

PI-PLC β 1 gene copy number alterations in breast cancer

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Abstract. Deregulation of signal transduction pathways frequently confers selective biological advantages to tumors. Phosphoinositides play an essential role in numerous cellular functions and, among the enzymes implicated in these processes, phosphoinositide-specific phospholipase C β 1 (PI-PLC β 1) is one of the key regulators. In the present study, a fluorescence *in situ* hybridization (FISH) approach was used to investigate PI-PLC β 1 gene copy number alterations in various types of breast cancer differing in their invasiveness and proliferative activity, according to their mitotic index. At the molecular level, we also performed both real-time PCR and immunohistochemical analyses on PI-PLC β 1 to further investigate its expression in primary breast cancers. Finally, we analyzed the correlation between PI-PLC β 1 gene copy number and clinicopathological parameters. Our results show that most of our cases had aneusomies on the PI-PLC β 1 locus (20p12) and amplification of this specific region was the most frequent alteration observed. Our findings also indicate that the amplification of the region containing the PI-PLC β 1 gene was mostly related to the mitotic index, rather than to the invasion status. Finally, even though our case series is limited, PI-PLC β 1 gene amplification seems to be correlated to clinicopathological parameters.

Introduction

Breast cancer is one of the most frequent malignancies in women, with the highest incidence rate reported in Western industrialized countries (1). The widespread occurrence and clinical heterogeneity of this disease, along with its significant social impact, has driven the continuous search for new biomole-

cular markers to better characterize this tumor. A number of different factors are involved in breast carcinoma pathogenesis, making a correct biopathological characterization essential to determine tumor aggressiveness and to identify the most appropriate therapy. Moreover, as numerous genetic alterations are preserved throughout the evolution of breast cancer and a strong correlation exists between the presence of precursor lesions and the risk of developing invasive carcinoma (2-5), the discovery of new molecular targets could play an important role in the early diagnosis and monitoring of this disease.

In breast cancer, as well as in other tumors, the deregulation of signal transduction pathways frequently confers selective biological advantages to tumor cells. Phosphoinositide signaling has been implicated in several cellular functions, and one of the key regulators of this pathway is phosphoinositide-specific phospholipase C β 1 (PI-PLC β 1), an enzyme that catalyzes the formation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), important second messengers that control numerous cellular functions (6-10). There are two alternative splicing variants of PI-PLC β 1, PI-PLC β 1a and PI-PLC β 1b (9), each of which has a different cellular localization (1a, both cytoplasmic and nuclear; 1b, predominantly nuclear) and modes of activation (11-13).

Aneuploidy and aneusomy are often correlated with low rates of cell differentiation and with proliferation (14). Furthermore, DNA amplification at specific chromosomal sites is a leading mechanism of oncogene overexpression and has an important impact on the deregulation of cell growth and survival (15). Several studies have focused on structural and copy number changes of chromosome 20 as it is often altered in prostate, ovarian, bladder, pancreatic, colon and breast cancer, and may be involved in disease initiation and progression. It is well known that amplifications along the long arm of chromosome 20 are present in 5-40% of breast cancers and are associated with cell immortalization, genomic instability and more aggressive phenotypes, although their prognostic value remains controversial (16-21). In addition, the short arm, where PI-PLC β 1 is located (20p12.3) (10), has often been found to be altered in several solid tumors, including breast cancer (20,21).

The aim of the present study was to investigate the status of PI-PLC β 1 in distinct classes of breast cancer which differ in their degree of invasiveness and proliferative activity.

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Materials and methods

Case series. The present study included 52 patients with breast cancer at first diagnosis recruited at the Morgagni-Pierantoni Hospital in Forlì, Italy, between 2000 and 2006. The study was approved by the local ethics committee and each patient gave informed consent in accordance with the Institutional guidelines. The median age of the patients at diagnosis was 61 years (range 39-86). All patients underwent surgery for primary breast cancer and none had previously received neoadjuvant treatment or had distant metastases at that time. Histological tumor sections were provided by the Pathology Unit of the same hospital, and fresh biopsy samples were sent to the Biosciences Laboratory of our institute (I.R.S.T., Meldola, Italy) and immediately stored at -80°C until use. Tumors were classified according to the World Health Organization (WHO) histological classification (22,23): 20 cases (38%) were ductal carcinomas *in situ* (DCIS) and the remaining 32 (62%) were invasive ductal carcinomas (IDC). Histological grading was performed using the criteria of Holland *et al* for DCIS (24) and of Bloom and Richardson for IDC (25) as modified by Elston and Ellis (26). The proliferative activity was determined using the mitotic activity index (MAI), calculated as the number of mitotic figures per 10 consecutive fields, chosen in high density areas (27). We used the same MAI cut-off criteria as those of standard clinical practice to determine proliferation indices: <10 mitotic figures/10 fields, ≥ 10 and ≤ 19 , and ≥ 20 .

