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SPECIAL ARTICLE

Expression of lysophosphatidic acid receptors and local invasiveness and metastasis in Chinese pancreatic cancers

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ABSTRACT

Background and Objectives

The present study evaluated the potential role of lysophosphatidic acid receptors (LPARs) in processes leading to local invasiveness and metastasis in Chinese pancreatic carcinoma.

Methods

Real-time reverse-transcriptase polymerase chain reaction and Western blot analysis were used to detect expression of LPARs in tumour and adjacent non-tumour tissues from patients with surgically resected pancreatic carcinoma. Surgical specimens from 50 patients were examined for relative expression of each receptor's messenger RNA (mRNA) and protein. Findings were analyzed for correlations with tumour size, pathologic classification, clinical stage, and infiltration of capsule and lymphonodi.

Results

Increased levels of mRNA of LPARs (LPAR1 ≈ LPAR3 < LPAR2) were found in the pancreatic cancer tissues examined. Low levels of transcripts for LPAR1, LPAR2, and LPAR3 receptors were detectable in adjacent non-tumour tissues. The difference in LPAR1 protein expression between tumour and adjacent non-tumour tissues does not seem significant, but the signals of LPAR2 expression in pancreatic cancer tumour tissues were significantly amplified compared with those in adjacent non-tumour tissues. Tumour and adjacent non-tumour tissues both weakly expressed LPAR3 protein with no statistical difference. However, expression of LPAR1, LPAR2, and LPAR3 showed an obvious correlation with infiltration of capsule cells, surrounding lymphonodi, and specific histopathologic features.

Conclusions

Lysophosphatidic acid receptor is a promising indicator for pancreatic cancer, and our findings suggested that LPAR2 might be a potential target for clinical treatment of pancreatic cancer.

KEY WORDS

Lysophosphatidic acid, LPARs, pancreatic cancer

1. INTRODUCTION

Pancreatic cancer is one of the most common malignant tumours of the human digestive system. Its initiation and progression are closely related to the excessive proliferation and uncontrolled apoptosis of its tumour cells [1]. Therapeutic approaches are disappointing, and prognosis is poor [2]. In China, the incidence of pancreatic cancer seems to be increasing. Early diagnosis and treatment are therefore of significance in prolonging survival and improving quality of life.

The pathogenesis of cancer is characterized by changes in cell growth, survival, differentiation, and attachment, leading to uncontrolled tumour growth, invasiveness, and metastasis. Lysophosphatidic acid (LPA) has been shown to modulate invasion and growth of tumour cells [3]. Pancreatic ductal adenocarcinoma has shown LPA-induced cellular responses, including cell proliferation and motility [4].

The naturally occurring phospholipid LPA has diverse effects on various cells and is widely distributed in various tissues. Like many other biomediators, LPA interacts with cells through specific cell-surface receptors (LPARs, G protein–coupled receptors) to induce biologic effects. The subfamily formerly known as Edg/LP (endothelial differentiation gene/lysophospholipid), consisting of LPAR1 (Edg2), LPAR2 (Edg4), and LPAR3 (Edg7), are the 3 most common lysophosphatidic acid receptors. The LPA of augmentation, which mediates signal transduction

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through LPARs, induces tumour cell proliferation, adhesion, migration, antiapoptotic function, invasion, and metastasis, suggesting a common path for carcinogenesis in various human malignancies [5]. In addition, LPA elicits upregulation of vascular endothelial growth factor, which promotes angiogenesis, indirectly influencing the initiation and progression of malignancies. It may also stimulate secretion of matrix metalloproteinases and tumour angiogenesis factor, which closely is related to local invasion and metastasis [6]. A previous study by our group suggested that matrix metalloproteinases and platelet-derived growth factor participate in the process of invasion and metastasis in human pancreatic cancer. The process of tumour initiation and progression is far more complicated, involving multiple steps and factors [7]. Thus, LPA might be a potential target for clinical diagnosis and therapy in pancreatic cancer.

In the present study, we evaluated the potential role of LPARs in the processes leading to local invasiveness and metastasis in human ductal adenocarcinomas of the pancreas by measuring the expression of LPARs in tumour and adjacent non-tumour tissue from patients undergoing resection for pancreatic cancer. We also analyzed correlations between LPAR expression and tumour mass size, pathologic classification, clinical stage, infiltration of capsule cells and surrounding lymphonodi, and specific histopathologic features, with a focus on the evaluation of LPA receptors for clinical diagnosis and therapy in pancreatic cancer.

