RESEARCH ARTICLE

Prostate Cancer Associated Lipid Signatures in Serum Studied by ESI-Tandem Mass Spectrometry as Potential New Biomarkers

Divya Duscharla1,2, Sudarshana Reddy Bhumireddy2,3, Sridhar Lakshetti3, Heike Pospisil4, P. V. L. N. Murthy5, Reinhard Walther6, Prabhakar Sripadi2,3, Ramesh Ummanni1,2*

1 Center for Chemical Biology, CSIR-Indian Institute of Chemical Technology (CSIR-IICT), Hyderabad, India, 2 Centre for Academy of Scientific & Innovative Research, CSIR-Indian Institute of Chemical Technology (CSIR-IICT), Hyderabad, India, 3 National Centre for Mass Spectrometry, CSIR-Indian Institute of Chemical Technology (CSIR-IICT), Hyderabad, India, 4 High Performance Computing in Life Sciences, Technical University, Wildau, Germany, 5 Department of Urology, Nizam’s Institute of Medical Sciences (NIMS), Hyderabad, India, 6 Department of Medical Biochemistry and Molecular Biology, University of Greifswald, Greifswald, Germany

* ummanni@iict.res.in

Abstract

Prostate cancer (PCa) is one amongst the most common cancers in western men. Incidence rate of PCa is on the rise worldwide. The present study deals with the serum lipidome profiling of patients diagnosed with PCa to identify potential new biomarkers. We employed ESI-MS/MS and GC-MS for identification of significantly altered lipids in cancer patient’s serum compared to controls. Lipidomic data revealed 24 lipids are significantly altered in cancer patient’s serum (n = 18) compared to normal (n = 18) with no history of PCa. By using hierarchical clustering and principal component analysis (PCA) we could clearly separate cancer patients from control group. Correlation and partition analysis along with Formal Concept Analysis (FCA) have identified that PC (39:6) and FA (22:3) could classify samples with higher certainty. Both the lipids, PC (39:6) and FA (22:3) could influence the cataloging of patients with 100% sensitivity (all 18 control samples are classified correctly) and 77.7% specificity (of 18 tumor samples 4 samples are misclassified) with $p$-value of $1.612 \times 10^{-6}$ in Fischer’s exact test. Further, we performed GC-MS to denote fatty acids altered in PCa patients and found that alpha-linolenic acid (ALA) levels are altered in PCa. We also performed an in vitro proliferation assay to determine the effect of ALA in survival of classical human PCa cell lines LNCaP and PC3. We hereby report that the altered lipids PC (39:6) and FA (22:3) offer a new set of biomarkers in addition to the existing diagnostic tests that could significantly improve sensitivity and specificity in PCa diagnosis.
Introduction

Despite the significant advances achieved in diagnosis and treatment of PCa, still it is the second most cause of cancer-related deaths, next to lung cancer among men [1]. Autopsy investigations have revealed that 30% of men above 50 years and 80% of men above 70 years have evidence for occurrence PCa [2]. Early diagnosis and aggressive treatment is the only option to cure PCa. Biomarkers play an important and decisive role in early diagnosis of PCa. Till date, screening of for PCa involves the digital rectal examination (DRE) and the prostate specific antigen (PSA) blood test. However, these two tests currently in use for PCa diagnosis are sub-optimal because PSA is abundantly produced by prostatic epithelium and also secreted by the epithelium of peri-urethral glands. PSA expression is not specific to tissue or gender, it will be secreted by both benign and cancer cells of prostate [3]. Advanced "Omics" technologies have identified altered genome, transcriptome and proteome related to PCa. These studies have provided a number of potential genomic and proteomic biomarkers for diagnostic purposes. However, none of these markers are translated into routine diagnostic and/or prognostic applications. Consequently, there are innumerable chances for either over diagnosis of patients with limited potential for cancer or under diagnosis of patients already suffering from the disease. Thus lack of current diagnostic methods for prostate diseases underscores the need for improvement in this area.

Diagnosing cancers based on serum profiling is an attractive concept. A number of studies have employed proteomic and genomic analysis of serum samples from cancer patients. Only sparse information is available on metabolome alterations particularly lipid composition in serum/plasma associated with PCa. Abnormal lipid metabolism has been shown to be associated with many diseases such as inflammation, diabetes, renal and heart failures as well as many cancers; thus indicating that lipid metabolites could be used as disease biomarkers [4]. Unfortunately, to the best of our knowledge, only a few reports have been found analyzing lipids from PCa patients associated with disease progression. One study reported that apolipoprotein and cholesterol together diagnosed ovarian cancer with 97% accuracy [5]. In colorectal cancer, altered linoleic acid (LA), alpha-linolenic acid (ALA), arachidonic and oleic acids were shown to be associated with cancer progression [6]. However, in these studies only a few classes of lipids were analyzed due to the technical limitations. Two recent studies have reported that the phospholipid alterations are associated with PCa [7,8]. Particularly cancer progression depends on the proliferation and invasion of tumor cells to distal organs. Tumor cells undergo functional and morphological alterations, many of which start with cell membrane releasing their components into blood stream. Lipidomics approach has been applied to identify biomarkers and study role of lipids in disease progression of many metabolic disorders such as obesity [9], atherosclerosis [10], hypertension [11], diabetes [12], cystic fibrosis [13] and cancers [14]. Recently Patel et al. have reported that a three lipid signature (phospholipids) can distinguish PCa patients from normal individuals [8]. Therefore, we sought to investigate complete lipid profiles covering various classes of lipids including fatty acids, TGs, DGs and phospholipids in serum from PCa patients. These lipid signatures might be used for screening of PCa patients along with existing diagnostic tests with better specificity and sensitivity. Furthermore, we believe that lipidomic approach will identify lipids and their associated pathways, which may play a role in PCa initiation and progression.

