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Original Article

Differential expression of lysophosphatidic acid receptors between benign and malignant tissues in humans

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Abstract: This study is aimed to simultaneously investigate the systemic distribution, subcellular localization, and differences in the expression level between human benign and malignant tissues for LPAR1, LPAR2, and LPAR3. Immunohistochemistry (IHC) was performed for LPAR1, LPAR2, and LPAR3 on 384 formalin-fixed paraffin-embedded human tissues from 33 organs/systems in tissue microarrays. All three LPARs were found to be widely expressed in major organs/systems of humans. The IHC signals for studied LPARs were mainly localized in the nucleus and cytoplasm, but rarely seen on cellular membranes. All three LPARs have higher nuclear IHC scores in overall malignancy compared to those in benign tissues. Of the three LPARs, LPAR1 exhibited the highest expression level in both benign and malignant tissues. Liver is the only organ that exhibited significant differences in expression levels of all three LPARs in nucleus between benign hepatocytes and hepatocellular carcinoma. Seven organs (bladder, brain, colon, kidney, lung, ovary and pancreas) exhibited significant differences in IHC scores for a single LPAR (cytoplasmic or nuclear), and twenty-six organs/tissues show no significant difference in expression of LPAR1, LPAR2 or LPAR3 (cytoplasmic or nuclear) between benign and malignant tissues. It is concluded that the expression of LPARs in human organs/tissues is varied, depending on the type of organ and pathological status. This is the first study to simultaneously investigate the systemic distribution, subcellular localization and differences in expression of multiple LPARS in human benign and malignant tissues.

Keywords: Lysophosphatidic acid (LPA) receptors (LPARs), cancer, tissue microarrays (TMAs), immunohistochemistry (IHC), hepatocellular carcinoma (HCC)

Introduction

Lysophosphatidic acids (LPA) comprise a group of small glycerophospholipids. These phospholipids act as messengers in multiple biological functions [1], and are important ligands for several closely-related G protein-coupled receptors (GPCRs) [2]. The GPCRs specific to LPA are named LPA receptors (LPARs) [3]. Six LPAR subtypes (LPAR1-LPAR6) have been identified since the first was discovered in 1996 [4]. LPA and LPARs are known to participate in a wide range of cellular activities including: cellular morphogenesis, proliferation, migration, and survival [5]. Alterations in the expression of LPARs have been shown to be involved in the pathogenesis of many diseases, including cancers [6].

LPARs, especially LPAR1, LPAR2 and LPAR3, have been implicated in migration, invasion, metastasis, proliferation and survival of different cancers [7-9]. Whether the activation of a LPAR promotes or inhibits cancer progression largely depends on the type of tissue. In human ovarian cancer cells, up-regulation of LPAR1, LPAR2 and LPAR3 increased the invasiveness of the cancer, whereas down-regulation of LPAR1 or LPAR2 inhibited migration, invasion, and production of IL-6, IL-8, and vascular endothelial growth factor (VEGF) [10]. Another study showed that LPAR1 and LPAR2 can induce migration and invasion through the beta-arrestin/Ral signaling pathway in breast cancer metastasis. The ectopic expression of LPAR1 in MCF-10A cells, a non-cancer source, helps these cells acquire an invasive phenotype [11].

Studies have shown that LPAR2 is related to invasion and metastasis of ovarian, endometrial and colon cancer cells [12-14]. Cross-talk between LPAR1 and epidermal growth factor receptor (EGFR) promotes the motility and invasion of gastric and colon cancer cells through the up-regulation of sphingosine kinase 1 (SPHK1) [15]. There is increasing evidence to suggest that LPARs have been associated with the activation and induction of various cancer signaling pathways, such as matrix metalloproteinases (MMPs), COX-2 and RhoA/Rac signaling [12, 16-19].

Studies on LPAR expression have used PCR, Northern blots, and in situ hybridization and Western blots on cell cultures or animal models [20]. Since LPA molecules are ligands common to all LPARs, it is important to distinguish which LPAR is up- or down-regulated in the pathogenesis or progression of a given cancer, as well to differentiate up- or down-regulation of a given LPAR among types of cancers. The aim of this study is to simultaneously determine the systemic distribution, subcellular localization and differential expression levels of LPAR1, LPAR2 and LPAR3 by performing immunohistochemistry on benign and malignant tissues from major human organ/systems.

Materials and methods

Tissue collection, tissue microarray (TMA) construction and grouping

This study was approved by the University of Mississippi Medical Center (UMMC) Institutional Review Board. All participants were patients enrolled into UMMC. A total of 384 formalin-fixed and paraffin-embedded (FFPE) specimens were obtained from the files at the Department of Pathology at UMMC (See Supplementary Table 1, ST1 for details in sample primary origin, diagnosis and classification). After review of the histological features on the hematoxylin and eosin (H&E) stained original glass slides, and confirmation of the pathological diagnosis by departmental pathologists, informative regions were selected on the original H&E stained slides and topographically correlated with the corresponding original FFPE tissue blocks. One 1-mm cylindrical core from each informative area on the primary FFPE blocks was removed by punch biopsy and transferred to composite paraffin blocks to construct the TMA using a Beecher MTA1 manual tissue arrayer. The resulting TMA blocks were heated at 40°C for 4 h in order to fuse the transferred cores with paraffin in the composite block. Each composite TMA block was sectioned at 5 μm in thickness and slides prepared. One slide from each TMA block was stained with H&E, in order to re-confirm the morphology and pathological diagnosis. The remaining slides were used for IHC study.

Three hundred and eighty-four (384) specimens were classified into thirty-three (33) human organs/systems/tissues, according to the original site of each specimen. Thus, specimens in some organs/systems, such as immune system and neural system may be heterogeneous in nature. Specimens in each system/organ are divided into benign and malignant groups. The benign group included non-malignant changes such as tissues with normal histology, inflammatory reactions, degenerative processes, and benign tumors such as adenoma, meningioma, etc. The malignant group included cancers and tumors of a given organ system with characteristics of uncontrollable growth and metastatic tendency. The malignant group in a given organ system may contain cancers in primary and metastatic sites, and may include cancers with different cell origins.

