

Expression of phospholipase C isozymes in human breast cancer and their clinical significance

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Abstract. Phospholipase C (PLC) regulates a number of cellular behaviours including cell motility, cell transformation, differentiation and cell growth. PLC plays a regulatory role in cancer cells partly by acting as signalling intermediates for cytokines such as EGF and interleukins. The current study examined the expression of the PLC isozymes in human breast cancer and corresponding clinical relevance. Transcript levels of human PLC- α , - β 1, - δ , - ϵ , and - γ 1 in human breast cancer tissues were quantitatively determined by real-time PCR. Immunohistochemical staining was performed for PLC- δ . The clinical relevance was analysed with clinic pathological information. Mammary tissues widely expressed PLC- α , - β 1, - δ , - ϵ , and - γ 1. Significantly high levels of PLC - β 1 and - ϵ were seen in breast cancer tissues in comparison with normal mammary gland tissues. PLC- γ 1 however, showed marginally low levels in tumour tissues. No significant difference was seen in the expression of the PLC isozymes in tumours with lymph node metastases. Moderately and poorly differentiated breast tumours (grade 2 and grade 3) had significantly higher levels of PLC- γ 1, compared with well differentiated tumours. High levels of PLC- δ were significantly correlated with a shorter disease-free survival. The altered expression of other isozymes had no correlation with the survival. It is concluded that mammary tissues differentially expressed PLC isozymes. These isozymes have certain implications in the disease development and progression, with PLC- δ showing a significant correlation with shorter disease-free survival.

Introduction

Phospholipase C (PLC) is a type of membrane-associated enzymes consisting of thirteen mammalian PLC isozymes that are classified into five isotype groups (β , γ , δ , ϵ , ζ) based on their different structures (1). PLC- α also known as protein disulfide isomerase family A member 3 (PDIA3) interacts with calnexin and calreticulin to coordinate the folding of newly synthesised glycoprotein (2). According to gene sequence analysis studies, the major homology shared by different isozymes is presented in N-terminus with around 250 amino acid residues (3). The core enzyme of PLC is composed of a triosephosphate isomerase (TIM) barrel, a pleckstrin homology (PH) domain, four tandem EF hand domains, and a C2 domain (4). Specifically, an insert in the TIM barrel named the X-Y linker interrupts its function by occluding the active site. This can be remedied with the removal of the X-Y linker (5). Activated by all types of cell surface membrane receptors, PLC cleaves the phospholipid, phosphatidylinositol 4, 5-bisphosphate (PIP₂), into diacyl glycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP₃). These two products of the PLC-catalyzed reaction are important second messengers that control diverse cellular processes and are substrates for synthesis of other important signalling molecules (6).

As a phospholipid-hydrolyzing enzyme, PLC plays a significant role in the metabolism of inositol lipids. The bioactive lipid mediators generated by PLC families are involved in a variety of cellular processes, which are implicated in promoting tumourigenesis through intercellular and extracellular signalling pathways (7). Several studies have been carried out to detect the function of PLC subfamilies and the result demonstrated their essential role in regulating proliferation, invasion, and migration in cancer development (8-11). PLC- γ and PLC- ϵ work as oncogenes in regulating cell proliferation which is associated with Ras activities (12-14). Conversely, the deletion or downregulation of PLC- β and PLC- δ isoforms has been shown in human leukaemia (15,16). PLC- γ -mediated cell spreading and motility is achieved by its binding with the complex GPCR kinase interacting ARF-GAP 1 (GIT1) and the RAC1 and CDC42 GEF β -Pix, and subsequently leads to the activation of the RHO family GTPases CDC42 and RAC1.

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Downregulation of PLC- γ 1 expression severely impairs the activation of RAC, as well as cell invasion in breast cancer, glioblastoma and head and neck cancer cell lines (17).

The occurrence and development of breast cancer is related to aberrant signalling pathways in cell proliferation, invasion and migration, but there is limited relevant therapy targeting these signalling pathways. The evidence indicating the relationship between PLC isoforms and breast cancer has suggested a possibility to develop target therapy for breast cancer. Some of the previous studies highlighted the alteration of PLC expression levels in breast tumour cells. For example, the expression level of PLC- γ 1 is seen to be changed in breast cancer cells, with PLC- γ 1 having a role in the regulation of cell migration by targeting EGFR (18,19). Furthermore, PLC- δ 4 is upregulated in breast tumour cells, and its overexpression enhances cell proliferation in those breast cancer cells with lower oncogenicity (20). It has been reported that a poor clinical outcome of breast tumour is associated with an increased expression level of PLC- β 2, which appears to be a molecular marker indicating the severity of breast cancer. In addition, PLC- β 2 provokes the transition from G0/G1 to S/G2/M cell cycle phase, which is important in cancer progression and inositol lipid-related modifications of the cytoskeleton architecture occurring during tumour cell division, motility and invasion (10). *In vivo*, dominant-negative PLC- γ 1 fragment reduced the metastatic potential of breast cancer in transgenic mouse (21). Metastasis assays also demonstrated that nude mice with knockdown of PLC- γ 1 presented inhibition of breast cancer-derived lung metastasis (8).

Based on a previous study, the aim of this work was to establish a correlation for the pattern of expression of 5 PLC isoforms (α , β , δ , ϵ , and γ) in human breast cancer tissues with clinical information (differentiation, tumour staging, histology type and clinical outcome). We demonstrated that mammary tissues widely expressed PLC- α , - β 1, - δ , - ϵ , and - γ 1. No significant difference in the levels of the different isozymes was seen between node positive and node negative tumours. Poorly differentiated breast tumours (grade 2 and grade 3) had significantly higher levels of PLC- γ 1. Over a 10-year period, high levels of PLC- δ were significantly correlated with a shorter disease-free survival.