Materials and Methods

Chemicals

Internal standards (heptadecanoic acid and methyl heptadecanoate) as well as saturated and unsaturated fatty acids used in the study were purchased from Sigma-Aldrich (St. Louis, MO,
Analytical grade solvents were used to prepare the stock solutions of fatty acids. Ethanol was purchased from Commercial Alcohols, Canada. Methanol used in the ESI-MS analyses was purchased from MERCK, Mumbai, India. Diazomethane in ether is used for esterification (methylation) of fatty acids by known method.

Clinical Samples and Ethics Statement

The serum samples and subsequent pathological data were collected after obtaining patient’s consent by filling the consent forms which were prepared and approved by the institutional ethics committee. The institutional ethics and bio safety committee of the Nizam’s Institute of Medical Sciences (NIMS) Hospital, Hyderabad, India approved the present study. For lipidomics, blood samples were collected from patients with high serum PSA values and pathological examination prior to any chemotherapy or surgery. Serum samples separated from whole blood were stored at -80°C until total lipids were isolated using solvent extraction procedure.

Selection of patients and sample collection

For the present study serum samples were collected from 18 patients with elevated PSA levels diagnosed with PCa from NIMS hospital sample archive for research purposes. All patients selected for this study did not undergo any treatment and/or surgery before collecting the blood samples. For confirmation of PCa, the diagnosis for each patient was established by histopathology of prostate biopsies. Each patient’s information including their age, serum PSA value and pathological diagnosis such as tumor grade and Gleason score is provided in Table 1. For the control group, 18 serum samples from male controls were obtained from institute dispensary where patients had their routine check for wellness or for diagnosis of other diseases. We had set a criteria including age matching with no history of the diagnosis of PCa for collecting these control samples. The serum samples were collected from both tumor and control group individuals in the same manner. From each individual, 5 ml of whole blood was collected in a vacutainer tube containing Trisodium citrate. The tubes were allowed to stand for 10 min for clotting and then centrifuged at 3000 RPM to collect serum. The clear supernatant from surface was collected and stored as separate aliquots at -80°C until use.

Isolation/extraction of total lipids from serum samples

Total lipids from prostate cancer and normal serum samples were extracted as reported previously by Bligh and Dyer with minor modifications [15]. Briefly, lipids were extracted from serum samples by substituting chloroform with dichloromethane (DCM)[16, 17]. A 30 μl aliquot of serum was spiked with an internal standard (1.5 μl of 50 μM methyl heptadecanoate for positive ion ESI-MS analysis or heptadecaanoic acid for negative ion ESI-MS analysis), and added with 190 μl of MeOH. The mixture was vortexed for 30 Sec and then 380 μl of DCM was added to the mixture and again vortexed for 30 Sec. Finally, to enhance the separation efficiency of two phases, 120 μl of water was added and mixed by vortexing for 15 Sec. Then the mixture was allowed to equilibrate at room temperature for 10 min and subjected to centrifugation at 8000g for 10 min at 10°C for separation of phases. The upper layer was carefully removed. The lower lipid-rich DCM layer was collected into a separate 1.5 ml micro tube and the solvent was evaporated in centrivap under vacuum at 4°C. Finally, the dried lipid extracts were reconstituted in 100 μl of buffer (ACN/IPA/H2O in 65:30:5 v/v/v) before subjecting to direct ESI-MS analysis. For GC-MS analysis the dried lipid extract were further subjected to methylation using diazomethane.
Electrospray Ionization Mass Spectrometry (ESI-MS) analysis

The experiments were performed using a quadrupole time-of-flight mass spectrometer (QSTAR XL, Applied Biosystems/MDS Sciex, Foster City, CA, USA) equipped with an ESI source, acquiring data using Analyst QS software (Applied Biosystems). All the samples were introduced into the source by flow injection (10 μl loop) using methanol as the mobile phase at a flow rate of 0.03 ml/min. The samples were analyzed under positive and negative ESI conditions. The typical positive ion ESI conditions were: capillary voltage, +5 kV; declustering potentials (DP1), 60 V; DP2, 10 V; focusing potential, 250 V. The typical negative ion ESI conditions were: capillary voltage, -4.5 kV; DP1, 60 V; DP2, 10 V; focusing potential, 250 V. Full scan mass spectra were recorded over the mass range of \( m/z \) 50–2000 using a time-of-flight (TOF) analyzer at a resolution of 10,000 Full Width Half Maximum. Nitrogen was used as the curtain gas and the collision gas, whereas air was used as the nebulizer. For structure identification, collision-induced dissociation (CID) spectra were recorded by selecting the precursor ion of interest using the quadrupole, allowing them to fragment in the collision cell, and separating the product ions by the TOF analyzer. The collision energies used were between 5 to 25 eV. The experimental conditions used for samples are as same as for reference standards. All the spectra reported were averages of 25 to 30 scans. Elemental compositions of all the precursor ions as well as product ions were obtained from the accurate mass values using the Analyst software.

