The Journal of Pathology: Clinical Research
J Path: Clin Res April 2017; **3:** 123–138
Published online 14 March 2017 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/cjp2.69

Original Article

PLA2G7 associates with hormone receptor negativity in clinical breast cancer samples and regulates epithelial–mesenchymal transition in cultured breast cancer cells

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Breast cancer is the leading cause of cancer-related deaths in women due to distinct cancer subtypes associated with early recurrence and aggressive metastatic progression. High lipoprotein-associated phospholipase A2 (*PLA2G7*) expression has previously been associated with aggressive disease and metastasis in prostate cancer. Here, we explore the expression pattern and functional role of *PLA2G7* in breast cancer. First, a bioinformatic analysis of genome-wide gene expression data from 970 breast samples was carried out to evaluate the expression pattern of *PLA2G7* mRNA in breast cancer. Second, the expression profile of *PLA2G7* was studied in 1042 breast cancer samples including 89 matched lymph node metastasis samples using immunohistochemistry. Third, the effect of *PLA2G7* silencing on genome-wide gene expression profile was studied and validated in cultured breast cancer cells expressing *PLA2G7* at high level. Last, the expression pattern of *PLA2G7* mRNA was investigated in 24 nonmalignant tissue samples and 65 primary and 7 metastatic tumour samples derived from various organs using qRT-PCR. The results from clinical breast cancer samples indicated that *PLA2G7* is overexpressed in a subset of breast cancer samples compared to its expression in benign breast tissue samples and that high *PLA2G7* expression associated with hormone receptor negativity as well as with poor prognosis in a subset of breast cancer samples. *In vitro* functional studies highlighted the putative role of *PLA2G7* in the regulation of epithelial–mesenchymal transition (EMT)-related signalling pathways, vimentin and E-cadherin protein expression as well as cell migration in cultured breast cancer cells. Furthermore, supporting the findings in breast and prostate cancer, high *PLA2G7* mRNA expression was associated with metastatic cancer in four additional organs of origin. In conclusion, our results indicate that *PLA2G7* is highly expressed in a subset of metastatic and aggressive breast cancers and in metastatic samples of various tissues of origin and promotes EMT and migration in cultured breast cancer cells.

Keywords: *PLA2G7*; epithelial-mesenchymal transition; EMT; vimentin; breast cancer; prognosis; metastasis; invasion

Received 2 January 2017; Accepted 10 March 2017

Microarray data: Data can be accessed at ArrayExpress (E-MTAB-5397 and E-MTAB-5398)

No conflicts of interest were declared.

Introduction

Breast cancer is the most frequent cancer in women worldwide accounting for 25% of all cancer cases among females [1]. Although survival rates of breast cancer patients have improved significantly during the last decade breast cancer is still the leading cause of cancer-related deaths in women due to distinct subtypes of breast cancer that are associated with early recurrence and an aggressive metastatic progression of the disease [1–4]. Identification of biomarkers and development of targeted therapies especially for these malignant tumours is important. Recently, the regulators of metastasis initiating epithelial-mesenchymal transition (EMT) have been suggested as important factors and possible diagnostic markers for aggressive and metastatic breast cancers, including triple negative breast cancers (TNBC) [5–9].

EMT, a vital process during embryogenesis and tissue repair, is characterised by specific gene expression changes, reduced intercellular adhesion, gain of mesenchymal characteristics and increased cell mobility to distant locations. Loss of immotile epithelial phenotype and gain of mesenchymal properties via EMT has recently been shown to be a key metastasis-promoting step in many cancers [10]. In addition to enabling metastasis EMT has been suggested to promote the generation and function of cancer stem cells as well as development of drug resistance [11]. EMT is induced by multiple oncogenic events and signalling pathways and requires the expression of a variety of regulators, including members of the Twist, Snail/Slug and Zeb transcription factor families. In addition, Tiwari *et al.* recently reported the SOX4-EZH2 pathway as one of the master regulators of EMT in metastatic breast cancer [12]. Two major hallmarks of EMT are the loss of expression of the epithelial cell adhesion molecule E-cadherin (CDH1) and gain of expression of the major mesenchymal cell cytoskeletal component vimentin (VIM). The cytoplasmic intermediate filament protein vimentin is a component of the cytoskeleton normally found in embryonic or mesenchymal cells. However, in cancer vimentin is a marker of EMT and is expressed in neoplastic epithelial cells with metastatic properties [13–15]. The expression of vimentin correlates with invasiveness and poor prognosis in clinical cancer samples [16–18].

PLA2G7, also known as lipoprotein-associated phospholipase A2 or platelet-activating factor (PAF) acetyl hydrolase, is a phospholipase that hydrolyses PAF and truncated phospholipids generated by oxidative attack [19]. *PLA2G7* was recently reported as a novel biomarker in 50% of primary and 70% of metastatic prostate cancers and associated with aggressive disease in prostate cancer [20]. Furthermore, PLA2G7 was identified as a potential drug target especially in *ERG* oncogene positive prostate cancers and the alterations induced by *PLA2G7* silencing highlighted the potential of *PLA2G7* inhibition as an anti-proliferative, pro-apoptotic and anti-migratory therapeutic approach [20,21]. Interestingly, in addition to *ERG* oncogene expressing prostate cancers, high *PLA2G7* expression has also been proposed to play a causal role in colon tumourigenesis downstream of loss-of-function *TP53* mutations and activating *RAS* mutations [22]. Supporting this finding, deletion of *PLA2G7* in $Apc^{Min/+}$ mice has been shown to decrease both intestinal polyposis as well as colon tumourigenesis [23]. In recent years, *PLA2G7* has also been under intensive research in the area of cardiovascular diseases. High PLA2G7 amount and enzyme activity have been associated with an increased risk of cardiovascular disease, and *PLA2G7* inhibitor darapladib has been studied in the prevention and treatment of coronary heart disease [24–29].

In this study, we focus on analysing the expression pattern of *PLA2G7* in breast cancer and investigate the functional role of *PLA2G7* in cultured breast cancer cells using genome-wide gene expression analysis, immunohistochemistry and functional assays. The results indicate a role for *PLA2G7* in the regulation of metastasis-enabling EMT and cell migration, associate high *PLA2G7* mRNA expression with hormone receptor negative aggressive disease and indicate that high PLA2G7 protein expression in lymph node metastases associates with unfavourable prognosis in breast cancer.

Methods

Transcriptomics analysis

In order to evaluate the *in vivo* relevance of PLA2G7 in breast cancer, we analysed *PLA2G7* mRNA expression levels in normal ($n = 13$) and malignant ($n = 957$) breast tissue samples using GeneSapiens database (www.genesapiens.org) [30]. Survival information was available for 395 and recurrence data for 159 breast cancer samples.

Clinical material

Breast tumour samples were obtained from patients at Hamburg University Medical Centre (Hamburg, Germany) between 1999 and 2007 and Tampere University Hospital (Tampere, Finland) between 1990 and

1999. Patient data and tumour characteristics have been described previously [31,32]. Informed consent for the scientific use of tissue materials was obtained from the patients and ethical permission was obtained from the local ethical committees. All procedures were in accordance with the Helsinki Declaration.