Materials and methods

Materials and reagents. A polyclonal antibody to human PLC- γ 1 was purchased from Santa Cruz Biotechnology Ltd. (Santa Cruz, CA, USA). RNA extraction kit and the first strand cDNA synthesis kit were from AbGene Laboratories (Surrey, UK). The master mix for conventional PCR and a customer quantitation PCR master mix for quantitative PCR were also purchased from AbGene Laboratories.

Breast cancer cell lines MCF-7 and MDA MB 231 were purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). Human umbilical vein endothelial cells (HUVEC) were purchased from TCS Biologicals Ltd. (Oxford, UK). Breast cancer tissues (n=120) and normal background tissues (n=32) were collected immediately after surgery and stored at -20°C until use. Patients were routinely followed clinically after surgery. The median follow-up period was 72 months. The presence of tumour cells in the collected

tissues was verified by examination of frozen sections using H&E staining by a consultant pathologist.

Tissue processing and extraction of RNA and generation of cDNA. Over 20 frozen sections from the each tissue sample were homogenised in an RNA extraction solution using a hand held homogeniser to extract total RNA. The concentration of RNA was quantified using a UV spectrophotometer. RNA (1 μ g) was used to generate cDNA using a commercially available RT kit (AbGene Laboratories).

Detection of PLC isoforms using RT-PCR. Routine RT-PCR was carried out using a PCR master mix that was commercially available (AbGene). Primers were designed using the Beacon Designer software (version 2, CA, USA) (17), to amplify regions of human PLC- γ 1 that have no significant overlap with other known sequences and to ensure that the amplified products span over at least one intron. The primer sequences are given in Table I. β -actin was used as a housekeeping control. Reactions were carried out using the following conditions: 94°C for 5 min, 36 cycles of 94°C for 15 sec, 55°C for 25 sec and 72°C for 15 sec. PCR products were separated on a 2% agarose gel and photographed using a digital camera mounted over a UV transluminator.

Quantitative analysis of the transcript of PLC isoforms. The level of PLC- γ 1 transcripts from the prepared cDNA described above was determined using real-time quantitative PCR, based on Amplifluor™ technology that was modified from previous studies (18,19). In brief, pairs of PCR primers were designed using the Beacon Designer software (version 2), with one of the primers having an additional sequence, known as the Z sequence (5'-actgaacctgaccgtaca) which is complementary to the universal Z probe (Intergen Inc., Oxford, UK). A Taqman detection kit for β -actin was purchased from Perkin-Elmer. The reaction was carried out using the following: Hot-start Q-master mix (Abgene), 10 pmol of the specific forward primer, 1 pmol of the reverse primer (which has the Z sequence), 10 pmol of FAM-tagged probe (Intergen Inc.), and cDNA transcribed from approximately 50 ng of RNA. The reaction was carried out using an iCycler iQ™ (Bio-Rad) which equipped with an optic unit that allows real-time detection of 96 reactions, using the following condition: 94°C for 12 min, 100 cycles of 94°C for 15 sec, 55°C for 40 sec and 72°C for 20 sec. The levels of the transcripts were generated from a standard that was simultaneously amplified with the samples.

Immunohistochemical staining of PLC- γ 1 protein. The procedure was carried out as previously reported with minor modifications (19,20). Frozen sections of breast tumour and background tissue were cut at a thickness of 6 μ m using a cryostat. The sections were mounted on Superfrost Plus (Gerhard Menzel, Germany), microscope slides, air-dried and fixed in a mixture of 50% acetone and 50% methanol. The sections were then placed in 'Optimax' wash buffer for 5-10 min to rehydrate before being incubated for 20 min in a 0.6% BSA blocking solution and probed with the primary antibody. Following extensive washings, sections were incubated for 30 min in the secondary biotinylated antibody (Multilink Swine anti-goat/mouse/rabbit immunoglobulin, Dako Inc.). Avidin Biotin