Alexa Fluor 555 PI-PLC β 1 probe preparation. The PI-PLC β 1 FISH probe was prepared by digesting 4 μ g of PAC Clone HS881E24 DNA (obtained from the P de Jong RPCI-5 PAC library) overnight at 37°C with 80 units of *Rsa*I endonuclease (Promega Corp., Madison, WI, USA). The next day, after enzyme inactivation at 65°C for 20 min, digested DNA was purified by the QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany) and about half of the product was labeled by random priming (BioPrime Plus ArrayCGH Genomic Labeling System, Invitrogen, Milan, Italy) using Alexa Fluor 555 labeled primers and nucleotides, according to the manufacturer's instructions. After a 2-h incubation at 37°C, the reaction was blocked on ice by adding 5 μ l of Stop Buffer (BioPrime kit, Invitrogen). To assess labeling efficiency, 3 μ l of DNA were electrophoresed on a 2% agarose gel, obtaining fragments between 100 and 500 bp. The labeled probe was then purified using a Purification Module (Invitrogen), according to the supplier's instructions and precipitated with 30 μ l of 1 mg/ml COT-1 DNA (Roche Diagnostics Inc., Mannheim, Germany), 1/10 v/v of 3 M sodium acetate (pH 5.6) and 3 volumes of 100% ethanol. The probes were stored for 30 min at -80°C and then centrifuged at 13000 rpm for 30 min.

Fluorescence in situ hybridization (FISH). PI-PLC β 1 and human epidermal growth factor receptor 2 (HER2) genes were analyzed by FISH using labeled PI-PLC β 1 (see above) and LSI-HER2/neu-CEP17 probes (PathVysion™ HER-2 DNA Probe kit; Vysis, Inc., Downers Grove, IL, USA). Briefly, after an overnight incubation at 56°C, slides were deparaffinized in xylene for 30 min at 56°C and 10 min at room temperature and then dehydrated in two changes of 100% ethanol for 5 min. The Paraffin Pre-treatment kit (Vysis, Inc.) was used to prepare the

tumor sections: after a 10-min incubation in 0.2 M HCl solution at room temperature, slides were washed in deionized water for 3 min, wash buffer for 3 min, pre-treatment reagent (1 M NaSCN) for 30 min at 80°C and then rinsed once in deionized water for 1 min and twice in wash buffer for 5 min. Samples were then incubated in a solution of 0.2 M HCl per 4 mg/ml protease at 37°C for 25 min, rinsed twice in wash buffer for 5 min, incubated in neutral buffered formalin for 5 min, rinsed twice in wash buffer for 5 min and then dehydrated. The HER2/neu or PI-PLC β 1 probes (10 μ l) were resuspended in 15 μ l of hybridization solution (50% formamide/2X SSC/10% dextran sulphate), incubated for 5 min at 37°C and then added to the tumor sections. Slides were then coverslipped, sealed and subjected to co-denaturation (85°C for 5 min) and hybridization (37°C for 18 h in a humid atmosphere) in a Hybrite System (Vysis, Inc.). The next day the rubber cement was removed and the coverslips were floated off by soaking the slides at room temperature in 2X SSC/0.3% Nonidet P-40. Slides were then washed in the same solution at 73°C for 2 min, dried in the dark and counterstained with 4,6-diamidino-2-phenylindole (DAPI) in antifade solution (Vysis, Inc.). The slides were examined under an Axioscope 40 microscope (Carl Zeiss) equipped with a triple filter (DAPI/Green/Orange; Vysis, Inc.).

At least 60 non-overlapping nuclei were counted in contiguous x1000 microscopic fields to determine HER2 gene copy number status. The number of signals per nucleus for the HER2 gene and CEP17 region were counted on a cell-to-cell basis, and tumors were defined as having HER2 gene amplification (HER2⁺) when the ratio between the total number of HER2 and CEP17 signals was ≥ 2.0 (28,29). For PI-PLC β 1 evaluation, about 150-200 cells were counted. A sample was defined as amplified for this gene when the percentage of cells with more than two signals was at least 1.5-fold higher than the percentage of cells with only one signal; otherwise, when this percentage was lower, the sample was defined as deleted. All remaining cases were defined as normal.