2. METHODS

2.1 Patients and Samples

The study enrolled 50 patients [34 men, 16 women; average age: 52 years (range: 32–64 years)] with pancreatic cancer who underwent surgical resection in the Department of Surgical Oncology at the Shenzhen Second People's Hospital and at Nanjing First Hospital from July 2006 to January 2010. Written informed consent was obtained for the use of the residual samples from all participants. The study was approved by the Institutional Research Ethics Committee of the Faculty of Medicine, Medical University of Nanjing.

By final pathology confirmation, the 50 pancreatic cancer specimens were classified as ductal carcinoma (n = 41), acinar carcinoma (n = 6), and others (n = 3). Of the tumours, 32 were located in the head, 14 in the body and tail, and 4 throughout the pancreas. In accordance with the National Comprehensive Cancer Network 2010 clinical practice guideline for pancreatic cancer (Chinese edition), the pathologic stages of the tumours were classified as follows: stage I (n = 2), stage II (n = 6), stage III (n = 27), and stage IV (n = 15). Adjacent non-tumour tissues were used as controls.

2.2 Isolation of Total RNA and Reverse Transcription

Pancreatic cancer tissues and paired non-tumour tissues (taken 2 cm away from the neoplasm) were immediately frozen in liquid nitrogen and kept at –80°C until extraction of RNA. Total RNA was extracted from each sample using Trizol (Sigma–Aldrich, St. Louis, MO, U.S.A.) according to the manufacturer's instructions. Then, 1 mg of total RNA was reverse-transcribed using a Superscript First-Strand Synthesis System on an ABI Prism 7000 sequence detector (Invitrogen, Carlsbad, CA, U.S.A.). The reverse-transcription reaction was performed in strict accordance with the manufacturer's protocol. The harvested complementary DNA was stored at –20°C for the subsequent experiments.

2.3 Quantitative Reverse-Transcriptase Polymerase Chain Reaction

To evaluate the expression of the LPAR messenger RNAs (mRNAs), quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was performed using real-time TaqMan technology (Invitrogen) with the ABI Prism 7000. The human LPAR1-, LPAR2-, LPAR3-, and GAPDH-specific primers were purchased from Applied Biosystems (Beijing, China). The expression level of the target mRNA was normalized to the relative ratio of the GAPDH mRNA expression.

2.4 Western Blot Analysis

Protein extracted from the 50 matched samples was electrophoresed in sodium dodecyl sulfate–15% polyacrylamide gel for 2 hours at 70 V. The protein was then transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA, U.S.A.) for sequential incubation with 4% reconstituted nonfat milk powder to block unspecific sites, dilutions of rabbit polyclonal anti-LPARs or beta-actin antibodies as the primary antibodies (1:1000), and then horseradish peroxidase–labelled goat anti-rabbit immunoglobulin G as a secondary antibody (1:3000) before development using a standard enhanced chemiluminescence kit (Amersham, Amersham, England). Films were exposed in a darkroom.

2.5 Statistical Analyses

Statistical comparisons were performed using the POM software (4th Military Medical University, Xian, China), with the t-test and chi-square test being used to determine the significance of the associations between the various variables. The level of significance was set at 0.05.

3. RESULTS

3.1 Expression of LPARs at the mRNA Level

The expression of mRNA for each LPAR was quantitatively evaluated against that for GAPDH (Figure 1). We detected a rather low level of LPAR1 mRNA in both tumour and adjacent non-tumour tissues, and the expression level did not differ significantly between the tissues (0.132% ± 0.029% vs. 0.142% ± 0.042%). However, the expression level of LPAR2 in tumour tissue was significantly greater than that in adjacent non-tumour tissue (0.471% ± 0.064% vs. 0.027% ± 0.015%, $p < 0.05$). In comparison, LPAR3 was moderately expressed in both tumour and adjacent non-tumour tissue without being different in the two tissues (0.163% ± 0.046% vs. 0.231% ± 0.043%).

3.2 Western Blot Analysis

Western blot analysis using polyclonal anti-LPAR antibody showed that LPAR1 protein expression in tumour and adjacent non-tumour tissues was same, but that expression of LPAR2 protein in tumour tissue was significantly higher than that in adjacent non-tumour tissue. Weak expression of LPAR3 was found in both tumour and adjacent non-tumour tissue, with no statistical difference. Figure 2 shows representative results of the Western blot analysis. Although Western blot is quantitatively less sensitive than real-time RT-PCR, the results from the two analyses are consistent.

