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Original Research

Phospholipase D activation mediates growth and migration of colon cancer cells interacting with cancer-associated fibroblasts

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Abstract: Cancer-associated fibroblasts of the stroma play a major role in tumor promoting processes. In this study we evaluated the significance of Phospholipase D (PLD) enzyme activity in promoting human colon cancer malignant potency when interacting with proximal colonic fibroblasts. Human colon cancer cell lines SW480 and HCT116, and colonic fibroblasts CCD-18Co were used as an *in vitro* model. PLD's activity was measured in resting cancer cells and after culturing with fibroblasts and cancer-associated fibroblasts (CAFs) conditioned medium. The viability and migration level of the cancer cells alone and after co-culturing with fibroblast or CAFs conditioned medium were evaluated, with and without adding a PLD inhibitor. Exposure of colon cancer cells to CAFs conditioned medium significantly increased the level of PLD activity in the cancer cells ($p<0.0001$). Exposure of colon cancer to resting and activated fibroblast conditioned medium significantly enhanced the number of viable cancer cells as well as its migration level measured following 24 and 48 hours. Adding a PLD inhibitor significantly reduced the elevation of cell viability and migration of the colon cancer cells exposed to fibroblasts conditioned medium ($p<0.005$). In this *in vitro* model, inhibition of PLD significantly decreased proliferation and migration levels of colon cancer cells generated by stromal fibroblasts. This provides evidence that the PLD signaling pathway is directly involved in stroma-cancer interactions in the colon, thereby promoting cancer progression. Further research is needed in order to evaluate PLD as a target in colon cancer prevention or therapy.

Key words: Phospholipase D; Colon cancer; Stroma; Cancer-associated fibroblasts.

Introduction

Carcinomas, the majority of human cancers, arise from the epithelial cell layer that under normal conditions is separated from the supporting connective tissue known as stroma (1). Stromal cells such as fibroblasts, myofibroblasts and endothelial cells, represent the tumor microenvironment and play a major role in tumor development and progression (2,3). Colorectal cancer (CRC) tissues expressing a high percentage of stromal cells are linked to worse patient prognosis; stages II and III CRC patients with many stromal cells were shown to have significantly lower overall and disease-free survival durations compared to patients with few stromal cells (4). In addition, cancer-specific survival was shown to be poorer for CRC patients who exhibit low proportion of tumor cells relative to epithelial stromal cells, at all disease stages. Furthermore, the proportion of tumor cells relative to stromal cells was found to be an independent prognostic marker for CRC patients (5).

Fibroblasts of the tumor stroma, termed cancer-associated fibroblasts (CAFs), support tumor growth and progression by secreting factors promoting carcinogenesis and interacting with the tumor cells and other components of the tumor stroma (3). CAFs are found in high proportion in CRC, as in other type of cancers. Its number is increased in CRC compared to normal colonic mucosa and they are the main cellular constituents of stroma associated with primary and metastatic CRC (6). CRC CAFs were shown to be directly involved in a wide range of tumor promoting processes such as angiogenesis, cell death resistance, sustaining proliferative signaling and avoiding immune distraction (6). Moreover, primary CAFs from CRC patients display direct pro-migratory effects on cancer cells (7). The molecular mechanisms that underlie these processes are not fully understood, however.

The Phospholipase D (PLD) enzyme super family mammalian isoforms include PLD1and PLD2. PLD signaling is activated by several types of G protein-coupled receptors and receptor tyrosine kinases (GPCRs and RTKs), this activation can be induced by a wide range of extracellular signals that include growth hormones, insulin, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), sphingosine 1-phosphate (S1P), and lysophosphatidic acid (LPA) (8). The extracellular signals usually stimulate PLD through the direct activation of GPCRs or the RTKs, which further catalyze the hydrolysis of phosphatidylcholine into phosphatidic acid and choline and induce a hierarchical and multi-layered signaling network (8). The phosphatidic acid produced via this signaling plays a role in central cellular functions such as regulation of cellular metabolism such as glucose and amino acids, and is involved in tumorigenesis (9,10).