RNA extraction, retro-transcription and real-time polymerase chain reaction (RT PCR). Total RNA was isolated from the breast cell line SKBR3 and frozen tissues by the RNeasy Mini kit (Qiagen) and cDNA was retrotranscribed from 500 ng of total RNA in a final volume of 20 μ l using the iScript cDNA Synthesis kit (Bio-Rad), according to the manufacturer's instructions. The reaction was performed at 42°C for 30 min, followed by a 5-min incubation at 85°C to block the enzyme. Transcript levels of both PI-PLC β 1 splicing variants (1a and 1b) were quantified using the MyiQ Single Color real-time PCR Detection System (Bio-Rad) and SYBR-Green I approach. PCR amplifications were carried out using the following oligonucleotide primers, designed using the Beacon Designer software (Version 4, Bio-Rad): PI-PLC β 1a forward primer, 5'-TGGATAAAAAGAGGCAGGAGAAGA-3' and reverse primer, 5'-GCAGCTTGGGCTTTTCATCC-3'; PI-PLC β 1b forward primer, 5'-GAAGAGGAGAAGACAGAGATG-3' and reverse primer, 5'-TGGCAAGTTTCCGACAAG-3'. A pool of 3 housekeeping genes was amplified as internal controls using the following primers: β -actin forward primer, 5'-CGCCGCCAGCTCACCATG-3' and reverse primer, 5'-CACGATGGAGGGGAAGACGG-3'; β_2 -microglobulin forward primer, 5'-CATTCCTGAAGCTGACAGCATTC-3'

Table I. Tumor characteristics.

A, Histotype and proliferation						
	Histological grade ^a % (n)			ER ⁺ % (n)	PgR ⁺ % (n)	HER2 ⁺ % (n)
	I	II	III			
DCIS low proliferation (n=10)	-	70 (7)	30 (3)	90 (9)	80 (8)	30 (3)
DCIS high proliferation (n=10)	-	-	100 (9)	30 (3)	10 (1)	60 (6)
IDC low proliferation (n=16)	40 (6)	60 (9)	-	94 (15)	56 (7)	6 (1)
IDC high proliferation (n=16)	7 (1)	27 (4)	66 (10)	50 (8)	31 (5)	44 (7)

B, Phenotype	% (n)
Luminal-like A: ER ⁺ /PgR ⁺ /HER2 ⁻	56 (29)
Luminal-like B: ER ⁺ /PgR ⁺ /HER2 ⁺	11.5 (6)
HER2-like: ER ⁻ /PgR ⁻ /HER2 ⁺	21 (11)
Triple negative basal-like	11.5 (6)

^aHistological grade was not available for tumors <1 cm. DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

and reverse primer, 5'-TGCTGGATGACGTGAGTAAACC-3'; hypoxanthine ribosyltransferase (HPRT) forward primer, 5'-AGACTTTGCTTTCCTTGGTCAGG-3' and reverse primer, 5'-GTCTGGCTTATATCCAACACTTCG-3'. The cDNA of each tumor sample was added to a PCR reaction mix containing 1X SYBR-Green mix (Bio-Rad) and primers in a 25- μ l reaction volume. Each gene was tested in duplicate within the same PCR run. The reactions were carried out as follows: an initial run of 90 sec at 95°C, followed by 40 cycles consisting of 30 sec at 95°C and an annealing step specific for each gene: 30 sec at 60°C for the housekeeping genes and 45 sec at 60°C for the two PI-PLC β 1 isoforms. For PI-PLC β 1 amplification, an additional step of 72°C for 45 sec was carried out to offset the small amounts of mRNA obtained. Melting curve analysis was performed after each experiment to verify amplification product specificity. Semi-quantitative analysis was performed by the $\Delta\Delta$ Ct method using the Gene Expression Macro Software (Version 1.1, Bio-Rad). The amount of mRNA expression in each sample was normalized to the pool of housekeeping transcripts and expressed as n-fold in relation to the calibrator level, i.e., cDNA of breast cancer cell line SKBR3, at known concentrations.