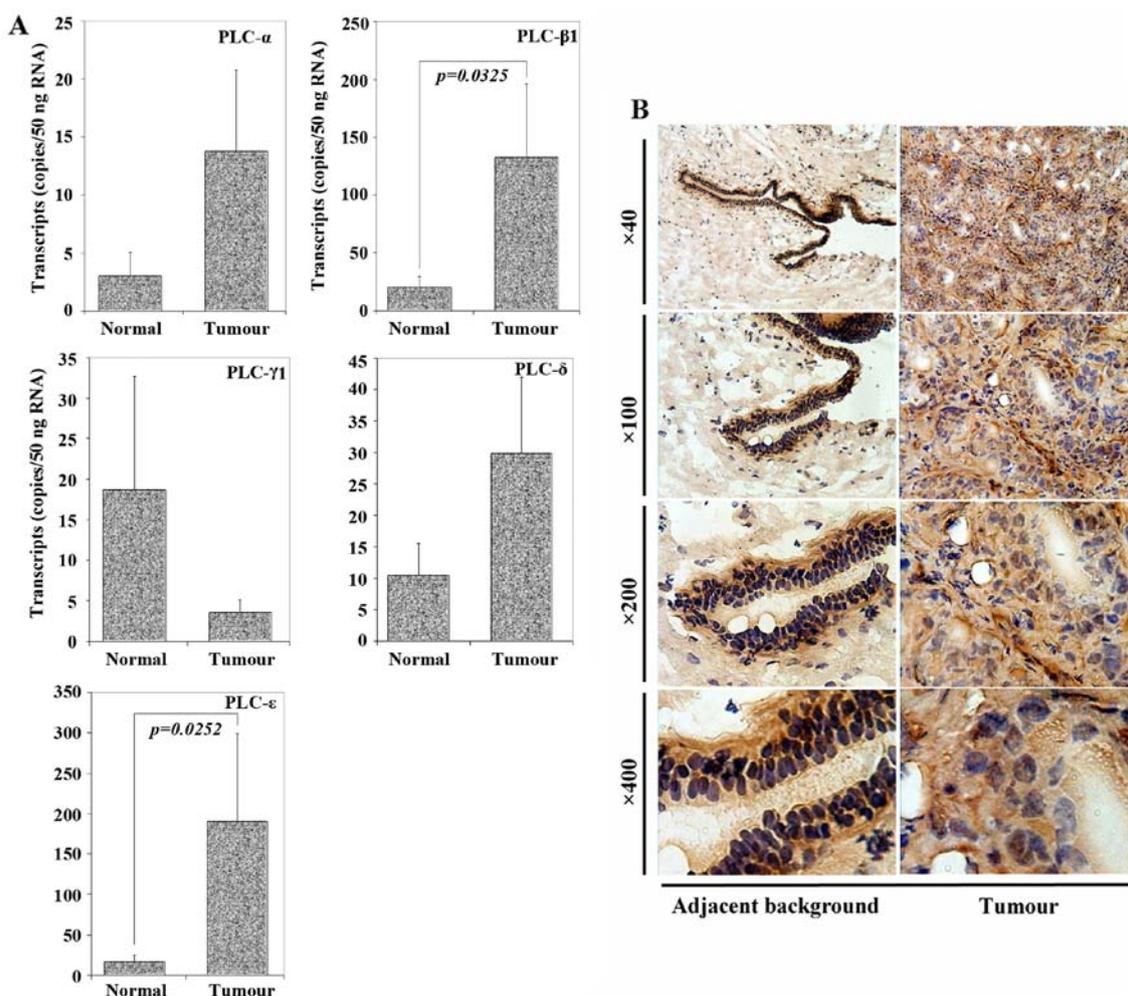


Figure 1. Levels of expression of PLC isoforms in non-neoplastic mammary tissues and breast cancer tissues. (A) PLC- α , - $\beta 1$, - δ and - ϵ expression increased in tumour tissues compared with normal tissues, with statistical significance shown in PLC- $\beta 1$ ($p=0.0325$) and PLC- ϵ ($p=0.0252$) levels. PLC- $\gamma 1$ presented a reduced level in breast tumour tissues. (B) Expression of PLC- $\gamma 1$ in human mammary tissues. Left panels: normal non-neoplastic tissues; right panels: breast cancer tissues. In normal tissues, PLC- $\gamma 1$ is largely seen in the cytoplasm of epithelial cells. Neither stromal fibroblasts nor vascular endothelial cells showed positive staining. However, in breast cancer tissues, the stromal cells displayed a substantial amount of staining. In contrast, the breast cancer cells showed little positive staining, thus indicating a shift of staining pattern between stromal and epithelial/cancer cell staining.

Complex (Vector Laboratories, Nottingham, UK) was then applied to the sections followed by more extensive washings. Diaminobenzidine chromogen (Vector Laboratories) was to the sections which were incubated in the dark for 5 min. Sections were then counter-stained in Gill's haematoxylin and dehydrated in ascending grades of methanol before clearing in xylene and mounting under a cover slip. Images were obtained using a digital camera.

Statistical analysis. Statistical analysis was carried out using Mann-Whitney U test and Kruskal-Wallis test. Survival analysis was performed using Kaplan-Meier survival and Cox proportional analysis.

Results

Expression of PLC isoforms in human breast cancer tissues. We first studied the levels of expression of the 5 PLC isoforms using quantitative analysis of the respective transcripts. As shown in Fig. 1A, PLC- α , - $\beta 1$, - δ and - ϵ showed increased levels in tumour tissues compared with normal tissues. On

the contrary, the expression level of PLC- $\gamma 1$ in tumour tissues was lower than in normal tissues. Notably, statistical significance was shown in PLC- $\beta 1$ and PLC- ϵ transcript levels, with $p=0.0325$ and $p=0.0252$, respectively, which demonstrated the considerable differences in the expression levels of these two isoforms between breast cancer and normal tissues.

In addition to the quantitative analysis of transcript levels, we used an anti-PLC- $\gamma 1$ as a case study to assess the protein expression and distribution in breast cancer. Immunohistochemical staining was performed for PLC- $\gamma 1$ in a small number of frozen sections of the breast tumour tissue samples together with some adjacent background mammary tissues. As seen in Fig. 1B, normal mammary epithelial cells stained very strongly for the PLC, but little staining was present in normal stromal cells. However, in breast cancer, the pattern of staining varied from that of normal epithelial cells. As shown in Fig. 1B (right panel), breast cancer cells stained rather weakly for the protein, whereas stromal cells stained strongly.

Levels of PLCs and nodal status. With the aim to investigate whether the expression level of PLC is associated with lymph

Table I. PCR primers.

Gene	Sense (5'-3')	Antisense (5'-3')
PLC- α	tagaactcacggacgacaa	actgaacctgaccgtacatactcaggtgcaagtctct
PLC- β 1	gcaaaaagcaaaccatattct	actgaacctgaccgtacagacttgctctgtttggag
PLC- δ	gttctcatcgaggacatt	actgaacctgaccgtacacttgaagacaatggagaagc
PLC- ϵ	ttgccaagattctctcaat	actgaacctgaccgtacacatgattttcccaacatt
PLC- γ 1	agacctccaggtcaagc	actgaacctgaccgtacagcggatctcctaatttca
β -actin	atgatatgccgcgctcg	cgctcgtgtaggatcttca