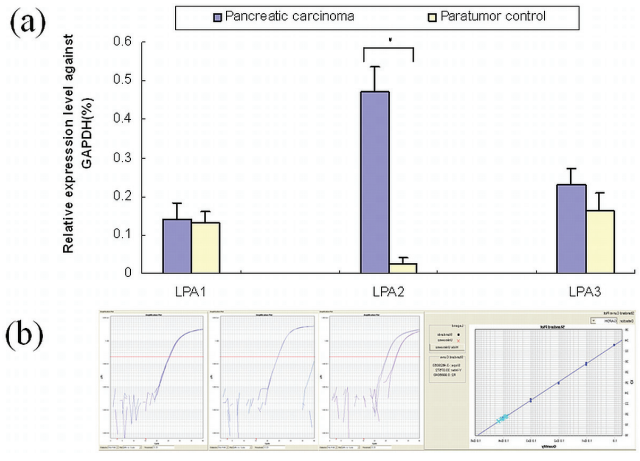

FIGURE 1 Relative expression level of lysophosphatidic acid (LPA) receptors (LPARS) in tumour and non-tumour tissue from patients with pancreatic carcinoma. (a) Expression of LPAR1, LPAR2, and LPAR3 messenger RNA measured by real-time reverse-transcriptase polymerase chain reaction. (b) Representative data of an average level selected for an LPAR calibration curve and a standard curve.

3.3 Expression of LPAR mRNA and Tumour Size

We allocated the 50 pairs of matched samples to two groups according to the diameter of the tumour sections (≤2 cm vs. >2 cm, Table I). For those two groups, we observed a relatively lower correlation in gene expression of LPAR1 and LPAR3 (LPAR1: 0.111% ± 0.036%; LPAR3: 0.199% ± 0.017%), but a rather higher correlation in gene expression of LPAR2 (0.412% ± 0.027%). Tissue from larger-diameter tumours showed a higher level of LPAR mRNA expression (LPAR1: 0.151% ± 0.043%; LPAR2: 0.489% ± 0.046%; LPAR3: 0.241% ± 0.023%) than did tissue from smaller-diameter tumours (LPAR1: 0.111% ± 0.036%; LPAR2: 0.412% ± 0.027%; LPAR3: 0.199% ± 0.017%).

3.4 Expression of LPAR mRNA and Pathologic Classification

Among the 50 tumour tissue samples were 39 cases of ductal carcinoma (the highest percentage at 78.0%). The percentage of acinar carcinoma was 8.0%, and of others, 14.0% (Table II). Expression of LPAR1 and LPAR3 mRNA was not different between the pathologic types. On the other hand, expression of LPAR2 mRNA was different for ductal carcinoma

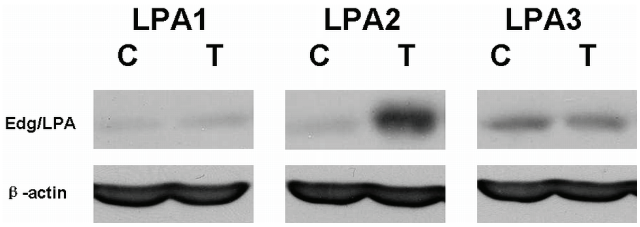

FIGURE 2 Western blot analysis using an antibody against lysophosphatidic acid (LPA) receptors (LPARS). Total cellular lysates of paired tumour and non-tumour tissues from patients with pancreatic carcinoma were analyzed for the presence of LPAR protein. Total protein in each sample was determined by Western blot analysis using antibodies to beta-actin (β-actin). In all, 50 matched samples were examined and representative cases are shown. T = tumour tissue; C = control (non-tumour) tissue; Edg = endothelial differentiation gene.

TABLE I Correlations between the expression of lysophosphatidic acid receptor (LPAR) messenger RNA[a] and tumour size

Tumour diameter	*Samples* (n)	*LPAR (%)*		
		1	*2*	*3*
≤2 cm	11	0.111±0.036	0.412±0.027	0.199±0.017
>2 cm	39	0.151±0.043	0.489±0.046	0.241±0.023
TOTAL	50	0.142±0.042	0.471±0.064	0.231±0.043

[a] The relative expression level of each LPAR's messenger RNA was quantitatively evaluated against that of GAPDH: $1/2^{\Delta t} \times 100\%$ (mean ± standard deviation).