The patient records contained information on clinicopathological parameters, primary treatments, site and time of tumour recurrences and survival. The maximum follow-up period was 19.8 years. Relapse–free survival was defined as time to any disease recurrence (local, regional or distant). The end point in breast cancer specific survival was death from breast cancer and in overall survival death from any cause.

Immunohistochemistry

The construction of tissue microarrays (TMA) has been described previously [31,32]. The paraffin embedded breast cancer TMAs containing in total 1042 samples from 897 breast cancer patients, including 89 cases with matched primary and lymph node metastatic samples, were stained with *PLA2G7* (1:150, rabbit polyclonal, Cayman Chemical) antibody. Immunostaining was performed with the automated immunostaining device BenchMark XT (Roche Diagnostics/Ventana Medical Systems, Tucson, AZ, USA) using ultraView Universal DAB Detection Kit (Roche Diagnostics/Ventana Medical Systems). PLA2G7 protein expression level in the epithelial breast cancer cells in each sample was evaluated independently in blinded fashion by two pathologists and graded into two groups based on staining intensity: low expression (no staining or weak staining), high expression (moderate or strong staining). After excluding the cases without residual tissue or tumour cells, 618 breast tumours and 65 lymph node metastasis samples remained eligible for evaluation.

Cell culture

The breast carcinoma cell lines MDA-MB-468, MCF-7, MDA-MB-231 and ZR-75-1 cell lines were from American Type Culture Collection (Manassas, VA, USA). BT-549 and KPL-1 cell lines were from Cell Lines Service (Eppelheim, Germany). All cell lines were maintained according to the distributor's instructions.

Gene silencing

For transient knockdown of *PLA2G7*, siRNA (SI00072177; siPLA2G7, Qiagen, Valencia, CA) was transfected into MDA-MB-468 cells using siLentFect Lipid Reagent (Bio-Rad Laboratories, CA) in Opti-MEM (Invitrogen, Carlsbad, CA) at a final concentration of 13 nM. Cells were incubated for 48 hours before further experiments. To generate stable PLA2G7 deficient MDA-MB-468 cells (MDA-468/PLA2G7- cells), two lentiviral shRNA constructs (NM_005084.2-1046s1c1; shPLA2G7_1 and NM_005084.2-684s1c1; shPLA2G7_2, Sigma-Aldrich, St. Louis, MO) were transduced into MDA-MB-468 cells as described [33]. All Stars scrambled siRNA (siScr, Qiagen) and non-targeting construct (SHC002V; shScr: Sigma-Aldrich) were used as negative control.

Quantitative RT-PCR

The confirmation of effective target gene silencing was performed with Taqman reverse transcriptase PCR (qRT-PCR). Total RNA from 70 000 cells was extracted using RNeasy kit (Qiagen) according to the manufacturer's protocol and processed to cDNA with Applied Biosystems cDNA synthesis kit. TaqMan gene expression probes and primers from the Universal Probe Library (Roche Diagnostics, Espoo, Finland) were used to study *PLA2G7* (forward: tggctctaccttagaaccctga; reverse: ttttgctctttgc cgtacct), and β-actin (ACTB, forward: ccaaccgcgagaagatga; reverse: ccagaggcgtacagggatag) mRNA expression. The quantitative PCR was done using ABI Prism 7900 (Applied Biosystems, Foster City, CA). Quantitation was carried out using the $_{\Delta\Delta}$CT method with RQ manager 1.2 software (Applied Biosystems). Average expression of the control samples was considered for the calculation of the fold changes and β-actin was used as an endogenous control. Three or more replicate samples were studied for detection of mRNA expression.

Gene expression analysis using bead arrays

500 ng of total purified RNA was used for amplification with Illumina RNA TotalPrep Amplification kit (Ambion, Austin, TX), and biotin labelled cRNA was hybridised to Sentrix HumanRef-12 vs.3 Expression Bead Chips (Illumina, San Diego, CA). Analysis was done as described [20]. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress, siRNA treatment: E-MTAB-5397 and shRNA treatments: E-MTAB-5398). Two replicate samples were studied for each treatment and gene expression profiles of the PLA2G7 silenced samples were compared with the scrambled control-treated samples. Ingenuity Pathway Analysis (IPA) Software (Ingenuity Systems Inc., Redwood City, CA, USA), DAVID Bioinformatics Resources [34,35] and

Molecular Signatures Database (MSigDB) Gene Set Enrichment Analysis Software [36,37] were used to assess the enrichment of functional and pathway annotations as well as gene sets for the genes differentially expressed by *PLA2G7* siRNA or both *PLA2G7* shRNAs.

Cell viability and apoptosis assays

The effects of *PLA2G7* silencing on cancer cell viability and induction of apoptosis were assessed with the CellTitre-Glo cell viability assay and ApoONE apoptosis assay (Promega Inc, Madison, WI, USA) according to the manufacturer's instructions. The EnVision multilabel plate reader (Perkin-Elmer, Waltham, MA) was used for signal quantification. Scrambled siRNA or shRNA was used as the negative control and *KIF11* siRNA as the positive control.

Western blotting

Western blot analysis was performed using antibodies against vimentin (1:1000, V6630, Sigma-Aldrich), E-cadherin (1:1000, Cell Signalling Technology, Danvers, MA), EGFR (1:100, Cell Signalling Technologies) and β-actin (1:5000, Sigma-Aldrich). The signals obtained were visualised with the enhanced chemiluminescence (ECL) detection system (Amersham) and normalised to β-actin signal.

Immunofluorescence staining

For the immunofluorescence staining, MDA-468/PLA2G7- cells were fixed with 4% paraformaldehyde (PFA) in PBS, permeabilised with 0.2% Triton X-100, and blocked with 3% bovine serum albumin in PBS. Cells were stained with vimentin antibody and Alexa-conjugated secondary antibody was used for secondary staining (1:300 dilution; Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA). Nuclei were stained with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing DAPI. Images were taken with Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany).

Wound healing invasion assay

MDA-468/PLA2G7- cells were utilized in the wound healing invasion assay using the IncuCyte real-time imaging system (Essen BioScience, Ann Arbor, Michigan, USA). The cells were plated on 96-well plates (ImageLock plate, Essen BioScience, MI) coated with 50 µl 10% Growth Factor Reduced Matrigel (BD Biosciences) after which the cells were allowed to attach overnight at +37°C. A wound was scratched across each well (Wound Maker, Essen BioScience) and the growth medium was removed. The cells were then carefully covered with 50 µl 25% Matrigel in normal growth medium and incubated in 37°C for 2–3 hours to allow gelling, after which 100 µl of growth medium was carefully added to each well. The rate of invasion (wound closure through the matrix) was monitored hourly with Incucyte imaging software (Essen BioScience) for 72 hours. Invasion efficiency was determined as percentage of the relative wound confluence compared to respective negative control (regarded as 100%).

cDNA tumour panel

TissueScan^{TM} Cancer Survey cDNA Array 96 – I (Origene, Rockville, MD, USA) consisting of 96 samples from 65 primary and 7 metastatic tumour samples as well as 24 non-malignant tissue samples derived from 8 different primary organs and provided with clinical information was utilised according to the manufacturer's instructions to analyse the expression pattern of *PLA2G7* mRNA in multiple malignancies. The expression of *PLA2G7* was quantified using qRT-PCR.

