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Original Article

Lipid quantification by Raman microspectroscopy as a potential biomarker in prostate cancer

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ABSTRACT

Metastatic castration-resistant prostate cancer (mCRPC) remains incurable and is one of the leading causes of cancer-related death among American men. Therefore, detection of prostate cancer (PCa) at early stages may reduce PCa-related mortality in men. We show that lipid quantification by vibrational Raman Microspectroscopy and Biomolecular Component Analysis may serve as a potential biomarker in PCa. Transcript levels of lipogenic genes including sterol regulatory element-binding protein-1 (*SREBP-1*) and its downstream effector fatty acid synthase (*FASN*), and rate-limiting enzyme acetyl CoA carboxylase (*ACACA*) were upregulated corresponding to both Gleason score and pathologic T stage in the PRAD TCGA cohort. Increased lipid accumulation in late-stage transgenic adenocarcinoma of mouse prostate (TRAMP) tumors compared to early-stage TRAMP and normal prostate tissues were observed. FASN along with other lipogenesis enzymes, and SREBP-1 proteins were upregulated in TRAMP tumors compared to wild-type prostatic tissues. Genetic alterations of key lipogenic genes predicted the overall patient survival using TCGA PRAD cohort. Correlation between lipid accumulation and tumor stage provides quantitative marker for PCa diagnosis. Thus, Raman spectroscopy-based lipid quantification could be a sensitive and reliable tool for PCa diagnosis and staging.

Introduction

Prostate cancer (PCa) is the second leading cause of cancer-related death in American men [1]. Despite recent advances in targeted therapies, specifically against the androgen receptor, metastatic castration-resistant prostate cancer (mCRPC) remains incurable [2]. By definition, mCRPC is resistant to androgen deprivation therapy (ADT), but most aggressive mCRPCs also develop resistance to various chemotherapeutic agents [3]. Thus, alternative approaches to detect this advanced disease state must be identified to prevent progression and recurrence of PCa.

It is established that tumor cells require more energy than normal cells and metabolic alterations are required to sustain the proliferative potential of growing tumors [4,5]. One such metabolic alteration is endogenous or *de novo* lipogenesis by cancer cells, which usually takes place in liver and adipose tissues but not in other normal tissues [6]. Several recent studies determined that cancer cells of numerous origins fulfill their requirement of fatty acids through *de novo* lipogenesis [7–9]. Due to their high proliferation rate, cancer cells require a substantial amount of metabolic energy in order to synthesize cellular and subcellular membranes, which are mainly composed of fatty acids. Lipids in cancer cells are

Abbreviations: FASN, fatty acid synthase; ACC, acetyl Co-A carboxylase; SREBP1, sterol regulatory element binding protein 1; TRAMP, transgenic adenocarcinoma of the mouse prostate; PCa, prostate cancer; mCRPC, metastatic castration-resistant prostate cancer; CRPC, castration-resistant prostate cancer; ADT, androgen deprivation therapy; AR, androgen receptor; BCA, biomolecular component analysis; WT, wild-type; FFA, free fatty acids; CPT1, carnitine palmitoyltransferase; PIN, prostatic intraepithelial neoplasia; NEPC, neuroendocrine prostate cancer; PRAD, prostate adenocarcinoma; TCA, tricarboxylic acid; AceCS1, acetyl-coA synthetase 1; ACSL1, acetyl-coA synthetase long chain family member 1; ACL, ATP-citrate lyase.