Immunohistochemical analysis. Protein expression levels were determined by immunohistochemistry (IHC) using the following primary antibodies: PI-PLC β 1 (sc-5291, Santa Cruz Biotechnology, Santa Cruz, CA, USA); estrogen receptor (ER), clone 6F11 (Novocastra Laboratories, Newcastle upon Tyne, UK) and progesterone receptor (PgR), clone 636 (Dako, Copenhagen, Denmark). Briefly, 5- μ m sections of formalin-fixed and paraffin-embedded breast cancer samples were deparaffinized by xylene and rehydrated through graded alcohols, according to standard protocols. Tissue sections were then quenched for endogenous peroxidases with 6% (v/v) H₂O₂ for 10 min, incubated in citrate buffer 10 mM (pH 6.0) at 98.5°C

for 40 min and left to cool in the solution for 20 min at room temperature. Next, the slides were washed in PBS for 5 min and incubated for 30 min at room temperature in specific pre-diluted primary antibody: 1:20 PI-PLC β 1, 1:200 ER and 1:200 PgR. Hormone receptors were identified using the Polymer Detection kit (Novocastra Laboratories), while PI-PLC β 1 expression levels were determined with the LSAB⁺ System-HRP kit (Dako) using an avidin-streptavidin reaction, according to the manufacturer's instructions. Peroxidase activity was detected by diaminobenzidine reaction for 10 min before counterstaining with Mayer's hematoxylin and each tissue sample was analyzed using an optical microscope (Axioscope, Carl Zeiss). The primary antibody was omitted to obtain negative controls.

ER and PgR staining was considered positive when >10% of tumor cells were distinctly stained. Semi-quantitative analysis of PI-PLC β 1 expression was performed by counting the percentage of stained cells and by scoring immunostaining intensity (ranging from 0 to 3), reflecting the intensity as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. The final mean score was then obtained by multiplying the intensity score by the percentage of stained cells.

Statistical analysis. Statistical analyses were carried out with SPSS software (SPSS Inc., Chicago, IL). χ^2 and Fisher's exact tests were performed to identify the association between patient characteristics and PI-PLC β 1 status. Multivariate analysis was conducted by running a backward stepwise regression.

Results

Tumor characteristics. Complete tumor characteristics are summarized in Table I. Tumors were divided into four classes according to their invasive status and proliferative activity: DCIS, low proliferation rate with a mitotic activity index (MAI)<10 (n=10); DCIS, high proliferation rate (MAI \geq 20;

Table II. Association between PI-PLC β 1 copy number status and clinicopathological characteristics.

	n (%) ^a		p-value
	Not amplified	Amplified	
Mitotic activity index			0.001
<10 (low proliferation)	16 (62)	10 (38)	
≥10 (high proliferation)	4 (15)	22 (85)	
Histological grade			0.032
G1	4 (57)	3 (43)	
G2	10 (50)	10 (50)	
G3	5 (23)	17 (77)	
ER (% positive nuclei)			0.049
<10	3 (19)	13 (81)	
≥10	17 (47)	19 (53)	
PgR (% positive nuclei)			0.07
<10	8 (28)	21 (72)	
≥10	12 (52)	11 (48)	
HER2			0.123
Not amplified	16 (46)	19 (54)	
Amplified	4 (24)	13 (76)	
Phenotype			0.161
Luminal-like A	15 (52)	14 (48)	
Luminal-like B	1 (17)	5 (83)	
HER2-like	3 (27)	8 (73)	
Triple negative basal-like	1 (17)	5 (83)	
Lymph node status			0.365
N ⁻	12 (36)	21 (64)	
N ⁺	3 (60)	2 (40)	
Histotype			0.857
DCIS	8 (40)	12 (60)	
IDC	12 (37)	20 (63)	

^aIndicates the n and % of breast cancer samples with an amplified or not amplified PI-PLC β 1 gene.

n=10); IDC, low proliferation rate (MAI<10; n=16); and IDC, high proliferation rate (MAI≥20; n=16). As Table I shows, low proliferation DCIS were classified as grades II (70%) or III (30%) and were mainly ER⁺ and PgR⁺, with only 30% (n=3) HER2⁺. For high proliferation DCIS, which were all grade III, 30% (n=3) were ER⁺, 10% (n=1) were PgR⁺ and 60% (n=6) were HER2⁺. In the IDC low proliferation class, 40% (n=6) were grade I and 60% (n=9) were grade II tumors: 94% (n=15) were ER⁺ and 56% (n=7) were PgR⁺, while only 6% (n=1) were HER2⁺. Finally, for high proliferation IDC, 7% (n=1) were grade I, 27% (n=4) were grade II and 66% (n=10) were grade III: 50% (n=8) were ER⁺, 31% (n=5) were PgR⁺ and 44% (n=7) were HER2⁺.