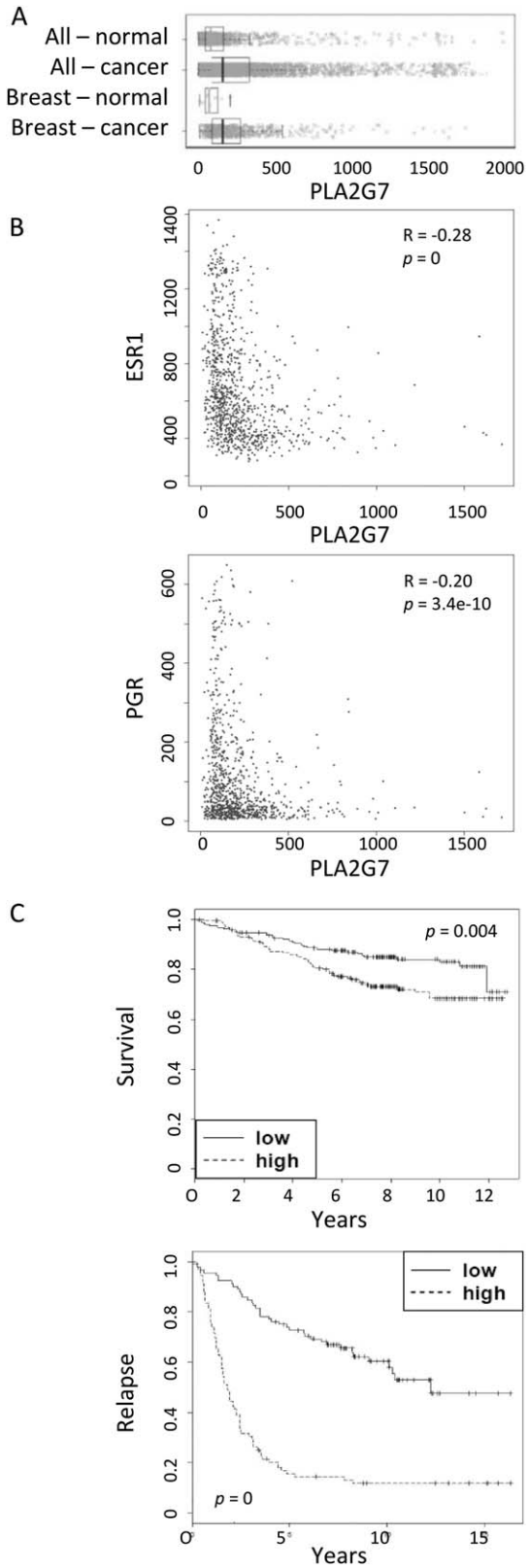
Statistical analysis

Statistical analysis of immunohistochemical samples was carried out using the SPSS program (SPSS Inc., Chicago, IL). Comparisons between categorical variables were performed using the χ^2test. Kaplan-Meier curves were used to estimate survival probability and patient survival differences were analysed by a log-rank test. Other statistical analyses were performed using Student's t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$) and the Pearson correlation coefficient. The results from cultured cells are presented as the mean ± SD of at least three replicates unless otherwise stated.

Results

Correlation of *PLA2G7* mRNA expression with hormone receptor expression and prognosis in breast cancer

To evaluate the expression pattern and possible association of *PLA2G7* with poor survival in breast cancer, the GeneSapiens database was utilised to analyse *PLA2G7* mRNA expression in 13 normal breast



samples and 957 breast cancer samples [30]. Comparison of *PLA2G7* expression levels in normal breast tissues and breast cancer samples indicated high *PLA2G7* mRNA expression in a subset of breast cancers (Figure 1A). Co-expression analysis indicated *PLA2G7* mRNA to be overexpressed especially in breast cancer samples with low oestrogen and progesterone receptor (ESR1 and PGR) expression ($p = 0$ and $p = 3.4E\text{-}10$, respectively; Figure 1B) whereas the association between high *PLA2G7* mRNA and low *HER2*/*ERBB2* expression was not statistically significant (supplementary material, Figure S1). To investigate whether *PLA2G7* may have prognostic significance, Kaplan–Meier analysis was performed. The results revealed a significant association between high *PLA2G7* mRNA expression and poor breast cancer survival ($p = 0.004$) as well as increased risk of disease relapse ($p = 0$; Figure 1C).

To validate the association between high *PLA2G7* expression and hormone receptor negativity as well as poor prognosis in breast cancer, two additional independent sets of clinical breast cancer tissue microarrays were studied with immunohistochemical staining. *PLA2G7* expression was analysed in a total of 683 breast cancer samples, including 65 samples derived from lymph node metastases (Figure 2A). High *PLA2G7* expression was detected in 21.7% ($n = 148$) of all breast cancer samples. Association between high *PLA2G7* expression and standard clinicopathological parameters is presented in Table 1. In accordance with the results from bioinformatic analysis of *PLA2G7* mRNA expression, high *PLA2G7* expression showed significant association with hormone receptor negativity in primary the breast cancer samples analysed. Furthermore, even though no significant association with survival or relapse was seen in the samples obtained from primary tumours, high PLA2G7 protein expression was associated with poor

Figure 1. High *PLA2G7* mRNA expression is associated with hormone receptor negativity and poor prognosis in clinical breast cancer samples. (A) Box plot analysis of normalized *PLA2G7* mRNA expression values in normal and malignant tissues. The box refers to quartile distribution (25–75%) range, with the median shown as a vertical line. Data observations which lie more than 1.5*inter-quartile range higher than third quartile are considered as outliers and indicated separately. (B) The mRNA co-expression pattern of PLA2G7 and oestrogen receptor (ESR1) or progesterone receptor (PGR) in breast cancer samples ($n = 957$). (C) Kaplan-Meier plot of breast cancer specific survival and risk of relapse based on *PLA2G7* mRNA expression in breast cancer. A log-rank test was performed between samples with high (50%) and low (50%) expression to evaluate the prognostic significance of *PLA2G7* mRNA expression level.