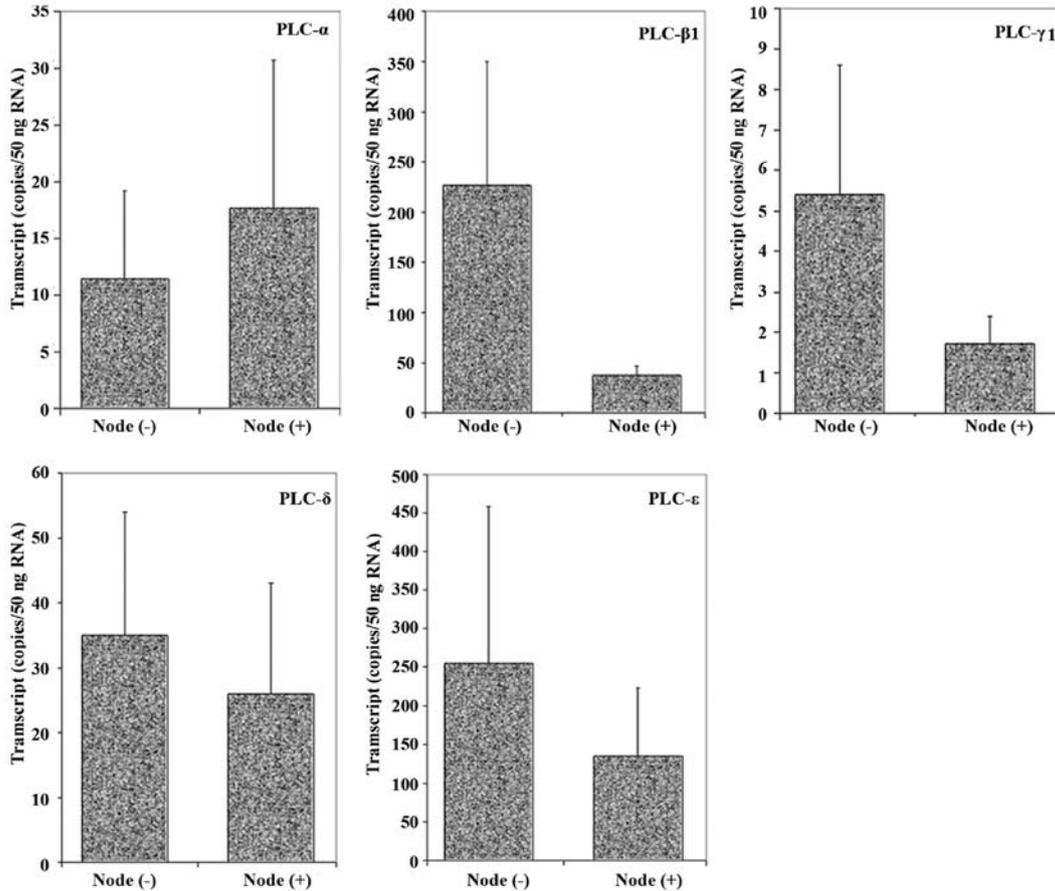


Figure 2. Expression of PLC isoforms in node-negative and node-positive breast tumours. There is a trend for reduced level of PLC- β 1, - γ 1, - δ , and - ϵ in node-positive tumour tissues in comparison with node-negative tumour tissues. Conversely, PLC- α presented an increased expression in node positive tumour tissues.

node status, cDNA samples from node-positive and node-negative breast tumour tissues were tested using real-time quantitative PCR. As shown in Fig. 2, node-positive tissues had lower PLC- β 1, - δ , - γ 1, and - ϵ levels, whilst higher PLC- α expression was shown. However, no statistically significant differences in the isoform levels were detected between the node-positive and node-negative breast tumour tissues.

Correlation of PLC levels with tumour grade and histology types. We examined PLC levels in breast tumours of different grades. As shown in Fig. 3A, grade 3 tumours had higher levels of PLC- β 1, - γ 1, - δ and - ϵ . Higher levels of PLC- α were detected in grade 1 tumours. A statistical difference was seen in PLC- γ 1 expression between grade 1 and grade 2 ($p=0.04$),

with the patients in grade 2 showing higher levels compared to those in grade 1.

When histology types were considered, there was a trend in ductal tumours to have higher levels of PLC- β 1, - γ 1, - δ and - ϵ , but lower levels of PLC- α (Fig. 3B). However, no statistical significance was found between these groups.

Levels of PLC in TNM stages. To evaluate the correlation between PLC levels and TNM stages, we quantified the levels of the different isoforms in breast tumours from TNM1 to TNM4. In particular, all the isoforms we examined presented the highest expression levels in TNM3 stage. There was also a significant difference in PLC- γ 1 levels when comparing tumours of the different TNM stages (TNM1 versus TNM2