Hormone receptors and HER2 status were also used to build four distinct phenotypes: ER⁺ and/or PgR⁺/HER2⁻, homologous to the luminal-like A phenotype; ER⁺ and/or

PgR⁺/HER2⁺, homologous to the luminal-like B phenotype; ER⁻/PgR⁺/HER2⁺, homologous to the HER2-like phenotype; and ER⁻/PgR⁻/HER2⁻, homologous to the triple-negative basal-like phenotype. On the basis of this classification, 56% of cases (n=29) were homologous to the luminal-like A, 11.5% (n=6) to the luminal-like B, 21% (n=11) to the HER2-like and 11.5% (n=6) to the triple-negative basal-like phenotype.

PI-PLC β 1 copy number status in breast cancer samples. We used the FISH assay to evaluate PI-PLC β 1 copy number status in different classes of breast cancer (Table II). On the basis of the previously reported criteria, the vast majority of cases exhibited aneusomies of the locus containing the PI-PLC β 1 gene (deletion or amplification). The percentage of cases showing copy number alterations in DCIS and IDC with the same proliferative index was similar for both deletions and amplifications, suggesting that the PI-PLC β 1 gene status could be independent of invasiveness. Conversely, the percentage of deleted or amplified cases in the distinct proliferative classes differed substantially, especially for amplification. Interestingly, the association between PI-PLC β 1 amplification and the MAI was statistically significant (p=0.001).

PI-PLC β 1 protein expression analysis in breast cancer samples. Immunohistochemical analyses, performed in 50 patient samples, showed that breast cancer tissue expressed higher levels of PI-PLC β 1 than normal breast tissue, which was generally PI-PLC β 1 negative. An example of various staining intensities, together with their relative scores, is presented in Fig. 1. We used a cut-off value of 120 for PI-PLC β 1 protein analysis, thus identifying a class that weakly or moderately expressed PI-PLC β 1 (44%, n=22) and another that strongly expressed the protein (56%, n=28). In contrast to copy number status, PI-PLC β 1 protein levels seemed to be more strongly associated with invasiveness than the proliferation index. In fact, the number of low or high proliferating tumors showing high protein expression was similar, while the difference in protein expression levels between DCIS and IDC was greater, albeit not statistically significant.

PI-PLC β 1 mRNA expression analysis in breast cancer samples. Quantification of PI-PLC β 1 mRNA splicing variants was performed only on 12 samples of IDC with different proliferative activities (50% low and 50% high proliferation) for which frozen neoplastic tissue was available. Material was not available for DCIS. The β -actin, β_2 -microglobulin and HPRT genes were chosen as reference standards, while cDNA from the breast cancer cell line SKBR3 was used as a calibrator. Interestingly, our results showed that the relative amount of the PI-PLC β 1b transcript was higher than that of PI-PLC β 1a. Taking into consideration the proliferative classes, no apparent differences were found in the amount of mRNA obtained for PI-PLC β 1 splicing variants. However, a slight difference was observed between tumors with PI-PLC β 1 gene amplification and those with a normal copy number profile, becoming more evident when the sum of both the transcripts was considered. Furthermore, using the median expression value as a cut-off, an association between PI-PLC β 1 gene amplification and expression level was observed. In fact, a higher expression of the PI-PLC β 1a and PI-PLC β 1b splicing variants was found in