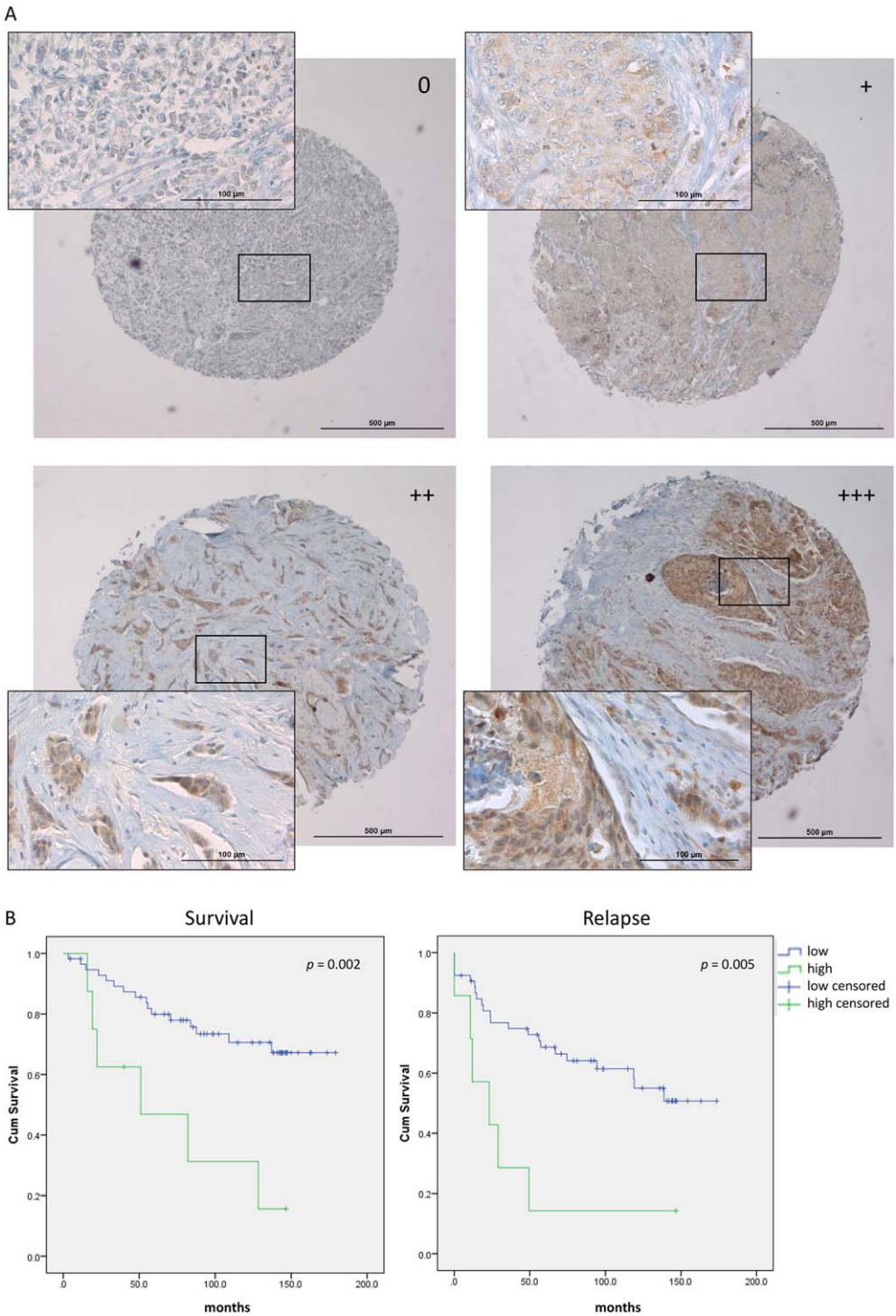


Figure 2. *PLA2G7* is expressed in a subset of breast cancers and associates with poor prognosis in breast cancer lymph node metastasis samples. (A) Staining intensity of *PLA2G7* in primary breast cancer and breast cancer lymph node metastasis was scored as follows: Low expression (0 or +), high expression (++ to +++). Representative section of staining intensities is presented. The areas presented at higher magnification have been indicated in the core images. (B) Kaplan-Meier curve presentation of overall survival and risk of relapse in the patient groups with no or weak *PLA2G7* staining (n = 57) or positive *PLA2G7* staining (n = 8) in the lymph node metastasis samples.

Table 1. *PLA2G7* expression and standard clinicopathological parameters in 618 breast cancer samples.

	TMA 1					TMA 2 (primary tumours)				
Variable	All (n)	All (%)	High PLA2G7 (n)	High PLA2G7 (%)	*p*	All (n)	All (%)	High PLA2G7 (n)	High PLA2G7 (%)	*p*
All samples	400	100	84	21.0		218	100	56	25.7	
Tumour type	309					218				
ILC	135	43.7	21	15.6	**0.028**	27	12.4	5	18.5	**0.048**
IDC	174	56.3	45	25.9		165	75.7	43	26.1	
Hormone receptor status	328					218				
negative	53	16.2	16	30.2	**0.041**	44	20.2	18	40.9	**0.010**
positive	275	83.8	51	18.0		174	79.8	38	21.8	
HER2 expression	330					187				
negative	285	86.4	54	18.9	0.061	160	85.6	41	25.6	0.706
positive	45	13.6	14	31.1		27	14.4	6	22.2	
WHO grade	260					213				
I	81	31.2	12	14.8	0.244	10	4.7	4	40.0	**0.018**
II	126	48.5	27	21.4		116	54.5	21	18.1	
III	53	20.4	14	26.4		87	40.8	30	34.5	
pT stage	342					218				
pT1	218	63.7	51	23.4	0.227	109	50.0	29	26.6	0.973
pT2	98	28.7	14	14.3		91	41.7	23	25.3	
pT3	12	3.5	1	8.3		10	4.6	2	20.0	
pT4	14	4.1	3	21.4		8	3.7	2	25.0	
Lymph node metastasis	322					217				
pN0	205	63.7	38	18.5	0.538	129	59.4	31	24.0	0.590
pN1	117	36.3	25	21.4		88	40.6	24	27.3	
Metastasis	341					214				
M0	330	96.8	68	20.6	0.350	205	95.8	50	24.4	0.543
M1	11	3.2	1	9.1		9	4.2	3	33.3	
Recurrence	345					208				
no	316	91.6	67	21.2	0.065	161	77.4	40	24.8	0.211
yes	29	8.4	2	6.9		47	22.6	16	34.0	

ILC, invasive lobular carcinoma; IDC, invasive ductal carcinoma. Numbers in bold indicate statistically significant *P* values.

survival ($p = 0.002$) as well as increased risk of disease relapse ($p = 0.005$) in the samples obtained from lymph node metastases (Figure 2B).

No significant difference was detected in *PLA2G7* expression between the paired primary tumour and metastasis samples. A staining result was obtained for 39 paired primary tumours and lymph node metastases and 72% (28) of the pairs showed similar staining intensity in primary and metastatic samples.

PLA2G7 mRNA expression in cultured breast cancer cells

In order to find a suitable breast cancer cell model for functional experiments the mRNA expression level of *PLA2G7* was studied in six breast cancer cell lines with qRT-PCR. Three of the cell lines studied were ER-positive (MCF-7, KPL-1 and ZR-75-1) and three were ER-negative (MDA-MB-231, BT-549 and MDA-MB-468). The results from qRT-PCR analysis are in accordance with the clinical data and show high *PLA2G7* expression in two hormone receptor negative cell lines, MDA-MB-468 and BT-549 (Figure 3A).

The effect of *PLA2G7* silencing on genome-wide gene expression in MDA-MB-468 breast cancer cells

To investigate the molecular processes regulated by *PLA2G7* in breast cancer cells, the effect of *PLA2G7* silencing on genome-wide gene expression profile was studied in MDA-MB-468 cells expressing *PLA2G7* at high level. The gene expression analysis was conducted using one siRNA and two different shRNA molecules silencing *PLA2G7* efficiently compared to the respective scrambled control (Figure 3B and supplementary material, Figure S2A). For MDA-468/PLA2G7- cells the genes uniformly differentially regulated by both of the shRNAs were selected for further analysis.