In cellular cancer models, elevated PLD expression

and signaling has been associated with metastasis and tumorigenesis such as actin cytoskeleton reorganization, proliferation, angiogenesis, inflammation, growth, and matrix metallo-proteinase (MMP) secretion. Moreover, elevated PLD activity and expression have been found in many types of human cancers, including colon, and its activity has been linked with oncogenic signals and tumorigenesis (11). PLD mRNA and protein analyses from tumors of breast, renal, colorectal, gastric, thyroid, and brain origin show elevated PLD1 and/or PLD2 expression relative to normal surrounding tissue (9) .More specifically, significant correlation of PLD expression levels with tumor size and survival in colorectal carcinoma patients has been demonstrated by clinical studies (12).

However, the role of PLD signaling in stroma-cancer interactions in the colon has not been examined. In this study, we evaluated the role of PLD in the crosstalk between human stromal fibroblasts and human colon adenocarcinoma cells that promotes malignant potency.

Materials and Methods

Cell lines and cell culture

The human colon cancer cell lines SW480 and HCT 116 (CCL-228™, CCL-247™) and the human colon-fibroblast cell line CCD18-Co (CRL-1459™) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf sera (FCS), penicillin (40 U/ml), and streptomycin (100 µg/ml; Biological Industries, Beit HaEmek, Israel) and kept in a 5% CO_2, air-humidified atmosphere at 37°C.

Conditioned medium preparation

Approximately 75×10^4 cells (SW480, HCT116 or CCD18-Co, each when relevant) were plated in a T25 flask with normal culture media. After 24 hr, cell monolayer was washed with sterile phosphate buffered saline (PBS, Biological Industries, Beit HaEmek, Israel), and added to serum-free (SF) media. After 48 hr, conditioned medium (CM) was collected from the flask directly into a sterile tube and centrifuged at 12,000 rpm for 1 hr to remove cells and any insoluble material. CM was stored frozen at -20°C until required.

Activated conditioned medium

Fibroblasts were activated to CAFs by adding SW480 or HCT 116 CM to its flask for 72 hr. Cells were then washed with sterile PBS and replenished with SF medium for an additional 24 hr. Medium was then pipetted out and centrifuged for 1 hr at 12,000 rpm. This medium was stored at -20°C until required and used as an activated conditioned medium (ACM).

PLD activity assay

SW480 and CCD18-Co cells ($35x10^4$) were plated and grown for 24 hr with normal culture media (6 mm x 15 mm dishes, Corning Inc., Corning, NY, USA). Medium was then aspirated. Cells were washed with sterile PBS and replaced with either CM or ACM with or without a PLD inhibitor (300 nano-molar, FIPI hydrochloride hydrate,F5807 Sigma-Aldrich, USA) for another 48 hr. Following the incubation, cells were washed with PBS and processed using CytoBuster™ Protein Extraction Reagent (Novagen, Madison, WI, USA) supplemented with EDTA-free Complete Protease Inhibitors, Calbiochem, San Diego, CA, USA). Lysate specimens were centrifuged at 13,000 rpm for 5 min at 4°C and 50 µg used to determine PLD activity using Amplex Red PLD assay kit (A12219, Invitrogen, Breda, The Netherlands), according to the manufacturer's protocol.

Migration level evaluation

The migration evaluation assay was adopted from the *in vitro* scratch assay protocol (13). Each experiment was conducted in triplicate. SW480 cells were cultured in cell culture dishes (6 mm x 15 mm, Corning Inc., Corning, NY, USA) and left overnight to form a confluent monolayer. The cell monolayer was then scored to leave a scratch 0.5 mm wide, rinsed with PBS and replaced with fresh media as follows: ACM or CM was added to the cell dishes and the effect on wound healing monitored. Negative control of this assay was evaluated using serum-free culture medium with L-glutamine, penicillin, and streptomycin supplementation only. Scratch areas representative for each culture dish were photographed using an Olympus TH4-200 Microscope (Olympus Corp., Center Valley, PA). Image-Pro Plus Version 6.0 software (Media Cybernetics, Bethesda, MD, USA) was used for the measurements and data analysis. For SW480, cells migrating into the scratched area were counted within a 400×400 µm frame, which was created by the region of interest (ROI) function, enabling selection of 2 random regions in the scratched area.

