were visualized by avidin-biotin binding to peroxidase-conjugated secondary antibodies (DAKO).

Cyclin D1 was positive in both IDC and ILC. ERBB2 was positive in IDC but negative in ILC (Fig. 1C). ER $\alpha$  was positive in more than 80% of nuclei of ILC but negative in the nuclei of IDC. PgR was negative both in the ILC and IDC tumor nuclei. Ki 67-positive nuclei were found in approximately 50% of IDC and ILC cells.

### MLPA

Two representative tumor areas of IDC and ILC, approximately 0.5 cm<sup>2</sup> and 1.0 cm<sup>2</sup> respectively, were identified on consecutive 6- $\mu$ m-thick sections by using adjacent HE-stained sections. DNA was extracted separately and manually according to the manufacturer's protocol. MLPA analysis was performed by using a kit (SALSA MLPA KIT P078-B1 Breast Tumour) from MRC-Holland (Amsterdam, The Netherlands). This kit contains 39 probes for 21 different genes (*ESR1*, *EGFR*, *FGFR1*, *ADAM9*, *IKBKB*, *PRDM14*, *MTDH*, *MYC*, *CCND1*, *C11orf30*, *CDH1*, *TRAF4*, *CPD*, *MED1*, *ERBB2*, *CDC6*, *TOP2A*, *MAPT*, *BIEC5*, *CCNE1*, and *AURKA*), including four probes for *ERBB2*, two probes for *CCND1*, two probes for *CDH1* and one probe for *MED1*. Data analysis was performed with Coffalyser MLPA-DAT software (version 9.4, MRC-Holland) to generate normalized peak values. Peak values below 0.7 were defined as lost, between 0.7 and 1.3 as normal, between 1.3 and 2.0 as gain, and values >2.0 as amplified, as previously established.<sup>11</sup>

The results showed that *CCND1* was amplified both in IDC and ILC, however *ERBB2* and *MED1* were amplified only in IDC, and *CDH1* was within the normal range in both IDC and ILC. MLPA values for the other 17 genes were within normal ranges. (Table 1)

### FISH

FISH probes for *ERBB2* (RP11-62N23), *CCND1* (RP11-775J10) and *CDH1*(RP11-354M1) were acquired from BACPAC Resources (Oakland, CA, USA) and labeled with SpectrumOrange<sup>TM</sup> or SpectrumGreen<sup>TM</sup> with a nick translation kit (Abbott Laboratories, Abbott Park, IL, USA). For the detection of gene amplification, SpectrumGreen<sup>TM</sup>-labeled pericentromeric probes (Abbott) specific to chromosomes 11, 16 and 17 on which the particular gene was located were co-hybridized to standardize the chromosome number.

By FISH on paraffin-embedded surgical specimens, clustered signals of *CCND1* corresponding to gene amplification on a homogeneously staining region (HSR)<sup>13</sup> were found in both IDC and ILC; however, similar clustered signals of *ERBB2* were only found in IDC. (Figs. 2A and B.). The copy numbers of *ERBB2* and CEP17 were

18.0±4.5 (mean±SD) and 3.0±2.7, respectively, and their ratio was 8.3±4.7. The copy numbers of *CCND1* and CEP11 were 9.1±2.6 and 1.4±0.6, respectively, and their ratio was 7.1±2.9. Dual-color FISH on a biopsy specimen showed that approximately a quarter of IDC cells had HSR signals of both *ERBB2* and *CCND1*; however 70% were negative for *ERBB2* and *CCND1*. (Figs. 2C and D)

Touch smears of fresh tumor prepared immediately after surgery were also used for the FISH analysis. Dual-color FISH for *CCND1* and *ERBB2* revealed two types of unequivocal tumor nuclei: nuclei with clustered signals of *CCND1* and *ERBB2*, and smaller nuclei with clustered signals of only *CCND1*. (Fig. 3A) Each signal corresponds to the nucleus of IDC and that of ILC in paraffin-embedded surgical specimens, respectively. Interestingly, the FISH images of amplified *CCND1* in IDC and ILC appeared similar in size and shape. (Fig. 3A) Dual-color FISH of *CDH1* and centromere 11 showed two predominant patterns for the number of *CDH1* signals/number of centromere 11 signals; 2/2 and 1/1, as shown in Figures 3B and C.