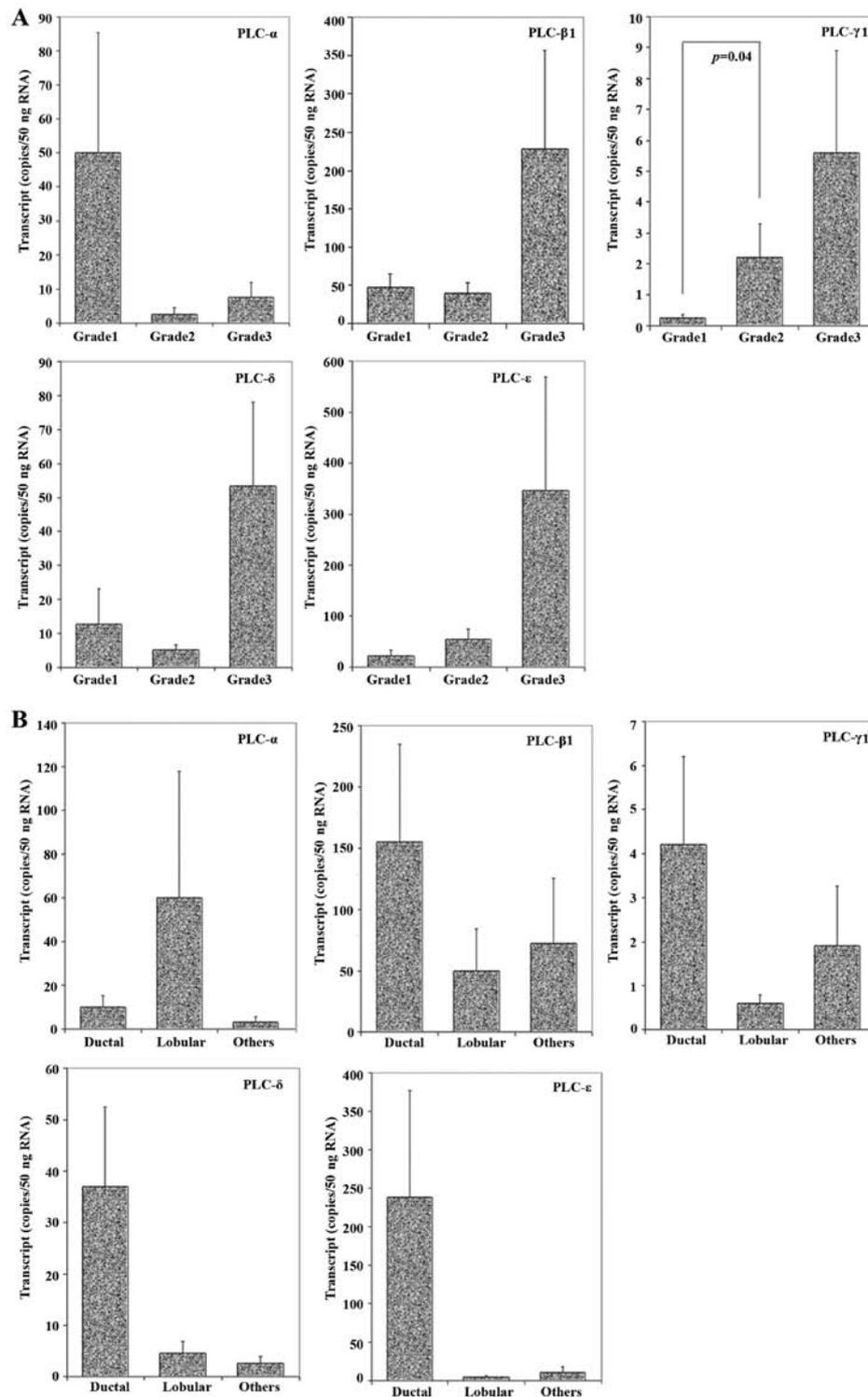


Figure 3. Expression of the PLC transcripts in different tumour grade and histological types. (A) PLC- $\beta 1$, - $\gamma 1$, - δ and - ϵ expression increased markedly in grade 3 breast tumour tissues (poorly differentiated). Higher levels of PLC- α was shown in grade 1 tumours (well differentiated). There is a reduction in PLC- $\gamma 1$ expression levels detected in grade 1 tumour tissues compared to breast tumours in grade 2 (moderately differentiated) ($p=0.04$). (B) There is a trend in ductal tumours showing higher levels of PLC- $\beta 1$, - $\gamma 1$, - δ and - ϵ , but lower levels of PLC- α ; however, there is no statistical significance between these groups.

$p=0.021$; TNM1 versus TNM4 $p=0.022$), with a decreased level in TNM2 and TNM4 in comparison with TNM1. The quantity of PLC- ϵ also reached statistical significance (TNM1 versus TNM3 $p=0.0265$; TNM3 versus TNM4 $p=0.002$), and the result showed that patients in TNM1 and TNM4 had a markedly reduced level of PLC- ϵ expression compared with the patients in TNM3 stages (Fig. 4).

PLC expression and predicted prognosis. In order to establish if a link exists between the isoforms of PLC and the predicted clinical outcome at the time of operation, the Nottingham Prognostic Index was used as an indicator, where $NPI < 3.4$ was designated as NPI1, and comprised of patients who had a good prognosis. NPI2 and NPI3, with the value 3.4-5.4 and > 5.4 , respectively, had moderate and poor prognosis. As shown