TABLE II Correlations between the expression of lysophosphatidic acid receptor (LPAR) messenger RNA[a] and pathologic classification

Pathologic classification	*Samples* (n)	LPAR *(%)*		
		1	*2*	*3*
Ductal carcinoma	39	0.151±0.041	0.482±0.030	0.239±0.017
Well differentiated	23	0.147±0.033	0.449±0.046	0.236±0.033
Poorly differentiated	16	0.154±0.035	0.529±0.071	0.241±0.028
Acinar carcinoma	4	0.123±0.016	0.469±0.041	0.195±0.011
Others	7	0.107±0.013	0.403±0.034	0.217±0.025
TOTAL	50	0.142±0.042	0.471±0.064	0.231±0.043

[a] The relative expression level of each LPAR's messenger RNA was quantitatively evaluated against that of GAPDH: $1/2^{\Delta t} \times 100\%$ (mean ± standard deviation).

(0.482% ± 0.030%), acinar carcinoma (0.469% ± 0.041%), and other carcinoma types (0.403% ± 0.034%). A higher level of LPAR2 mRNA expression was observed in the cases of ductal carcinoma with poor differentiation (0.529% ± 0.071%) than in those with good differentiation (0.449% ± 0.046%, $p < 0.05$). Our findings suggested that LPAR2, but not LPAR1 and LPAR3, might be involved in tumorigenesis in pancreatic carcinoma.

3.5 Expression of LPAR mRNA and Invasive Metastasis

Local invasion and distant metastasis often occur at the early stage of pancreatic carcinoma. Capsule infiltration, lymphoid invasion, and abdominal visceral metastasis are the most notable characteristics of pancreatic cancer. Among the 50 cases, there were 43 with capsule infiltration, lymphoid invasion, and abdominal visceral metastasis. Tumour-tissue samples from the latter cases showed a higher level of LPAR mRNA expression than did samples from the 7 cases free of invasion and metastasis (Table III, $p < 0.05$). Among the various LPARs, LPAR2 showed the highest level of mRNA expression, followed by LPAR1, suggesting that those receptors might play separate and different roles whose mechanism is uncertain.

TABLE III Correlations between the expression of lysophosphatidic acid receptor (LPAR) messenger RNA[a] and invasive metastasis

Invasive metastasis	*Samples* (n)	LPAR *(%)*		
		1	*2*	*3*
No	7	0.074±0.017	0.386±0.026	0.195±0.019
Yes	43	0.153±0.037	0.486±0.042	0.238±0.028
TOTAL	50	0.142±0.042	0.471±0.064	0.231±0.043

[a] The relative expression level of each LPAR's messenger RNA was quantitatively evaluated against that of GAPDH: $1/2^{\Delta t} \times 100\%$ (mean ± standard deviation).

4. DISCUSSION

The molecular mechanism of local invasion and distant metastasis in pancreatic cancer is uncertain [8]. In the course of this metastasis, LPAR-mediated LPA may play a role, leading to the induction of biologic effects such as cellular proliferation, survival, and migration.

Lysophosphatidic acid is the simplest small-molecule lipid of glycerophosphate originating from membrane [9]. Platelets, some inflammatory cells, nerve cells, injury cells, endothelial cells, and tumour cells are stimulated to release LPA by autocrine and paracrine pathways, with the induction of various biologic effects [10].

Lysophosphatidic acid has a high affinity for G protein membrane receptors, which commonly include the LPAR1, LPAR2, and LPAR3 subfamily, originally called endothelial differentiation gene (Edg) receptors, which are a class of G protein–coupled receptors. Edg1, -3,-5, -6, and -8 bind the bioactive lipid sphingosine-1-phosphate as their primary signalling ligand. Edg2, -4, and -7 bind LPA [11].

In addition to LPARS 1–3, LPAR4 (formerly GPR23 or P2Y9) of the purinergic receptor family and the related LPAR5 (formerly GPR92) and LPAR6 (formerly P2Y5) have been identified as new LPARS. It has been proposed that GPR87 is the 7th LPAR, but further evidence is needed to validate that proposed role. This novel non-Edg subgroup of LPARS are structurally distant from the Edg LPARS. The homology among them is also low [12].