This study was approved by the Institutional Review Board at Kanazawa University Hospital (approval no. 265), and written informed consent was obtained from the patient.

### Discussion

The present case was a morphologically and immunohistologically typical MDLC. The MLPA analysis of the IDC revealed clear gene amplification of *ERBB2* and *CCND1*; furthermore, the FISH analysis showed that the IDC contained amplified *CCND1* and *ERBB2* in HSRs although the ILC contained amplified *CCND1* alone. In addition, the FISH analysis on touch smears showed that the IDC and ILC appeared to share similar HSR-type gene amplification of *CCND1*. High-level gene amplification associated with such well recognized abnormal chromosomal structure as HSR is mechanistically distinct from other forms of DNA copy number change and an important cytogenetic marker of cancer clones.<sup>13</sup> Thus, in terms of tumorigenesis, it is most likely that the two cancer subclones found in the present tumor share a common precursor cell as drawn in Figure 4.

The co-existence of two HSRs of *CCND1* and *ERBB2* in single nuclei of the IDC indicates the present tumor has a chromosomal instability phenotype; however, the cytogenetic mechanisms of co-amplification of *ERBB2* and *CCND1*, which are located on different chromosomes, are enigmatic. At the present time, the selective merit, if any, of co-amplification of *ERBB2* and *CCND1* is unknown. A FISH study examining 93

cases of IDC reported high-level amplification of *CCND1* and *ERBB2* in 15% and 17%, respectively; however, there was no statistical significance for the frequent coamplification.<sup>9</sup> A recent MLPA study identified *ERBB2* and *CCND1* coamplification in 9% of the examined invasive breast cancers,<sup>10</sup> but it was not determined if the coamplifications occurred in a single nuclei or on the same amplicon. Different from *CCND1* and *ERBB2*, genes such as *MED1* located very closely to *ERBB2* are occasionally co-amplified with *ERBB2*, as found in the present case as a molecular variant of *ERBB2* amplicons.<sup>14</sup> Whether it simply represents co-amplification of a ‘passenger gene’ or it implicates reduced sensitivity to hormone therapy of *ERBB2*-amplified tumors remains to be clarified.<sup>14</sup>

Negative expression of E-cadherin in ILC is considered to be caused by missense mutation or methylation silence of E-cadherin of an allele of *CDH1* in combination with loss of the other normal allele by physical deletion of the chromosomal foci of 16p22.<sup>5</sup> The cancer cells with a single copy of *CDH1* found by FISH on touch smear in the present study most probably correspond to the ILC. Deletion of one copy of the gene, which occurred in a subset of tumor cells, could not be detected by MLPA, which examines the net numerical changes.

In the present study, three different cancer clones with different genetic profiles were found in specimens obtained pre- and post-systemic therapy: IDC with amplification of both *CCND1* and *ERBB2*, IDC without the gene amplifications, and ILC with *CCND1* amplification. We speculate that systemic therapy caused clonal selection in the tumor, as depicted in Figure 4. The IDC cells without amplification of *ERBB2* and *CCND1* found predominantly in the biopsy specimen may have been killed in response to the chemotherapy. However, the *CCND1*-positive cells were more resistant to the chemotherapy and survived. In fact, *CCND1*-amplified tumors are associated with treatment resistance and reduced survival times as compared to *CCND1*-positive [Is this correct?] cells.<sup>8</sup> ILCs are generally less responsive to chemotherapy in relation to their biological characteristics.<sup>1, 15</sup> As a result, the *CCND1*-amplified and *ERBB2*-non-amplified ILC, which is chemotherapy-resistant and trastuzumab-insensitive, became predominant as shown in Table 1. Interestingly, a recent study has reported that it was not infrequent that IDC carcinomas diagnosed on the core biopsy turned out to only have either lobular or another type of tumor in the post-chemotherapy excision samples.<sup>16</sup>



The present case showed that the semi-comprehensive analysis of amplification status by MLPA could be useful to plan individualized molecularly targeted therapy against breast cancers.

