

CCND1- and ERBB2-Gene Deregulation and PTEN Mutation Analyses in Invasive Lobular Carcinoma of the Breast

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Because of the relatively low incidence of lobular breast carcinoma, there are very few studies on the molecular characteristics of this breast cancer. In an attempt to improve its characterization, we investigated in a large collection of invasive lobular carcinomas (ILCs) the status of markers known to be involved in the better-studied invasive ductal carcinomas (IDC). In the current study we disposed of 80 well-characterized ILC cases. Gene amplification of cyclin D1 (*CCND1*) and c-erbB2-encoding gene (*ERBB2*) and expression of their gene products were studied by differential polymerase chain reaction (PCR) and immunohistochemistry, respectively. A comprehensive point mutation study of the phosphatase and tensin homolog tumor suppressor gene (*PTEN*) was pursued by single strand conformation polymorphism (SSCP)/sequencing analysis. The *CCND1* gene was rarely amplified in ILC in spite of showing over-expression of the protein in 41% of tumors. Hence, unlike IDC, increase in gene dosage did not account for the protein excess. *PTEN* mutations were detected in ILC (truncating mutations) in around 2% of the tumors. Unlike IDC, ILC did not display *ERBB2* overexpression and expression of the transcription factor E2F1 correlated inversely with tumor grade. The observed discrepancy in the pattern of the human oncogenes *CCND1* and *ERBB2*, which are involved in the process of carcinogenesis of ductal tumors, appears to suggest a different molecular basis for development and progression of ILC. © 2002 Wiley-Liss, Inc.

Key words: invasive lobular carcinoma; breast cancer; genetics; *CCND1*; *ERBB2*; *PTEN*

INTRODUCTION

Breast cancer comprises a variety of tumor types with well-characterized histopathological features. Two types account for most malignancies, i.e., invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC), which arises from the epithelial lining of the glandular elements of the mammary lobules and comprise a fraction estimated at 2–10% of breast cancers [1]. Molecular studies in IDC have unveiled a number of alterations in cell proliferation-related genes such as cyclin D1 (*CCND1*) and the c-erbB2 protein-encoding oncogene (*ERBB2*) [2,3]. Because of their confirmed role in IDC, we hypothesized that these alterations occur during the development of ILC.

Alterations in the cyclin-cyclin dependent kinase-retinoblastoma pathway that increase the proliferative potential of the cell are known to play a central role in breast cancer. In a recent study, one of our laboratories found that expression of the transcription factor E2F1, released after functional inactivation of the retinoblastoma protein, correlates

positively with tumor stage in IDC [4]. The phosphatase and tensin homolog tumor suppressor gene (*PTEN*) encodes a dual-specificity phosphatase that is also involved in phosphate-mediated growth signaling through the retinoblastoma protein [5,6]. Loss of the gene occurs in sporadic IDC [7]. Moreover, the germline *PTEN* mutations of Cowden's syndrome [8,9], a condition predisposing to breast cancer, enhance the interest in discerning the involvement

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Abbreviations: IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; *CCND1*, cyclin D1 gene; *ERBB2*, gene encoding c-erbB2 oncoprotein; *PTEN*, phosphatase and tensin homolog gene deleted on chromosome 10; PCR, polymerase chain reaction; fdPCR, fluorescent differential PCR; *DD2r*, dopamine-D2 receptor gene; γ -*IFN*, gamma interferon gene; F, fluorescein; SSCP, single strand conformation polymorphism.

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of this gene in the different varieties of breast tumors. Overexpression of *CCND1* due to a large extent to the presence of extra gene copies contributes to aberrant cell-cycle activation in IDC [10–12] and together with abnormal copy number increases of *ERBB2* gene, as a consequence of genetic gains on 17q12, is among the most frequent genetic alterations in IDC; overexpression of this member of the epidermal growth factor receptor family is present in approximately 1/4 of tumors [2,3,13–15]. Nevertheless, these abnormalities are poorly documented in other breast tumors. It thus remains unclear whether or not the molecular alterations that characterize IDC are shared by ILC.