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the main fuel source for membrane synthesis and act as one of the major drivers in tumor cells, especially PCa, for growth and membrane synthesis [10]. Moreover, the rate of fatty acid synthesis and accumulation has been reported to accelerate in hypoxic PCa cells and tumor core as a survival response to hypoxic stress [11]. Following re-oxygenation, the accumulated lipids support growth and aggressiveness of PCa [8,11]. Recently, Rysman et al. reported that *de novo* lipogenesis protects cancer cells from endogenous and exogenous stresses like ROS and chemotherapeutic drugs. Specifically, *de novo* lipogenesis protects cancer cells from lipid peroxidation and subsequent cell death due to free radicals by enhancing saturation of membrane lipids [12]. These evidences further emphasize the importance of *de novo* lipogenesis in averting cancer cell death causing cancer progression [12]. A brief overview of the key lipogenic steps is highlighted in Fig. 1. Briefly, pyruvate is synthesized from glucose by glycolysis, and this pyruvate is converted to Acetyl-CoA either inside mitochondria (via pyruvate dehydrogenase complex) or in the cytosol through citrate derived from TCA cycle. In the cytosol, Acetyl-CoA is carboxylated by acetyl-CoA carboxylase (gene: *ACACA*, protein: ACC) to form malonyl-CoA. In the next rate-limiting step, malonyl-CoA is synthesized into palmitic acid and various other fatty acids via fatty acid synthase (FASN). These fatty acids are accumulated and stored as lipid droplets, which can be used as fuel source in proliferating tumor cells. FASN and other enzymes involved in *de novo* lipogenesis are under control of the lipogenic transcription factor sterol regulatory element binding protein 1 (SREBP-1). In addition, miRNAs miR-27b and miR-222 have also been predicted to negatively regulate lipogenesis, as miR-27b targets *FASN* and miR-222 targets both the *ACACA* and *FASN* mRNA for degradation [2].

A link between lipogenesis and PCa progression has been investigated in the past few decades [8,12–15]. Various studies have determined that inhibition of the key lipogenic enzyme FASN in PCa cells slows growth and proliferation, while increasing apoptosis of PCa cells [16–18]. In addition, ACC inhibition in PCa cells resulted in sensitization to chemotherapeutic agents [12]. *De novo* lipogenesis is also crucial for development of mCRPC because of reactivation of AR signaling by *de novo* steroidogenesis [19,20]. In this manuscript, we implemented a systemic approach to verify the role of lipogenesis in PCa progression and validate the impact of lipogenic transcript levels for the evaluation of PCa biomarkers. Specifically, we employed vibrational Raman Microspectroscopy and Biomolecular Component Analysis (BCA) approach to quantify expression of major types of biomolecules: DNA, proteins and lipids in prostate tumor tissues [21]. This label-free optical approach has unraveled a significant correlation between lipid content and PCa progression. We suggest that macromolecular lipid quantification by microRaman-BCA approach can serve as quantitative marker for general categorization of the lesions as well as for clinical prognosis.

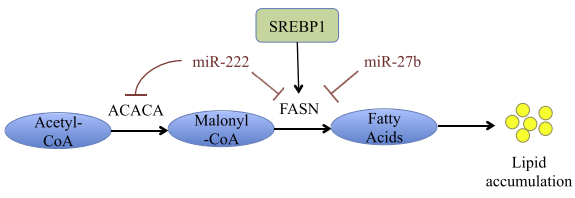

Fig. 1. Schematic of key steps in lipogenesis. Acetyl-CoA is carboxylated by acetyl-CoA carboxylase (ACC) to form malonyl-CoA. In the rate-limiting step, malonyl-CoA is synthesized into palmitic acid and various other fatty acids via fatty acid synthase (FASN). These fatty acids are accumulated and stored as lipid droplets, which can be used as fuel source in proliferating tumor cells.

Materials and methods

Analysis from publicly available datasets

The TCGA prostate adenocarcinoma (PRAD) dataset (version 2016-08-16) was retrieved from the UCSC Xena Browser. The polyA+ IlluminaHiSeq gene expression dataset was downloaded in its normalized format. RNA-seq values were grouped between tumor and matched-normal samples and the tumor samples were further divided according to Gleason score or pathologic T-score. The average transcript reads were calculated for each group. The RPKM method was used to quantify gene expression from RNA sequencing data by normalizing for total read length and the number of sequencing reads. A student's T-test was performed between matched normal and each Gleason score or pathologic T-score. cBioportal [22,23] was used to download the Trento/Cornell/Broad Neuroendocrine (2016) dataset and the PRAD TCGA (provisional) dataset for heat map generation, OncoPrint analyses, co-expression analyses, and survivorship analysis.