The distribution of LPAR1 is relatively wide, appearing in various tissues, but LPAR2 and LPAR3 are relatively local.

We used real-time RT-PCR to examine the relative expression levels of each receptor's mRNA against GAPDH mRNA in tumour and adjacent non-tumour tissues surgically removed from patients with pancreatic carcinoma. We also used Western blot to analyze the LPAR proteins.

The results showed that the tumour and adjacent non-tumour tissues expressed LPAR1 mRNA at similar levels, but that the expression level of LPAR2 mRNA was significantly higher in tumour than in matched non-tumour tissue ($p < 0.05$). In both tumour and adjacent non-tumour tissue, LPAR3 was weakly expressed (Figure 1). Results from the Western blot analysis were compatible with those from the real-time RT-PCR analysis (Figure 2). These findings show that the expression of LPARs in tumour tissue from pancreatic cancer is significantly different than that in matched adjacent non-tumour tissue. We conclude that overexpression of LPAR2 in tumour cells may be involved in the initiation and progression of pancreatic carcinoma. The receptor LPAR1 is present both in tumour tissue and in adjacent normal tissue. No change in LPAR1 expression is evident from normal function to malignant transformation [13]. That observation is consistent with the fact that LPAR couples to Gi, which links to activation of the downstream target Rac. Rac, together with Rho downstream of G12/13 (Figure 3), mediates cell migration in a coordinated manner. Rac promotes lamellipodia protrusion and forward movement, and RhoA regulates actomyosin-driven cytoskeleton contraction and detachment of the rear of migrating cells [14].

Unlike LPAR1, LPAR2 does not seem to be a major mediator of the cellular migratory response to LPA. However, strong evidence indicates that expression of this receptor is commonly increased in human malignancies. The mRNA of LPAR2 or LPAR3, or both, is highly expressed in ovarian cancer cell lines, but is not present in normal ovarian epithelial cells [15].

Many other groups have reported abnormal expression of LPARs, particularly LPAR2 in various human malignancies [16]. In breast cancer, overexpression of LPAR2 was observed in more than half (57%) of the most common invasive ductal carcinomas [17]. Overexpression of LPAR2 was also observed overall in gastric cancer [18]. In colorectal cancer compared with matched normal intestinal mucosa, LPAR2 mRNA was found consistently to be increased, and LPAR1 mRNA, to be decreased [19]. Studies of breast, gastric, and colorectal cancers also showed increases in immunohistochemical staining for LPAR2 protein in carcinoma cells compared with cells from surrounding normal epithelial tissues.

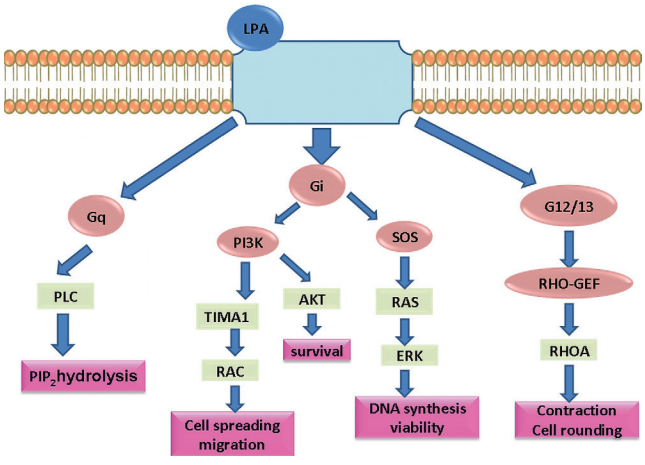

FIGURE 3 Diverse pathways of lysophosphatidic acid (LPA) signal transduction.

The increased LPAR2 expression in malignancy does not establish a causal role for this receptor in oncogenesis. The overexpression could be a consequence of cell transformation. However, several recent studies have provided ample evidence that LPAR2 is causally linked to tumorigenesis in animal models. Ectopic expression of LPAR2 in ovarian cancer cell lines enhances their tumorigenicity and aggressiveness in subcutaneous and intraperitoneal xenograft models in nude mice [20]. An LPAR2 transgenic model in the ovary has been also reported [21]. The directed expression of LPAR2 is not sufficient to induce ovarian tumorigenesis, but resulted in overexpression of oncogenic factors such as vascular endothelial growth factor and urokinase-type plasminogen activator [22].