MATERIALS AND METHODS

Specimens

The present study was performed on 80 ILCs from patients diagnosed at the Hospital Clínico, Madrid, Spain, from January 1990 through December 1996. Most tumors (80%) showed the classic or strict ILC patterns, whereas the remaining 20% were of tubulo-alveolar, alveolar, or mixed variant forms. According to their nuclear features, 16% of tumors were of low tumor grade (grade 1), 62% of intermediate malignancy (grade 2), and 22% of grade 3. Twenty IDCs included for comparative purposes were from Departamento de Especialidades Médico-Quirúrgicas, Universidad del País Vasco, Leioa, Spain.

Immunohistochemistry

Paraffin sections were used for immunohistochemical detection of *CCND1*, *ERBB2*, *E2F1*, and *Ki-67*. Once deparaffinized and rehydrated with graded ethanol, sections were microwaved for antigen retrieval, except for *ERBB2*, in 1 mM EDTA or 0.1 M sodium citrate (*Ki-67*), treated with 3% H_2O_2 and normal horse serum to minimize nonspecific signals, and left at room temperature for 1 h with monoclonal antibody (1:100): anti-mouse *CCND1* (NCL-CYCLIN D1-GM, Novocastra, Newcastle upon Tyne, UK), anti-mouse *ERBB2* (MU-134-UC, Biogenex, San Ramon, CA), anti-human *E2F1* (sc-25, Santa Cruz Biotechnology, CA), or anti-mouse *Ki-67* (Immunotech, Westbrook, ME); normal mouse immunoglobulin G was used as negative control. Immunocomplexes were visualized by use of a biotinylated ABC kit (Vectastain Elite, Vector Laboratories, Inc., Burlingame, CA), diaminobenzidine as chromogen, and hematoxylin counterstaining. Labeling indices (percentage of stained/total cells), when indicated, were determined counting more than 500 cells at 400 \times magnification.

DNA Extraction

Paraffin-embedded tissue sections were dewaxed in xylene, rehydrated with graded ethanol, immersed briefly in acetone, and air-dried. In the case

of ILCs, DNA was extracted from selected areas of extended tumor carefully localized by comparison with hematoxylin and eosin-stained sections of each specimen. Tumor material was scraped off and suspended in 0.1 mL of 10 mM Tris–0.1 mM EDTA (pH 7.4) containing 5% Tween-20. Tumor from three adjacent 7- μ m sections was pooled for each sample and digested with 0.4 mg/mL proteinase K (55°C, 2 h; 95°C, 5 min), and the debris was removed by centrifugation. DNA was estimated in the supernatants from their absorbance at 260 nm and used as template for polymerase chain reaction (PCR). Blood DNA used as PCR assay control was extracted by a standard phenol-chloroform procedure.

Fluorescent Differential PCR (fdPCR) Analysis

Short fragments within either the *CCND1* (157 bp) or *ERBB2* (98 bp) genes were coamplified by fdPCR along with regions of the dopamine D2 receptor (*DD2r*) (128 bp) or gamma interferon (γ -*IFN*) (82 bp) genes, respectively. The sequences of the forward and reverse primers (Genset, Paris, France) were (from 5' to 3' end): *CCND1*: fluorescein (F)-ACCAGCTCC-TGTGCTGCGAAGTG and GACGGCAGGACCTCC-TTCTGCACA; *DD2r*: F-TGATGATGATCTGGAGAG-GCAGAAC and TGCCGAAGACGATGACAGCGAT-GAG; *ERBB2*: F-CCTCTGACGTCCATCATCTC and ATCTTCTGCTGCCGTCGCTT; and γ -*IFN*: F-GCAGAGCCAAATTGTCTCCT and GGTCTCCACACT-CTTTTGGGA. fdPCR was carried out in a Biometra T3 Thermocycler (Gottingen, Germany) by heating 25 μ L containing 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 200 μ M dNTPs (Boehringer, Mannheim, Germany), 2 mM $MgCl_2$, 0.5 μ M each primer, 1 U of *Thermus aquaticus* DNA polymerase (Taq) (Bioline, London, UK), and 200 ng of DNA, at 95°C (5 min), 85°C (Taq addition); then 94°C (50 s), 59°C (30 s), and 72°C (50 s) (28 cycles); and 72°C for 10 min. The pairs of products of each fdPCR, repeated twice, were electrophoresed through 2.5% agarose and visualized under ultraviolet light.