DAVID Bioinformatics Resources and Ingenuity Pathway Analysis (IPA) software was used to assess enrichment of functional and pathway annotations for

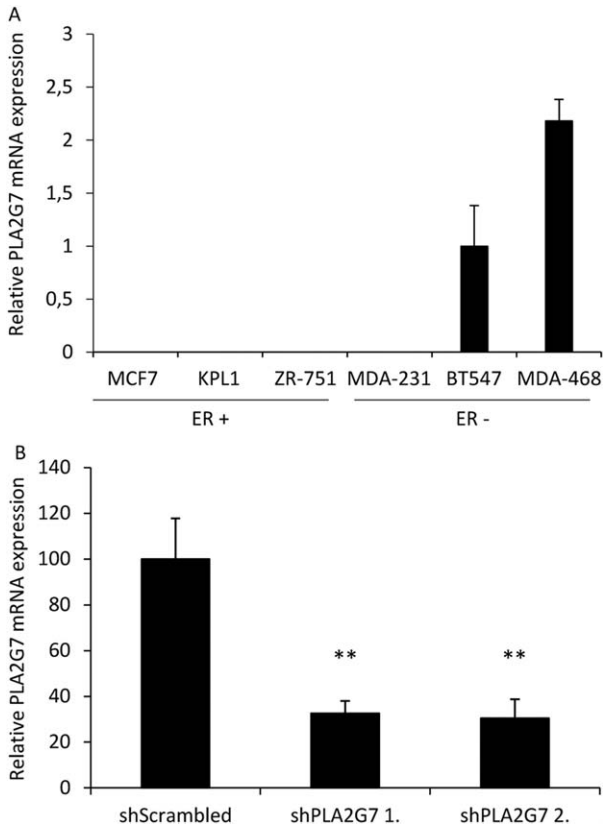


Figure 3. *PLA2G7* mRNA is highly expressed in triple negative breast cancer cell lines. (A) *PLA2G7* mRNA expression in oestrogen receptor positive and oestrogen receptor negative breast cancer cell lines. (B) Validation of stable *PLA2G7* gene silencing in MDA-MB-468 breast cancer cells at the mRNA level.

the genes differentially expressed by *PLA2G7* impairment. In accordance with earlier functional studies in prostate cancer cells [20], in transiently silenced *PLA2G7* siRNA transfected breast cancer cells, the gene expression profiling highlighted the effect of *PLA2G7* on proliferation (cell cycle) and apoptosis in addition to cell motion and adhesion (supplementary material, Table S1). However, functional cell viability and apoptosis assays with siRNA treated MDA-468 breast cancer cells showed only a minor decrease in cell viability at the 48 hours time point (supplementary material, Figure S2B). Interestingly, the results from genome-wide gene expression profiling using MDA-468/PLA2G7- cells indicated that *PLA2G7* silencing decreases the expression of genes associated with regulation of cell migration, epithelial cell differentiation and EMT (Table 2). Supporting the role of *PLA2G7* in the regulation of EMT as well as migration and invasion, *PLA2G7* impairment induced gene expression changes associated with IL-17A (interleukin 17A), Notch and oncostatin M signalling. These processes have all been previously associated with tumour metastasis or EMT [38–42].

Validation of the effect of *PLA2G7* impairment on vimentin, E-cadherin and EGFR expression

Two major hallmarks of EMT are the loss of expression of the epithelial cell adhesion molecule E-cadherin and gain of expression of the major mesenchymal cell cytoskeletal component vimentin. To validate the role of *PLA2G7* in EMT, MDA-468/PLA2G7- cells were analysed for vimentin and E-cadherin protein expression. The results indicated clearly reduced vimentin and increased E-cadherin protein levels (Figure 4A). In addition, immunofluorescence staining of MDA-468/PLA2G7- cells showed a clear decrease in vimentin staining intensity in comparison to the corresponding control (Figure 4B).

Table 2. The effect of stable *PLA2G7* silencing on gene expression profile in MDA-MB-468 breast cancer cells

Down (n = 19)	
Biological processes	*P* value
Epithelial cell differentiation	6.3E-3
Regulation of cell migration	9.4E-3
Epidermis development	1.1E-2
Regulation of cell motion	1.2E-2
Ectoderm development	1.3E-2
Canonical Pathways	
Bile Acid Biosynthesis, Neutral Pathway	1.28E-2
Regulation of the Epithelial-Mesenchymal Transition Pathway	1.36E-2
Methylglyoxal Degradation III	1.48E-2
IL-17A Signalling in Gastric Cells	2.45E-2
Notch Signalling	3.7E-2
Up (n = 39)	
Biological processes	
Immune response	2.5E-3
Canonical Pathways	*P* value
Interferon Signalling	1.35E-13
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	2.34E-5
Activation of IRF by Cytosolic Pattern Recognition Receptors	1.83E-4
Oncostatin M Signalling	1.63E-3
Role of IL-17A in Psoriasis	2.26E-2

The functional gene ontology and pathway annotations were analysed for the sets of uniformly differentially expressed genes (FC >1.5 or FC <0.666) in response to two shRNAs. The analysis was conducted using DAVID Bioinformatics Resources and Ingenuity Pathway Analysis software

