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THE EXPRESSION LEVEL OF LYSPHOSPHATIDYLCHOLINE ACYLTRANSFERASE 1 (LPCAT1) CORRELATES TO THE PROGRESSION OF PROSTATE CANCER

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Introduction

It is estimated that there will be 217,730 new cases of prostate cancer and 32,050 deaths in 2010 (1), ranking prostate cancer as the most frequently diagnosed cancer and the second leading cause of cancer death in the United States. Important issues in care of patients with prostate cancer are how to screen and diagnose prostate cancer earlier, and how to determine a therapeutic strategy based on outcome prediction. Biomarkers could play a pivotal role in accomplishing these tasks. However, none of the currently used biomarkers for prostate cancer, including PSA, is satisfactory. Therefore, it is important in seeking new biomarkers that are able to reflect biological activities and are able to be used in diagnosis and prognosis of prostate cancer.

Phosphatidylcholine (PC) accounts for 40–60% of the phospholipids of eukaryotic membranes and plays important roles in cellular structure and biological functions (2). PC, like other phospholipids, is synthesized *de novo* in tumor tissue through the Kennedy pathway (3). PC's fatty acyl composition at the *sn*-2-position is then altered in a remodeling pathway, known as Lands' cycle (4), through which diverse individual PC species differing in the length of carbon chain and the degree of saturation of fatty acid are produced (5–7). Therefore, the enzymes controlling these biochemical processes might greatly affect biological activities of prostate cancer.

Lysophosphatidylcholine transferase 1 (LPCAT1) is a key enzyme for remodeling phospholipids, including phosphatidylcholine (PC), in Lands' cycle which was proposed 50 years ago (4). Until recently, LPCAT1 was cloned and characterized by two independent research groups in mouse alveolar type II cells demonstrating a preference of lysophosphatidylcholine (LPC) as its substrate/recipient and palmitoyl-CoA as an acyl donor in production of dipalmitoyl phosphatidylcholine (DPPC), the major phosphatidylcholine in pulmonary surfactant (8–9). A human homolog of mouse LPCAT1, 1-acylglycerol-3-phosphate-O-acyltransferase isoform 9 (AGPAT9) was cloned and characterized by Agarwal et al. AGPAT9 was demonstrated to be able to catalyze the synthesis of phosphatidic acid

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(PA), however, it failed to show LPCAT activity (10). Subsequently, human LPCAT1 was reported to exhibit activities of both lysophosphatidylcholine acyltransferase (LPCAT) and lysophosphatidylglycerol acyltransferase (LPGAT) by Harayama et al (11). In addition to its significance in production of DPPC (8–9, 12) and in retinal photoreceptor homeostasis (13–14), LPCAT1 was found to be overexpressed in colorectal adenocarcinomas as compared to normal rectal mucosa and shown a weak immunohistochemical signal in prostate cancer (15). However, this study was limited in sample size, lacked a statistical analysis, and did not correlate the expression level of LPCAT1 to the existing indicators of prostate cancer prediction.

To reveal the association of LPCAT1 with the progression of prostate cancer and to demonstrate diagnostic and prognostic values of LPCAT1 for prostate cancer, the expression level of LPCAT1 is determined by immunohistochemistry (IHC) on tissue microarray (TMA) slides and correlated with various prostatic disorders including prostate cancer.

Patients and Methods

Patients, specimen and data collection

The protocol was approved by the University of Mississippi Medical Center Institutional Review Board. Clinical information and pathology reports were retrieved from the University database. This study included 148 patients who had undergone prostatectomy, transurethral resection of the prostate, or resection of tissue with metastatic prostate cancer at the University of Mississippi Medical Center from 1992 to 2010. Ninety-two out of 148 patients were African American with a mean age of 60.4 ± 7.9 ; fifty-six were Caucasian with a mean age of 62.2 ± 8.8 .

In formalin-fixed and paraffin-embedded (FFPE) surgical specimens from 148 patients, 251 sites were selected including: 39 with benign prostatic changes, 37 with high grade prostatic intraepithelial neoplasia (HGPIN), 154 with primary prostate cancers and 21 with metastatic prostate cancers.