Mice with LPAR2 deficiency do not exhibit any physiologic abnormalities. However, compared with wild-type mice, LPAR2$^{-/-}$ deficient mice are more resistant to intestinal tumour formation induced by colitis or by ApcMin mutation. The mechanism for the oncogenic action of LPAR2 is not well understood. Most studies have focused on the ability of LPAR2 to stimulate the expression of oncogenic protein factors, including interleukin-6, vascular endothelial growth factor, hypoxia-inducible factor 1 alpha, c-Myc, cyclin D1, Kruppel-like factor 5, and Cox-2 [23,24].

In addition to LPAR1 and LPAR2, LPAR3 is unique in being expressed in few normal tissues. There is inconsistency with respect to its expression in cancer. For instance, it is overexpressed in ovarian cancer and ovarian cancer cell lines, but overexpression was not observed in other human cancer types [25].

Recently, receptors for insulin-like growth factor 1 were shown to be functionally expressed in pancreatic cancer [26]. The crosstalk between the Edg LPA-mediated signal and the insulin-like growth factor or insulin-mediated signal needs further study.

Taken together, aberrant expression of LPARs might be a key event in processes leading to local invasiveness and metastasis in human ductal adenocarcinomas of the pancreas. Lysophosphatidic acid receptors may be promising indicators for pancreatic cancer, given that our findings suggest that LPAR2 might be a potential target for clinical diagnosis and therapy of pancreatic cancers.

In this study, we analyzed relationships between the levels of Edg LPAR and tumour size, pathologic classification, infiltration of capsule cells and surrounding lymphonodi, and specific histopathologic features.

Larger tumour size (diameter > 2 cm) was associated with a higher level of expression of Edg LPAR mRNA than was seen with smaller tumours (diameter < 2 cm, $p < 0.05$, Table I). The results suggested that upregulation of Edg LPARs contributed to LPA-induced biologic effects promoting proliferation in tumour cells. On the other hand, a higher and significant level of LPAR2 mRNA expression was observed in cases of ductal carcinoma with poor differentiation (0.529% ± 0.071%) than in cases with good differentiation (0.449% ± 0.046%, Table II). Our findings suggested that LPAR2, but not LPAR1 and LPAR3, might participate in tumorigenesis in pancreatic ductal carcinoma.

We infer that tumour cells in pancreatic ductal adenocarcinoma develop differently than metastatic cells do. Although the size of the tumour is much smaller in the early stage, distant metastasis and postoperative recurrence might develop, and the prognosis-related pathologic classification is dismaying in some poorly differentiated pancreatic duct carcinoma. Our data also show that pancreatic cancer cells with a lower degree of differentiation are more susceptible to invasion and metastasis, and that Edg LPAR expression increases as the disease progresses.

Poorly differentiated tumour cells progress more easily in the early stage. Capsule infiltration and lymphoid invasion are both characteristic signs involved in local invasiveness and distant metastasis in pancreatic ductal adenocarcinoma. The present study showed that Edg LPAR mRNA expression was higher in 43 samples with capsule infiltration, lymphoid invasion, and abdominal visceral metastasis than in 7 samples free of invasion and metastasis (Table III). Among the elevated levels of mRNA expression of the various Edg LPARs, LPAR2 was the highest and LPAR1 was the next highest, suggesting that those receptor might play separate and different roles in a mechanism that is currently uncertain.

With the acknowledgment that Edg LPARs mediate LPA signal transduction, the potential role of LPA in the development of human pancreatic cancer—namely, the pathophysiology and pathogenesis of the tumour—has gradually become a focus of concern. However, it is now possible to alter the expression of specific LPARs both *in vivo* and *in vitro*. With the help of biologic techniques, the research into targeted therapy for Edg LPARs may provide a novel strategy for pancreatic cancer.

5. CONCLUSIONS

An understanding of alterations in the levels of Edg LPARs and the resulting biologic effects might help physicians to assess the metastatic potential of tumours and the prognosis of individual patients. The Edg LPARs may be promising biologic indicators for the course of invasion and metastasis. The LPA-mediated induction of cellular proliferation, survival, and migration may play a significant role in human pancreatic cancer.

6. ACKNOWLEDGMENTS

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7. CONFLICT OF INTEREST DISCLOSURES

The authors declare that they have no competing interests.

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