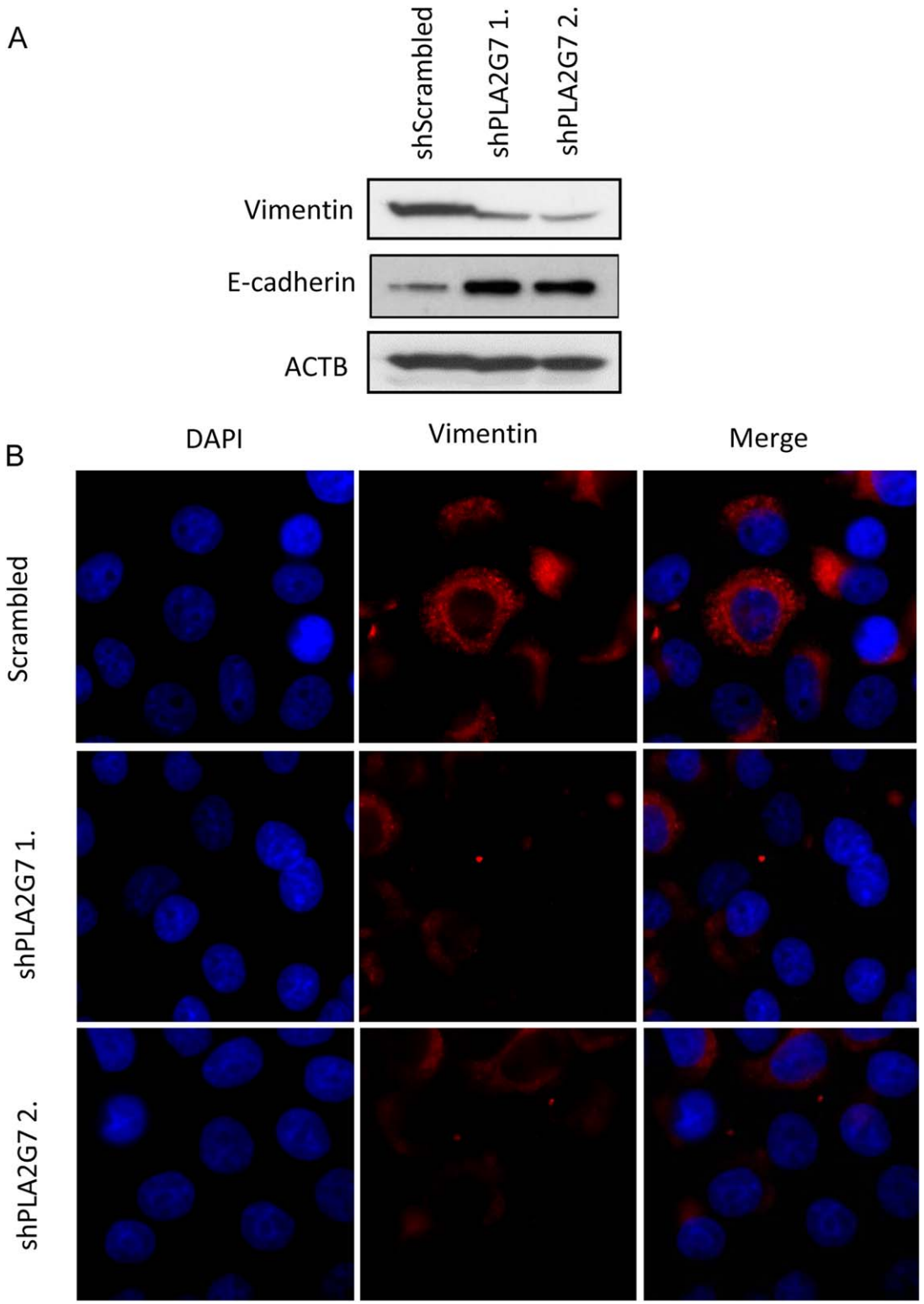


Figure 4. *PLA2G7* impairment decreases the protein expression of vimentin and increases the protein expression of E-cadherin. (A) The effect of stable *PLA2G7* silencing on vimentin and E-cadherin protein expression in MDA-MB-468 cells. β-actin expression was used as an endogenous control. (B) Immunofluorescence staining of MDA-MB-468 cells without (shScrambled) or with stable PLA2G7 silencing (shPLA2G7) using vimentin antibody (red). DAPI staining (blue) was used to visualize nuclei. 63× objective magnification.

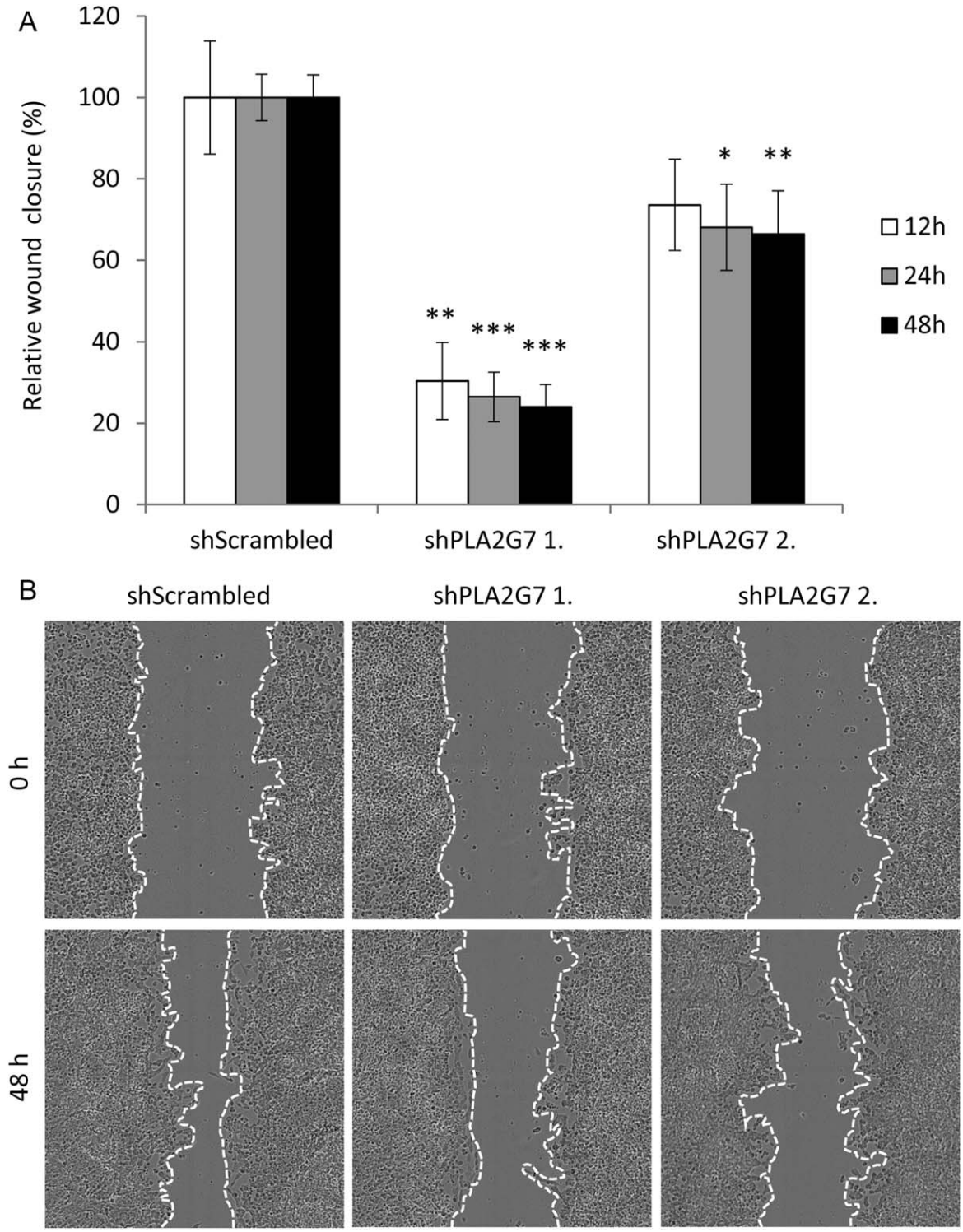


Figure 5. *PLA2G7* impairment reduces breast cancer cell invasion. (A) The effect of stable PLA2G7 silencing on MDA-MB-468 cell motility in wound healing invasion assay at 12, 24 and 48 hours after wound scratch. The relative wound closure effect was calculated as wound confluence in comparison to shScrambled control. (B) Wound closure at 48 hours in comparison to 0 hours. Scale bar = 300 μm. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