Out of 148 patients, 134 patients had well- documented information for the stage of prostate cancer. Sixty-nine patients had organ-confined prostate cancer staged as stage I or stage II (TMN system) or stage A or stage B (Whitmore-Jewett stage). Sixty-five patients had non organ-confined prostate cancer, which had broken the prostate capsule, invaded into adjacent tissues or organs, or metastasized to lymph nodes or other organs, and staged as stage III or stage IV (TMN system) or stage C or stage D (Whitmore-Jewett stage).

In addition, out of 148 patients, 23 patients were demonstrated to have biochemical recurrence defined as two consecutive rising PSA values more than 0.4 ng/ml at least 6 months after prostatectomy; 5 patients to have clinical metastases after prostatectomy. In the study cohort, each of these 28 patients were matched to a patient, who did not have biochemical recurrence and clinical metastasis during equal or longer observation time after prostatectomy, had the same race and Gleason grade, and had similar age, PSA value and stage at the time of diagnosis.

Tissue microarray and immunohistochemistry (IHC)

After histological features in hematoxylin and eosin stained slides were re-confirmed by two researchers including one pathologist, they were topographically correlated with the corresponding paraffin blocks. A 2 mm cylindrical core from each selected site of the primary FFPE block was transferred to the composite paraffin blocks to construct TMA blocks by using a Beecher MTA1 Manual Tissue Arrayer. The resulting TMA blocks were then sectioned at 5 μ m in thickness for IHC study. The primary anti human LPCAT1

antibody was a rabbit anti-human IgG polyclonal antibody (16112-1-AP), purchased from Protein Tech Group, Inc. (Chicago, IL). ABC kit (VECTASTAIN® Elite PK-6101) and substrate kit VECTOR® NovaRed (SK-4800) were purchased from Vector Laboratories (Burlingame, CA). IHC stain protocols followed manufacturer instructions. Briefly, 5- μ m thick tissue microarray sections were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide solution (Sigma-Aldrich, St. Louis, MO) for 20 min. Antigens were retrieved by Citrate plus antigen retrieval solution (BioGenex, San Ramon, CA) with a high pressure cooker for 30 min. After slides were blocked by normal serum (provided with ABC kit), 1:100 diluted primary antibodies (anti-human LPCAT1, 16112-1-AP; stock solution concentration: 37 μ g/150 μ l) were added and incubated at 4°C over night. Biotinylated secondary antibody (one drop/10 ml diluted block serum) was then added for 30 min followed by avidin-biotin-peroxidase complex for 30 min. The chromogen substrate, NovaRed, was added for 3 min to produce a pink-red visible reaction product. The slides were finally counter-stained with hematoxylin.

IHC scoring system

All slides stained with LPCAT1 were reviewed jointly by two of the authors, followed by a second, independent review by another pathologist. LPCAT1 IHC stains were only analyzed in benign and cancerous prostatic glandular cells. The scoring system included the extent and intensity of staining. The score for the extent of the IHC stained area was scaled as 0 for no IHC signal at all, 1 for <10%, 2 for 10–50%, and 3 for >50% of tumor cells stained. The score for IHC intensity was also scaled as 0 for no IHC signal, 1 for weak, 2 for moderate, and 3 for strong IHC signals. The final score used in the analysis was calculated by multiplying the extent score and intensity score, with a maximum score of 9.

Statistical analysis

SPSS 18 software was employed for statistical analysis. One way ANOVA was used in the analysis of intergroup difference in LPCAT1 IHC mean score. T-Test was used in the analysis of difference in the mean score between any two groups. Bivariate correlation and linear regression were used in the correlation of the mean IHC score to PSA value at the time of diagnosis of prostate cancer. Significant p value was set at <0.05.