Immunohistochemistry (IHC) and scoring system

The protocol for immunohistochemical staining and scoring was described previously [21]. Briefly, TMA slides were deparaffinized in an oven at 56°C overnight on the day before immunohistochemical staining was performed. The slides were further deparaffinized in xylene, and rehydrated in graded ethanol. Antigens were retrieved with antigen retrieval solution (Citric-plus, BioGenex, Fremont CA, USA), and endogenous peroxidase was quenched with 3% Hydrogen Peroxide for 30 min. A blocking serum corresponding to each primary antibody in ABC kits (Vector Laboratories, Burlingame, CA, USA) was incubated at room temperature 1 h to block nonspecific binding. Then, the slides were incubated with primary antibodies, including, 1) anti-LPAR1 (EDG2) antibody: ab77940, N-terminal, Abcam Inc., MA, USA, 1:400; 2) anti-LPAR2 (EDG4) antibody: ab38322, N-terminal, Abcam Inc., MA, USA, 1:400; and, 3) anti-LPAR3 (EDG7) antibody: ABT113, N-terminal, EMD Millipore Corporation, CA, USA, 1:500, for 2 hours at room temperature for polyclonal anti-

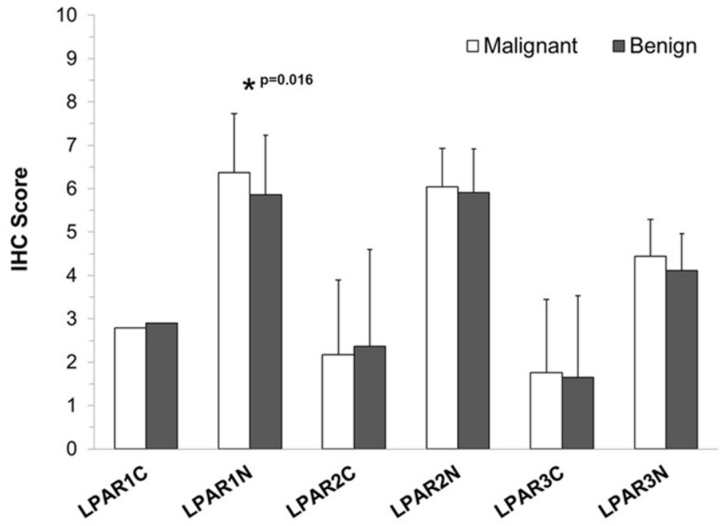

Figure 1. Comparison of immunohistochemistry (IHC) score of LPA receptors (LPARs) expressed in the cytoplasm and nucleus in benign and malignant tissues.

bodies, or overnight at 4°C for monoclonal antibodies. The slides for negative controls were incubated with the blocking serum replacing the primary antibodies. Following extensive washing in phosphate-buffered saline (PBS), antigen-antibody complexes were detected using the ABC Elite kits and NovaRed peroxidase substrate kits (Vector Laboratories, Burlingame, CA, USA). Then the slides were counterstained and mounted.

Subcellular localization for each LPAR was determined microscopically. The expression of each LPAR in different subcellular locations was quantified by IHC score system described previously [21]. Briefly, the extent of IHC-staining in the relevant areas was recorded as area score as follows: 0 for no cell stained, 1 for < 10%, 2 for 10% to 50%, and 3 for > 50% cells stained. The IHC intensity score was also recorded as 0 for no IHC signal at all, 1 for weak, 2 for moderate, and 3 for strong IHC signals. The final IHC score used in the analysis was calculated by multiplying the area score and intensity score, with a maximum score of 9. Any LPA receptor expressed in the cytoplasm or nucleus was defined as a low expression pattern if final IHC score is < 3, or as high expression pattern if final IHC score is ≥ 3.

Statistical analysis

The mean IHC scores of each LPAR were compared between benign and malignant tissues in overall, and in individual organ/system, using 2-sample t test in SPSS22 software (IBM, USA). The percentage of high expression pattern for each LPAR was compared between its expression in nucleus and cytoplasm, and between benign and malignant changes, using Fisher Exact Test. The difference in IHC score and percentage of high expression pattern is statistically significant between two compared groups if *p* value was less than 0.05.

Results

Comparison of LPAR expression between overall benign and malignant tissues

IHC for LPAR1, LPAR2 and LPAR3 was performed on 384 FFPE specimens (183 overall benign and 201 overall malignant) from 33 human organs/systems. Microscopically, IHC signals for LPAR1, LPAR2 and LPAR3 were seen in the nucleus and cytoplasm, but rarely in cellular membranes. The differences in cytoplasmic and nuclear expressed LPARs in overall benign and malignant tissues are illustrated in **Figure 1**. All LPARs were more highly expressed in the nuclei than cytoplasm. Both cytoplasmic and nuclear expressions were highest in LPAR1, and lowest in LPAR3. The differences in cytoplasmic LPARs (LPAR1C, LPAR2C, and LPAR3C) were not statistically significant between overall benign tissues and malignant tissues. The nuclear LPARs (LPAR1N, LPAR2N and LPAR3N) were all higher in malignant tissues than in benign. The difference between malignant and benign tissues was statistically significant for only LPAR1N (P=0.016). It was noted that the subcellular localization and expression of LPAR1, LPAR2 and LPAR3 varied among individual organs/systems.