GC-EIMS analysis

GC-EIMS analyses were performed using an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a 5973N mass selective detector (MSD). HP-5MS capillary column (30 m x 250 μm, i.d. 0.25 μm of film thickness) was used for the chromatographic separation of the fatty acid methyl ester (FAME) derivatives. 1 μl of the sample aliquot was injected into the GC-MS instrument in the splitless type of injection mode. The GC oven was

| S.No | ID   | Pre/Post | Age | Weight | PSA | Gleason Score | Metastasis |
|------|------|----------|-----|--------|-----|---------------|------------|
| 1    | Tumor-1 | Pre     | 60  | 58     | 100 | 3+3 = 6      | No         |
| 2    | Tumor-2 | Pre     | 75  | 47     | 100 | 4+5 = 9      | Yes        |
| 3    | Tumor-3 | Pre     | 65  | 70     | 31.2| 4a+4b = 8    | Yes        |
| 4    | Tumor-4 | Pre     | 60  | 50     | 89.5| 3+4 = 7      | No         |
| 5    | Tumor-5 | Pre     | 65  | 72     | 32  | 3+3 = 6      | No         |
| 6    | Tumor-6 | Pre     | 75  | 70     | 114.6| 3+4 = 7     | Yes        |
| 7    | Tumor-7 | Pre     | 75  | 75     | 13.2| 3+4 = 7      | No         |
| 8    | Tumor-8 | Pre     | 68  | 75     | 44.1| 3+3 = 6      | No         |
| 9    | Tumor-9 | Pre     | 55  | 65     | 155.4| 3+4 = 7     | Yes        |
| 10   | Tumor-10| Pre    | 61  | 72     | 0.1 | 5+4 = 9      | No         |
| 11   | Tumor-11| Pre    | 78  | 50     | 6   | 4+3 = 7      | No         |
| 12   | Tumor-12| Pre    | NA  | NA     | 6   | 3+3 = 6      | No         |
| 13   | Tumor-13| Pre    | NA  | NA     | 20  | 3+4 = 7      | No         |
| 14   | Tumor-14| Pre    | NA  | NA     | 28  | 5+4 = 9      | Yes        |
| 15   | Tumor-15| Pre    | NA  | NA     | 8   | NA           | No         |
| 16   | Tumor-16| Pre    | 64  | NA     | 12  | 5+4 = 9      | Yes        |
| 17   | Tumor-17| Pre    | 60  | 75     | 0.01| 5+4 = 9      | No         |
| 18   | Tumor-18| Pre    | NA  | 56     | 21  | 3+3 = 6      | No         |

doi:10.1371/journal.pone.0150253.t001
programmed to increase from 50°C to 280°C at a rate of 10°C/min ramp and initial and final temperatures were held for 2 min and 5 min respectively. Total run time was 30 min. Helium was used as the carrier gas at a constant flow rate of 1 ml/min. The inlet and GC-MS interface temperatures were kept at 250°C and 280°C respectively. EI source and quadrupole analyzer were kept at 230°C and 150°C respectively. Data processing was done using MSD ChemStation software (Agilent Technologies, USA). The mass spectrometer was operated in the both full scan and selected ion monitoring (SIM) modes. The mass spectrometer was scanned from m/z 29 to 600 in full scan mode of analysis.

Methylation of fatty acids for GC-EIMS analysis

Extraction method of total lipids from serum sample was same as discussed above except the last reconstitution step. For GC-MS analysis, dried serum lipid extracts were directly subjected to methylation using diazomethane reagent, by which the non-volatile fatty acids present in the serum samples were converted into volatile FAMEs. The sample vials containing dried lipid extracts were added with 1 ml of freshly prepared diazomethane in ether solution. The vials were quickly vortexed for 10 sec and left at room temperature for 10 min. The samples were concentrated to 50 μl using ScanVac. The standard fatty acids in ethanol (0.3 ml of 5 μM) subjected to methylation using the above mentioned procedure after evaporating ethanol. For identification of compounds, the same experimental conditions were maintained for the samples and reference standards.

Data base search

The accurate mass values of all the detected peaks (m/z) in both positive and negative modes of ESI-MS analysis of extracts were taken into account, and they were searched in the metabolite databases with a mass tolerance of 5–10 ppm. The databases include Lipid Maps, Metlin (http://metlin.scripps.edu/index.php) and the Human Metabolome Database (HMDB) (http://www.hmdb.ca/spectra/ms/search) [18]. The database hits (with less ppm values) together with isotope distribution patterns were used for peak identification. Some of the critical metabolites were further confirmed by MS/MS analysis. The MS/MS spectra were compared with the data available in the literature as well as the American Oil Chemists Society (AOCS) lipid library (http://lipidlibrary.aocs.org/ms/ms16/index.htm) [7]. A few lipids were also confirmed from GC-MS data by comparing either the retention times of standards or the EI mass spectra available in AOCS lipid library.

Statistical and bioinformatics analysis

The t-test was performed to compare the concentrations of identified lipid species between cancer patient and control (without PCa) groups. The lipid signatures showing differences with more than 1.5 fold increase or decrease with observed p value < 0.05 were considered as differentially regulated lipids among PCa patients. Further an un-supervised analysis was performed to identify the best lipid signatures that could discriminate normal and tumor sample groups. Hierarchical clustering analysis (HCA), principal component analysis (PCA), correlation plots and partition analysis have been applied for the quantification data obtained. In addition, based on the abundance of lipids individually and/or in combinations, formal concept analysis (FCA) was used to predict classification of samples. FCA method is appropriate to find out significant relationships between altered lipids and clinical data of patients mathematically by graphical illustration[19]. These illustrations could envisagetheoretical hierarchy across the samples and data thus produced could be used to find out data dependency between sample attributes (clinical data and altered serum lipids). Recently, FCA is commonly used for
theoretical clustering to identify combinatorial biomarkers and co-regulation of genes, proteins and metabolites [20,21]. In the present study, the FCA method was applied to identify any correlation between abundance of serum lipids and the classification (tumor and control groups) of samples. In FCA analysis theoretical lattices were built based on low or high abundance of lipids to understand the reliance of serum lipids abundance with PCa and normal samples. The lattices were drawn in combinations or single lipid species classified sample groups with different specificities.

