PHLDA1 (pleckstrin homology-like domain, family A, member 1) knockdown promotes migration and invasion of MCF10A breast epithelial cells

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ABSTRACT

PHLDA1 (pleckstrin homology-like domain, family A, member 1) is a multifunctional protein that plays distinct roles in several biological processes including cell death and therefore its altered expression has been identified in different types of cancer. Progressively loss of PHLDA1 was found in primary and metastatic melanoma while its overexpression was reported in intestinal and pancreatic tumors. Previous work from our group showed that negative expression of PHLDA1 protein was a strong predictor of poor prognosis for breast cancer disease. However, the function of PHLDA1 in mammary epithelial cells and the tumorigenic process of the breast is unclear. To dissect PHLDA1 role in human breast epithelial cells, we generated a clone of MCF10A cells with stable knockdown of PHLDA1 and performed functional studies. To achieve reduced PHLDA1 expression we used shRNA plasmid transfection and then changes in cell morphology and biological behavior were assessed. We found that PHLDA1 downregulation induced marked morphological alterations in MCF10A cells, such as changes in cell-to-cell adhesion pattern and cytoskeleton reorganization. Regarding cell behavior, MCF10A cells with reduced expression of PHLDA1 showed higher proliferative rate and migration ability in comparison with control cells. We also found that MCF10A cells with PHLDA1 knockdown acquired invasive properties, as evaluated by transwell Matrigel invasion assay and showed enhanced colony-forming ability and irregular growth in low attachment condition. Altogether, our results indicate that PHLDA1 downregulation in MCF10A cells leads to morphological changes and a more aggressive behavior.

KEYWORDS

aggressive phenotype; breast cancer; invasion; mammary epithelial cells; migration; MCF10A; PHLDA1

Introduction

Pleckstrin homology-like domain, family A member 1 (PHLDA1) gene encodes an evolutionarily conserved 401-amino acid proline-histidine rich protein. Its deregulation has been correlated with cancer progression in clinical samples and in vitro studies. In breast cancer, growth-inhibitory effect of PHLDA1 was described for transformed HME16C breast cells, triple-negative MDA-MB-231, ER+ T47D, and ErbB2-positive SKBR3 breast cancer cells. In a previous work from our group with a series of 699 invasive breast cancer patients, negative expression of PHLDA1 protein was a strong predictor of poor prognosis for breast cancer with rates of 5-year overall survival of 52.7% for patients with PHLDA1 negative tumor samples against 74.8% for patients with positive PHLDA1 tumor samples. Multivariate analysis showed that PHLDA1 protein expression was an independent prognostic factor of overall survival of breast cancer patients even after adjusting for clinical stage and lymph nodal status. Otherwise, PHLDA1 was reported as a follicular stem cell marker in a set of studies and, adding controversy over PHLDA1 role in breast, previous report suggested that PHLDA1 upregulation is associated with cancer stem cell properties in ER+ MCF7 breast cancer cell line.11 Thereby, the role of PHLDA1 in breast cancer remains to be clarified.

Breast cancer is essentially a genetic disease where tumorigenesis involves alterations in oncogenes, tumor-suppressor genes and DNA stability genes. It is estimated that 5 to 10% of all breast cancers are attributable to well-defined breast cancer susceptibility genes. Notably, BRCA1 and BRCA2 are arguably the most well characterized genes in which germline mutations are responsible for the majority of hereditary breast cancers. Mutations in BRCA1/2 and other genes of low, middle or high penetrance are believed
to account for 30% of familial breast cancer.\textsuperscript{14,15} Apart from familial breast cancer, the remaining majority of breast cancer cases are considered sporadic, and molecular alterations contributing to the disease have not been fully identified yet.\textsuperscript{16}