Expression of LPAR1 in benign and malignant groups in individual organs/systems

LPAR1 was observed in both the nuclei and cytoplasm of all organs examined except in myocardium (**Table 1**). In general, LPAR1 is expressed much higher in nucleus (LPAR1N) than in cytoplasm (LPAR1C). The higher expression pattern (IHC score ≥ 3) of LPAR1N was

Table 1. The expression level (IHC score) of LPAR1 in different organs/tissues in humans

Organs/Tissues	Cytoplasmic Stain (LPAR1C)							Nuclear Stain (LPAR1N)						
	Benign			Malignant			*p* Value	Benign			Malignant			*p* Value
	N	Mean	SD	N	Mean	SD		N	Mean	SD	N	Mean	SD	
Adrenal Gland	5	3.90	0.82	3	4.50	3.97	0.74	5	4.80	1.25	3	7.00	1.73	0.08
Appendix	2	2.25	1.06					2	5.25	1.06				
Artery, Adult	4	1.88	1.44					4	4.88	1.44				
Artery, Umbilical	2	2.25	1.06					2	7.50	0.00				
Bone				3	2.00	0.87					3	2.50	4.33	
Breast	4	2.63	1.44	14	2.63	1.13	0.70	4	6.38	1.89	14	5.89	1.61	0.62
Cervix	5	2.10	0.82	3	2.50	1.73	0.67	5	5.60	0.89	3	7.00	1.73	0.17
Colon	9	2.50	1.06	16	3.00	1.22	0.32	9	7.33	1.17	16	5.16	0.94	0.00004
Esophagus	3	3.00	1.50	2	1.50	0.00	0.27	3	6.50	0.87	2	8.25	1.06	0.13
Fallopian Tube	2	2.25	1.06					2	5.00	1.41				
Gallbladder	1	3.00		2	3.00	2.12	1.00	1	6.00		2	6.75	1.06	0.67
Immune System	4	1.75	2.06	4	3.38	1.89	0.29	6	6.42	1.93	4	6.75	1.94	0.80
Kidney	3	2.50	0.87	10	3.15	1.65	0.53	3	5.50	0.87	11	6.36	2.56	0.59
Liver	6	3.50	1.82	12	2.38	0.77	0.08	6	0.92	1.20	12	7.50	0.90	0.000000001
Lung	4	3.00	1.22	7	2.57	1.43	0.63	4	7.88	1.44	7	5.79	1.82	0.08
Myocardia	4	1.50	0.00					4	0.00	0.00				
Laryngopharynx	4	2.63	0.75	10	2.70	0.95	0.89	4	6.38	0.75	10	6.15	1.13	0.76
Neural System	9	2.50	1.06	3	3.50	0.87	0.17	9	6.17	1.90	3	8.00	0.87	0.15
Ovary	4	2.63	1.44	5	3.30	1.25	0.48	4	5.88	1.89	5	6.30	0.67	0.65
Pancreas	9	3.33	1.46	10	2.10	0.77	0.03	9	6.50	1.68	10	5.85	1.11	0.33
Pituitary	2	3.00	0.00					2	6.00	0.00				
Prostate	4	2.63	0.75	14	3.21	1.42	0.44	4	6.00	0.00	14	6.97	1.39	0.19
Oral Gland	6	2.26	0.81	4	3.00	0.00	0.11	6	5.33	1.40	4	6.63	2.14	0.28
Small Intestine	3	4.00	1.73					3	8.50	0.87				
Skin	4	3.75	0.87	5	3.00	1.06	0.29	4	6.38	0.75	5	6.60	0.82	0.68
Skeletal Muscle	4	1.88	0.75					4	2.00	2.31				
Soft Tissue	2	3.00	0.00	7	2.36	1.18	0.49	2	7.50	2.12	7	7.50	2.12	1.00
Stomach	8	3.56	2.53	9	2.67	1.64	0.39	8	7.13	1.33	9	7.50	1.30	0.57
Testis	4	4.50	1.22	4	2.63	1.44	0.09	4	7.13	1.44	4	7.13	1.44	1.00
Thyroid	8	3.19	0.96	4	4.13	1.44	0.20	8	5.25	1.13	4	5.63	1.44	0.63
Urothelium	5	2.40	0.82	9	2.50	1.50	0.89	5	7.40	2.04	9	6.00	0.75	0.08
Uterus	5	2.40	0.82	11	3.00	1.34	0.38	5	6.90	1.71	11	5.86	1.70	0.28
Wilm's Tumor				2	2.25	1.06					2	8.25	1.06	

seen in malignant tissues of 96% (24/25) organs/systems, whereas the higher expression pattern of LPAR1C was seen in malignant tissues of 28% (7/25) organs/systems ($P < 0.0001$). Similarly, the higher expression pattern (IHC score ≥ 3) of LPAR1N was seen in benign tissues of 91.3% (28/31) organs/systems, whereas that of LPAR1C was seen in benign tissue of 25.8% (8/31) organs/systems ($P < 0.0001$). These results imply that the nucleus might be the principle subcellular localization for LPAR1 to play its biological roles. The percentages of organs/systems with high expression patterns of both LPAR1N and LPAR1C was higher in malignant tissues versus benign.

A potentially significant difference in IHC score for both LPAR1C and LPAR1N is seen between benign and malignant groups in a few individual organs/systems. The IHC scores of LPAR1C are lower in malignances of liver, pancreas and testis as compared with their benign counterparts: the IHC score of LPAR1C is 2.38 in hepatocellular carcinoma (HCC) cells vs. 3.5 in benign