Results

Expression level of LPCAT1 in the diagnosis of prostate cancer

Morphologically, LPCAT1 IHC signals were granular and localized in the cytoplasm. The extent and intensity of LPCAT1 IHC signals showed an ascending pattern along with the evolution of prostate cancer. Staining was rare in benign prostatic tissues, scattered with weak to moderate intensity in PIN, diffuse with weak intensity in primary prostate cancer with a Gleason score <7, diffuse with moderate intensity in primary prostate cancer with a Gleason score of 7, diffuse with strong intensity in primary prostate cancer with a Gleason score >7 (especially in the cancer cells with prominent nucleoli), and diffuse with strong, sometimes clumped or condensed staining in metastatic prostate cancer, especially the metastatic cancer cells with mitotic figures (Figure 1). In addition, IHC signals were also present in some nuclei of high grade primary prostate cancer and metastatic prostate cancer (Fig 1F-1I). ANOVA analysis showed a significant difference in LPCAT1 IHC mean scores among the groups (benign prostate, HGPIN, primary and metastatic prostate cancer; $F=21.66$, $p=1.55E-12$). Further analysis of the differences in LPCAT1 IHC mean scores between any two groups indicated that metastatic prostate cancer had the highest LPCAT1 IHC mean score (8.00), which was dramatically higher than that in primary prostate cancer (4.63, $p=9.73E-07$), HGPIN (2.72, $p=1.02E-12$) and benign prostatic tissues (2.68, $p=6.17E-12$). Similarly, the LPCAT1 IHC mean score in primary prostate cancer was

significantly higher than that in PIN and benign prostatic tissues ($p=0.000274$, $p=0.000409$, respectively). There was no statistical significance in LPCAT1 IHC mean score between HGPIN and benign prostatic tissues (Table 1). To correlate LPCAT1 IHC mean score to tumor grade, primary prostate cancer was further stratified by the Gleason score into three subgroups (Gleason score <7 , $=7$ and >7 , respectively). One way ANOVA analysis indicated a significant difference in LPCAT1 IHC mean score among these subgroups ($F = 7.442$, $p = 8.27E-04$). The LPCAT1 IHC mean score in the subgroup with high grade primary prostate cancer (Gleason score > 7) was 5.57, which was significantly higher than that in the subgroup with a Gleason score of 7 (3.91, $p=0.00105$) and the subgroup with a Gleason score <7 (3.68, $p=0.00399$). There was no statistical difference in LPCAT1 IHC mean score between the subgroups with a Gleason score of 7 and with a Gleason score < 7 (Table 2). These results demonstrated that the expression level of LPCAT1 is able to differentiate prostate cancer from noncancerous prostatic changes, and correlates to the tumor grade of prostate cancer.

The expression level of LPCAT1 in association with the pathologic stages of prostate cancer

To demonstrate the association of the expression level of LPCAT1 with the stages of prostate cancer, the LPCAT1 IHC mean score in non organ-confined prostate cancers was compared with that in organ-confined prostate cancer. Non organ-confined prostate cancer had a significantly higher LPCAT1 IHC mean score than organ-confined prostate cancer (6.55 ± 2.54 in non organ-confined vs. 3.89 ± 2.99 in organ-confined, $p < 0.0001$). In addition, patients with non organ-confined prostate cancer were significantly older, and had higher serum PSA levels and tumor grade at the time the specimens were obtained (Table 3). Thus, the expression level of LPCAT1 is consistent with existing tumor staging systems, which may be useful in predicting the prognosis of prostate cancer.

The influence of existing prognostic indicators of prostate cancer on the expression level of LPCAT1

It is well known that African American race, advanced age, and high PSA level at the time of diagnosis as well as positive surgical resection margins in radical prostatectomy are indicators for an unfavorable prognosis of prostate cancer. In order to determine the influence of these existing indicators on the expression level of LPCAT1, LPCAT1 IHC scores were compared between different groups of patients according to race (African American and Caucasian), age (≤ 60 year old or > 60 year old), positivity of surgical resection margins (positive or negative), and PSA level at the time of diagnosis of prostate cancer. The results indicated that there was no statistical difference in LPCAT1 IHC score between African American and Caucasian patients, younger and older, and with positive and negative surgical resection margins (Figure 2). Also, there was no correlation between the expression level of LPCAT1 and PSA level at time of diagnosis ($y = 1.00031x + 3.478$, where y is the LPCAT1 IHC score and x is the PSA value, $R^2 = 0.026$, $p > 0.05$, figure not shown). These results suggest that the association of the LPCAT1 expression level with grade, stage and later outcomes is independent of existing prognostic indicators of prostate cancer.