The development of breast cancer is commonly postulated to be a multi-step process that progressively evolves from non-diseased to preclinical cancer, then clinical cancer states and ultimately metastasis.\textsuperscript{17-19} As a longitudinal observation of this process is not tangible, inferences are only elusive and do not rule out the possibility that normal cells give rise to ductal carcinoma \textit{in situ} or invasive ductal carcinoma, for example. In this context, the use of \textit{in vitro} models for breast cancer investigation has emerged, as they are systems that allow mimicking the \textit{in vivo} situation in a controlled manner at the same time that provide the possibility of testing each genetic change individually. The human mammary epithelial cell line MCF10A is a reliable and widely used model for studying normal breast cell function. MCF10A cells are mammary epithelial cells derived from human fibrocystic mammary tissue of a 36-years-old woman who neither had cancer nor a family history of cancer.\textsuperscript{20} Remarkably, MCF10A cell line was sub-derived from MCF10, which is the unique cell line that is diploid and contains only a reciprocal translocation between chromosomes 3 and 9.\textsuperscript{21} Also, MCF10A is near-diploid and became spontaneously immortalized, without viral infection, cellular oncogene transfection or exposure to carcinogens or radiation, preserving a variety of cell characteristics that mimic normal mammary epithelial cells in culture.\textsuperscript{19,20,22}

The central hypothesis of our study was that PHLDA1 has tumor suppressive properties in breast cancer. Despite PHLDA1 had been reported deregulated in breast cancer studies, it has not yet been determined whether these changes are responsible for the initiation and/or the progression of the disease, nor its functional role or significance in those processes. In this sense, we believe that PHLDA1 relation with mammary epithelial transformation and tumorigenesis can be better understood if its imbalance appears as an individual event in non-tumoral breast cells, helping to avoid possible biases from the deeply distinct molecular characteristics of each breast tumor cell lineage. In the current study, we aimed to further dissect the role of PHLDA1 in breast cells, performing functional studies in MCF10A cells stably transfected with PHLDA1 shRNA. Our data revealed that PHLDA1 downregulation increases cell proliferation, migration and invasiveness contributing to a more aggressive phenotype in MCF10A cells.

**Results**

**PHLDA1 knockdown induces morphological changes in MCF10A cells**

Morphological changes were observed in MCF10A cells with reduced expression of PHLDA1 compared with control cells. Plasmid vectors containing 2 different PHLDA1 specific shRNAs (named shPHLDA1 #1 and #2) were used to downregulate PHLDA1 in MCF10A cells. The knockdown efficiency was confirmed by Western blot analysis (Fig. 1A). Sub-confluent MCF10A cells grown in monolayer formed clusters that presented lamellipodia at the edges and showed cobblestone morphology with tight cell-to-cell contact upon confluence, which is characteristic of mammary epithelial cells (Fig. 1B). By contrast, MCF10A cells with reduced expression of PHLDA1 showed distinct nucleus/cytoplasm ratio, more pronounced lamellipodia formation at sub-confluence, and somewhat looser cell-to-cell contact in comparison with the control cells (Fig. 1B).

**PHLDA1 knockdown increases proliferation and enhances mammosphere formation in MCF10A cells**

We conducted CyQuant cell proliferation assay to examine whether reduced PHLDA1 expression could lead to changes in proliferation of MCF10A breast epithelial cells under attached conditions. We found that PHLDA1 downregulation in MCF10A cells significantly increased the proliferation rate compared with control cells for both knockdown sub-clones (shPHLDA1 #1 and #2) along 96 h (Fig. 2A). We further evaluated the effect of PHLDA1 down regulation on anchorage-independent growth conditions. After 9 d of culture, the majority of the cells died. However, a small fraction survived and formed mammospheres for both control and knockdown cells (Fig. 2B). Control MCF10A cells formed spheres with regular shape and exhibited low mammosphere-forming ability. In contrast, the number of spheroids for MCF10A cells with reduced expression of PHLDA1 was marginally increased, and these were more irregular compared with control cells. No differences in the size of the mammospheres were observed (Fig. 2B, b). The results showed that PHLDA1 knockdown increases proliferative behavior in attached conditions and change the growth pattern of mammospheres under anchorage-independent conditions corroborating with a negative regulation of chemotaxis for PHLDA1 function in breast cells.

**PHLDA1 knockdown increases migration and invasiveness in MCF10A cells**

The effect of PHLDA1 downregulation was further evaluated on cell motility, determined by wound-healing assay.
We found that PHLDA1 downregulation significantly increased cell migration (Fig. 3). Within 18 hours, the area of the wound was significantly recovered by the migrating of MCF10A cells with reduced expression of PHLDA1; by 24 hours, the wound area had been almost completely recovered (Fig. 3A, right). In marked contrast, the wound closure of MCF10A control cells exhibited comparatively less difference at the same imaged times (Fig. 3A, left), dramatically distinct from knockdown cells.