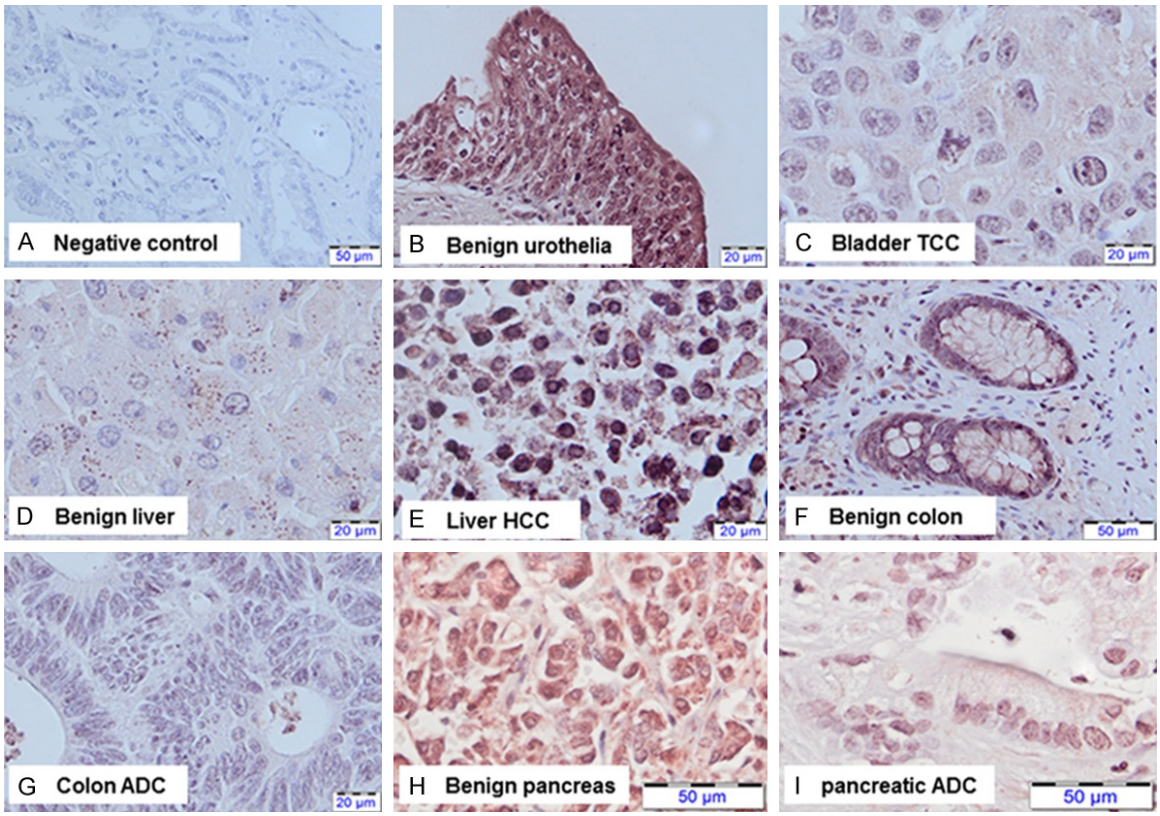

Figure 2. IHC staining for LPAR1 in representative systems/organs. Negative control of IHC staining for LPAR1 in prostate cancer with Gleason's score of 6 (A); the IHC signal for nuclear LPAR1 (LPAR1N) is stronger in benign urothelium (B) than in transitional cell carcinoma of bladder (C); the IHC signal for LPAR1N is not seen in benign hepatocytes (D), but is strong in hepatocellular carcinoma (HCC, E); the IHC signal for LPAR1N is strong in benign colon (F) than in colon adenocarcinoma (G); and the IHC signal for cytoplasmic LPAR1 (LPAR1C) is stronger in benign pancreas (H) than in pancreatic ductal adenocarcinoma (I).

hepatocytes (P=0.08); 2.10 in pancreatic ductal adenocarcinoma cells vs. 3.33 in benign pancreatic glandular cells (P=0.03); and 2.63 in seminoma cells vs. 4.5 in benign germinal epithelia in testis (P=0.08). No obvious difference for LPAR1C expression level is observed between malignant and benign groups in other organs/systems. Similarly to LPAR1C, the IHC score of LPAR1N are lower in malignancies of colon, lung and bladder as compared with their benign counterparts: the IHC score of LPAR1N is significantly lower colonic adenocarcinoma (5.16) as compared with benign colon epithelia (7.33, P=0.00004); the IHC score of LPAR1N in lung adenocarcinoma (5.78, not including small cell lung cancer, SCLC) is obviously lower than that in benign alveolar epithelium of lung (7.88, P=0.08); and the IHC score of LPAR1N in bladder transitional cell carcinoma (6.0) is also obviously lower than that in benign urothelial cells in bladder and ureter (7.4, P=0.08). Dissimilar to LPAR1C, the IHC score of LPAR1N are higher in malignancies of the adrenal gland and liver as compared with benign counterparts: the IHC score of LPAR1N is increased in pheochromocytoma cells in adrenal gland vs. benign cells in adrenal gland (7.0 vs. 4.8, P=0.08); and in HCC cells vs. benign hepatocytes (7.5 vs. 0.92, P=0.000000001). In addition, the IHC score of LPAR1N is higher in the nuclei of smooth muscle of the umbilical vein than in that of adult artery (7.5 vs. 4.88, P=0.07).

These results indicate that LPAR1 correlates with malignancies in liver, pancreas, testis, colon, lung, bladder and adrenal gland. However, alteration in subcellular localizations and in expression patterns (down- or up-regulated) varies among these malignancies. The representative IHC staining for LPAR1C and LPAR1N are shown in **Figure 2**.

Table 2. The expression level (IHC score) of LPAR2 in different organs/tissues in humans