#### Acknowledgements

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No conflicts of interests were declared.

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### Figure legends

Figure 1.

Representative histology of the MDLC (A). E-cadherin (B) and ERBB2 (C) were positive in IDC, but negative in ILC.

Figure 2.

FISH on FFPET. In the resect tumor, IDC and IDC had gene amplification of *CCND1* (A: *CCND1*, orange fluorescence; centromere 11, green fluorescence); however, amplification of *ERBB2* was confined to IDC (B: *ERBB2*, orange fluorescence; centromere 17, green fluorescence). The biopsy showed IDC with both *CCND1*- and *ERBB2*-amplification (C) and those without amplification (D) (*CCND1*, orange

fluorescence; *ERBB2*, green fluorescence).

Figure 3.

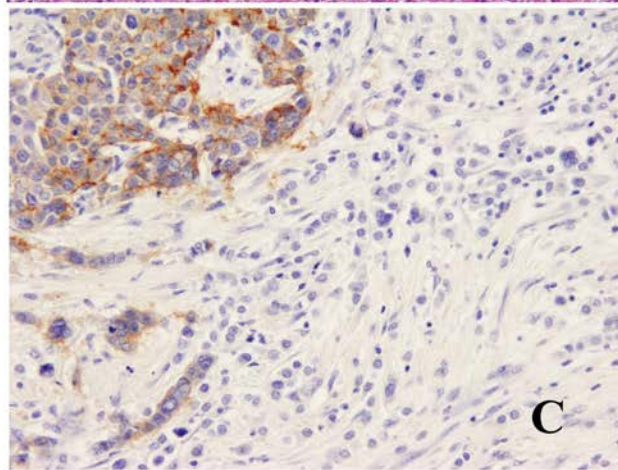
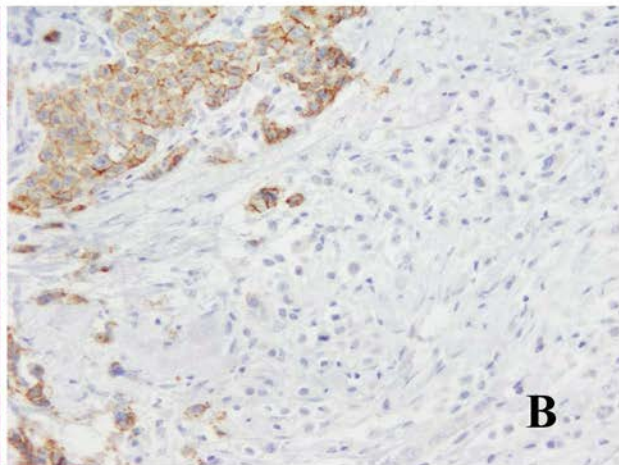
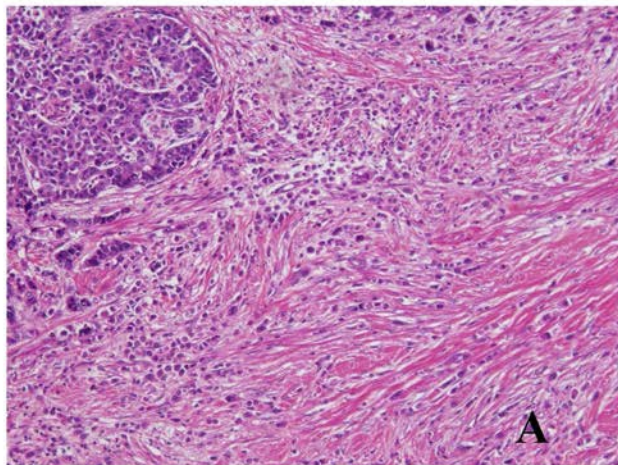
FISH on touch smears. Cancer nuclei with a cluster of amplified *CCND1* (orange signals) and a cluster of amplified *ERBB2* (green signals) and cancer nuclei with a clustered signal of *CCND1* were found (A). In panels B and C, cancer cells with two copies and one copy of *CDHI* were found, respectively. (Orange signal, *CDHI*; green signal, centromere 11.)