For quantitation, a fluorescein compatible-LASER system beaming throughout the gel of an automated DNA sequencer (A.L.F. Pharmacia, Uppsala, Sweden) was used. Each fdPCR coamplification product was diluted 1:4 in 90% formamide, 10 mM EDTA, and 0.3% bromophenol blue; heated at 95°C; and quickly chilled on ice. Ten microliters of denatured product was loaded onto a 7 M urea–6% acrylamide gel (19:1 acrylamide:bisacrylamide) (Gibco BRL, Scotland, UK) and run at constant voltage. The areas of the peaks of fluorescence determined by use of Pharmacia software (Fragment Manager, FM 1.2, Uppsala) served as semiquantitative estimations, and gene dosages were calculated by dividing the peak areas of the oncogene fdPCR product and its control. Ratios of control DNA were checked from 24 to 28 PCR cycles to assure the exponential range of our one-point measurement. Basal gene ratios in peripheral

lymphocytes and paraffin-embedded nontumor breast tissue (0.78 ± 0.07 ($n = 23$) and 0.81 ± 0.16 ($n = 45$) for *ERBB2*/ γ -*IFN* and 0.65 ± 0.25 ($n = 23$) and 0.60 ± 0.27 ($n = 45$) for *CCND1/DD2r*), defined basal variation. The cut-off points, set at 1.5 times the basal ratio (2 standard deviations were added to the mean to allow for variation), were 1.4 for *ERBB2* and 1.7 for *CCND1*. Higher ratios were scored as evidence for gene amplification.

Southern Blot Analysis of *CCND1*

DNA (15 μ g/tumor), extracted by standard protocols from frozen-stored pieces of 15 additional cases of ILC retrieved from the Tumor Bank Facility at Fox Chase Cancer Center, Philadelphia, PA, was digested with EcoRI, size-sorted through 0.6% agarose, capillary-transferred onto Hybond-XL nylon filters (Amersham Pharmacia Biotech, UK), and heat-linked in a vacuum oven. After prehybridization with ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA), blots were hybridized overnight at 60°C with purified [α - 32 P]-dCTP-labeled cDNA probes of a 1.4-Kb *CCND1* fragment or β -actin (Clontech) as loading control. Blots were washed under stringent conditions and exposed to Kodak films at -70°C.

Single Strand Conformation Polymorphism (SSCP) and Sequence Analysis of *PTEN*

Fourteen *PTEN* gene fragments encompassing the entire coding region were analyzed for mutations by PCR-SSCP. Concentration of PCR reagents was as for fdPCRs. After denaturation and Taq addition, PCR amplification was accomplished by 30 cycles of denaturation (94°C, 50 s), annealing (Table 1, 30 s), and extension (72°C, 50 s) and a final extension at 72°C for 10 min. PCR products were diluted 1:4

in 90% formamide, 10 mM EDTA, and 0.3% bromophenol blue; heat-denatured; and cooled on ice. Ten microliters was loaded onto a 5% glycerol-12% acrylamide minigel (19:1 acrylamide:bisacrylamide) and electrophoresed at constant 10 W for 8 h in a cool room in 0.5 \times TBE (1 \times TBE, 90 mM Tris, 90 mM boric acid, 8 mM EDTA (pH 8)). Gels were silver-stained [16], dried, and examined for bands with altered mobility. Variant and control samples were sequenced with an ABI Prism 310 Genetic Analyzer of Perkin Elmer (Wellesley, MA). For this purpose, 1 μ L of product was reamplified and purified (High Pure PCR product Purification Kit, Boehringer). Sequencing in both directions was performed following directions of the ABI Prism dRhodamine terminator cycle sequencing kit (Perkin Elmer).

RESULTS

Expression of E2F1 and Ki-67 in ILC

Immunohistochemical staining of the transcription factor E2F1 was noted in all cases examined (73 tumors), with $12 \pm 9\%$ of positively stained cells. The mean percentages of E2F1-stained cells showed a tendency to decline as tumor grade increased (12.7% for grade 1 ($n = 8$), 10.3% for grade 2 ($n = 46$), and 5.7% for grade 3 ($n = 19$)), significant between tumor grades 2 and 3 and close to significance for grades 1 and 2 (Figure 1). No association was found with the Elston grading of malignancy or presence of signet-ring cells. The mean percentage of E2F1-stained nuclei was above sixfold higher in tumor areas than in nontumor breast tissue of the same samples. Tissue sections of 15 nonparaffin samples of ILC examined were immunoreactive for Ki-67 in $17 \pm 9\%$ of the nuclei.