Organ/Tissue	Cytoplasmic Stain (LPAR2C)							Nuclear Stain (LPAR2N)						
	Benign			Malignant			*p* Value	Benign			Malignant			*p* Value
	N	Mean	SD	N	Mean	SD		N	Mean	SD	N	Mean	SD	
Adrenal Gland	5	3.30	1.25	3	3.50	2.29	0.88	5	5.40	1.71	3	6.00	2.60	0.70
Appendix	2	1.50	0.00					2	4.50	0.00				
Artery, Adult	4	1.88	1.44					4	4.38	1.25				
Artery, Umbilical	2	2.25	1.06					2	5.75	2.47				
Bone				3	2.00	0.87					3	3.00	5.20	
Breast	4	2.25	1.50	14	2.25	0.78	1.00	4	6.00	1.22	14	5.46	1.51	0.53
Cervix	5	1.51	0.01	4	2.25	0.87	0.91	5	5.90	1.24	4	6.75	0.87	0.29
Colon	9	2.50	0.75	16	2.16	0.77	0.29	9	5.83	2.42	16	5.34	0.94	0.48
Esophagus	2	2.25	1.06	2	2.25	1.06	1.00	2	6.00	0.00	2	7.50	0.00	
Fallopian Tube	2	2.25	1.06					2	6.00	0.00				
Gallbladder	1	1.50		2	3.00	0.00		1	7.50		2	6.00	0.00	
Immune System	3	2.00	0.87	4	2.63	0.75	0.35	5	6.00	1.84	4	6.75	1.94	0.57
Kidney	3	3.00	0.00	11	2.05	2.56	0.59	3	6.00	0.00	11	6.77	2.18	0.56
Liver	7	2.57	1.13	12	2.13	0.77	0.32	7	1.64	2.04	12	5.29	1.14	.00002
Lung	4	3.75	0.87	8	2.06	0.78	0.01	4	8.00	2.00	8	6.38	1.06	0.09
Myocardia	4	1.50	0.00					4	3.75	0.50				
Laryngopharynx	4	2.25	0.87	10	1.95	0.72	0.52	4	6.75	0.87	10	6.45	1.23	0.67
Neural System	8	2.25	0.80	3	3.00	0.00	0.15	8	6.44	1.24	3	8.00	0.87	0.08
Ovary	4	2.63	0.75	5	2.70	0.67	0.88	4	5.63	0.75	5	4.80	1.25	0.29
Pancreas	8	2.25	0.80	8	2.06	0.78	0.64	8	6.75	1.13	8	6.19	0.96	0.30
Pituitary	2	1.50	0.00					2	5.25	1.06				
Prostate	4	1.50	0.00	15	1.90	0.89	0.39	4	6.00	0.00	15	6.40	1.55	0.62
Oral Gland	6	2.50	1.22	4	3.00	0.00	0.45	6	6.33	2.04	4	8.25	1.50	0.15
Small Intestine	3	3.00	0.00					3	7.00	0.87				
Skin	4	2.63	1.44	6	2.00	0.77	0.39	4	6.38	0.75	6	7.00	0.77	0.24
Skeletal Muscle	4	1.50	0.00					4	4.38	2.81				
Soft Tissue	2	1.50	0.00	7	1.93	0.73	0.46	2	6.75	1.06	7	6.21	3.17	0.83
Stomach	7	2.36	0.80	9	1.67	0.50	0.05	7	6.64	1.18	9	6.28	1.54	0.61
Testis	4	3.75	1.50	3	3.50	1.73	0.85	4	7.50	1.22	3	6.00	0.00	0.09
Thyroid	9	2.17	1.09	4	3.00	2.12	0.36	9	5.78	1.25	4	5.25	0.87	0.46
Urothelium	4	1.88	0.75	9	2.00	0.75	0.79	4	6.75	0.87	9	6.33	1.25	0.56
Uterus	5	1.80	0.67	12	2.13	0.77	0.43	5	5.40	1.34	12	5.33	2.09	0.95
Wilm's Tumor				2	1.50	0.00					2	6.00	0.00	

Expression of LPAR1 in benign and malignant groups in individual organs/systems

The IHC signals for LPAR2 are also found in both nuclei and cytoplasm of all tissues studied as shown in **Table 2**. LPAR2 is expressed much higher in nuclei (LPAR2N) compared to cytoplasm (LPAR2C). In malignant tissues, the high expression pattern of LPAR2N (IHC score ≥ 3) is seen in 96% (24/25) organs/systems, whereas the high expression pattern of LPAR2C is seen in 8% (2/25) organs/systems, $P < 0.0001$. In benign tissues, high LPAR2N expression pattern was observed in 96.8% (30/31) organs/systems, which is significantly higher than that of LPAR2C (9.7%, 3/31 organs/systems, $P < 0.0001$). The percentage of organs/systems with high expression pattern for LPAR2N and LPAR2C are not obviously different between benign and malignant groups.

In individual organ/system, malignancies in lung and stomach are associated with decreased expression of LPAR2C. The IHC

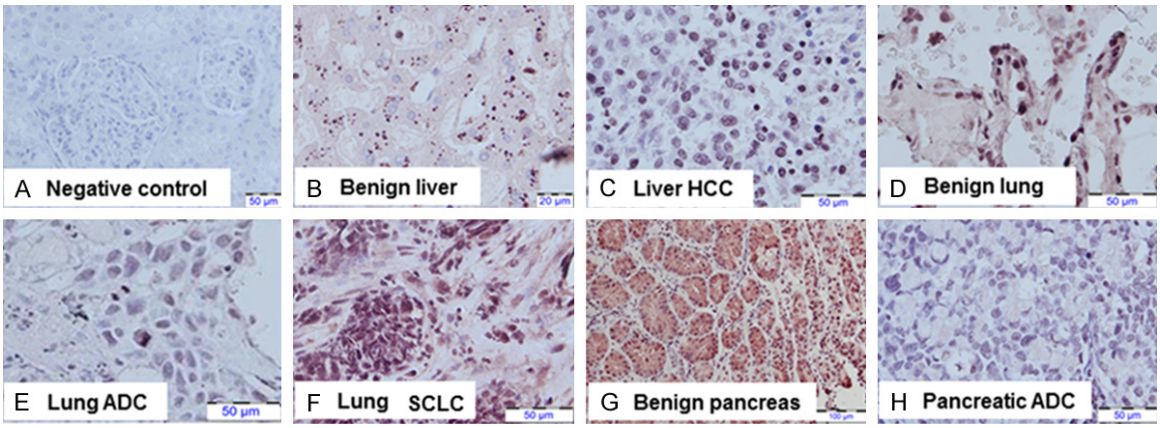

Figure 3. IHC staining for LPAR2 in representative organs/systems. Negative control of IHC staining for LPAR2 in benign kidney (A); the IHC signal for nuclear LPAR2 (LPAR2N) is not seen benign hepatocytes (B), but is moderate in HCC (C); the IHC signal for LPAR2N is strong in type I and type II alveolar epithelial cells of lung (D), weak in lung adenocarcinoma (E), and strong in small cell lung cancer (SCLC, F); and the IHC signal for cytoplasmic LPAR2 (LPAR2C) is strong in benign gastric glands (G), but very weak in gastric adenocarcinoma (H).