To independently investigate the role of PHLDA1 on migration and invasiveness capabilities of MCF10A cells we conducted the Transwell assay. As expected, MCF10A breast cells showed almost no invasive ability (Fig. 3B, left). Interestingly, downregulation of PHLDA1 induced an invasive behavior to these non-malignant and non-invasive cells, being able to degrade and invade through the Matrigel matrix (Fig. 3B, right), with an increment of 62 and 28 times to shPHLDA1 #1 and #2 subclones, respectively, in comparison to control cells (p < 0.01).

PHLDA1 downregulation enhances colony formation ability of MCF10A cells

The ability of cells to form colonies at low density was assessed. After 8 d of plating, PHLDA1 knockdown cells sub-clone #2 formed significantly more colonies than control cells in a clonogenic assay, p < 0.05 (Fig. 4). Similar results were found to shPHLDA1 #1 sub-clone, although not significantly (data not show). Moreover, colonies appearance differed markedly. Colonies of MCF10A cells with reduced PHLDA1 expression were weakly stained due to scattered morphology compared with control cells (Fig. 4B). MCF10A cells with reduced PHLDA1 expression exhibited a spindle-like morphology and lacked obvious cell-cell contacts when observed at high power magnification (Fig. 4C), confirming our observations of morphological changes assessed with phase-contrast morphology (Fig. 1B).
Down-regulation of PHLDA1 changes actin filaments distribution in MCF10A cells

Development of migratory and invasive properties involves a dramatic reorganization of the actin cytoskeleton and the concomitant formation of membrane protrusions required for invasive growth. Morphological changes in PHLDA1 knockdown cells such the presence of more lamellipodium in monolayer together with acquired invasive behavior and enhanced migration capacity, suggested a possible involvement of actin filaments reorganization. As assessed by immunofluorescence (Fig. 5A), cells with PHLDA1 downregulation (lower) showed highly actin-rich membrane projections...
that were not observed in control cells (upper). Additionally, images taken after phalloidin staining allow seeing the scattered morphology and the loose cell-cell contact of shPHLDA1 cells when compared with control cells (Fig. 5B).

Discussion

Previously we have demonstrated that reduced expression of PHLDA1 is strongly associated with poor outcome in breast cancer patients. In the present study, we demonstrate that PHLDA1 downregulation is related to the development of an aggressive phenotype in MCF10A cells, inducing morphological alterations, increasing proliferation rate, migration and clonogenic ability as well as inducing the acquisition of invasive behavior, in accordance with a putative tumor suppressor activity. Also, when stemness capacity was investigated with anchorage-independent mammosphere forming assay we found that MCF10A cells with reduced expression of PHLDA1 showed higher mammosphere-forming capacity in comparison to control cells.

Previous reports showed that PHLDA1 negatively regulates cell motility and proliferation in breast cancer
Despite that, one study has found opposite results for growth under unattached conditions.\(^\text{11}\) We, therefore, tested if these effects could be overcome by the PHLDA1 knockdown in non-malignant breast epithelial cells MCF10A in which cellular proliferative controls are intact.\(^\text{20}\) Phase contrast microscopy observations of MCF10A cells with PHLDA1 knockdown showed distinct nuclei/cytoplasm ratio as they exhibited larger cytoplasm and cells at the edge of clusters had larger and more frequent lamellipodia protrusions than control cells. These projections are known to facilitate cell movement and act as sensory extensions of the cytoskeleton.\(^\text{23}\) Also, actin-rich invadopodia exert a proteolytic function in ECM degradation, thus facilitating cell invasion.\(^\text{24,25}\) In our clonogenic assay, morphological differences became more evident, scattered colonies and even spindle-shaped individual cells were observed for PHLDA1 knockdown cell line. These differences in morphology suggested a distinct pattern in actin distribution. Indeed, when we used immunofluorescence to assess actin organization, we observed actin-rich invadopodia in PHLDA1 knockdown cells, which play a proteolytic function in ECM degradation, thus facilitating cell invasion.\(^\text{26}\) These findings are in accordance with the increased migratory activity and acquired invasive properties observed in MCF10A cells with PHLDA1 knockdown, phenomena that involve a dramatic reorganization of the actin cytoskeleton and the concomitant formation of membrane protrusions that are required for invasion.\(^\text{26}\)

PHLDA1 is a pleckstrin homology-like domain protein.\(^\text{27}\) Interestingly, pleckstrin homology (PH) domains are known to interact with phosphoinositides, a property shared by some PH-like domains (as reviewed by Scheffzek\(^\text{28}\)). Phosphoinositides are known to play key roles in the regulation of the actin cytoskeleton and control membrane dynamics\(^\text{29}\) and lamellipodia-dependent cell migration by inducing actin filament assembly at the plasma membrane and by regulating the direction of cell movement during chemotaxis.\(^\text{30,31}\) However, a relationship between PHLDA1 and actin filament assembly at the plasma membrane has not been reported before, and this is also the first evidence that PHLDA1 knockdown can induce breast epithelial cell migration and invasiveness.