Cell proliferation assay

The human PCa cell lines LNCaP (androgen dependent cells) and PC3 (androgen independent cells) were obtained from ATCC and maintained in normal growth media (RPMI-1640 (Sigma) supplemented with 10% fetal bovine serum (FBS) along with 100 units/ml penicillin and streptomycin). Both the cell lines were cultured in humidified incubator at 37°C with a constant supply of 5% CO₂. Mycoplasma contamination of cells in culture was controlled by regular testing using specific primers in RT-PCR. To determine the effect of alpha-linolenic acid on proliferation of PCa cell lines, LNCaP and PC3 cells were seeded in 12 well plates (1.0 X 10⁶ cells per well) in complete medium. Cells were allowed to grow for 24 h allowing them to attach on well surface. Then the cells were treated with indicated concentrations of ALA (0–25 μM) or vehicle control (solvent in which ALA is prepared). To measure cell count, cells were harvested at indicated time intervals and cell viability was estimated using countess (Invitrogen) in cell viability analyzer (Invitrogen). Both time dependent as well as concentration dependent effect of ALA on cell viability was determined in proliferation assays. The effect of ALA on cell morphology was determined by imaging the cells under microscope (Olympus Xi72, Japan).

Results

The serum samples from normal (control) and cancer patients were processed identically as described in the experimental section. The sample aliquots were then subjected for direct high resolution ESI-MS analysis under positive and negative ion modes.

Positive ion ESI-MS analysis

The positive ion ESI mass spectra exhibited ions in the range of m/z 100–1000 due to various metabolites (Fig 1A). As the sample aliquots were analyzed by direct ESI-MS method, which avoids chromatography and desalting steps, the Na⁺ and K⁺ ions present in the serum remain in the sample aliquots. Thus, the detected ions could be protonated, sodiated and/or potas-siated molecules, i.e., [M+H]⁺, [M+Na]⁺ and/or [M+K]⁺ ions, respectively. Hence, the detected ions in the positive ion mode were searched in the databases for all the possible ion species mentioned above.

The database search results show that the detected peaks in the ESI mass spectra include [M+H]⁺ ions of phosphatidylycholines (PCs), sphingomyelins (SMs), and phosphatidylethanol-amine (PEs); [M+K]⁺ ions of fatty acids (FAs), diglycerides (DGs), triglycerides (TGs) and phosphatidic acid (PA); [M+Na]⁺ ions of DGs. Some of the key metabolites were confirmed by comparing their MS/MS data with that of corresponding standards available in the databases. The collision energy value used for the MS/MS experiments was 28 eV for PEs, 30 eV for PCs and 20 eV for SMs. Where the precursor ion intensities were lower, about 100–150 scans were combined (in MCA mode) to obtain their MS/MS spectra. The MS/MS spectra of the [M+H]⁺ ions of PC and PE and [M-H]⁻ ion of FA are presented in Fig 2A–2C. The MS/MS spectra of [M+H]⁺ ions of phosphatidylcholine molecules generate the product ion at m/z 184
corresponding to its polar head group [22]. The ESI-MS/MS spectrum of PC (37:3) is shown in Fig 2A as an example. The spectrum showed the ion at m/z 184, the characteristic ion of the PC, and the ion at m/z 615 which provide the information about the acyl moieties attached to the glycerol. The ion at m/z 104 in the low mass region corresponds to the choline group. The MS/MS spectrum of PE (42:4) is shown in the Fig 2B, which showed the characteristic product ion due to the loss of 141 Da (ethanolaminephosphate) from the [M+H]+ ion, and this ion known to be specific for PE [23,24]. The other product ion in the spectrum appeared at m/z 279 provides the information about one of the acyl moiety of the PE as FA (18:3). The MS/MS spectrum of [M-H]- ion of FA (20:4) is shown in Fig 2C. This spectrum matched well with the MS/MS spectrum of the standard FA (20:4) available in the Metline database.

Negative ion ESI-MS analysis

The negative ion ESI mass spectra of lipid extracts showed [M-H]- ions of different classes of lipid species (Fig 1B). Similar to positive ion ESI data, the accurate mass values of the detected peaks were searched against databases for the peak identifications. Based on database search results, the detected peaks in the spectra were found to be the [M-H]- ions of fatty acids (FAs), DGs and Phosphatidic acid (PA). Some of the fatty acids were further confirmed by the MS/MS experiments on the [M-H]- ions. The collision energies used were 25–30 eV for FAs. The
MS/MS spectrum of FA 20:4 is shown in Fig 2D as a typical example and the spectrum showed characteristic product ions due to the loss of H₂O (18 Da) and CO₂ (44 Da) from [M-H]⁻ ions in addition to the product ion at m/z 59 corresponding to the acetate ion (CH₃COO⁻) [25].