score of LPAR2C in lung adenocarcinoma (2.06) was significantly lower than that in benign alveolar epithelium (3.75, P=0.01). The IHC score of LPAR2C in gastric adenocarcinoma (1.67) was significantly lower than that in benign gastric epithelia (2.36, P=0.05). No difference in LPAR2C was observed between benign and malignant groups in other organs/systems. The IHC score of LPAR2N was decreased in malignancies of lung and testis. It was slightly lower in lung adenocarcinoma (6.38) than in benign alveolar epithelia (in 8.0, P=0.09), and was somewhat lower in seminoma (6.0) than in benign germinal epithelia in testis (7.5, P=0.09). The IHC score of LPAR2N was significantly higher in HCC (5.29) than in benign hepatocytes (1.64, P=0.00002), and higher in astrocytoma (8.0) than in benign brain tumors such as meningioma (6.44, P=0.08).

Therefore alteration in expression of LPAR2 correlates with malignant progression in lung, stomach, testis, liver and nervous system. Representative IHC stains for LPAR2C and LPAR2N are shown in **Figure 3**.

Expression of LPAR3 in benign and malignant groups in individual organs/systems

The IHC signals for LPAR3 are also present in cytoplasm (LPAR3C) and in nucleus (LPAR3N) in all benign and malignant groups (except LPAR3N in myocardia) as shown in **Table 3**. Similar to LPAR1 and LPAR2, LPAR3 is expressed much higher in nucleus (LPAR3N) than in cytoplasm (LPAR3C): in malignant group, the high expression pattern (IHC score ≥ 3) of LPAR3N is seen in 96% (24/25) organs/systems studied, which is significantly higher than that of LPAR3C (0%, 0/25 organs/systems studied, P < 0.0001); similarly, in benign group, LPAR3N is seen in 91.3% (28/31) organs/systems studied, which is significantly higher than that of LPAR3C (3.2%, 1/31 organs/systems studied, P < 0.0001). The percentage of organs/systems with high expression pattern for LPAR3N and LPAR3C are not obviously different between benign and malignant groups.

Although LPAR3C has a low expression level in overall, obvious difference in IHC score of LPAR3C is seen in three organs/systems: the IHC score of LPAR3C is significantly lower in bladder transitional cells carcinoma (1.36) than in benign Urothelium in bladder and ureter (1.64, P=0.045); potentially higher in lymphoma (2.25) than in benign lymphocytes in immune system (0.75, P=0.07); and significantly higher in ovarian cancer (2.63) than in benign ovary (1.13, P=0.03). The IHC score of LPAR3N is significantly lower in squamous carcinoma as compared with benign squamous cells of laryngopharynx (4.2 vs. 5.63, P=0.05). Reversely, the IHC score of LPAR3N is significantly higher in HCC as compared with benign hepatocytes (4.15 vs. 0.86, P=0.00001). The IHC scores are potentially higher in malignancies in the nervous system (6.0) as compared with benign changes in the nervous system (3.6, P=0.07), and in ovary cancers (4.88) as compared within

Table 3. The expression level (IHC score) of LPAR3 in different organs/tissues in humans

Organs/Tissues	Cytoplasmic Stain (LPAR3C)							Nuclear Stain (LPAR3N)						
	Benign			Malignant			*p* Value	Benign			Malignant			*p* Value
	N	Mean	SD	N	Mean	SD		N	Mean	SD	N	Mean	SD	
Adrenal Gland	5	2.10	0.82	3	1.50	0.00	0.27	5	3.90	2.01	3	5.00	0.87	0.41
Appendix	2	1.50	0.00					2	3.75	1.06				
Artery, Adult	3	1.33	0.29					3	3.00	0.00				
Artery, Umbilical	2	1.50	0.00					2	4.50	0.00				
Bone				3	0.50	0.87					3	2.00	3.46	
Breast	7	1.36	0.94	14	1.93	0.92	0.20	7	3.43	2.07	14	4.61	1.61	0.17
Cervix	5	1.50	0.0000	4	1.50	0.00		5	4.20	1.25	4	4.50	0.00	0.65
Colon	9	1.83	0.66	16	2.06	1.08	0.57	9	5.17	1.52	16	4.13	1.60	0.13
Esophagus	3	2.00	0.87	4	2.63	1.44	0.54	3	4.50	1.50	4	5.63	1.89	0.44
Fallopian Tube	2	1.50	2.12					2	3.00	4.24				
Gallbladder	2	1.50	0.00	2	1.50	0.00	1.00	2	4.50	0.00	2	4.50	2.12	
Immune System	6	0.75	0.82	4	2.25	1.50	0.07	6	4.00	1.82	4	5.25	1.50	0.29
Kidney	3	1.50	0.00	11	1.23	0.61	0.46	3	4.50	0.00	11	3.68	1.94	0.49
Liver	7	1.50	0.00	13	1.85	0.66	.186	7	0.86	1.70	13	4.15	0.66	0.00001
Lung	5	1.50	0.00	7	1.93	0.73	0.23	5	5.40	0.82	7	4.93	1.43	0.52
Myocardia	4	1.50	0.00					4	0.00	0.00				
Laryngopharynx	4	1.88	0.75	10	1.65	0.47	0.51	4	5.63	0.75	10	4.20	1.18	0.05
Neural System	10	1.20	0.63	3	2.00	0.87	0.10	10	3.60	2.02	3	6.00	0.00	0.07
Ovary	4	1.13	0.75	4	2.63	0.75	0.03	4	2.63	1.89	4	4.88	0.75	0.07
Pancreas	8	1.56	0.68	10	1.35	0.34	0.40	8	4.56	1.68	10	4.40	1.37	0.82
Pituitary	2	1.50	0.00					2	6.00	0.00				
Prostate	4	1.13	0.75	14	1.96	1.01	0.14	4	3.38	2.25	14	4.18	1.92	0.49
Oral Gland	7	1.57	0.73	4	0.75	0.87	0.13	7	3.64	2.27	4	3.50	1.22	0.91
Small Intestine	4	3.38	0.75					4	6.38	0.75				
Skin	4	2.25	1.50	7	1.71	0.57	0.41	4	4.88	1.44	7	4.93	1.67	0.96
Skeletal Muscle	4	2.25	1.50					4	4.88	1.44				
Soft Tissue	2	1.50	0.00	7	1.36	1.28	0.88	2	3.75	1.06	7	3.64	2.87	0.96
Stomach	8	1.88	0.69	11	1.64	0.45	0.38	8	4.25	1.69	11	5.32	1.03	0.10
Testis	4	1.88	0.75	3	2.00	0.87	0.85	4	4.50	1.22	3	5.50	0.87	0.29
Thyroid	9	2.33	0.79	4	2.25	0.87	0.87	9	5.33	1.09	4	5.25	1.94	0.92
Urothelium	7	1.64	1.07	11	1.36	0.45	.045	7	4.71	2.51	11	4.23	1.88	0.64
Uterus	7	1.29	0.57	13	1.62	0.74	0.32	7	3.43	1.67	13	4.04	1.77	0.46
Wilm's Tumor				2	3.00	2.12					2	5.25	1.06	