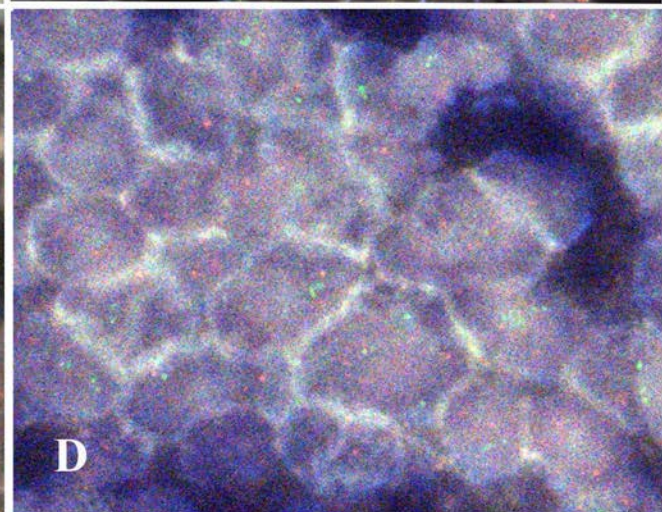
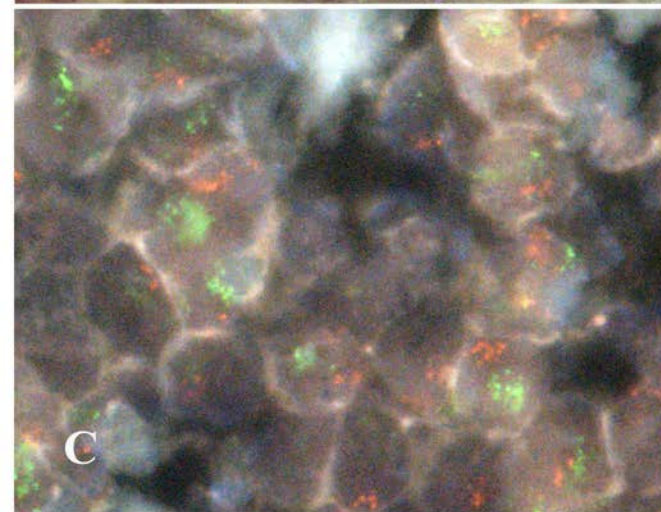
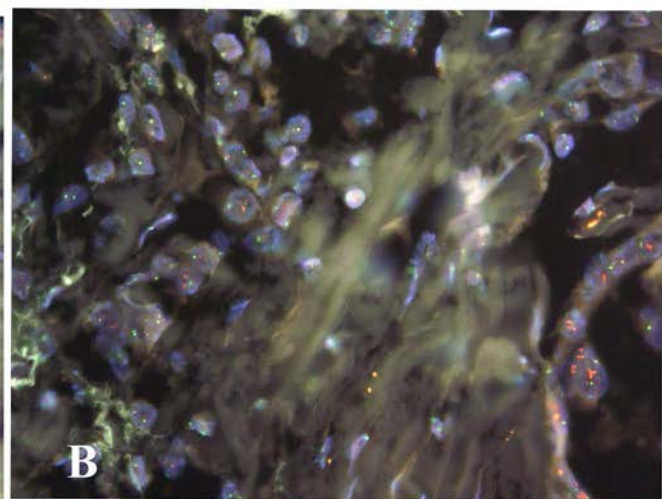
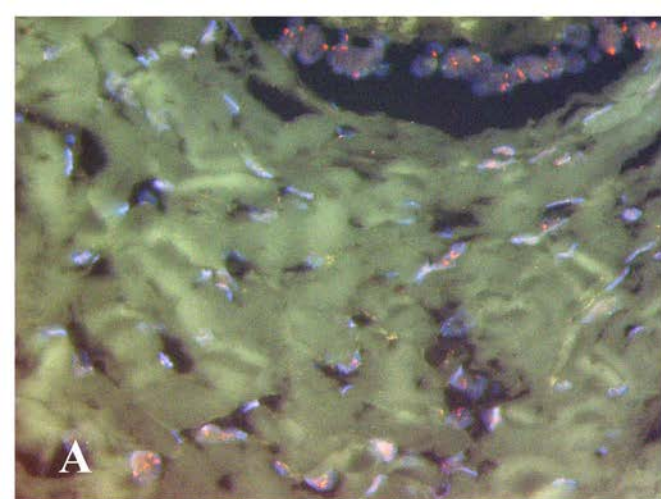
Figure 4.

