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Identification of upregulated phosphoinositide 3-kinase γ as a target to suppress breast cancer cell migration and invasion

Yan Xie [a], Peter W. Abel [a], Joseph K. Kirui [a], Caishu Deng [b], Poonam Sharma [b], Dennis W. Wolff [a], Myron L. Toews [c], Yaping Tu [a,*]

[a] Creighton University School of Medicine, Department of Pharmacology, Omaha, NE 68178, USA
[b] Department of Pathology, Omaha, NE 68178, USA
[c] University of Nebraska Medical Center, Department of Pharmacology and Experimental Neuroscience, Omaha, NE 68198, USA

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ABSTRACT

Metastasis is the major cause of breast cancer mortality. We recently reported that aberrant G-protein coupled receptor (GPCR) signaling promotes breast cancer metastasis by enhancing cancer cell migration and invasion. Phosphatidylinositol 3-kinase γ (PI3Kγ) is specifically activated by GPCRs. The goal of the present study was to determine the role of PI3Kγ in breast cancer cell migration and invasion. Immunohistochemical staining showed that the expression of PI3Kγ protein was significantly increased in invasive human breast carcinoma when compared to adjacent benign breast tissue or ductal carcinoma *in situ*. PI3Kγ was also detected in metastatic breast cancer cells, but not in normal breast epithelial cell line or in non-metastatic breast cancer cells. In contrast, PI3K isoforms α, β and δ were ubiquitously expressed in these cell lines. Overexpression of recombinant PI3Kγ enhanced the metastatic ability of non-metastatic breast cancer cells. Conversely, migration and invasion of metastatic breast cancer cells were inhibited by a PI3Kγ inhibitor or by siRNA knockdown of PI3Kγ but not by inhibitors or siRNAs of PI3Kα or PI3Kβ. Lamellipodia formation is a key step in cancer metastasis, and PI3Kγ blockade disrupted lamellipodia formation induced by the activation of GPCRs such as CXC chemokine receptor 4 and protease-activated receptor 1, but not by the epidermal growth factor tyrosine kinase receptor. Taken together, these results indicate that upregulated PI3Kγ conveys the metastatic signal initiated by GPCRs in breast cancer cells, and suggest that PI3Kγ may be a novel therapeutic target for development of chemotherapeutic agents to prevent breast cancer metastasis.

1. Introduction

Recent preclinical and clinical studies have demonstrated that specific G-protein coupled receptor (GPCR) systems are excessively activated in malignant breast cancer due to over-expression of receptors [1–4], abnormally elevated levels of ligands for GPCRs [4–6] and/or down-regulation of their regulators [7], which contributes to the progression and spread of breast cancer [8]. For example, signaling initiated by CXC chemokine receptor 4 (CXCR4) and protease-activated receptors (PARs) on breast cancer cells drives cancer cells to migrate and invade through surrounding tissues and spread to distant organs [5,9]. Unfortunately, clinical trials with drugs inhibiting specific GPCR activation show limited efficacy, presumably because metastasis could be driven by several different classes of GPCR simultaneously, thereby generating metastatic signal redundancy.

GPCRs convey signals via heterotrimeric G-proteins (classified into G_s, G_i, G_q, G_{12}) in the form of activated Gα-GTP and Gβγ subunits. We recently demonstrated that Gβγ released from G_i-proteins promotes migration and invasion of metastatic breast cancer cells by generating the lamellipodia protrusions at the leading edge of migrating cancer cells [10], suggesting that blockade of Gβγ could attenuate breast cancer metastasis. However, Gβγ cannot be blocked indiscriminately because of its diverse physiological roles. Therefore, the challenge is to target Gβγ effectors that are vital to breast cancer metastasis but inconsequential for physiologically normal cells.