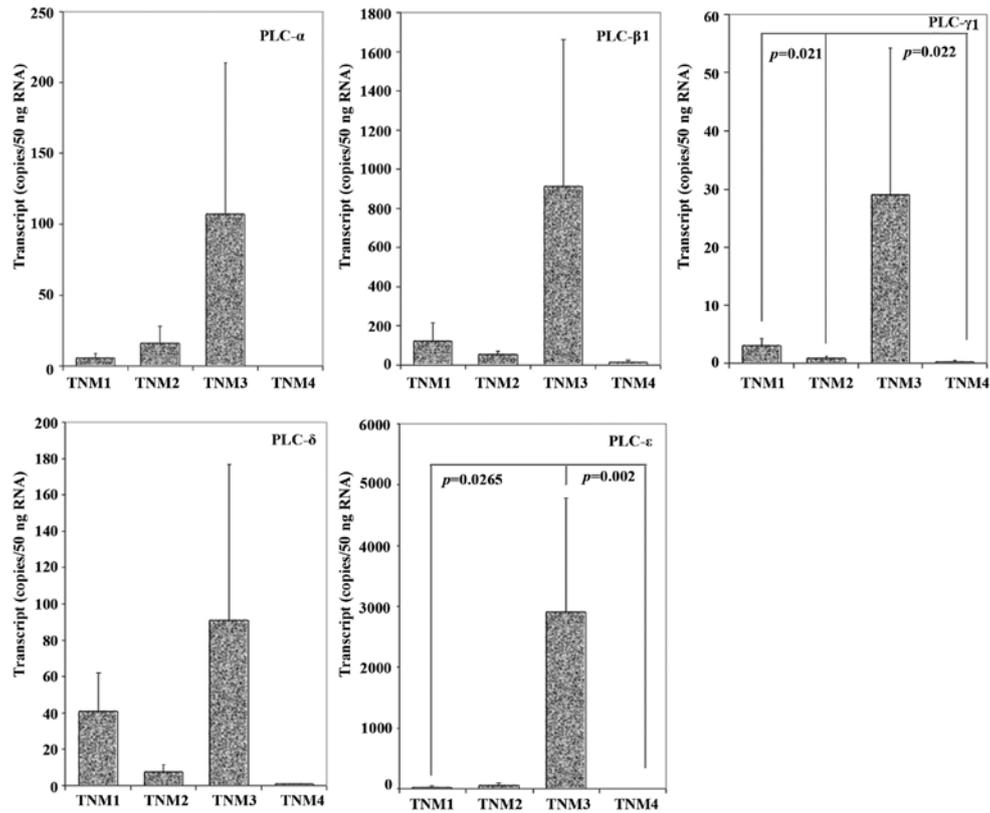


Figure 4. PLC isoforms in patients with different TNM tumours. Breast cancer patients in TNM3 showed an increased level of all PLC isoforms. The results show evidence of a relationship between PLC- $\gamma 1$ and the different TNM stages, with a decreased level of PLC- $\gamma 1$ seen in TNM2 and TNM4 in comparison with TNM1 (TNM1 versus TNM2 $p=0.021$; TNM1 versus TNM4 $p=0.022$); Statistically significant differences were also seen for expression levels of PLC- ϵ . (TNM1 versus TNM3 $p=0.0265$; TNM1 versus TNM4 $p=0.002$), with reduced level in TNM1 and TNM4 compared with TNM3.

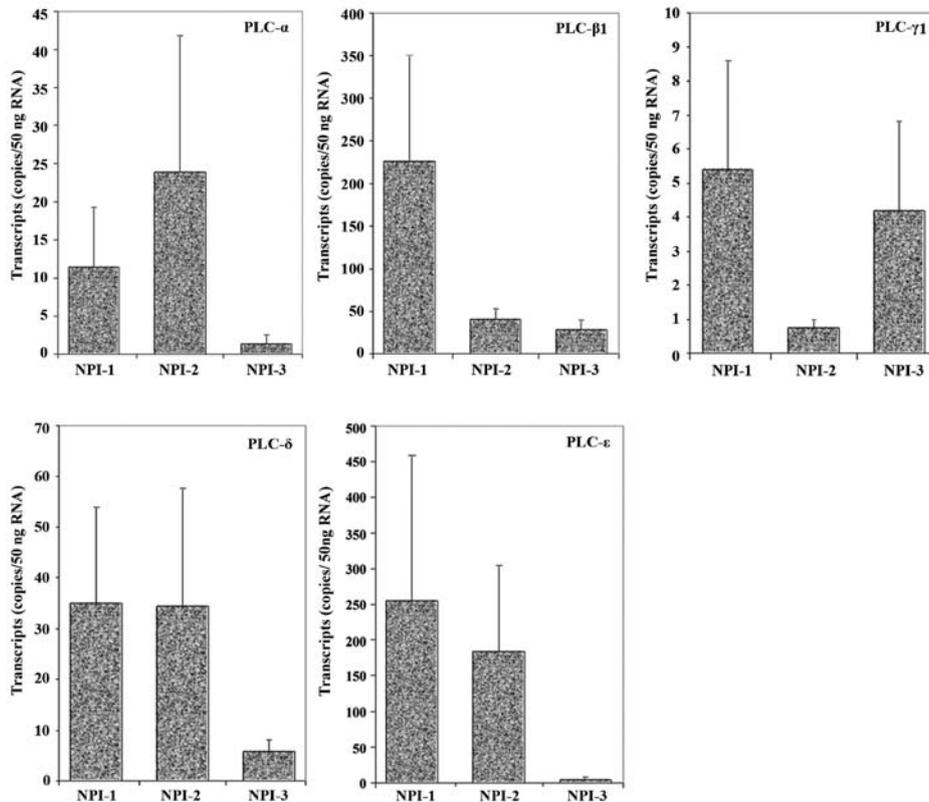


Figure 5. Transcript levels of PLC isoforms in patients with predicted outcome, using the Nottingham Prognostic Index as an indicator. NPI1=NPI < 3.4 (with good prognosis), NPI2=NPI 3.4-5.4 (with moderate prognosis), NPI3=NPI > 5.4 (with poor prognosis). Levels of PLC isoforms appeared to be at lower levels in NPI3 tumours compared with NPI1 tumours, although differences were not statistically significant.