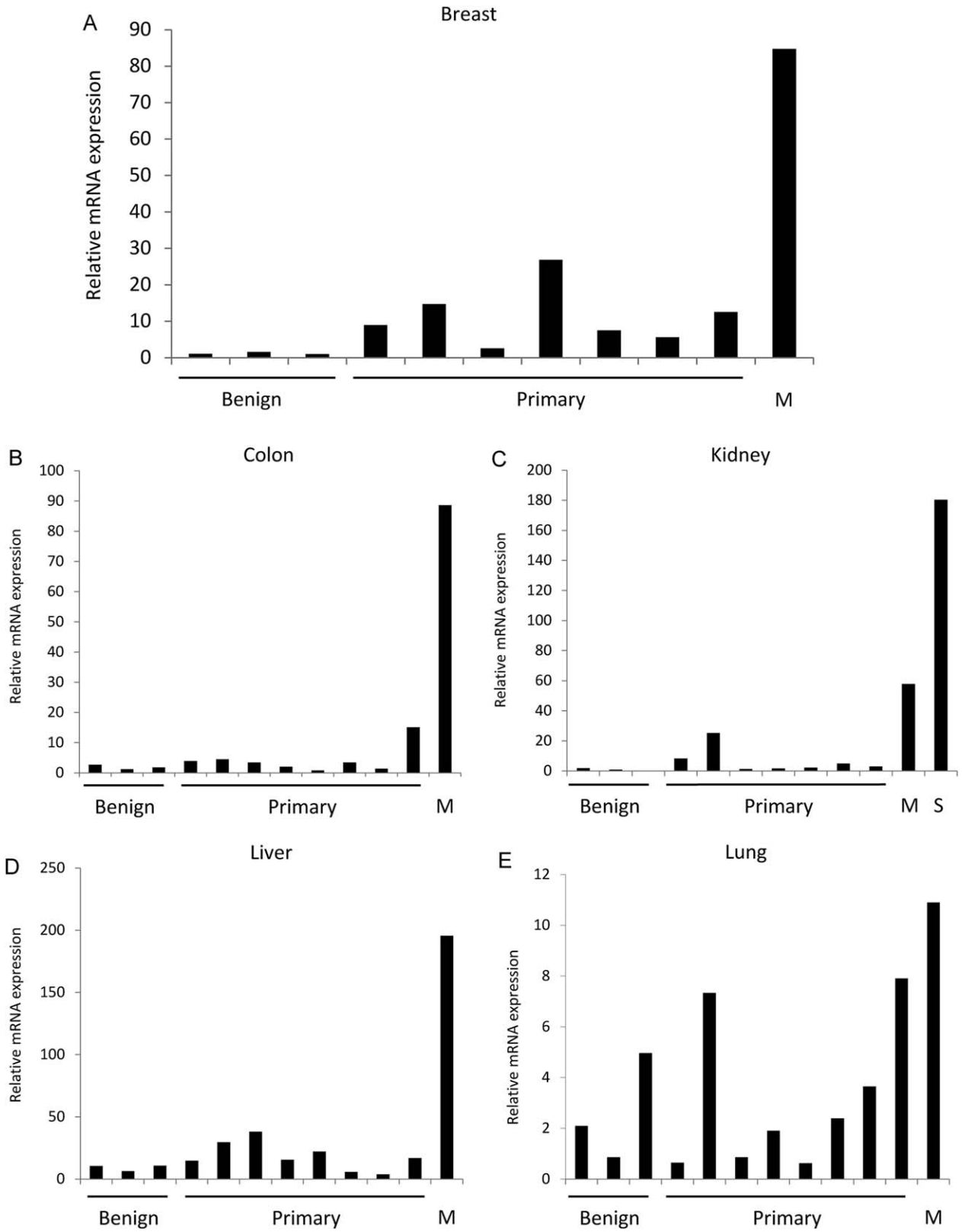


Figure 6. Relative *PLA2G7* mRNA expression in non-malignant and malignant breast, colon, kidney, liver and lung tissue samples based on TissueScan™ Cancer Survey cDNA panel. M, metastasis; S, sarcomatoid.

Epidermal growth factor receptor (EGFR) is known to be frequently overexpressed and suggested as a potential drug target in aggressive TNBCs [43,44]. In the current study, gene expression analysis suggested *PLA2G7* as a regulator of EGFR expression in MDA-468 breast cancer cells expressing EGFR at high level. Western blot analysis of EGFR showed a significant decrease in protein expression in response to stable *PLA2G7* silencing (supplementary material, Figure S3).

Analysis of the effect of PLA2G7 impairment on breast cancer cell invasion

To validate the putative regulatory role of PLA2G7 on breast cancer cell migration and invasion, a wound healing invasion assay was performed with MDA-468/PLA2G7- cells. The results showed that stable PLA2G7 knockdown significantly reduced cell invasion in comparison to scrambled control at 12, 24 and 48 hours (Figure 5A and B). To confirm that the effect was not due to changes in cell viability the effect of stable PLA2G7 silencing on cell viability was studied (supplementary material, Figure S4). In accordance with the experiments conducted with PLA2G7 siRNA transfected MDA-MB-468 cells (supplementary material, Figure S2B) stable PLA2G7 knockdown only moderately affected MDA-468 cell viability thus confirming that the effect on cell invasion was not due to reduced cell viability.

PLA2G7 mRNA expression in cancers of various origins

The expression of *PLA2G7* mRNA was further analysed in a cDNA tumour panel consisting of 65 primary and 7 metastatic tumour samples as well as 24 non-malignant tissue samples derived from 8 different primary organs. Interestingly, the results from breast, colon, kidney, liver and lung derived samples showed the highest *PLA2G7* expression in the aggressive and metastatic cancer samples (Figure 6A–E). In ovarian cancer samples, no similar pattern was seen (supplementary material, Figure S5A). In accordance with earlier reports [20], the prostate cancer sample with the highest expression was the only true cancer sample in the set (supplementary material, Figure S5B). Surprisingly, in thyroid samples the expression pattern was the opposite and normal tissues showed higher *PLA2G7* expression than tumour tissues (supplementary material, Figure S5C).

Discussion

Breast cancer is the most frequent cancer in women worldwide and the leading cause of cancer-related deaths in women due to distinct cancer subtypes associated with aggressive progression [1–4]. Here, we evaluated the expression pattern of *PLA2G7* in breast cancer and investigated the molecular processes regulated by *PLA2G7* in cultured breast cancer cells.

The results from three independent clinical breast cancer sample sets showed that *PLA2G7* is highly expressed in hormone receptor negative breast cancers. Analysis of transcriptomic data indicated a significant association between high *PLA2G7* mRNA expression and poor prognosis in primary breast cancer samples. However, the association between high PLA2G7 protein expression and unfavourable disease outcome was not seen at the protein level in samples derived from primary breast cancers. Surprisingly, when evaluating the protein expression of PLA2G7 in the lymph node metastases, high PLA2G7 protein expression was significantly associated with poor prognosis. The reason for the inconsistent results in survival analyses of primary breast cancer samples using either PLA2G7 mRNA or protein expression remain to be investigated. The qRT-PCR primers and antibody used for evaluation of *PLA2G7* expression detect both of the transcripts listed in the current release of the Ensembl database [45]. It can be hypothesised that the results may be affected by possible PLA2G7 protein secretion or the expression of *PLA2G7* by stromal and inflammatory cells present in the samples. However, the association between high *PLA2G7* expression and poor prognosis using samples from lymph node metastases was acquired using the protein expression of PLA2G7 only in the tumour cells, supporting the role for *PLA2G7* in cancer progression. Furthermore, *PLA2G7* was also highly expressed in metastatic or aggressive tumours derived from cancers of other origins, indicating a wider range of significance in cancer progression. However, although high *PLA2G7* expression associates with aggressive tumours and metastasis in many tissues, the results were opposite in thyroid tissue, suggesting that the role of PLA2G7 is multifaceted and dependent on the nature of the disease and biological context.