The most studied type I phosphatidylinositol 3-kinases (PI3Ks), including α, β, γ and δ, play a pivotal role in numerous cellular functions [11]. PI3Kγ is especially intriguing because it is normally expressed primarily in hematopoietic cells [12], which have a physiological need to migrate. In addition, PI3Kγ is only activated

* Corresponding author at: Department of Pharmacology, Creighton University School of Medicine, 2500 California Plaza, Omaha, NE 68178, USA.
Tel.: +1 402 280 2173; fax: +1 402 280 2142.
E-mail address: Yat60399@creighton.edu (Y. Tu).

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by Gβγ following stimulation of GPCRs, whereas PI3Kα, β and δ are stimulated by receptor tyrosine kinases [11,13]. In fact, GPCR-dependent activation of PI3Kγ in neutrophils causes its accumulation at the leading edge of migrating cells, which is a critical determinant of cell migration [12,14]. Although somatic mutations of PI3Kα are very common in cancers [15,16], and may promote cancer cell growth and invasion in colorectal and breast cancer [17,18], the contribution of PI3Kγ to human cancer is much less clear with different studies showing conflicting results [19–22]. Brazzatti et al. [22] recently reported that knockdown of PI3Kγ inhibited lung colonization of human breast cancer MDA-MB-231 cells in xenografts and suppressed primary tumor growth, metastases and lung colonization caused by mouse 4T1.2 mammary carcinoma allografts. While this suggests an important role for PI3Kγ in breast cancer tumor growth and metastasis, these studies did not explore the molecular mechanisms associated with PI3Kγ signaling or whether PI3Kγ protein levels were correlated with the metastatic potential of various human breast cancer cell lines or human breast cancer specimens.

In the present study, we show that PI3Kγ is aberrantly expressed in invasive human breast carcinoma and its expression level correlates with the metastatic potential of established breast cancer cell lines. Most importantly, we show that silencing PI3Kγ with its siRNA or treatment with a PI3Kγ-selective inhibitor, but not with inhibitors or siRNAs of PI3Kα and β, attenuates lamellipodia formation and suppresses migration and invasion of breast cancer cells. Thus, targeting dysregulated PI3Kγ may provide a novel strategy for development of chemotherapeutic agents to suppress breast cancer metastasis.

2. Materials and methods

2.1. Cell lines, reagents and plasmids

MCF-10A, MCF-7, T47D, MDA-MB-231 and MDA-MB-436 cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). MDA-MB-231 and -436 cells were cultured in DMEM with 10% fetal bovine serum (FBS). MCF-7 cells were cultured in IMEM and 10% FBS with 10 μg/ml insulin. MCF-10A cells were grown in MEBM with additives (ATCC), and T47D cells were maintained in RPMI 1640 with 10% FBS.N-((1E)-(6-bromoimidazo-[1,2-a]pyridin-3-yl)methylene)-N′-methyl-N″-(2-methyl-5-nitrobenzene)sulfonohydrazide, HCl (PI3Kα inhibitor VIII or PIK-75, IC_{50} = 5.8, 1300, and 78 nM for lipid kinase activity of PI3Kα, β and γ isoforms, respectively) [23], 7-methyl-2-(morpholin-4-yl)-9-(1-phenylaminoethyl)-pyrido[1,2-a]-pyrimidin-4-one (PI3Kβ inhibitor VI or TGX-221, IC_{50} = 0.005, 5, and ≥3.5 μM for PI3Kβ, α and γ isoforms, respectively) [24], 5-(2,2-difluoro-benzo[1,3]-dioxol-5-ylmethylene)-thiazolidine-2,4-dione (PI3Kγ inhibitor II or AS-604850, IC_{50} = 0.25, 4.5, and > 20 μM for PI3Kγ, α and β) [25], and a broad spectrum PI3K inhibitor 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (Ly294002) were obtained from EMD Biosciences (San Diego, CA). Rabbit PI3Kα antibody and PI3Kγ antibody were from Cell Signaling Technology (Danvers, MA) and IRDye800-labeled anti-rabbit IgG was from LI-COR Bioscience. Rabbit PI3Kβ antibody and mouse PI3Kδ antibody were purchased from Millipore (Billerica, MA) and Santa Cruz (Santa Cruz, CA), respectively. ON-TARGETplus SMARTpool siRNAs targeting human PI3Kα, PI3Kβ or PI3Kγ, were purchased from Thermo Scientific Dharmacon (Lafayette, CO). Epidermal growth factor (EGF) was from BD Biosciences (San Jose, CA). CXCL12 was from R&D Systems (Minneapolis, MN) and protease activated receptor 1 (PAR1) agonist TFLLR was from Peptides International (Louisville, KY). Unless indicated otherwise, remaining reagents were purchased from either Sigma–Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA).