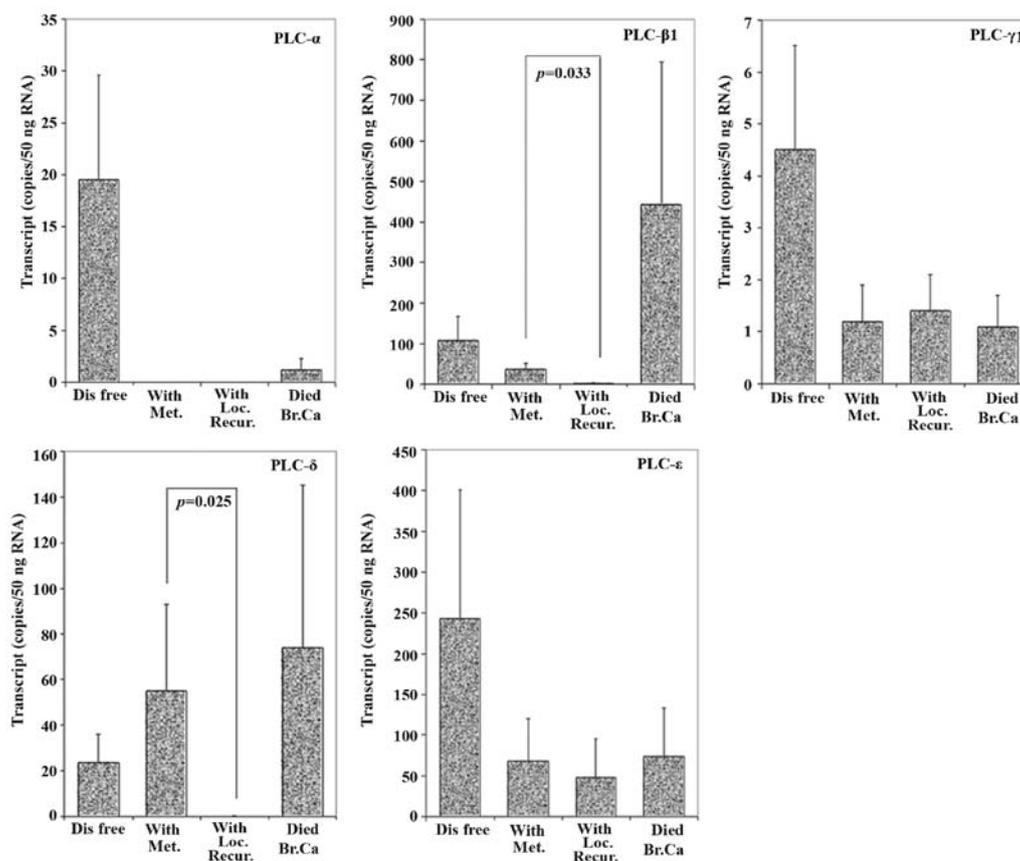


Figure 6. Levels of expression of PLC in tumours from patients with different clinical outcomes after a 10 year follow-up: patients who remained disease-free, patients who developed metastasis, patients who developed local recurrence and those who died from breast cancer. Patients who died from other unrelated causes were excluded from the analysis. There was a statistically significant difference in PLC- β 1 and PLC- δ levels between metastasis and recurrence tumour tissues ($p=0.033$ and $p=0.025$, respectively).

in Fig. 5, all the isoforms appeared to be at lower levels in NPI3 tumours compared with NPI1 tumours. However, the results were not statistically significant.

PLC expression and clinical outcomes. To determine the correlation between PLC and clinical outcome, patients were divided into four groups: patients who remained disease-free, patients with metastasis, patients with local recurrence and patients who died from breast cancer. As shown in Fig. 6, after a 10-year follow up, patients who suffered from complications had low expression levels of PLC- α , - γ 1 and - ϵ , but high levels of PLC- β 1 and - δ . This was particularly evident when all the patients with complications were considered together. Moreover, the differences of expression between metastasis and recurrence tumour tissues in PLC- β 1 level and PLC- δ level were statistically different ($p=0.033$ and $p=0.025$, respectively).

Levels of PLC and long-term survival. We assessed the association between PLC levels and breast cancer-related death using Kaplan-Meier survival analysis. An average transcript level of NPI2 group was set as a threshold. Higher levels of PLC- δ transcripts were associated with a shorter disease-free survival, but not for overall survival (Fig. 7). Patients with higher PLC- δ transcript level had an average disease-free survival of 96.7 months (95% confidence interval 67.7-124.4 months) compared to 137.4 months (95% confidence

interval 127.6-147.2 months) of patients who had lower expression of PLC- δ . No correlation with either overall survival or disease-free survival was observed for other PLC isozymes (data not shown).

Discussion

Globally, breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death in women. Considerable evidence has suggested that diverse molecules can induce aberrant signalling events in breast cancer and lead to breast tumour proliferation, invasion, and migration. The recent identification of phospholipase C (PLC), a phospholipid-hydrolyzing enzyme, has a role in the regulation of a number of cellular behaviours and promotes tumourigenesis by regulating cell motility, transformation and cell growth, partly by acting as signalling intermediates for cytokines such as EGF and interleukins in cancer cells. A number of PLC isoforms have been investigated with studies focusing on their functions and expression profiles in human cancers, however, the importance of PLC in clinical diagnosis is largely unknown. Our study aimed to examine the pattern of expression of 5 PLC isoforms, namely, α , β , γ , δ and ϵ in human breast cancer, and to predict their correlation with clinical significance.

Oestrogen exposure is one of the risk factors for the development of breast cancer (22). Ovarian hormones induce cell proliferation, and the uncorrected signal molecules result in