Differential analysis & Quantification of lipids

The relative abundances of the peaks detected in control and patient samples were normalized based on the internal standard peak intensities. The normalized values were subjected for determining differentially regulated lipid signatures with minimum of 1.5 fold increase or decrease in cancer patients compared to control group. ANOVA test was performed for statistical significance (< 0.05) of altered lipids across the PCa patients. HRMS data of the differentially regulated lipid species with corresponding fold differences are summarized in Table 2. All identified lipids were classified into seven different classes PC, PA, PE, TG, DG, SPL and FA. Except fatty acids, remaining six classes of lipids were higher in patient serum samples than
those of control samples. Among the individual classes, TGs were elevated in PCa patients compared to normal healthy individuals.

Hierarchical clustering and partition analysis

The hierarchical clustering of samples based on altered serum lipids is presented in Fig 3. On heat map, samples with higher expression are colored red while samples with lower expression are colored green. The columns represent samples and the rows indicate altered lipids in cancer patients serum compared to healthy individuals. The distances among the clusters are measured by dendrograms between the clusters. From the observed heat map two distinct clusters divide the tumor and normal samples with only minor exception. Nonetheless, hierarchical clustering revealed that 17 of 18 tumor samples (red) formed a discrete cluster. In case of controls, 14 samples (green) clustered together whereas the remaining four samples became part of the tumor samples cluster. In two-dimensional clustering, we also made an attempt to identify any specific lipid class forming a cluster that is mainly determining the tumor and control groups. The observed dendrograms confirm that no cluster is formed with single lipid class (FA, PE, PA, DG, PC and TG) (Fig 3). Based on the observed clusters, auxiliary PCA and partition analysis was performed. From the obtained relative abundance of lipids, a scatterplot with

Table 2. High resolution mass spectral data. The identified metabolites in the lipid extracts of the serum samples.

| S.No | Lipid species | Molecular formula | Ion | Theoretical m/z | Observed m/z | Error (ppm) | Fold change (P/C) | Regulation |
|------|--------------|-------------------|-----|-----------------|--------------|-------------|-----------------|------------|
|      | Phospolipids  |                   |     |                 |              |             |                 |            |
| 1    | PA(14:0)     | C_{13}H_{25}O_5P  | [M-H]^* | 381.2048        | 381.2066     | -0.67       | 0.67            | Down       |
| 2    | PA(32:1)     | C_{32}H_{67}O_5P  | [M+K]^* | 685.4205        | 685.4238     | -0.41       | 4.09            | UP         |
| 3    | PC(37:3)     | C_{42}H_{95}O_9P  | [M+H]^* | 798.6007        | 798.5964     | 0.53        | 2.08            | UP         |
| 4    | PC(39:6)     | C_{40}H_{90}O_9P  | [M+H]^* | 820.5851        | 820.5815     | 0.38        | 1.99            | UP         |
| 5    | PE(29:1)     | C_{29}H_{60}O_8P  | [M+K]^* | 686.4158        | 686.4191     | -0.48       | 7.8             | UP         |
| 6    | PE(31:3)     | C_{30}H_{60}O_8P  | [M+H]^* | 672.4599        | 672.4642     | -0.63        | 0.69            | Down       |
| 7    | PE(42:4)     | C_{42}H_{84}O_8P  | [M+H]^* | 824.6164        | 824.6108     | 0.67        | 4.19            | UP         |
| 8    | PE(42:5)     | C_{42}H_{84}O_8P  | [M+H]^* | 822.6007        | 822.5968     | 0.47        | 5.04            | UP         |
|      | Fatty acids   |                   |     |                 |              |             |                 |            |
| 9    | FA(16:3)     | C_{16}H_{26}O_2   | [M-H]  | 249.1855        | 249.1846     | 0.69        | 0.69            | Down       |
| 10   | FA(18:3)     | C_{18}H_{30}O_2   | [M-H]  | 277.2168        | 277.2155     | 0.68        | 1.51            | UP         |
| 11   | FA(19:1)     | C_{19}H_{31}O_2   | [M-H]  | 295.2637        | 295.2621     | 0.43        | 2.06            | UP         |
| 12   | FA(20:2)     | C_{20}H_{35}O_2   | [M-H]  | 307.2643        | 307.2654     | 0.38        | 0.66            | Down       |
| 13   | FA(20:4)     | C_{20}H_{34}O_2   | [M-H]  | 303.2324        | 303.2309     | 0.94        | 0.58            | Down       |
| 14   | FA(22:0)     | C_{22}H_{44}O_2   | [M-H]  | 339.3263        | 339.3248     | 0.43        | 0.28            | Down       |
| 15   | FA(22:3)     | C_{22}H_{38}O_2   | [M+K]^* | 373.2509        | 373.2496     | 0.35        | 0.68            | Down       |
|      | Di and Tri glycerides |       |     |                 |              |             |                 |            |
| 16   | DG(40:9)     | C_{43}H_{65}O_5   | [M+K]^* | 701.4512        | 701.4487     | 0.36        | 4.18            | UP         |
| 17   | DG(30:2)     | C_{33}H_{65}O_5   | [M-H]  | 535.4368        | 535.4392     | 0.48        | 0.61            | Down       |
| 18   | DG(32:3)     | C_{33}H_{65}O_5   | [M-H]  | 561.4525        | 561.4508     | 0.52        | Down            |            |
| 19   | DG(41:0)     | C_{44}H_{85}O_5   | [M-H]  | 693.6403        | 693.6355     | 0.92        | 1.5             | UP         |
| 20   | TG(52:3)     | C_{55}H_{106}O_6 | [M+K]^* | 895.7152        | 895.7098     | 0.59        | 4.69            | UP         |
| 21   | TG(59:5)     | C_{62}H_{110}O_6 | [M+H]^* | 951.8375        | 951.8322     | 0.56        | 4.38            | UP         |
| 22   | TG(60:11)    | C_{63}H_{110}O_6 | [M+H]^* | 953.8532        | 953.8588     | 0.08        | 4.43            | UP         |
| 23   | TG(61:5)     | C_{64}H_{114}O_6 | [M+H]^* | 979.8688        | 979.8645     | 0.38        | 10.72           | UP         |
| 24   | TG(45:2)     | C_{48}H_{88}O_6  | [M+K]^* | 799.6212        | 799.6164     | 0.60        | 2.26            | UP         |