The pEYFP-PI3Kγ-CAAX plasmid encoding YFP-tagged PI3Kγ with the CAAX motif for constitutive association with the plasma membrane was a gift from Dr. Bernd Nürnberg (University of Tübingen, Germany) [26]. PI3Kγ consists of the catalytic subunit p110γ and two regulatory subunits, p101 and p84 [27–29]. A constitutively membrane targeted PI3Kγ (p110γ-CAAX) could compensate for the lack of its regulatory subunits [26].

2.2. Conventional RT-PCR and quantitative real-time RT-PCR analysis

The procedures for conventional RT-PCR and real-time RT-PCR were described previously [30]. β-actin was used as the internal control. The PI3Kγ primers resulting in a 98-bp product are: 5′-ttgtgatgggaacttctgga-3′, 5′-ggtttgtgtgatgacgaagg-3′. The conventional RT PCR cycling conditions were 1 cycle at 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, followed by 1 cycle of 72 °C for 7 min.

2.3. Western blot analysis

Protein was extracted from cultured cells using 1x RIPA lysis buffer (Santa Cruz). Samples (40 μg) were electrophoresed and subjected to Western blot using β-actin and PI3K antibodies as we previously described [31]. Images were captured with an Odyssey (LI-COR Biosciences).

2.4. Immunofluorescence staining

MDA-MB-231 cells and MCF-7 cells transfected with control vector or vector encoding PK3Kγ were seeded on coverslips overnight, and then fixed and stained with PI3Kγ antibody as previously described [31]. To investigate the effects of different inhibitors on lamellipodia formation, serum-starved MDA-MB-231 cells seeded on coverslips were pretreated with PI3Kα and PI3Kβ inhibitors or PI3Kγ inhibitor for 1 h, and then stimulated without or with TFLLR (25 μM), CXCL12 (50 ng/ml) or EGF (5 ng/ml) for 30 min at 37 °C. F-actin was visualized with rhodamine-labeled phalloidin (Cytoskeleton, Denver, CO) as previously described [7,10]. Lamellipodia, the flattened F-actin-rich leading edge of migrating cells, were outlined and measured in length using the Image-Pro Plus software from Media Cybernetics, Inc. (Rockville, MD). The summed length of lamellipodia was expressed as a percentage of total cell circumferences [7].

2.5. Immunohistochemistry analysis of human breast tissues

Archived formalin-fixed, paraffin-embedded human breast tissue blocks were from the Creighton University Department of Pathology as approved by the Creighton University Institutional Review Board. Immunohistochemistry was performed as we previously reported [7], using a rabbit PI3Kγ antibody (Cell Signaling Technology). The negative control used non-immune rabbit IgG as the primary antibody. Expression levels of PI3Kγ protein were graded using a four-tier system from 0 (negative) to 3 (high) based on overall staining intensity by two pathologists, independently. A total of 40 sets of pair-matched patient samples were included.

2.6. RNA interference and overexpression of PI3Kγ

MDA-MB-231 cells (1×10^6 in 100 μl) were electroporated with 300 nM of control siRNA (negative control #1 siRNA, Ambion) or ON-TARGETplus SMARTpool siRNA for human PI3Kα, PI3Kβ or PI3Kγ using Nucleofector kits with an Amaxa Nucleofector System (Lonza) (Allendale, NJ). Cells were seeded on 6-well plates. The following day, adherent MDA-MB-231 cells were re-transfected