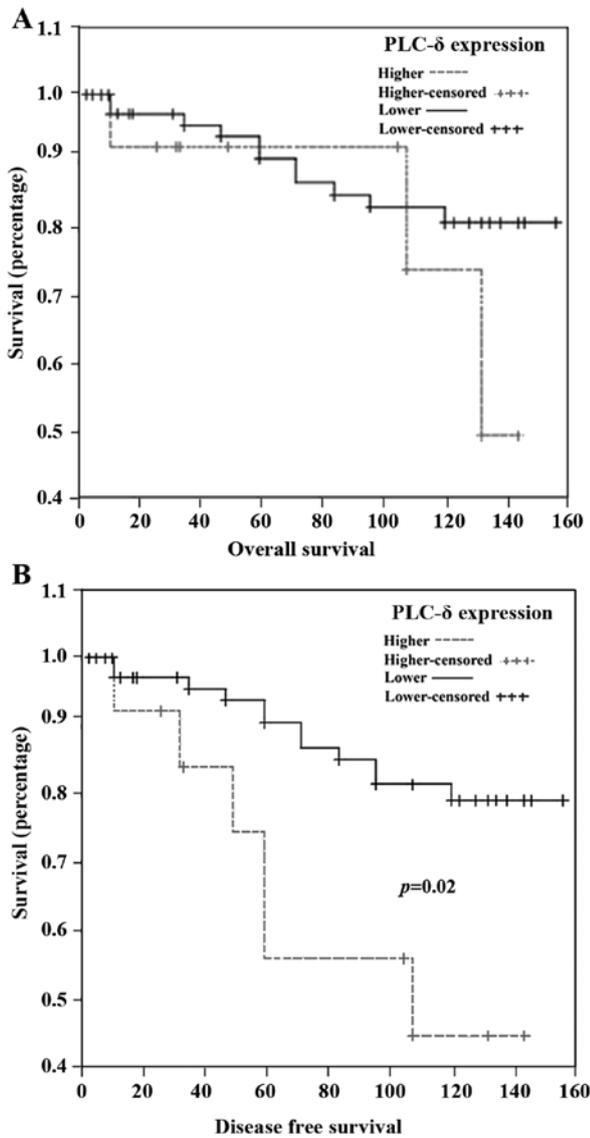


Figure 7. Association of PLC with long-term survival. Shown are the Kaplan-Meier survival curves for PLC- δ .

the generation of malignant phenotypes (23). Up to now, there have been limited studies focusing on the functions of PLC- α in breast cancer, but it has been reported that PLC- α is able to mediate the phosphatidylinositol (PI) second messenger pathway and the components in this pathway can increase some effects of estrogen (24). However, there needs to be further studies to investigate if there is a direct correlation between PLC- α and oestrogen.

The important factors for the prediction of clinical outcomes in breast cancer patients are tumour size, lymph node status and tumour grade, moreover, the involvement of molecular markers is beneficial to develop a treatment plan for patients with different tumour stages. Previous studies presented PLC- β 2, an isoform of PLC family, as a potential marker to predict the malignancy of breast cancer, with upregulated expression levels being detected in different types of breast tumours (25). In addition, breast cancer cell lines with high invasive potential show higher expression levels of PLC- β 2 in comparison with less-tumourigenic breast cancer cell lines (11).

In the present study, we detected isoform of the PLC- β subfamily and showed that there is a significantly increased level of PLC- β 1 in breast tumour tissues. Tissue microarray (TMA) analysis demonstrated a poor expression of PLC- β 2 in normal tissues and significantly higher expression levels in most tumour tissue specimens. Furthermore, there is no relationship between node status and PLC- β 1 level in breast tumour tissues. This result was also verified in our study focused on PLC- β 1. PLC- β 2 expression closely correlated with tumour grading, with an increase of staining intensity from grade 1 to grade 3. Similarly, our study demonstrated that grade 3 tumours showed the highest expression levels of PLC- β 1 (11). Lower overall survival has been demonstrated in patients whose primary tumours expressed high levels of PLC- β 2. The above findings therefore indicate a strong correlation between PLC- β levels and poor prognosis of breast cancer. Moreover, our present study demonstrated a significant difference in PLC- β 1 expression between metastasis and recurrence tumour tissues ($p=0.033$), which may indicate its role in promoting migration in breast cancer.

The expression of PLC, primarily PLC- γ , has been studied in a number of cancer types, although most of these studies have concentrated on established cell lines. In terms of clinical cancers, studies are available on squamous cell carcinoma, lung cancer, thyroid cancer, and limited on breast cancer (26-28). In a small-scale study in human breast cancer, PLC- γ was shown to be overexpressed in the majority of tumours in comparison with normal mammary tissues. The role of PLC in cells and indeed in cancer cells has been long studied. As already discussed, it seems that the prime role of the enzyme is the regulation of cell migration and adhesion by acting downstream of a number of cellular signalling pathways (29).

The current study was unable to provide further details of the link between PLC- γ and its associated signalling complexes. Future studies would need to address these points. The relationship between the PLC isoforms and long-term survival for patients with breast tumours was examined here. Our observations are interesting and suggest that these PLC isoforms may be potential therapeutic targets. Therapeutic applications of PLC- γ have been studied. PLC- γ inhibitors have been shown to inhibit PLC- γ mediated cell function in cancer cells. For example, the PLC- γ inhibitor, U73122, is able to inhibit the activation of PLC- γ , and hence PLC- γ mediated cell migration and invasion in a number of cancer cell types. The inhibitor has also the potential of reducing the growth of mammary tumours *in vivo* (30).

The loss of PLC- δ expression is highly associated with its role as a tumour suppressor in esophageal squamous cell carcinoma (ESCC). The tumourigenic ability of ESCC cells is suppressed both *in vitro* and *in vivo* assays. PLC- δ has been suggested to have a significant role in ESCC metastasis since its downregulation has been shown to have a reduction in cell mobility and an increase in cell adhesion (16). In addition, decreased PLC- δ expression correlated with poor clinical outcome in patients with acute or chronic myeloid leukaemia (16). In our study, patients with tumour metastasis expressed higher levels of PLC- δ than those with local recurrence. Significantly, breast cancer patients with higher expression levels of PLC- δ experience a shorter disease-free

survival period, and this result might indicate a correlation between PLC- δ and recurrence for breast cancer patients.

Taken together, the findings of the current study demonstrate for the first time that isoforms of PLC are aberrantly expressed in human breast cancer. The abnormalities are linked to both prognosis and to some degree to clinical outcomes. The study strongly indicated the potential therapeutic value of these signalling intermediates in human breast cancer and therefore warrants further investigation.

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