Cell proliferation assays

XTT assay was carried out as recommended by the kit manufacturer. 1×10^3 cells per well were grown in 96-well plates (Nunc, Sigma-Aldrich, Copenhagen, Denmark) for 24 hr. Medium was then replaced with CM or ACM with the addition of the appropriate treatment and the plate was incubated in a 5% CO_2 air-humidified atmosphere at 37°C. Cell proliferation was measured 24 and 48 hr after treatment using a XTT based cell proliferation kit (Biological Industries, Beit HaEmek, Israel). Absorbance against a background control as a blank (450 nanometer) was measured using ELISA reader (Tecan infinite® F200). Crystal Violet Assay was adopted from Feoktistova M. et al (14).

Crystal violet staining

HCT116 cells were cultured in cell culture dishes (3 mm , Corning Inc., Corning, NY, USA) and left overnight to form a confluent monolayer. The cell monolayer was then rinsed with PBS and replaced with fresh ACM or CM media with or without a PLD inhibitor (300 nano-molar, FIPI hydrochloride hydrate,F5807 Sigma-Aldrich, USA) for another 48 hr. Cells were then fixed in 4% paraformaldehyde (sigma, USA) for 10 minutes washed in PBS and stained with crystal violet (0.5%). Following 20 minutes excess stain was washed in water and plates were dried and photographed.

Statistical analysis

The results are represented as mean ± standard error. t-test or repeated measures test were used to analyze

the differences between 2 groups. One-way ANOVA or Kruskal-Wallis non-parametric test was used to determine whether there were any significant differences between the means of two or more independent groups, using SPSS-23 (SPSS Inc., IBM USA). Statistical significance was defined as $p<0.05$.

Results

Interaction between colon fibroblasts and cancer cells activates PLD

PLD activity level was significantly increased in SW480 cells following two days of exposure to activated CCD18-Co conditioned medium, ($p<0.0001$, Figure 1). A moderate elevation in PLD activity was also observed after exposure to resting CCD18-Co cell conditioned medium, but this effect was not significant.

The increased PLD activity in the colon cancer cells was significantly reduced when PLD inhibitor was added to the activated CCD18-Co conditioned medium, bringing the activity to levels similar to control baseline. Exposure of CCD18-Co fibroblasts to SW480 cell conditioned medium did not alter the PLD activity level in the fibroblast cells (data not shown).

PLD inhibition attenuates the pro-proliferative effect of colon fibroblasts on colon cancer cells

Colon cancer cell viability was evaluated following exposure to resting (CM) and activated (ACM) CCD18-Co cells conditioned media. Both conditioned media increased the number of cultured cancer cells, in 2 folds or over, when measured after 24 and 48 h of exposure (Figure 2). The effect on viability was similar to exposure to conditioned medium of resting and activated CCD18-Co cells.

Adding PLD inhibitor to the conditioned media significantly reduced these pro-proliferative effects to levels similar to baseline control ($p<0.005$). The effects of the conditioned media on the cell proliferation were similar after 24 and 48 h ($p>0.05$, repeated measures test).

PLD inhibition attenuates the migration capacity of colon cancer cells following interaction with colon fibroblasts

SW480 cell migration level was evaluated after exposure to resting and activated CCD18-Co cells conditioned medium. ACM significantly enhanced the num-

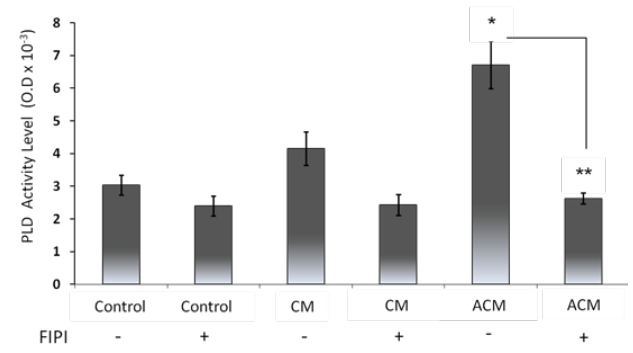

Figure 1. Conditioned medium of colonic fibroblasts elevated the phospholipase D activity level of colon cancer cells. Mean values and standard errors are displayed. *p<0.0001 relative to all other groups, **p<0.0001 relative to ACM; one-way ANOVA.