benign cells in ovary (2.63, P=0.07), respectively.

Thus, alteration in expression level of LPAR3 (in nucleus, and/or in cytoplasm) correlates with malignancies in bladder, immune system, ovary, laryngopharynx, liver and nervous system. The representative IHC staining for LPAR3C and LPAR3N are shown in **Figure 4**.

Discussion

The three LPA receptors studied were widely distributed in benign and/or malignant tissues in multiple organs/systems. The LPARs were more highly expressed nucleus than in the cytoplasm, and not detected in cellular membranes. However, each LPAR is unique in its systemic distribution and subcellular localization. The individual LPARs exhibited differences in expression between benign and malignant tissues of the same organs.

Studies to date suggest that LPAR play diverse roles in human cancers [22-24]. Whether activation of LPAR promotes or inhibits the progression of cancers is largely dependent on the specific LPAR as well as the tissue type. While

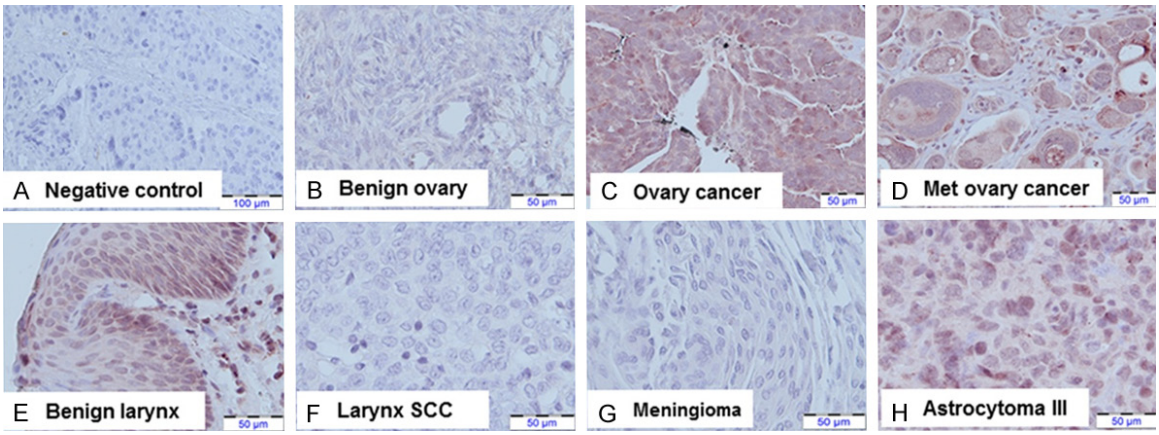

Figure 4. IHC staining for LPAR3 in representative organs/systems. Negative control of IHC staining for LPAR1 in prostate cancer with Gleason's sore of 9 (A); the IHC signal for cytoplasmic LPAR3 (LPAR3C) is not seen in benign ovary stromal (B), but is moderate in ovarian cancer (papillary carcinoma, C) and strong in metastatic ovarian cancer (D); the IHC signal for nuclear LPAR3 (LPAR3N) is moderate to strong in larynx benign squamous cells (E), but not seen in larynx squamous cell carcinoma (SCC, F); and the IHC signal for LPAR3N is not seen in benign brain tumor, meningioma (G), but is moderate to strong in brain astrocytoma III (H).

pancreatic cancer cells are stimulated with lysophosphatidic acid (LPA) and malignant ascites, both LPAR1 and LPAR2 are activated, their migration is induced by activated LPAR1, but inhibited by activated LPAR2 [25]. Such opposing effects on cell migration are also observed between LPAR1 and LPAR3 in hamster pancreatic cancer cells [26], and in rat neuroblastoma cells [27]. The current study further demonstrates a diverse effect of LPAR1, LPAR2, and LPAR3 on human cancers. First, the same LPAR shows opposite effects in different organs/systems: for example, both cytoplasmic and nuclear expressions of LPAR1, LPAR2, and LPAR3 are all up-regulated in malignancies in immune system and neural system, whereas they are all down-regulated in pancreatic malignancy; nuclear expression of LPAR1 is significantly higher in HCC than in benign hepatocytes, but significantly lower in colon adenocarcinoma as compared with benign colon epithelia. Second, different LPARs show opposing effects on malignancy in a given system/organ: for example, cytoplasmic and nuclear expression of LPAR1 is up-regulated, whereas that of LPAR3 is down-regulated in renal cell carcinoma as compared with normal kidney cortex. Third, same LPAR is inversely expressed between different subcellular localizations (cytoplasm and nucleus) in the same malignancy in some cases. It is not clear why a same LPAR varies in the expression level in different organs/tissues/cancers, and why a same malignancy varies in the expression levels of different LPA receptors. Possibly, LPARs are different in metabolic pathways, in affinity to LPA species (with different fatty acid chains), in distribution among organs/tissues, in pathophysiological statuses, and in signaling pathways downstream to each of them.