To gain biological insights into the role of *PLA2G7* in breast cancer we performed functional studies with breast cancer cells and identified a regulatory link between *PLA2G7* and metastasis-enabling EMT. The genome-wide gene expression profile

indicated that *PLA2G7* impairment altered IL-17A (interleukin 17A), Notch and oncostatin M signalling, supporting the role of *PLA2G7* in the regulation of EMT as well as migration and invasion. The pro-inflammatory cytokine IL-17A has recently emerged as a critical promoter of breast cancer metastasis, chemoresistance, angiogenesis as well as cell proliferation, whereas Notch signalling has been associated with the activation of EMT in cancer cells [38–42]. Furthermore, inhibition of Notch signalling causes growth arrest and inhibition of EMT in both breast cancer stem cells and breast cancer cells [46]. Cytokine oncostatin M has been associated with poor outcome in breast cancer [47]. In addition to regulating oestrogen receptor expression and breast cancer metastasis to bone, oncostatin M has been reported to mediate mesenchymal and stem cell-like differentiation in breast cancer [47–49].

The effect of *PLA2G7* depletion on the protein levels of key EMT regulators, vimentin and E-cadherin as well as the invasive phenotype was then monitored in cultured breast cancer cells expressing *PLA2G7* at high level. The MDA-468 and BT-549 cell lines were derived from aggressive triple-negative breast tumours harbouring inactivating mutations in tumour suppressor genes *PTEN*, *RB1* and *TP53* [50]. Mutations in *TP53* are found in the majority of TNBCs and targeted deletion of both *Trp53* and *Rb1* in mouse mammary stem progenitor cells has been reported to induce aggressive EMT-type mammary tumours [51–53]. Inhibition of *PLA2G7* expression resulted in decreased expression of vimentin, increased expression of E-cadherin as well as reduced cell invasion. The cytoplasmic intermediate filament protein vimentin is a central marker of EMT in cancer. Vimentin is expressed in neoplastic breast epithelial cells with metastatic properties as well as in aggressive and metastatic clinical breast cancers, supporting the association between *PLA2G7* and aggressive disease in breast cancer [14–16]. Furthermore, these results indicate that *PLA2G7* is required for cytoskeletal alterations enabling cell migration and EMT and highlight a significant role for *PLA2G7* in breast cancer metastasis. Regulators of EMT have been recently suggested as major therapeutic targets of aggressive and metastatic breast cancers, including triple negative breast cancers (TNBC) [5–9,12]. Further studies are needed to understand the mechanisms behind the ability of *PLA2G7* to regulate EMT and invasion in more detail.

In addition to regulating EMT and invasion, *PLA2G7* impairment was found to inhibit EGFR expression *in vitro*. Although recent results from clinical trials studying the combinatorial effect of monoclonal anti-EGFR antibody cetuximab and carboplatin or cisplatin in the treatment of TNBC have not been successful, combining different therapies has been suggested as a necessary step to reach significant therapeutic progress [54–59]. Interestingly, darapladib, a novel *PLA2G7* inhibitor, has recently been under intensive research in the area of cardiovascular diseases. In addition, lipid-lowering statins are known to reduce *PLA2G7* activity in plasma, atherosclerotic plaques and cultured prostate cancer cells [20,25,60,61]. Thus, although the first phase III trials with darapladib did not produce much anticipated promising results in the treatment of chronic coronary heart disease, pharmacologically well-studied and well-known molecules inhibiting *PLA2G7*, such as darapladib and statins, could be readily translated to *in vivo* preclinical studies and clinical trials also in the area of oncology. Our results propose a possible beneficial effect from the combination of *PLA2G7* inhibition with other therapies (ie EGFR targeted therapy) in advanced breast cancer.

In conclusion, results from this study propose *PLA2G7* as an EMT and metastasis-promoting enzyme in a subset of breast cancers and link the expression of *PLA2G7* with hormone receptor negativity and poor prognosis especially in metastatic breast cancer. Furthermore, in addition to breast and prostate cancer, results from this study also suggest a possible association between high *PLA2G7* expression and metastasis in four other malignancies; namely colon, kidney, liver and lung cancer. Recent studies further link *PLA2G7* with nasopharyngeal carcinoma invasiveness and early relapse in ovarian cancer [62,63]. Thus, these results present a rationale for further exploring the role of *PLA2G7* in cancer progression and the putative potential of *PLA2G7* targeted therapy in cancer management.

Acknowledgements

We thank Sinikka Kollanus for performing immunohistochemical staining and Taina Korpela for excellent technical assistance. We thank the Finnish DNA Microarray Centre for performing microarray and TaqMan qRT-PCR analysis, and Vidal Fey as well as Arho Virkki for the normalisation of gene expression analysis results. John-Patrick Mpindi is acknowledged for help in bioinformatics analyses from clinical sample data. This work was supported by Cancer Organizations of Finland, Sigrid Juselius Foundation, TIME collaborative project, Maud Kuistila Memorial Foundation, K. Albin Johanssons Stiftelse, Research Foundation for Clinical Chemistry, Finnish Cultural Foundation and the Academy of Finland.

Author contributions

Conceived and designed the experiments: LL, PV, KI. Performed the experiments: LL, PV. Analysed the data: LL, PV, HW, HH, PK. Contributed reagents/materials/analysis tools: HW, KP, VM, AK, KI, OK, OC. Wrote the paper: LL, PV, KI. All authors reviewed the manuscript and had final approval of the submitted version.

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary figure legends

Table S1. The effect of transient PLA2G7 silencing on breast cancer cell gene expression profile

Figure S1. The mRNA co-expression pattern of *PLA2G7* and *HER2/neu* (ERBB2) in breast cancer samples (n = 957)

Figure S2. (A) Validation of transient *PLA2G7* gene silencing in MDA-MB-468 breast cancer cells at the mRNA level. (B) The effect of 48 h *PLA2G7* and *KIF11* silencing on MDA-MB-468 cell viability. (C) The effect of 48 h *PLA2G7* and *KIF11* silencing on induction of apoptosis in MDA-MB-468 cells

Figure S3. The effect of stable *PLA2G7* silencing on EGFR protein expression in MDA-MB-468 cells. actin expression was used as an endogenous control

Figure S4. The effect of stable *PLA2G7* silencing and shScrambled expression on MDA-MB-468 cell viability at 48 h after plating equal numbers of cells

Figure S5. Relative *PLA2G7* mRNA expression in non-malignant and malignant ovarian, prostate and thyroid tissue samples based on TissueScan™ Cancer Survey cDNA panel