Table 1. Sequences of Primers Used to Amplify *PTEN* From Genomic DNA

Exon	5'-3' forward/reverse primers	Flanking nucleotides*	Annealing (°C)	Base pairs
1	AGAAGAAGCCCCGCCACCAG/GAGGAGCAGCCGCAGAAATG	-71, +25	59	174
2	GTTTGATTGCTGCATATTTCA/TCTAAATGAAAACACAACATGAA	-51, +66	50	201
3	TGTTAATGGTGGCTTTTTG/GCAAGCATACAAATAAGAAAAC	-39, +30	53	113
4	TTCCTAAGTGCAAAGATAAC/TACAGTCTATCGGGTTAAGT	-50, +53	50	146
5	TTTTTCTTATTCTGAGGTTATC/TCATTACACCAGTTCGTCC	-35	49	184
5	TCATGTTGCAGCAATTCAC/GAAGAGGAAAGGAAAAACATC	+35	53	176
6	CTTCTCTTTTTTCTGTCC/AAGGATGAGAATTTCAAGCA	-25, +25	50	191
7	TTCCTGTGAAATAATACTGG/GAACTCTACTTTGATATCAC	-39	50	175
7	AGTTCATGTACTTTGAGTTC/TCCTAATGAAAGTAAAGTAC	+24	50	115
8	CAGATTGCCTTATAATAGTC/TCCTGGTATGAAGAATGTAT	-181	50	225
8	AGGACAAAATGTTTCACTTTTGG/GTAAGTACTAGATATTCCTTGTC		50	156
8	GAAATCGATAGCATTGTCAG/ATACATACAAGTCACCAACC	+46	52	177
9	AGATGAGTCATATTTGTGGG/ATGATCAGGTTTCATTGTAC	-47	52	148
9	CAGTCAACTTCGTAACAC/ATGGTGTTCCTTCTTG	+32	50	164

*Last nucleotide positions targeted in flanking introns, according to *PTEN* intron/exon sequence reported in Genbank (accession no. AF067844), where positions -1, 1, and +1 refer to last 5'-intron base, first exon base, and first 3'-intron base, respectively.

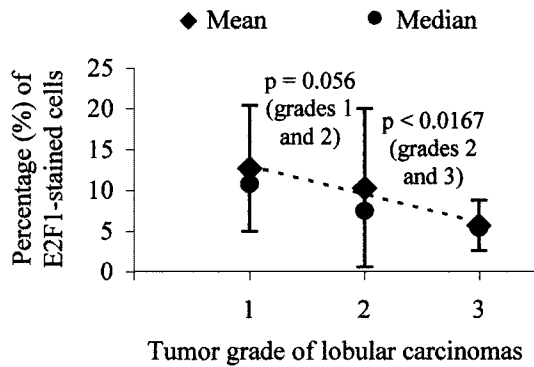


Figure 1. Summary results for the percentage of E2F1-stained nuclei in ILCs. The correlative link and the corresponding *P* value are shown.

CCND1 Gene Amplification/Overexpression

CCND1 gene amplification was detected by fdPCR in two ILCs (gene ratios of 1.8 ± 0.1 and 1.9 ± 0.1) of 80 cases studied (Table 2). The same fdPCR method showed exceeding CCND1 gene dosage in 19% of IDCs. CCND1 gene amplification in ILC was also investigated by Southern blotting DNA from 15 tumors obtained as tissue blocks. Densitometric analysis disclosed one case of high malignancy with CCND1 amplification, confirming sporadic occurrence in ILC (Figure 2).