Johnson et al.\(^\text{3}\) have investigated PHLDA1 and Aurora A kinase relation with invasiveness in MDA-

**Figure 4.** PHLDA1 knockdown enhances the ability of MCF10A cells to form colonies. (A) Representative images taken from 6 well plates showing colonies of MCF10A control cells, shPHLDA1 #1 and #2 cells. (B) Bar graph showing number of colonies after 8 d in culture. Data are expressed as mean ± s.e.m. \(^*\) P < 0.05 (unpaired t test). (C) Morphological differences between MCF10A control and PHLDA1 knockdown cells colonies stained with crystal violet. (Left) Representative photomicrograph of control cells. (Right) Representative photomicrograph of PHLDA1 knockdown cells.
MB-231 breast adenocarcinoma cells. They found that knockdown of PHLDA1 enhanced invasion whereas its overexpression had the opposite effect. Indeed, when using Ser98 phosphorylation-resistant PHLDA1 mutant, cell motility was decreased even upon Aurora A overexpression (Aurora A cells were highly motile). The authors also report decreased proliferation rates after overexpression of PHLDA1. Consistent with these findings we found that PHLDA1 knockdown also increases cell proliferation in MCF10A cells, corroborating with the tumor suppressor role of PHLDA1. Li et al. described similar effects on HER2 positive breast cancer cell line SKBR3 after overexpression of PHLDA1, where decreased motility and proliferation rates was seen. Under low attachment conditions, the authors report a similar inhibitory effect. In our study knockdown of PHLDA1 lead to a slight increase in the number of mammospheres formed under low attachment conditions in comparison to control MCF10A cells.

Although clinical and experimental studies are required to elucidate the molecular and cellular functions of PHLDA1, evidence reported in the literature indicate that PHLDA1 expression can be modulated by various stimuli, resulting in pleiotropic effects regulating different biologic
processes that might impact tumor initiation, progression, and therapeutic response. In a previous study, we raised the possibility of PHLDA1 being useful as a predictor of prognosis for breast cancer: its paucity was related to worse outcome. Here, we showed that PHLDA1 downregulation in non-malignant breast epithelial cells led to morphological and behavioral changes conferring a more aggressive phenotype. We show for the first time that PHLDA1 knockdown induces actin filaments reorganization and acquisition of migration and invasion capacities in MCF10A cells allowing us to speculate that PHLDA1 downregulation could be useful for early detection of breast cancer. Moreover, Li et al., showed that PHLDA1 plays a role in the negative feedback regulation of ErbB2 activity and its upregulation enhances sensitivity to lapatinib in SKBR3 cells, indicating that PHLDA1 could be a potential therapeutic response indicator. Based on that we could formulate the hypothesis that patients with different levels of expression of PHLDA1 may exhibit differences in drug response, helping to provide information for patients that may or may not be beneficial for one such treatment scheme.

**Material and methods**

**Cell culture**

MCF10A cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured at 37°C in an atmosphere of 5% CO2 and 95% air and passaged weekly in DMEM/F-12 supplemented with 5% horse serum, 50 ng/ml epidermal growth factor (EGF), 10 μg/ml insulin, 0.5 μg/ml hydrocortisone, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.1 μg/ml cholera toxin.

**Cellular transfection**

MCF10A cells were transfected with pRS vector empty or expressing shRNA targeting PHLDA1 mRNA (OriGene Technologies, Inc.) using Fugene HD reagent (Roche Applied Science). Polyclonal populations of transfected cells were selected with Puromycin 0.7 μg/ml. After selection, subclones were maintained with 0.5 μg/ml puromycin.

**Migration assay**

For migration assays, confluent monolayers of MCF10A control and shPHLDA1 stable transfected subclones were scratched with a sterile 20 μl pipette tip. The plates were then washed and incubated at 37°C in 5% Horse serum and DMEM-F12 supplemented medium. Images were taken after 18 and 24 hours.