LPAR belong to a superfamily of G protein-coupled receptors (GPCR), which are also known as seven-transmembrane domain receptors. Thus, it is logical that LPAR should be detected by IHC on the cellular membrane. However, only one study reported that using a monoclonal antibody against epitope within C-terminal of LPAR2, IHC signal of LPAR2 was seen on cellular membrane of colon cancer cells, while it was mostly found in cytoplasm of normal colon epithelium [28]. Our study fails to find IHC signals for LPAR1-3 on the cellular membranes. Instead, we found that IHC signals for LPARs are commonly localized in the nucleus and cytoplasm of benign and malignant cells. This could be a result of the antibodies used in the two studies targeted different epitopes. It could be also due to a function of kinetics: LPARs are rapidly internalized and translocated into the cytoplasm or nucleus upon binding with LPA ligands. This notion is supported with an experiment that indicated approximately 40% of LPAR1 is internalized within 15 minutes after LPA treatment [29].

The anti-LPA antibodies used in this study all targeted epitopes within the N-terminus, which is closer to the extracellular matrix and contains LPA binding sites. The IHC signals for these antibodies appearing in the nucleus implies a possibility that the entirety of the LPA receptor is translocated into the nucleus, rather than just a portion of cleaved C-terminal. Intriguingly, both ligands (LPA) and receptors (LPARs) are biologically active. Whether LPARs function as transporters in transporting lysophospholipids (including LPA) from the matrix into intracellular organelles for de novo synthesis of phospholipids in meeting cancer need, or serve as signal transducers, which are activated by ligands on cell membrane, and internalized into cytoplasm and nucleus, in triggering their downstream pathways critical to the pathogenesis and progress of cancers.

At the organ system level, significant differences in IHC scores between benign and malignant tissues were observed in the digestive system (liver, colon, and pancreas), lung, ovary, bladder, kidney and brain. It is especially noted that the nuclear expression levels of all LPAR1-3 are significantly higher in hepatocellular carcinoma than in benign hepatocytes. Actually, IHC signals of LPAR1-3 are rarely seen in the nuclei of benign hepatocytes. These results are in agreement with the concept that benign liver lacks these LPARs [30-32], and that their expression levels are only elevated in HCC [33, 34]. Thus, down-regulating the expression of LPARs, blocking their binding with ligands, and inhibiting activation of their downstream pathways, as well as decreasing production of ligands are all potential interventions to treat HCC, a rapid progressing lethal disease. Indeed, the knockdown of LPAR can reduce the tumorigenic effects and inhibit metastasis of some cancers [35, 36].

Previous studies on colon cancer cells suggest that elevated expression levels of LPAR1-3 are associated with pathogenesis or progression of colon cancer [14, 15, 36-39]. Except for one study reported by Shida [28], however, other studies lacked information on the expression of studied LPAR in normal colon in parallel. In our study, we found that the expression levels of LPARs are high in human colon adenocarcinoma, but, they are even higher in benign colon epithelia. Thus, the background level of LPA receptors in benign tissues needs to be evaluated in carcinogenesis, tumor progression, metastasis, and in tumor treatment.

The concentration of LPA was elevated in the blood and ascites of patients with aggressive ovarian cancer [40]. Among LPAR, LPAR2 is most relevant to the pathogenesis of ovarian cancer [10, 12, 41-43]. However, studies on LPAR in ovary cancer were mostly carried out in cell lines and animal models, without evaluation of expression levels of these receptors in benign ovary tissues. Our study shows that nuclear expressed LPAR2 is even lower in ovarian cancer than in benign ovarian tissues, and the expression level of LPAR1 is not significantly higher in malignant than in benign ovary tissues. However, the expression level of LPAR3 is higher in ovarian cancer: the IHC score for LPAR3C is 2.63 in ovarian cancer, which is significantly higher than that in benign ovary tissues (1.13, P=0.03); and that for LPAR3N is 4.88 in ovarian cancer, which is potentially higher than that in benign ovary tissues (2.63, P=0.07). These results imply that LPAR3, rather than LPAR2 or LPAR1 perhaps plays a more important role in the pathogenesis of ovarian cancer. These results also remind an importance of studying different LPA receptors in parallel for their roles in tumorigenesis.

Previous studies suggested that elevated expression of LPA receptors were related to malignancies in stomach, breast and prostate [44-46]. In our study, the expression levels of all three LPA receptors (both cytoplasmic and nuclear) were very close in benign and malignant tissues of stomach, breast, and prostate. The correlation of LPA receptors with pathogenesis in these cancers should be further evaluated in large sample sets and with other experimental methods.

Limitations of this study include: small sample size for each organ system, heterogeneity of analyzed samples in benign (different pathological conditions) and in malignant (different cellular origins or pathological stages) tissues, IHC as sole experimental method, and no functional studies for each LPAR. Nevertheless, we must first perform IHC for LPAR1, LPAR2 and LPAR3 simultaneously on benign and malignant tissues of major human organs/systems to illustrate their systemic distribution, subcellular localization and differences in the expression levels between benign and malignant

changes in most human systems/organs. Such information will be valuable as we continue to study the roles of LPA/LPARs in various cancers.

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Disclosure of conflict of interest

None.

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