The expression level of LPCAT1 in prognosis of biochemical recurrence and metastasis of prostate cancer

To reveal the correlation of the expression level of LPCAT1 at the time of prostatectomy with future biochemical recurrence or metastasis of prostate cancer, LPCAT1 IHC mean scores were compared between two pair-matched groups. One group contained prostatectomy specimens from patients with primary prostate cancer who experienced biochemical recurrence and/or metastasis during follow-up (Recurrence/metastasis). Another group included prostatectomy specimens from patients with primary prostate cancer

who did not experience biochemical recurrence and metastasis in the same or longer follow-up period (No recurrence/metastasis). The LPCAT1 IHC mean score was significantly higher in the initial prostatectomy specimens in the Recurrence/metastasis group than that in the No recurrence/metastasis group (5.96 ± 2.4 vs. 2.64 ± 2.46 , $p=0.00000409$, Figure 3). These results suggest that the expression level of LPCAT1 in initial specimens of primary prostate cancer might be used as an indicator of future biochemical recurrence and/or metastasis.

Discussion

Using TMA and IHC technologies, this study demonstrates that the expression level of LPCAT1 differentiates prostate cancer from benign prostatic changes, correlates to the grade and stage of prostate cancer, and may be predictive for future biochemical recurrence or metastasis after prostatectomy for primary prostate cancer. Its utility in diagnosis and prognosis of prostate cancer is independent of existing prognostic indicators such as patient race and age, PSA level at the time of diagnosis, and positivity of surgical resection margins at the time of prostatectomy for cancer. Therefore, LPCAT1 could serve as a new and an independent biomarker in the diagnosis and prognosis of prostate cancer.

Prior to cloning and characterization of LPCAT1, increased enzymatic activity of LPC acyltransferase had been demonstrated in animal and human prostate cancer (16–17), which was of little help for understanding the mechanism of the expression level of LPCAT1 in association with the progression of prostate cancer. Based on overexpression of LPCAT1 in human colorectal carcinomas, Mansilla et al suggested that LPCAT1 may contribute to total choline metabolite accumulation via phosphatidylcholine remodeling (15). Whether this occurs in prostate cancer requires further studies. Previous studies showed that PC was decreased in the plasma from patients with prostate cancer (18) and in prostate cancer tissues in a transgenic mouse model (19). In the study of lipid profiles in prostatic tissues, we found that all of the lysophosphatidylcholine (LPC) molecules, the preferable substrates of LPCAT1 (8–9), dramatically increased in prostate cancer tissues as compared to benign prostatic tissues, which makes the reaction catalyzed by LPCAT1 favorable towards the production of PC. However, the major PC species like PC34:1 and PC34:2 that constitute 99% of total PC did not increase in the prostate cancer tissues. Instead, a few minor PC species, such as PC28:0, PC32:2 and PC36:6 that constitute less than 1% of total PC were significantly increased in prostate cancer tissues as compared to benign prostatic tissues (data not shown). Thus, we propose that the possible mechanism of LPCAT1 in association with the progression of prostate cancer is through controlling production of some special minor PC species. This may have an irreplaceable function such as epigenetic regulation in the evolution of prostate cancer rather than through influencing synthesis of major structural lipids common to both benign and malignant prostatic cells. Recently, it has been observed that cytosolic LPCAT1 is able to shift into the nucleus in lung epithelia in response to exogenous Ca^{2+} . Nuclear Lpcat1 colocalizes with and binds to histone H4, where LPCAT1 catalyzes histone H4 palmitoylation, which further regulates mRNA synthesis (20). Whether nuclear localization of LPCAT1 in high grade primary and metastatic prostate cancer in our study is also associated with the progression of prostate cancer in such a way requires further study. PC production and conversion with LPC are also influenced by other enzymes, such as phospholipase A2 (different types), choline-phosphate cytidylyltransferase in other metabolic pathways.

Over the past decades, numerous studies have shown that African American patients have an earlier onset of prostate cancer and worse outcomes than do Caucasians (21–22). Older patients had a higher chance of being diagnosed with more advanced prostate cancer with worse outcomes (23–24). Positivity of surgical resection margins and high levels of PSA at

the time of diagnosis have been associated with worse outcomes following prostatectomy (25–27). All these existing predictive indicators are actually confounding or risk factors; however, they may not represent biological behaviors of prostate cancer. Therefore none of them has been used as an independent indicator in the diagnosis and prognosis of prostate cancer. PSA, for example, is a normal component in prostate glandular secretions, and itself is not a character of prostate cancer. Actually, prostate cancer cells have a decreased ability to secrete PSA as compared to normal glandular cells (28). This may explain why elevated PSA levels are unable to differentiate prostate cancer from benign hyperplasia and inflammation. Positive surgical resection margins can be due not only to tumor location, grade, and stage, but also to the surgeon's expertise (29). In this study, the association of the expression level of LPCAT1 with the progression of prostate cancer was not influenced by these existing confounding factors. This may be explained because up-regulated LPCAT1 is solely related to growth of prostate cancer, but not to the confounding factors and benign prostatic changes.