**Invasion assay**

Invasion assays were performed using BD BioCoat growth factor reduced Matrigel invasion chambers following manufacturer’s instructions (BD Biosciences). Briefly, control or shPHLDA1 cells (1 × 10³) were introduced into the upper compartment. After 18 h cells were wiped off from the upper surface of each insert. The cells on the lower surface, which represented the cells that migrated and invade through control insert membrane, were fixed with methanol, stained with DAPI and counted by microscopic examination in 10 representative fields. Cell invasion data was expressed as the mean number of cells per field.

**Cell proliferation assays**

CyQUANT kit (Invitrogen) assay was performed following manufacturer’s instructions. Briefly, cells in 5% horse serum and DMEM-F12 supplemented medium were seeded in triplicate in 96 multi-well plates (Corning) at a density of 1 × 10⁴ cells/well. Following 24, 48, 72 and 96 h medium was removed from the plates and cells were incubated with 1x dye binding solution at 37°C for 1 h in the dark. The fluorescence was measured using the Fluostar Optima microplate reader (Fluostar Optima, BMG Labtech) with excitation maximum at 485 nm and emission maximum at 530 nm.

**Anchorage independent growth assay**

Cells were seeded at a single-cell density of 526 cells/cm² into wells of ultralow attachment 6-well-plate (Corning) with serum-free supplemented DMEM/F12 medium (Gibco) and 1 x B27 supplement (Gibco) and incubated in a humidified atmosphere with 5% CO2 at 37°C. Mammospheres were evaluated after 9 d. For secondary spheres, the cells were collected, enzymatically disaggregated with trypsin and single-cell suspensions were seeded at the same prior density and culture conditions as described above, cultivated for further 9 d. Images were taken and mammospheres were counted in each final day.

**Clonogenic assay**

Single cells were plated onto 6 well plates and were allowed to grow for 8 d. Next, the cells were fixed with ice-cold methanol, stained with crystal violet for 30min and the number of colonies with more than 50 cells in each plate was determined.

**Western blot**

Whole-cell lysates were prepared from cells cultured in monolayers. Thirty μg of lysates were analyzed on 10%
SDS-polyacrylamide gels followed by transfer at 2.5 V for 30 min to nitrocellulose membranes (Pierce Biotechnology, Rockford, IL, USA). Protein concentration was measured using the Coomassie Plus Protein Assay Reagent™ (Pierce Biotechnology). Blots were blocked with 5% skim milk in Tris-buffered saline containing 0.1% of Tween 20 (TBS-T) for 1 h at room temperature and then incubated with primary antibody overnight at 4°C for all proteins but PHLD1 which membrane was blocked overnight (4°C) and incubated with primary antibody for 1 h at room temperature. Membranes were then washed and incubated with a peroxidase-conjugated secondary antibody for 1 h. Antibodies were diluted in 5% skim milk in TBS-T. After washing the membranes, the signals of reactive proteins were developed using the Western Lightning® Plus-ECL enhanced chemiluminescence substrate (PerkinElmer) and visualized in the ImageQuant LAS 4000 (GE Healthcare Life Sciences).

**Immunofluorescence**

Cells were plated on 8-well chamber slides at a density of 1.5 × 10^4 cells per well. Once 40–60% of confluence was reached cells were immunostained using actin primary antibody followed by Alexa-488-labeled secondary antibodies. Nuclear staining was performed with Hoechst 33342 (Invitrogen) for 15 min. After washing with PBS, coverslips were mounted on microscope slides with PBS/Glycerol or PBS/Glycerol/rhodamine-phalloidin staining. Images were taken using Zeiss LSM Meta 510 scanning confocal microscope.

**Statistical analysis**

Statistical analyses were performed using t-student test or ANOVA as appropriate, with P-value of < 0.05 considered statistically significant. Data are expressed as mean ± SD. The data are representative of 3 separate experiments.