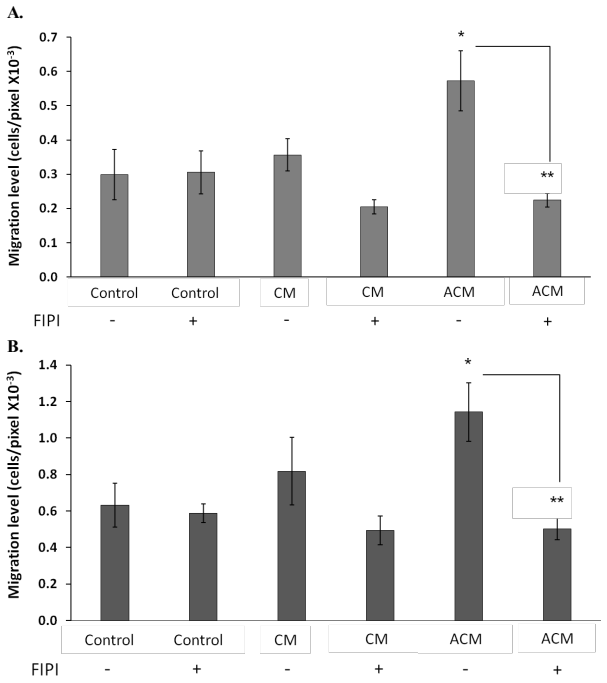

Figure 3. Effect of colonic fibroblasts on SW480 colon cancer cell migration capacity. Means and standard error values are displayed. A. Evaluation after 24 h exposure. B. Evaluation after 48 h exposure. *p=0.002 relative to all other groups, **p=0.001 relative to ACM, Kruskal-Wallis test.

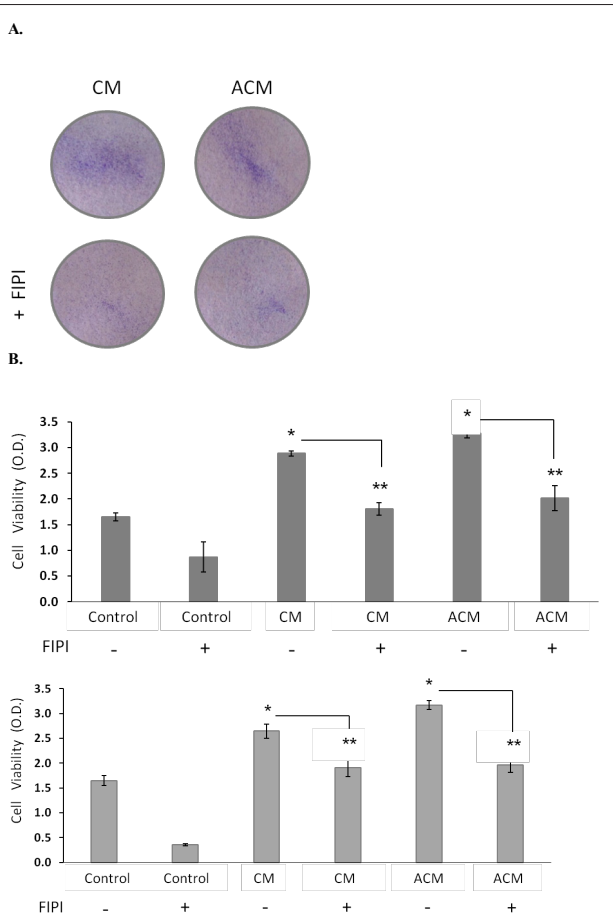

Figure 2. Effect of colonic fibroblasts on colon cancer cell proliferation. A. Crystal violet staining of HCT116 cells after 48hr exposure. B. Evaluation of SW480 viability after 24 h exposure. C. Evaluation of SW480 viability after 48 h exposure. Means and standard errors values are displayed. *p<0.005 relative to all other groups (±FIPI), **p<0.005 relative to the matched conditioned medium; one-way ANOVA.

ber of migrating colon cancer cells measured (Figure 3). Following 24 and 48 hours of exposure to ACM there two fold increase in the number of migrating cancer cells were measured, compared to the control non treated cells. The migration level was significantly increased after 48 h of exposure to both conditioned media relative to 24 h of exposure ($p<0.0001$; repeated measures test).