doi:10.1371/journal.pone.0150253.t002
The first three principal components show a good partition between cancer patients and controls (Fig 4A). From the PCA analysis it is clearly visible that all tumors formed a unique component (blue), only one tumor sample became part of the control group component (red) based on 24 identified lipids. However, as reported by Lukk et al. only one individual differential biomarker between cancer and normal groups is not satisfactory to classify clinical samples with 100% specificity and sensitivity[26]. Therefore, partition analysis has been performed to determine potential lipid signatures for classification of samples into respective labeled group. Partition analysis of the relative abundance values highlighted two lipids, FA (22:3) and PC (39:6) which can classify samples with higher specificity (Fisher test with p-value 1.612e-06). Under this
partition function if FA (22:3) is $\geq 0.045$ ppm and PC (39:6) is $\geq 0.145$ ppm, 100% sensitivity (18 control samples classified correctly PCa) and 77.7% specificity (of 18 tumor samples 4 samples are misclassified) could be achieved in classification of cancer patients from the corresponding normal group (Fig 4B). Partition analysis results also have indicated that the other identified lipids in combinations can classify all samples appropriately. From these results it is evident that more than one lipid of the same class or different class can distinguish tumor and control samples with higher specificity and sensitivity.

To better understand the variation of lipids that altered in PCa patients, a correlation analysis was performed in a pair-wise manner to visualize dependency between abundance levels of identified lipids and serum samples. Correlation analysis allows identification of co-regulated lipids and identifies relationships between the samples within a study. The resulting correlation heat maps of log-transformed abundance levels of lipids using the Pearson correlation coefficients clearly differentiated major number of cancer patients with only one exception (Fig 5). In addition to sample-sample correlation, correlation between lipids determining their co-regulation has identified a major cluster of lipids containing 3 PEs, 2 PCs, 3 TGs and 1 DG. In particular both PCs and PEs clustered together showing good co-regulation of their serum abundance (Fig 6).

**Gas chromatography with electron ionization mass spectrometry (GC-EIMS) analyses**

Fatty acids can be analyzed by GC-MS after derivatization (e.g., esterification, silylation etc.). In the present work, fatty acids were methylated by diazomethane and the FAMEs were subjected to GC-MS analyses under electron ionization (EI) conditions. The EI mass spectra of FAMEs are available in the AOCS lipid library (http://lipidlibrary.aocs.org/ms/ms16/index.htm). The spectra available include characteristic structure indicative fragment ions. The spectra are also available in commercial EI libraries (Wiley and NIST) as well as in the previously published reports [27]. Hence it is easy to identify the FAMEs in the GC-MS analysis by
searching the target spectra against the EI library. We have used the EI library and/or standards to identify the fatty acids. In the present study, as expected, the saturated fatty acids showed predominantly two characteristic fragment ions at $m/z$ 74 and 87 whereas polyunsaturated fatty acids showed the fragment ion at $m/z$ 79 in the low mass region of the spectra. The EI spectra of methyl esters of heptadecanoic acid (17:0), linolenic acid (18:3) and arachidonic acid (20:4) are shown in S1A to S1B Fig. We could easily characterize high abundant fatty acids from their EI mass spectra (full scan mode analysis). In the case of low abundant fatty acids, we have performed GC-MS experiments in SIM mode by selecting specific fragment ions of target fatty acids. In this type of analysis, the retention parameters of target compounds are crucial. Consequently, before SIM experiments, the retention times of the target fatty acids were confirmed using the standards. By applying the GC-MS (SIM) method, we have confirmed

**Fig 5. Sample-Sample correlation heat map for lipidomics data obtained.** Clustering on correlation coefficients clearly demonstrates samples group (tumor or normal) based on the abundance levels of differential lipids.

doi:10.1371/journal.pone.0150253.g005
The methyl esters of linolenic acid (18:3) and arachidonic acid (20:4) were eluted at the retention times of 20.9 and 22.3 min respectively (Fig 7). Presence of these two fatty acids were further confirmed by spiking the standards in the lipid extract and perform GC-SIM mode analysis after esterification, where these two fatty acids appeared at the same RTs as that of sample (Fig 7).

Alpha-linolenic acid (ALA) triggers the growth of prostate cancer cell lines

To determine the effect of alpha-linolenic acid (18:3) on prostate cancer cell growth, we have treated the LNCaP and PC3 prostate cancer cells with alpha-linolenic acid both in dose and time dependent manner. In our investigations, we observed that alpha-linolenic acid induced growth of LNCaP cells significantly at lower concentrations even at 1 μM where as its effect on PC-3 cells growth was significant at higher concentrations. As presented in Fig 8A–8C, the
dose and time dependent effect of ALA on the growth of both PCa cells is clearly suggesting its role in the proliferation of tumor cells. Further to determine the effect of ALA on morphology of proliferating PCa cells, the cells were imaged upon post treatment to ALA. The observed result showed that the cells are more proliferative and form clumps due to cell to cell interactions which could play an important role in increased cell proliferation up on ALA treatment (S2 Fig). Contrary to its pro proliferative activity, the concentration dependent effect of ALA on cancer cells demonstrate that the higher concentration of ALA at 100 μM inhibit cell proliferation thereby causing cell death (S3 Fig).