In all figures, asterisks denote significance levels as follows: *P < 0.05, **P < 0.01, and ***P < 0.001.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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**References**

[1] Nagai MA. Pleckstrin homology-like domain, family A, member 1 (PHLDA1) and cancer. Biomed Rep 2016; 4 (3):275-81; PMID:26998263
[2] Oberst MD, Beberman SJ, Zhao L, Yin JJ, Ward Y, Kelly K. TDAG51 is an ERK signaling target that opposes ERK-mediated HME16C mammary epithelial cell transformation. BMC Cancer 2008; 8:189; PMID:18597688; http://dx.doi.org/10.1186/1471-2407-8-189
[3] Johnson EO, Chang KH, de Pablo Y, Ghosh S, Mehta R, Badve S, Shah K. PHLD1A is a crucial negative regulator and effector of Aurora A kinase in breast cancer. J Cell Sci 2011; 124(Pt 16):2711-22; PMID:21807936; http://dx.doi.org/10.1242/jcs.084970
[4] Moad AI, Muhammad TS, Oon CE, Tan ML. Rafapycin induces apoptosis when autophagy is inhibited in T-47D mammary cells and both processes are regulated by Phlda1. Cell Biochem Biophys 2013; 66(3):567-87; PMID:23300262; http://dx.doi.org/10.1007/s12013-012-9504-5
[5] Li G, Wang X, Hibshoosh H, Jin C, Halmos B. Modulation of ErbB2 blockade in ErbB2-positive cancers: the role of ErbB2 Mutations and PHLDA1. PLoS One 2014; 9(9):e106349; PMID:25238247; http://dx.doi.org/10.1371/journal.pone.0106349
[6] Nagai MA, Fregnani JHTG, Netto MM, Brentani MM, Soares FA. Down-regulation of PHLDA1 gene expression is associated with breast cancer progression. Breast Cancer Res Treat 2007; 106(1):49-56; PMID:17211533; http://dx.doi.org/10.1007/s10549-006-9475-6
[7] Ohyama M, Terunuma A, Tock CL, Radonovich MF, Pise-Masison CA, Hopping SB, Brady NJ, Udey MC, Vogel JC. Characterization and isolation of stem cell – enriched human hair follicle bulge cells 2006; 116(1):249-60.
[8] Sellheyer K, Nelson P. Follicular stem cell marker PHLDA1 (TDAG51) is superior to cytokeratin-20 in differentiating between trichoepithelioma and basal cell carcinoma in small biopsy specimens. J Cutan Pathol 2011; 38(7):542-50; PMID:21352265; http://dx.doi.org/10.1111/j.1600-0560.2011.01693.x
[9] Sellheyer K, Krahl D. PHLDA1 (TDAG51) is a follicular stem cell marker and differentiates between morphoeic basal cell carcinoma and desmoplastic trichoepithelioma. Br J Dermatol 2011; 164(1):141-7; PMID:20846311; http://dx.doi.org/10.1111/j.1365-2133.2010.10045.x
[10] Battistella M, Peltre B, Cribier B. PHLD1A, a follicular stem cell marker, differentiates clear-cell/granular-cell trichoblastoma and clear-cell/granular cell basal cell carcinoma: a case-control study, with first description of granular-cell trichoblastoma. Am J Dermatopathol 2014; 36(8):643-50; PMID:23719479; http://dx.doi.org/10.1097/DAD.0b013e31828a31ae
[11] Kastrati I, Canestragi E, Frasor J. PHLDA1 expression is controlled by an estrogen receptor-NFκB-miR-181 regulatory loop and is essential for formation of ER-

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mammospheres. Oncogene 2015; 34(18):2309-16; PMID:24954507; http://dx.doi.org/10.1038/onc.2014.180

[12] Klutitig A, Schmidt-Pokrzywnia A. Established and Suspected Risk Factors in Breast Cancer Aetiology. Breast Care (Basel) 2009; 4(2):82-7; PMID:17498966; http://dx.doi.org/10.1016/j.brec.2006.12.004

[13] Oldenburg R A, Meijers-Heijboer H, Cornelisse CJ, Devilee P. Genetic susceptibility for breast cancer: how many more genes to be found?. Crit Rev Oncol Hematol 2007; 63(2):125-49; PMID:17498966; http://dx.doi.org/10.1016/j.critrevonc.2006.12.004

[14] Easton DF. How many more breast cancer predisposition genes are there? Breast Cancer Res 1999; 1(1):14-7; PMID:11250676; http://dx.doi.org/10.1186/bcr6

[15] Melchor L, Benitez J. The complex genetic landscape of familial breast cancer. Hum Genet 2013; 132(8):845-63; PMID:23552954; http://dx.doi.org/10.1007/s00439-013-1299-y