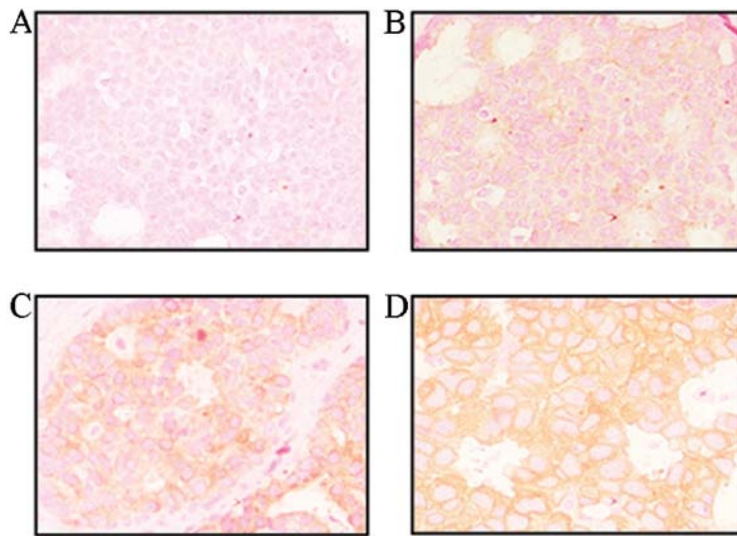


Figure 1. Immunohistochemical analysis of PI-PLC β 1 expression in sections of normal and cancerous human breast tissue. (A) Normal breast tissue with negative staining. (B-D) Ductal tumors with respectively weak, moderate and strong staining. Magnification x20 (A-C). (D) Magnified section (x40) of a strongly-stained ductal sample to note the specificity of the immunoreaction.

amplified tumors. Moreover, considering both isoforms, 82% of samples showed gene amplification and mRNA overexpression ($p=0.061$).

PI-PLC β 1 gene copy number and clinicopathological parameters. The association between PI-PLC β 1 status and the main tumor characteristics (histological grade, ER or PgR expression, HER2 or lymph node status, phenotype) is reported in Table II. Considering all analyzed samples, independently from MAI or histotype, univariate analysis highlighted a significant correlation between gene amplification and histological grade ($p=0.032$), with 77% ($n=17$) grade III tumors exhibiting PI-PLC β 1 amplification. Conversely, in our case series, the majority of samples that were ER $^-$ or PgR $^-$ displayed concomitant PI-PLC β 1 gene amplification (81 and 72%, respectively). However, statistical significance was not reached, probably due to the small number of cases analyzed. Although there was no clear association between HER2 and PI-PLC β 1 gene status, 13 cases showed concomitant HER2 and PI-PLC β 1 gene amplification. Furthermore, taking into account the four phenotype classifications, a higher number of PI-PLC β 1-amplified tumors was observed in the most aggressive phenotypes. No significant association was found between the PI-PLC β 1 gene and lymph node status. Overall, multivariate analysis indicated that the proliferative index was the only independent variable ($p=0.003$).

Discussion

Breast cancers frequently show evidence of aberrations in signal transduction pathways, which can lead to increased cellular proliferation, angiogenesis and metastasis, along with inhibition of apoptosis. In the present study, we focused on nuclear phosphoinositide signaling, which plays a crucial role in cell proliferation and differentiation in normal and pathological conditions. Several phospholipase isoforms have been associated with breast cancer cell motility and invasiveness, even though their exact function in tumor transformation

and prognosis has yet to be fully explored. Amongst these, PI-PLC γ 1 and PI-PLC δ 4 up-regulation is related to cell migration, proliferation and EGFR-directed tumor progression (30,31). Moreover, the PI-PLC β 2 isoform is overexpressed in a high percentage of breast cancers and is associated with histological grade and proliferative index, suggesting that its up-regulation could influence tumor differentiation and patient prognosis (32,33). More recently, a specific role of isoforms PI-PLC δ 1 and PI-PLC δ 3 in the growth and migration of normal and neoplastic mammary epithelial cells has been identified (34).

Several studies have shown that PI-PLC β 1 could play an important role in cancer progression. In fact, this enzyme has been identified as the principal mediator of the nuclear phosphoinositide cycle, which is involved in cell cycle progression (35). Moreover, chromosome band 20p12, where PI-PLC β 1 is mapped, is rearranged in numerous solid tumors, but the role of this alteration in cancer progression is still unknown.

The majority of our breast cancer cases showed aneusomies in the PI-PLC β 1 locus, and the most common genetic alteration was amplification ($n=32$). To further investigate the role of PI-PLC β 1 amplification in breast cancer, we analyzed PI-PLC β 1 gene and protein expression. Quantification of PI-PLC β 1 mRNA by real-time PCR in IDCs showed that PI-PLC β 1b transcript levels were higher than those of PI-PLC β 1a. Although no significant association was found between the mRNA levels of each splicing variant and gene copy number or proliferative activity, the sum of the 1a and 1b transcripts seemed to be correlated with gene amplification ($p=0.061$), even though we only analyzed a small number of cases. As for PI-PLC β 1 protein expression, although several tumors with PI-PLC β 1 amplification also showed high protein levels by immunohistochemical analyses, the association between PI-PLC β 1 gene amplification and protein overexpression was not significant.