Adding PLD inhibitor to the conditioned medium significantly reduced this effect, bringing down the mirgation to levels similar to control baseline ($p<0.005$).

Discussion

Several studies demonstrated the direct involvement of CRC CAFs in a wide range of tumor promoting processes (6,7). However, little is known about the specific mechanisms involved in that crosstalk. In this study, we demonstrated the effect of increased colon cancer cell proliferation and migration promoted by colonic stromal fibroblasts in an *in vitro* co-culture model. Colon cancer cell migration induced by culturing with activated colonic fibroblasts was significantly higher than the effect measured with resting colonic fibroblasts. Nonetheless, increased colon cancer cell proliferation was similar after exposure to activated or resting colonic fibroblasts.

Clinical findings have demonstrated that PLD is over-expressed and has increased activity in a wide variety of cancers, including CRC (11,13,15). Expression levels of PLD2, a PLD isoform, correlate significantly with tumor size and survival among patients with colorectal carcinoma (16). PLD is also involved in survival signals, migration, adhesion and invasion processes in cancer cells (12). All of these processes are increased during PLD up-regulation and conversely, decreased during loss of PLD function.

In this study, we found that PLD enzyme activity is involved in mediating the induction of proliferation and migration potential of CRC by colonic fibroblasts. Increased PLD activity was measured in the colon cancer cells grown with activated CCD18-Co cell conditioned medium.

Numerous factors derived from CAFs were shown to support proliferative signaling in CRC cells and tissues as well as to help the cells evade growth-suppressors (6). Approximately 170 genes were up-regulated in CAFs isolated from a liver metastasis of CRC as compared to resting fibroblasts, including many genes encoding cell adhesion molecules, growth factors, and COX2 (17). Additional identified CAF-derived regulators of colon cancer progression include epidermal growth factor (EGF), hepatocyte growth factor (HGF), IGF1/2, PGE-2, PDGF, fibroblast growth factor -1, and vascular endothelial growth factor (VEGF) (17). These growth factors act through activation of the mitogen activated proteinkinase (MAPK) and phosphatidyl inositol 3-kinase (PI3K)/AKT pathways, which mediate cell proliferation and cell survival, protein synthesis, cytoskeletal rearrangements and invasion processes (6). Specifically, growth factors that bind to receptor tyrosine kinases and activate Arf, Rho and PI3K, are able to up regulate PLD signaling and downstream related pathways (9). One of the best described factor in this context is EGF, however, any CAF-derived growth factor secretion in our cellular model may explain at least in part the effects mediated by the fibroblasts conditioned medium on the cancer cells.

Moreover, in our model, inhibition of PLD using its specific inhibitor (FIPI) significantly decreased the cancer cell proliferation and migration generated by the stromal fibroblasts. These results indicate that PLD plays a significant role in the tumor microenvironment interaction in the colon, which promotes cancer growth and metastasis.

PLD inhibitors have been studied as potential metastasis inhibitors for breast cancer in mice models and have been suggested for clinical applications (12,19). *In vivo* administration of PLD inhibitor (FIPI) to wild-type mice or loss of PLD via PLD gene knockout in mice, has led to a significant decrease in tumor metastases of human breast cancer xenographs, and further supported its role in promoting cancer virulence (19). In addition, the PLD signaling pathway has been shown to mediate the therapeutic effect of 5-aminosalicylic acid (5-ASA) in protecting against the development of colitis-associated CRC in humans (20).

This accumulating data extended by our results provides basis for further clinical research to determine the value of the PLD signaling pathway as a therapeutic target in the prevention and treatment of CRC.

We found that inhibiting PLD *in vitro* significantly reduced colon cancer cell proliferation and migration that is increased by stromal fibroblasts. This provides evidence that the PLD signaling pathway is directly involved in stroma-cancer interactions in the colon, thereby promoting cancer progression and potential metastasis of colon cancer.

This study could provide a basis for further research to evaluate the role of PLD as a therapeutic target in the treatment of cancer patients.

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Compliance with Ethical Standards

The study was not externally funded. The authors have no conflicts of interest to declare.