Immunohistochemical expression of CCND1 was detected in 27 of 66 cases of ILC (41%) (Figure 3A and B), including the tumors showing amplification of CCND1. No staining was seen in stromal cells, and staining was only occasionally noted in normal epithelial structures. The mean percentage of stained nuclei for the proliferative marker E2F1 in tumors positive for CCND1 expression was not significantly different from that proliferative index in tumors showing not detectable expression of the cyclin (10.4% and 8.6% of E2F1-stained cells, respectively) by unequal-variance *t* and Mann-Whitney tests. Interestingly, in three variant ILC cases we could detect an increase in expression of the cyclin in the tumor's invasive front and a relatively lower expression in the center of the tumor. Ki-67 staining of contiguous sections showed no difference between the invasive front and the more central portions of the same tumor (Figure 3D). Eleven of 20 tumor sections of IDC showed CCND1 immunoreactivity; the percen-

Table 2. Comparison of Number of Cases of CCND1 and ERBB2 Gene Amplification/Overexpression Detected in Lobular and Ductal Tumors

	ILC (%)	IDC (%)
CCND1 amplification	2/80 (3)	3/16 (19)
CCND1 overexpression	27/66 (41)	11/20 (55)
ERBB2 amplification	1/80 (1)	4/18 (22)
ERBB2 overexpression	0/44 (-)	12/20 (60)

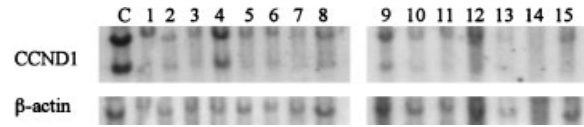


Figure 2. Southern blot detection of CCND1 amplification in ILCs. The CCND1 probe hybridized with two EcoRI fragments of 2 and 4 kb; the filter was also hybridized with a β -actin-specific probe to ascertain differences in DNA loading. DNA from a head and neck tumor was used as positive control (lane C). One case of amplification was detected, in lane 4.

tage of positively stained nuclei was slightly higher in IDC ($15\% \pm 12$) but not significantly different from ILC ($11\% \pm 9$).

PTEN Point Mutations

DNA from 54 paraffin cases of ILC was investigated for the presence of mutations in the nine exons of PTEN. SSCP analysis of the variant denaturation products allowed to identify an alteration in one specimen characterized as an adenine deletion at the second base of codon 197 (Figure 4). The deletion was not found in adjacent normal tissue, suggesting that it was somatic in origin.

ERBB2 Gene Amplification/Overexpression

ERBB2 gene copy number in ILC was assessed by fdPCR. One tumor surpassed the cut off for the presence of extra gene copies of this specific growth factor receptor gene (Table 2). ERBB2 overexpression was undetectable by immunohistochemistry in a set of 44 samples of ILC (Figure 3E); the lack of staining for this cell-surface receptor persisted regardless of the concentration of primary antibody employed. Neither membrane-bound nor cytoplasmic immunostaining, could be seen in the tumor sample with gene amplification, which therefore was considered equivocal. In contrast, 22% of IDC showed gene amplification, and more than half of equivalent paraffin IDC sections stained strongly by the same immunohistochemical procedure (Figure 3F).

DISCUSSION

Lobular carcinoma is a breast malignancy with a well-described diffuse histo-architecture, different from the usually compact morphology of ductal tumors. Although there is a clear morphological difference between these two types of breast carcinomas, little is known about the differences or similarities in molecular characteristics. Much is known about IDC, but there is in general a lack of knowledge of the underlying molecular alterations in lobular breast tumors. Several molecular alterations are known to progressively accumulate during IDC tumor progression [2,3]. However, with exception of the inactivating mutations in the adhesion molecule E-cadherin, a molecular hallmark of lobular carcinoma that accounts for its typical scattered pattern of growth [17], the altered genes that