[16] Ellsworth RE, Decewicz DJ, Shriver CD, Ellsworth DL. Breast cancer in the personal genomics era. Curr Genomics 2010; 11(3):146-61; PMID:21037853; http://dx.doi.org/10.2174/138920210791110951

[17] Beckmann MW, Niederacher D, Schnurch HG, Gusterson BA, Bender HG. Multistep carcinogenesis of breast cancer and tumour heterogeneity. J Mol Med 1997; 75(6):429-39; PMID:9231883; http://dx.doi.org/10.1007/s001090050128

[18] Tlsty TD, Crawford YG, Holst CR, Fordyce CA, Zhang J, McDermott K, Kozakiewicz K, Gauthier ML. Genetic and epigenetic changes in mammary epithelial cells may mimic early events in carcinogenesis. J Mammary Gland Biol Neoplasia 2004; 9(3):263-74; PMID:15557799; http://dx.doi.org/10.1023/B:JOMG.0000048773.95897.5f

[19] Russo J, Russo IH. Molecular Basis of Breast Cancer [Internet]. 1st ed. Intergovernmental Panel on Climate Change, editor. Vol. 53, Springer Berlin Heidelberg, Berlin, Heidelberg: Springer Berlin Heidelberg; 2004. XIV, 448. Available from: http://ebooks.cambridge.org/ref/id/CBO9781107415324A009

[20] Soule HD, Maloney TM, Wolman SR, Peterson WDJ, Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF, Brooks SC. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer Res 1990; 50(18):6075-86; PMID:1975513

[21] Tait L, Soule HD, Russo J. Ultrastructural and immuno-cytotoxic characterization of an immortalized human breast epithelial cell line, MCF-10. Cancer Res 1990; 50(18):6087-94; PMID:1697506

[22] Russo J, Barnabas N, Higgy N, Salicioni AM, Wu YL, Russo IH. Molecular basis of human breast epithelial cell transformation. In: Calvo F, Crepin M, Magdeletan H, editors. Breast Cancer Advances in Biology and Therapeutics. Paris: John Libbey Eurotext; 1996. p. 33-43.

[23] Gross SR. Actin binding proteins: their ups and downs in metastatic life. Cell Adh Migr 2013; 7(2):199-213; PMID:23302954; http://dx.doi.org/10.4161/cam.23176

[24] Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Natl Rev Mol Cell Biol 2014; 15(3):178-96; http://dx.doi.org/10.1038/nrm3758

[25] Saykali BA, El-Sibai M. Invadopodia, regulation, and assembly in cancer cell invasion. Cell Commun Adhes 2014; 21(4):207-12; PMID:24930891; http://dx.doi.org/10.3109/15419061.2014.923845

[26] Yilmaz M, Christoforoi G. EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev 2009; 28(1-2):15-33; PMID:19169796; http://dx.doi.org/10.1007/s10555-008-9169-0

[27] Park CG, Lee SY, Kandala G, Choi Y. A novel gene product that couples TCR signaling to Fas(CD95) expression in activation-induced cell death. Immunity 1996; 4(6):583-91; PMID:8673705; http://dx.doi.org/10.1016/S1074-7613(00)80484-7

[28] Scheffzek K, Welta S. Pleckstrin homology (PH) like domains - versatile modules in protein-protein interaction platforms. FEBS Lett Federation Eur Biochem Societies 2012; 586(17):2662-73; http://dx.doi.org/10.1016/j.febslet.2012.06.006

[29] Schink KO, Tan KW, Stenmark H. Phosphoinositides in control of membrane dynamics. Annu Rev Cell Dev Biol 2016; 32:143-71; PMID:27576122; http://dx.doi.org/10.1146/annurev-cellbio-111315-125349

[30] Saarikangas J, Zhao H, Lappalainen P. Regulation of the actin cytoskeleton-plasma membrane interplay by phosphoinositides. Physiol Rev 2010; 90:259-89; PMID:20086078; http://dx.doi.org/10.1152/physrev.00036.2009

[31] Gorai S, Paul D, Haloi N, Borah R, Santra MK, Manna D. Mechanistic insights into the phosphatidylinositol binding properties of the pleckstrin homology domain of lamellipodin. Mol Biosyst 2016; 12(3):74-57; PMID:26726010; http://dx.doi.org/10.1039/C5MB00731C