References

1. Weinberg RA. The Biology of Cancer, Garland Science. 2007.
2. Pietras K, Ostman A. Hallmarks of cancer: interactions with the tumor stroma. Exp Cell Res. 2010;316:1324-31.
3. Räsänen K, Vaheri A. Activation of fibroblasts in cancer stroma. Exp Cell Res. 2010;316:2713-22.
4. Huijbers A, Tollenaar RA, Pelt GW, Zeestraten EC, Dutton S, McConkey CC, et al. The proportion of tumor-stroma as a strong prognosticator for stage II and III colon cancer patients: validation in the VICTOR trial. Ann Oncol. 2013;24:179-185.
5. West NP, Dattani M, McShane P, Hutchins G, Grabsch J, Mueller W, et al. The proportion of tumour cells is an independent predictor for survival in colorectal cancer patients. Br J Cancer. 2010;102:1519-23.
6. Tommelein J, Verset L, Boterberg T, Demetter P, Bracke M, De Wever O. Cancer-associated fibroblasts connect metastasis promoting communication in colorectal cancer. Front Oncol. 2015;5:63.

7. Herrera M, Islam AB, Herrera A, Martín P, García V, Silva J, et al. Functional heterogeneity of cancer-associated fibroblasts from human colon tumors shows specific prognostic gene expression signature. Clin Cancer Res. 2013;19:5914-26.
8. Cho JH, Han JS. Phospholipase D and Its Essential Role in Cancer. Mol Cells. 2017 ;30:40(11):805-813.
9. Bruntz RC, Lindsley CW, Brown HA. Phospholipase D signaling pathways and phosphatidic acid as therapeutic targets in cancer. Pharmacol Rev. 2014;66:1033-1079.
10. Kandori S, Kojima T, Matsuoka T, Yoshino T, Sugiyama A, Nakamura E, Shimazui T, Funakoshi Y, Kanaho Y, Nishiyama H. Phospholipase D2 promotes disease progression of renal cell carcinoma through the induction of angiogenin. Cancer Sci. 2018 ;109(6):1865-1875.
11. Huang P, Frohman MA. The potential for phospholipase D as a new therapeutic target. Expert Opin Ther Targets. 2007;11:707-16.
12. Su W, Chen Q, Frohman MA.Targeting phospholipase D with small-molecule inhibitors as a potential therapeutic approach for cancer metastasis. Future Oncol. 2009;5(9):1477-86.
13. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protocols. 2007;2:329-33.
14. Feoktistova M, Geserick P, Leverkus M. CrystalViolet Assay for Determining Viability of Cultured Cells. Cold Spring Harb Protoc. 2016; 1;2016(4):pdb.prot087379.
15. Gomez-Cambronero, J. Phosphatidic acid, phospholipase D and tumorigenesis. Advances in biological regulation. 2014;54:197-206.
16. Saito M, Iwadate M, Higashimoto M, Ono K, Takebayashi Y, Takenoshita S. Expression of phospholipase D2 in human colorectal carcinoma. Oncol Rep 2007; 18: 1329–1334.
17. Nakagawa H, Liyanarachchi S,Davuluri RV,Auer H, Martin EWJr , DeLa Chapelle A, Frankel WL. Role of cancer-associated stromal fibroblasts in metasta- tic colon cancer to the liver and their expression profiles. Oncogene. 2004; 23:7366–77.
18. De Boeck A, Hendrix A, Maynard D, Van Bockstal M, Daniëls A, Pauwels P, Gespach C, Bracke M, De Wever O. Differential secretome analysis of cancer-associated fibroblasts and bone marrow-derived precursors to identify microenvironmental regulators of colon cancer progression. Proteomics. 2013;13(2):379-88.
19. Henkels KM, Boivin GP, Dudley ES, Berberich SJ, Gomez-Cambronero J. Phospholipase D (PLD) drives cell invasion, tumor growth and metastasis in a human breast cancer xenograph model. Oncogene. 2013;32:5551-62.
20. Baan B, Dihal AA, Hoff E, Bos CL, Voorneveld PW, Koelink PJ, et al. 5-aminosalicylic acid inhibits cell cycle progression in a phospholipase D dependent manner in colorectal cancer. Gut 2012;61:1708–15.