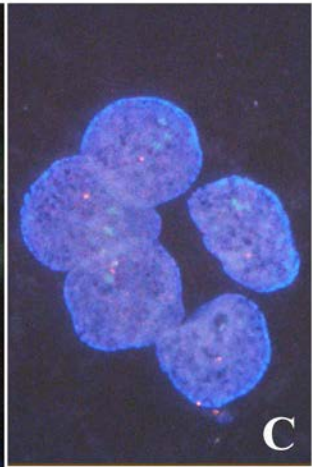
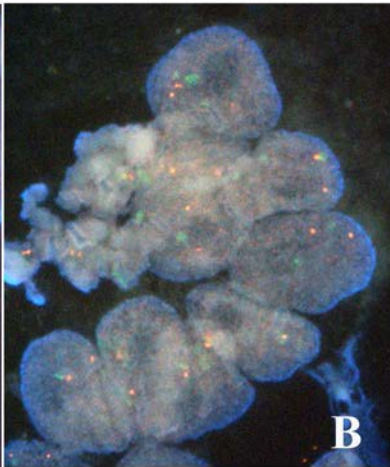
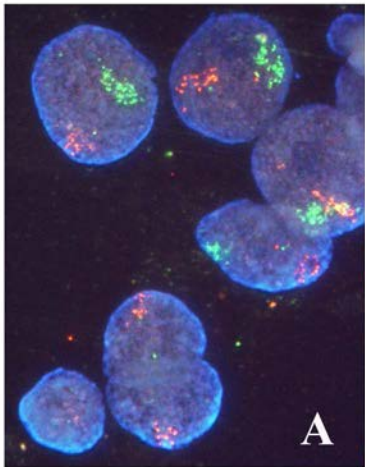
Speculated sequences of carcinogenesis and clonal selection in this case.