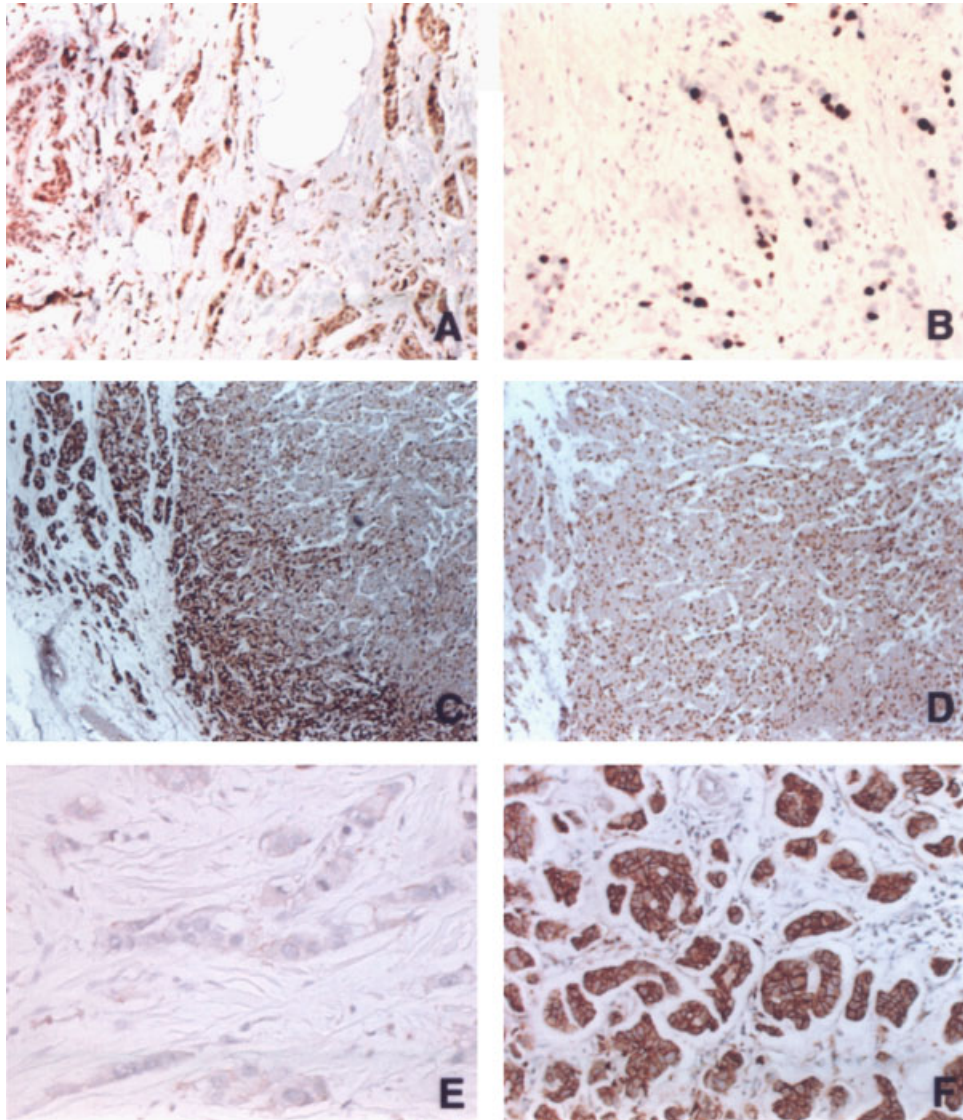


Figure 3. Immunohistochemistry. (A) Immunohistochemistry for CCND1 in a representative ILC; note nuclear and some cytoplasmic stain in the invading tumor cells. (B) Higher magnification showing intense and selective nuclear staining in approximately 1/3 of tumor cells. (C) CCND1 overexpression in the invading margin of a variant

type ILC. (D) Homogeneous Ki-67 immunostain distribution in the periphery and center of the tumor shown in panel C. (E) Absence of detectable expression of ERBB2 in ILC. (F) Detection of ERBB2 overexpression in IDC. Magnification: 100 \times (panels A, C, D, and F) and 250 \times (panels B and E).

participate in the development of ILC are not fully identified. This study, centered on a large sample of carefully selected lobular carcinomas, revealed distinct characteristics of ILC with respect to two major human breast cancer oncogenes, *CCND1* and *ERBB2*.

The presence of high levels of CCND1 in ILC was consistent with previous immunohistochemical data [18,19]. Thus, upregulation of the amount of CCND1 protein seems quite similar in ILC to that described in IDC [11,12,18–22]. ILC with overexpression levels of CCND1 protein did not show a significantly different percentage of cells stained for E2F1, a downstream cell-division regulator, suggesting a lack of coordination between the CCND1

protein excess of ILC and the cell-cycle machinery. This was particularly evident in a few cases in which CCND1 staining was very strong along the invasive front of the tumor and much less in the noninvasive component while staining for Ki-67, a protein expressed in cycling but not in resting (G_0 phase) cells, was equally distributed. In DNA from archival tissue, *CCND1* gene amplification had much greater incidence in IDC than in ILC. This result, by use of short differential PCRs with selected inner control genes in combination with a sensitive method of direct quantitation, indicated a significant molecular difference between both tumors, and thus, amplification of the gene seems of minor importance in ILC