Discussion

PCa is the most frequent type of cancer in men and is the second foremost cause of cancer deaths. The mortality rate of PCa has decreased dramatically with advent PSA test and DRE diagnostic methods. The PSA test is not prostate specific as a small amount of it is also detectable in other cancer types such as endometrial, breast, adrenal and renal cancers. As a result, routine screening of PCa patients with serum PSA test is still an open debate. Therefore, new biomarkers with a higher specificity and sensitivity may overcome limitations in PCa diagnosis. In this context, during the past decade a number of studies have performed genomic and proteomic profiling on radical prostatectomy tissues as well as extracellular fluids such as serum, plasma, urine and prostatic fluid [28–35]. These authors reported several potential markers that are less systemically studied for their sensitivity, specificity and accuracy in the diagnosis of PCa. Diagnosing cancer based on serum profiling is an attractive concept. Several studies have been performed protein and gene analysis on serum samples from cancer patients. Only sparse information is available for lipid composition in serum/plasma from cancer patients. However, research on lipids is still lacking compared to those of genes and proteins. Lipids are
cellular metabolites and essential components of an organism. Lipids and lipid metabolism play an important role in many physiological functions involved in cellular signal transduction regulating cell proliferation, cell death and differentiation. However, profiling of disease related lipids specific to cancers is still lagging behind when compared to those of genes and proteins. This may be due to the very sparse information available on the functional role of lipids and/or limited technology for analyzing the complex lipids in biological specimens. With recent advances more attention has been drawn to blood serum/plasma lipids (lipidome) to investigate their role in cancer progression, diagnosis and cancer therapy. Lipids, including phospholipids and fatty acids have been found to be involved in PCa progression. In this study we focussed on lipidome analysis of serum samples collected from patients diagnosed with PCa and age matched healthy individuals as control. To avoid data variations due to loss of sample during extraction of lipids and normalize the instrument response, known amount of internal standard has been added to serum samples before extraction. Qualitative and quantitative comparison of lipid profiles between cancer and healthy individuals identify cancer-associated lipids that will be further evaluated for clinical relevance and to further understand their role in cancer progression. Extracellular fluids such as serum and plasma contain a variety of lipid species. A recent study described the composition of plasma lipids determined by lipidomics on

Fig 8. Effect of ALA on proliferation of LNCaP and PC-3 cells. (A&B) Proliferation of LNCaP and PC-3 cells treated with different concentrations of ALA or Ethanol (solvent control) measured by using countess in cell viability analyzer (Invitrogen) over 72 h. (C&D) Time dependent effect of ALA on proliferation of LNCaP and PC-3 cells has been performed by measuring cell proliferation of ALA treated cells for 0 to 96 h. Initial number of cells seeded for assay was considered for 0 h.

doi:10.1371/journal.pone.0150253.g008
pooled plasma samples [36]. The lipid species identified from our study overlapped with the list reported. Further normalization of apparent peak areas to internal standards identified differential lipid species between PCa and normal individuals.

Lipidomic data from the current study showed altered levels of 24 lipids in serum from cancer patients compared to healthy controls. The list of identified lipids is heterogeneous belonging to different classes such as PC, PE, PA, FA, di and triglycerides. A recent report suggests that all phosphoglycerides, namely phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol were up regulated in PCa patients [7]. A three lipid signature including ePC 38:5, PC 40:3 and PC 42:4 is potential to screen patients for diagnosis of PCa [8]. However, in the present study, we have applied positive ion ESI, negative ion ESI and GC-MS approach to analyze different classes of lipids including fatty acids. Interestingly, we found that the fatty acids, tri and diglycerides are altered in PCa patient’s serum along with phospholipids. Other reports have shown that the plasma triglycerides are decreased in ovarian cancer whereas increased in breast cancer patients [37,38]. A higher TG level was associated with increased risk of breast cancer [37]. Similarly the serum triglyceride levels are high in colorectal carcinoma patients and their risk is associated with elevated triglycerides in serum [39]. There is also evidence that more risk for PCa is positively associated with elevated TG levels in men over 60 years compared to younger males [40]. Differential phospholipid classes such as PA, PE and PC have been reported in breast cancer [41]. A case–cohort analysis study reported a positive association between plasma phospholipids, saturated fatty acids and PCa risk. But no significant association was observed to tumor aggressiveness [42]. Therefore, from the collected lipidomic data we sought to identify potential lipid signature with combination of different lipids which can classify tumor samples from healthy controls.