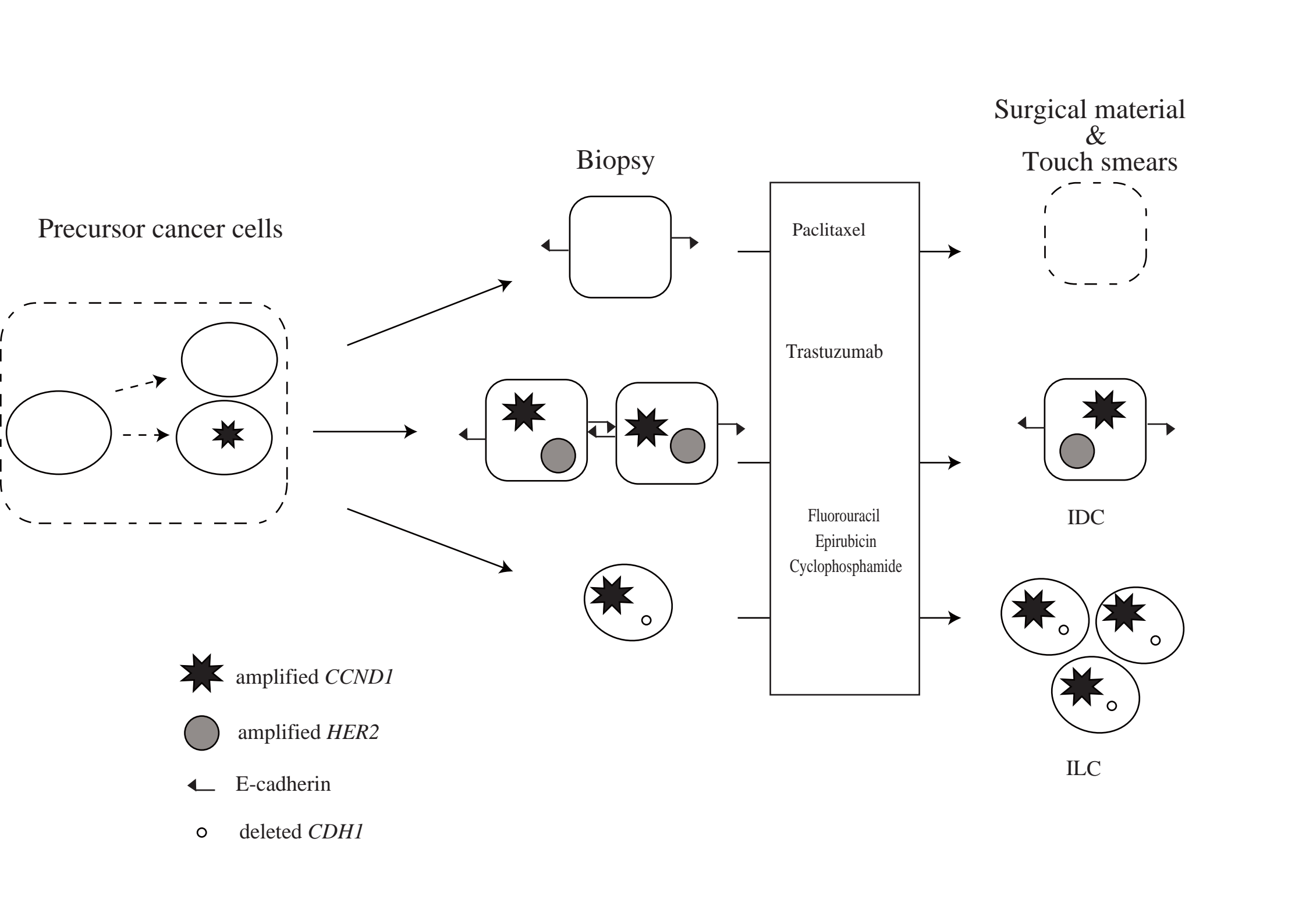
Table 1.

Result of the MLPA analysis. Bold font indicates 'amplification' by MLPA; Italic font indicates 'gain' by MLPA.











<i>Gene</i>	<i>ESR-1</i>		<i>EGFR</i>		<i>FGFR1</i>		<i>ADAM9</i>	<i>IKBKB</i>		<i>PRDM14</i>	<i>MTDH</i>		<i>MYC</i>			<i>CCND1</i>		<i>C11orf30</i>	
Chromosomal position	6q25		7p11		8p11		8p11	8p11		8p21	8q22		8q24			11q13		11q13	
IDC	0.97	0.94	1.15	0.93	1.21	1.13	0.88	0.96	1.01	1.2	0.98	1.02	0.75	1.06	1.03	<b>3.11</b>	<b>3.28</b>	1.17	1.05
ILC	0.85	0.95	1.04	0.91	1.11	1.05	0.85	0.87	1.01	1.05	1.01	0.97	0.99	1.13	0.95	<b>2.86</b>	<b>2.97</b>	1.11	1.04

<i>CDH1</i>	<i>TRAF4</i>	<i>CPD</i>	<i>MED1</i>	<i>ERBB2</i>			<i>CDC6</i>	<i>TOP2A</i>		<i>MAPT</i>	<i>BIRC5</i>		<i>CCNE1</i>		<i>AURKA</i>				
16q22	17q11	17q11	17q12	17q12			17q21	17q21		17q21	17q25		19q12		20q13				
1	0.95	1.26	1.1	<b>2.19</b>	<b>2.24</b>	<b>1.64</b>	<b>2.2</b>	<b>1.92</b>	0.85	1.04	1.03	0.92	0.81	0.84	1.12	0.98	0.92	0.93	0.85
0.87	0.86	1.08	1.05	1.11	1.07	0.8	1.11	0.88	0.85	1.08	1.02	0.95	0.8	0.7	0.93	0.9	0.87	0.86	0.9