In summary, demonstrating the association of the level of LPCAT1 expression with prostate cancer progression in this study suggests that LPCAT1 might be a new and independent biomarker in the diagnosis, prognosis and investigation of the pathogenesis of prostate cancer. Future expanded investigations with greater numbers of study subjects are required to confirm our findings.

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Research Highlights

LPCAT1 IHC signals were found in cytoplasm and nuclei of prostate cancer cells

LPCAT1 correlates to the progression of prostate cancer

LPCAT1 predicts outcomes of prostate cancer after prostatectomy

Figure 1.

LPCAT1 IHC stain. A: negative control for LPCAT1 IHC stain showing no IHC signal in a primary prostate cancer with a Gleason score of 6, 20×; B: rare LPCAT1 IHC signals in benign prostate, 20×; C: scattered cytoplasmic LPCAT1 IHC signals in a high grade prostatic intraepithelial neoplasia (HGPIN), 20×; D: weak and diffuse cytoplasmic LPCAT1 IHC signals in a primary prostate cancer with a Gleason score of 5, 20×; E: moderate and diffuse cytoplasmic LPCAT1 IHC signals in a primary prostate cancer with a Gleason score of 7, 20×; F: strong and diffuse cytoplasmic LPCAT1 IHC signals in a primary prostate cancer with a Gleason score of 10, nuclei with prominent nucleoli unstained, 20×; G: strong and diffuse cytoplasmic (also some nuclear) LPCAT1 IHC signals in a metastatic prostate cancer in spinal dura, condensed IHC stain around cancer cell nuclei with mitosis, 20×; H: strong and diffuse cytoplasmic and nuclear LPCAT1 IHC signals in a bone metastatic prostate cancer, 20×; I: strong and diffuse cytoplasmic and nuclear LPCAT1 IHC signals in a bone metastatic prostate cancer, 20×.

Figure 2.
Comparison of LPCAT1 IHC score in primary prostate cancer between different groups of age, race and surgical resection margin

Figure 3.
Comparison of LPCAT1 IHC score between patients with and without biochemical recurrence metastasis of prostate cancer after prostatectomy

Table 1

Comparison of LPCAT1 IHC scores among different pathological categories (2-tailed p value)

Category	<u>Benign Prostate</u>	<u>PIN</u>	<u>Primary Pca</u>	<u>Metastatic Pca</u>
	n/Mean±SD 39/2.68±2.67	n/Mean±SD 37/2.72±2.47	n/Mean±SD 154/4.63±3.00	n/Mean±SD 21/8.00±1.28
Benign Prostate	–	0.951	2.74E-04	6.17E-12
PIN		–	4.09E-04	1.02E-12
Primary Pca			–	9.73E-07
Metastatic Pca				–

Table 2

Comparison of LPCAT1 IHC scores between subgroups of in primary prostate cancer (2-tailed p value)

Subgroup	Gleason score<7	Gleason score=7	Gleason score>7
	n/Mean±SD 46/3.68±3.19	n/Mean±SD 35/3.91±2.50	n/Mean±SD 73/5.57±2.83
Gleason score<7	–	0.727	1.05E-03
Gleason score=7		–	3.99E-03
Gleason score>7			–

Table 3

Comparison of LPCAT1 IHC scores between organ-confined and non organ-confined prostate cancer

	Organ-confined Prostate cancer	Non Organ-confined Prostate cancer	p Value
Total numbers	69	65	>0.05
Age (Mean±SD)	58.6±6.35	63.4±9.37	0.0005
Black/White	47/22	39/26	>0.05
Serum PSA (median, ng/ml)	7	18	<0.0001
Gleason score			<0.0001
≤7	46	13	
>7 and Metastasis	23	52	
LPCAT1 IHC score (Mean±SD)	3.89±2.99	6.55±2.54	<0.0001