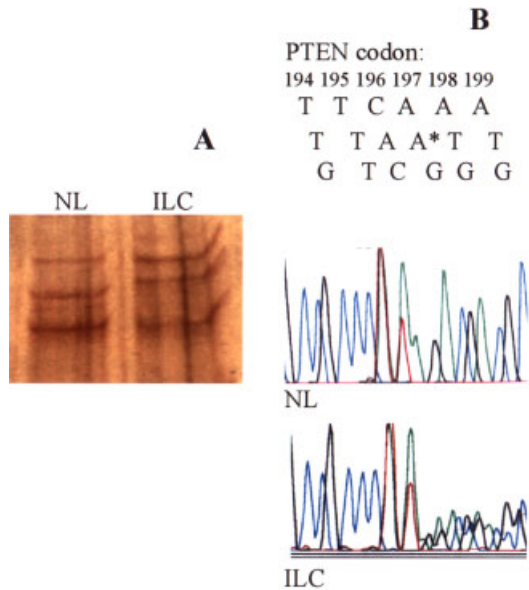


Figure 4. Detection of *PTEN* 590delA in ILC by intron based-PCR/SSCP/sequencing analysis. (A) SSCP gel showing altered mobility in a case of ILC with respect to the pattern of the product from a normal lymphocyte (NL) control DNA sample, suggesting an alteration in exon 6 of *PTEN*. (B) Sequencing profiles showing a deletion of the second adenine in codon 197 that changes the reading frame such that a premature stop signal arose at the next amino acid position.

while it represents an important mechanism in driving *CCND1* overexpression in ductal tumors even at early stages [22]. Therefore, the correlation between overexpression and gene amplification in IDC [10] cannot be directly extended to lobular breast tumors, in which aberrations other than gene amplification are at play. In this context, it is worth noting that other genes of the cyclin family undergo overexpression without gene amplification in breast cancer [23]. Overexpression without gene amplification might be due to alterations of the gene's regulatory sequences or due to factors other than the gene itself.

TP53, *BRCA1*, and *BRCA2* are the best-known breast cancer susceptibility genes [3]. However, interest has recently focused on *PTEN* because of its association with Cowden syndrome [8,9] and the presence of mutations in breast cell lines and high proportion of the primary tumors with loss of heterozygosity encompassing the locus. Inherited mutations were not found in the present study, and therefore, *PTEN*-related susceptibility seems unlikely. Although sporadic mutations were found in ILC, their low occurrence did not allow to assign to *PTEN* a relevant role in this human tumor. One mutation was noticed, predicted to change the reading frame and elicit a premature stop codon preceding the phosphate-acceptor regulatory sites located in the C-terminal region. Rhei et al. [8] described a similar mutation in another lobular tumor, whereas so far, no truncating *PTEN* mutations are known in IDC [24,25].

The divergence between ILC and IDC with regard to *ERBB2* overexpression was remarkable. The marginal or absent immunoreaction in most ILCs and the infrequent amplification of the gene suggested a minor role for this oncogene. Given the strong evidence for the primary involvement of this gene in ductal carcinogenesis and its established role as a direct-acting oncogene [13,15,26], the expression of negligible amounts of *ERBB2* in lobular breast cancer might be indicative of a different pathway of genetic evolution for this tumor. *ERBB2* amplification in breast carcinomas correlates with pathohistological characteristics such as larger tumor size [14]. The absence of significant overexpression of this cell-surface receptor in ILC may define a different phenotypic subset of breast cancer with a different range of extracellular signals for growth of tumor cells. Interestingly, expression of the transcription factor E2F1 in lobular carcinomas correlated inversely with tumor grade, in contrast to IDC [4]. A distinct pattern of allelic imbalance leads to the postulate that ILC might develop through a different carcinogenesis mechanism than IDC [27]. Although other candidate genes should be explored in the future, the observed discrepancy in two critical human oncogenes involved in the process of carcinogenesis of ductal tumors further supports the molecular divergence between these two histopathological types of breast cancer.

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