Hierarchical clustering of samples based on abundance of lipids led to clusters separating tumor specimen from normal. But four control samples became part of the tumor sample cluster. Interestingly, in two-dimensional clustering individual lipids identified did not lead to any specific cluster consisting same lipid species and/or class. Therefore, we were not able to identify a specific lipid class to consider as potential signature in distinguishing tumor from control specimens. To determine the effect of co-regulation of lipids on identification of biomarker, an unsupervised PCA has been applied to the abundance levels of altered lipids in cancer patients serum compared to corresponding controls. Here, as observed in cluster analysis, we could detect a clear separation of tumor with only one exception from normal patients and a separation of two distinct tumor groups based on the individual lipid abundance found in serum. Further data analysis with partition algorithms to find a lipid signature which can classify all samples with high certainty (distinguishing PCa patients and healthy controls) revealed more than one lipid are required to define sample identity. Among them serum lipid signatures identified in this study PC (39:6) and FA (22:3) together showed higher sensitivity, specificity and accuracy in differentiation of PCa specimens from normal compared to other lipids in possible combinations. Therefore, these lipids PC (39:6) and FA (22:3) may be possible serum biomarkers along with PSA test for diagnosis of PCa. Of course, further validation with large cohort of samples is needed to demonstrate sensitivity, specificity and accuracy of these lipids in classifying the tumors from normal.

Epidemiology reports have suggested an association of fatty acids with risk of prostate cancer [43]. From the studies presented so far it appears that some fatty acids are pro-tumorigenic, promoting tumor growth and metastasis [44,45], whereas others show anti-tumor effect [46]. Particularly, fatty acids such as palmitic acid are known to play a key role in initiation and progression of PCa. Therefore, we made an attempt to assign representative peaks to fatty acids in GC-MS with known standards. Of all differential lipids, FA (18:3) was identified as α linolenic acid (ALA). High level of palmitic, myristic, linolenic, and eicosapentaenoic acids enhances the
risk of prostate cancer and it has an inverse association with stearic acid [47]. Individual studies examining the association between ALA and PCa reported a positive association for the role of ALA in prostate carcinogenesis [48–51] except in one case which showed no association [52]. Reported data suggests that Arachidonic Acid, and linoleic acid (LA), an omega-6 polyunsaturated FA stimulates prostate cancer cell growth in vitro [53,54]. A recent study highlighted that the essential fatty acids promotes proliferation of both human PCa (PC3, LNCaP and TSU) cells as well as rat PCa (Mat-Ly-Lu (metastatic), EPYP2 and EPYP3 (non metastatic) cells [55]. Our results also showed an increase in proliferation of LNCaP and PC3 cells up on treatment with ALA in in-vitro. Interestingly, we observed that the higher concentration of ALA (50 and 100 μM) inhibiting proliferation of both LNCaP and PC3 cells contrary to the pro proliferative effect up to 25 μM. These observations are in agreement with the results reported by Pandalai et al. [55]. The lower concentration of fatty acids such as eicosapentanoic acid and arachidonic acid promotes growth of hormone dependent (LNCaP) and independent (PC3) PCa cells [53,56]. Contrarily, higher concentrations act negatively on proliferation of both cell types suggesting that the pro tumorigenic effect of essential fatty acids is concentration dependent [55,56]. However many mechanisms of action for fatty acids to different types of cancers progression have been hypothesized. ALA cannot be synthesized in humans. Therefore, serum levels of ALA are dependent on dietary intake only. The elevated levels of ALA in serum and prostate tissue may be due to altered ALA metabolism by desaturases [57,58]. Genetic variations particularly single nucleotide polymorphisms (SNPs) in gene encoded for delta-6-desaturase (FADS2) leading to higher linoleic acid (LA)/ALA ratio due to impaired metabolism [50]. In prostate cancer patients higher prostatic ALA is positively associated with serum PSA levels and cancer cell proliferation [59]. Fatty acids can regulate many cellular proteins in cancer cells directly or indirectly and there by influence DNA damage, tumor growth, angiogenesis and metastasis. The detailed mechanisms involved in ALA induced prostate carcinogenesis are worth pursuing.

Conclusions

In conclusion, the current study using ESI-MS/MS reported serum lipids that are altered in PCa patients. Systematic analysis of the altered lipids helped us identify lipids PC 39:6 and FA 22:3 distinguishes cancer patients from controls free from PCa. We also found that serum ALA levels are elevated in PCa. Moreover, LNCaP and PC-3 cells showed increased proliferation rate upon exposure to ALA in in vitro. Functional characterization of altered lipids will further substantiate their role in pathophysiology of prostate carcinogenesis. However, prospective clinical studies with large cohort of samples confirming diagnostic and prognostic potential of the lipid signatures are needed. With the results obtained from this study, we will continue to validate the identified signatures in a larger set of PCa and normal serum samples so that the findings will be useful in developing potential biomarker signatures.

Supporting Information

S1 Fig. GC-EIMS spectra of standard compounds of I.S (17:0) (a), (18:3) (b) and (20:4) (c) respectively. (TIF)

S2 Fig. Effect of ALA on proliferation of LNCaP and PC3 cells. Both LNCaP and PC3 cells were treated with 25μM of ALA and cell morphology was observed after 24, 72 and 96 h of post treatment to determine effect of the ALA on proliferation of PCa cells. (TIF)
**S3 Fig. Effect of ALA on proliferation of LNCaP and PC3 cells.** The LNCaP and PC3 cells were treated with ALA of varying concentration (1 to 100 μM) for 48 h and cell morphology was observed to determine effect of the ALA on proliferation of PCa cells. (TIF)

**Acknowledgments**

DD Acknowledges Council for Scientific and Industrial Research (CSIR), India for CSIR-JRF and AcSIR for registering as graduate student.

**Author Contributions**

Conceived and designed the experiments: DD RU RW. Performed the experiments: DD SRB SL RU. Analyzed the data: RU PS HP. Contributed reagents/materials/analysis tools: RU PM RW HP PS. Wrote the paper: DD RU RW PS.

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