To determine whether this alteration could affect tumor characteristics, we investigated the association between PI-PLC β 1 copy number and clinicopathological parameters,

such as proliferative activity and invasiveness. However, the lack of any apparent difference in PI-PLC β 1 gene amplification between DCIS and IDC within the same proliferative classes suggests that PI-PLC β 1 allelic status is independent from tumor invasiveness. In contrast, PI-PLC β 1 gene amplification seemed to be closely related to MAI ($p=0.001$), supporting the theory that in breast cancer this enzyme could be involved in cell cycle processes (35). In fact, our results also highlighted a statistically significant correlation with histological grading ($p=0.032$), confirming that altered DNA content frequently results in rapid cell proliferation and/or low differentiation (14). Moreover, there was a borderline correlation with hormone receptor status (ER, $p=0.049$; PgR, $p=0.07$) and an inverse association between PI-PLC β 1 gain and the luminal-like A phenotype ($p=0.027$). The presence of low PI-PLC β 1 amplification in luminal-like A type tumors, which have a relatively good prognosis among the four phenotypes (36), is consistent with the hypothesis that this enzyme or other genes within the 20p12 locus could be related to breast cancer aggressiveness, given that we cannot define the amplification as solely affecting the PI-PLC β 1 gene.

In conclusion, our preliminary results show that PI-PLC β 1 may be altered in breast cancer. Further studies are needed to define its real role in this tumor and to elucidate the molecular mechanisms regulating its activity in breast cancer.

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References

- Wilson CM, Tobin S and Young RC: The exploding worldwide cancer burden: the impact of cancer on women. *Int J Gynecol Cancer* 14: 1-11, 2004.
- Beckmann MW, Niederacher D, Schnurch HG, Gusterson BA and Bender HG: Multistep carcinogenesis of breast cancer and tumour heterogeneity. *J Mol Med (Berl)* 75: 429-439, 1997.
- Van de Vijver MJ and Peterse H: The diagnosis and management of pre-invasive breast disease: pathological diagnosis - problems with existing classifications. *Breast Cancer Res* 5: 269, 2003.
- Allred DC, Hilsenbeck SG and Mohsin SK: Biological features of human premalignant breast disease. In: *Diseases of the Breast*. Harris JR, Lippman ME, Morrow M and Osborne CK (eds). 3rd edition. Lippincott Williams and Wilkins, Philadelphia, pp507-520, 2004.
- Allred DC, Wu Y, Mao S, *et al*: Ductal carcinoma in situ and the emergence of diversity during breast cancer evolution. *Clin Cancer Res* 14: 370-378, 2008.
- Deraniche RM and Greenberg ML: Cellular consequences of inositol depletion. *Biochem Soc Trans* 37: 1099-1103, 2009.
- Ramazzotti G, Faenza I, Fiume R, *et al*: The physiology and pathology of inositol signaling in the nucleus. *J Cell Physiol* 226: 14-20, 2011.
- Follo MY, Mongiorgi S, Finelli C, *et al*: Nuclear inositol signaling in myelodysplastic syndromes. *J Cell Biochem* 109: 1065-1071, 2010.
- Peruzzi D, Aluigi M, Manzoli L, *et al*: Molecular characterization of the human PLC β 1 gene. *Biochim Biophys Acta* 1584: 46-54, 2002.
- Peruzzi D, Calabrese G, Faenza I, *et al*: Identification and chromosomal localisation by fluorescence in situ hybridisation of human gene of phosphoinositide-specific phospholipase C β 1. *Biochim Biophys Acta* 1484: 175-182, 2000.
- Martelli AM, Billi AM, Manzoli L, *et al*: Insulin selectively stimulates nuclear phosphoinositide-specific phospholipase C (PI-PLC) β 1 activity through a mitogen-activated protein (MAP) kinase-dependent serine phosphorylation. *FEBS Lett* 486: 230-236, 2000.
- Xu A, Suh PG, Marmy-Conus N, *et al*: Phosphorylation of nuclear phospholipase C β 1 by extracellular signal-regulated kinase mediates the mitogenic action of insulin-like growth factor I. *Mol Cell Biol* 21: 2981-2990, 2001.
- Faenza I, Billi AM, Follo MY, *et al*: Nuclear phospholipase C signaling through type 1 IGF receptor and its involvement in cell growth and differentiation. *Anticancer Res* 25: 2039-2041, 2005.
- Silvestrini R: Relevance of DNA-ploidy as a prognostic instrument for solid tumors. *Ann Oncol* 11: 259-261, 2000.
- Albertson DG: Gene amplification in cancer. *Trends Genet* 22: 447-455, 2006.
- Reis-Filho JS and Lakhani SR: The diagnosis and management of pre-invasive breast disease: genetic alterations in pre-invasive lesions. *Breast Cancer Res* 5: 313-319, 2003.
- James LA, Mitchell EL, Menasce L and Varley JM: Comparative genomic hybridisation of ductal carcinoma in situ of the breast: identification of regions of DNA amplification and deletion in common with invasive breast carcinoma. *Oncogene* 14: 1059-1065, 1997.
- Letessier A, Sircoulomb F, Ginestier C, *et al*: Frequency, prognostic impact, and subtype association of 8p12, 8q24, 11q13, 12p13, 17q12, and 20q13 amplifications in breast cancers. *BMC Cancer* 6: 245, 2006.
- Ginestier C, Cervera N, Finetti P, *et al*: Prognosis and gene expression profiling of 20q13-amplified breast cancers. *Clin Cancer Res* 12: 4533-4544, 2006.
- Nakopoulou L, Tsimpa I, Giannopoulou I, Trafalis D, Katsarou S and Davaris P: Aneuploidy of chromosome 20 in invasive breast cancer correlates with poor outcome. *Cancer Genet Cytogenet* 134: 127-132, 2002.
- Hodgson JG, Chin K, Collins C and Gray JW: Genome amplification of chromosome 20 in breast cancer. *Breast Cancer Res Treat* 78: 337-345, 2003.
- The World Health Organization: Histological Typing of Breast Tumours. World Health Organization, Geneva, 1981.
- Tavassoli FA and Devilee P (eds): World Health Classification of Tumours. Pathology and Genetics of Tumours of the Breast and Female Genital Organs. IARC Press, Lyon, 2003.
- Holland R, Peterse JL, Millis RR, *et al*: Ductal carcinoma in situ: a proposal for a new classification. *Semin Diagn Pathol* 11: 167-180, 1994.
- Bloom HJ and Richardson WW: Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years. *Br J Cancer* 11: 359-377, 1957.
- Elston CW and Ellis IO: Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* 19: 403-410, 1991.
- Medri L, Volpi A, Nanni O, *et al*: Prognostic relevance of mitotic activity in patients with node-negative breast cancer. *Mod Pathol* 16: 1067-1075, 2003.
- Carles J, Lloreta J, Salido M, *et al*: Her-2/neu expression in prostate cancer: a dynamic process? *Clin Cancer Res* 10: 4742-4745, 2004.
- Montironi R and Mazzucchelli R: HER-2 expression and gene amplification in high-grade PIN and prostate cancer. *Arch Ital Urol Androl* 78: 135-139, 2006.
- Kassis J, Moellinger J, Lo H, Greenberg NM, Kim HG and Wells A: A role for phospholipase C-gamma-mediated signaling in tumor cell invasion. *Clin Cancer Res* 5: 2251-2260, 1999.
- Leung DW, Tompkins C, Brewer J, *et al*: Phospholipase C delta-4 overexpression upregulates ErbB1/2 expression, Erk signaling pathway, and proliferation in MCF-7 cells. *Mol Cancer* 3: 15, 2004.
- Bertagnolo V, Benedusi M, Querzoli P, *et al*: PLC- β 2 is highly expressed in breast cancer and is associated with a poor outcome: a study on tissue microarrays. *Int J Oncol* 28: 863-872, 2006.
- Bertagnolo V, Benedusi M, Brugnoli F, *et al*: Phospholipase C- β 2 promotes mitosis and migration of human breast cancer-derived cells. *Carcinogenesis* 28: 1638-1645, 2007.
- Rebecchi MJ, Raghubir A, Scarlata S, Hartenstine MJ, Brown T and Stallings JD: Expression and function of phospholipase C in breast carcinoma. *Adv Enzyme Regul* 49: 59-73, 2009.
- Faenza I, Matteucci A, Manzoli L, *et al*: A role for nuclear phospholipase C β 1 in cell cycle control. *J Biol Chem* 275: 30520-30524, 2000.
- Sorlie T, Wang Y, Xiao C, *et al*: Distinct molecular mechanisms underlying clinically relevant subtypes of breast cancer: gene expression analyses across three different platforms. *BMC Genomics* 7: 127, 2006.