Whole tissue lysates preparation and Western blotting

Murine prostate tumors (TRAMP) were generated as previously described [24]. Briefly, mice hemizygous for the Pb-Tag transgene were crossed with non-transgenic FVB mice to obtain non-transgenic (WT) and transgenic (TRAMP) males. Animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee at the department of Laboratory Animal Resources, Roswell Park Cancer Institute, Buffalo, NY. Whole tissue lysates from WT prostate and TRAMP tumor tissues were prepared by homogenization and lysed in RIPA buffer (50 mM Tris, pH 7.5), 150 mM NaCl, 0.1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 1 mM EDTA, 1 mM dithiothreitol (DTT) supplemented with protease inhibitors (1 mM PMSF, 1% aprotinin, 1 mM leupeptin, 1 mg/ml pepstatin A and 1 mg/ml chymostatin). Thirty micrograms protein samples were loaded on 4–20% SDS polyacrylamide gels for Western blotting. The membrane was probed with indicated primary antibodies and corresponding HRP-conjugated secondary antibodies followed by immunodetection using ECL reagent (BioRad, Hercules, CA). Antibodies against SREBP1 and β-actin-HRP were procured from Santa Cruz Biotech, Dallas, TX. FASN, ACC, AceCS1, ACL, and ACSL1 antibodies were procured from Cell signaling, Danvers, MA.

Raman micro-spectroscopy

A confocal Raman microscope used to acquire spectra from individual tissue cells is equipped with an inverted Nikon TE200 microscope, fiber-coupled MS3501i imaging monochromator/spectrograph (Solar TII), a Hamamatsu S9974 CCD camera cooled down to −60 °C, and a 532 nm DPSSL excitation laser. This configuration enables the measurements within the range of Raman shifts of 600–3000 cm^{-1}. The spectral resolution for the fixed diffraction grating position (wave number interval of 1210 cm^{-1}) was ~1.5 cm^{-1}. An excitation laser beam of ~20 mW power was focused onto the sample in a spot of ~0.8 μm, using a 100 × NA = 1.3 Nikon oil-immersion objective lens. A 100 μm pinhole ensures for confocal acquisition of Raman signal. The integration time for spectra acquisition was 20 s for all our experiments. The measurements performed in bovine serum albumin solution demonstrated that experimental error of Raman spectra measurements does not exceed 5% within the Raman shift range of 700–1700 cm^{-1}.

Biomolecular component analysis

For assessment of intranuclear macromolecular content the biomolecular component analysis (BCA) [27–29] was applied to pre-processed Raman spectra. This method is based on an accurate spectral fit of a model spectrum – the linear summation of the weighted spectra of the basic components (Linear Combination Modeling (LCM) [30–33]), into a pre-processed Raman spectrum of nuclei. The spectral weights (coefficients), which are varied during the fitting procedure, are considered as the specific contributions of the basic spectra into the resulting spectrum and relate directly to the concentrations of basic macromolecules. In our case LCM of the acquired Raman spectra was utilized for experimental evaluation of the local biomolecular composition tissue cells nuclei by generating a model spectrum through a linear summation of weighted Raman spectra of the basic classes of bio-molecules, DNA, RNA, proteins, lipids, which make the largest contribution to nuclear Raman spectrum [28]. In other words it means to obtain a numerical value of the fractional fit contribution (weight, C_i) of each component, i, to Raman spectrum of measured nuclear domain (r_{total}): $r_{total} = C_1r_1 + C_2r_2 + C_3r_3 + C_4r_4$, where r_i is Raman spectrum of i-component. More detailed description of BCA used in this study can be found in our previous publications [28,29]. For initial step of BCA we used protein component (r_1), obtained from our previous studies of HeLa cell culture corresponding to 100 ml/mg of bovine serum albumin ($C_1 = 1$) equivalent of concentration. Preprocessed Raman spectra of extracted DNA, RNA, and lipid droplets from HeLa cells, calibrated to 20 mg/ml of calf thymus DNA, *Saccharomyces cerevisiae* RNA and bovine heart lipid extract equivalents of concentration accordingly, were used as the reference spectra of the DNA, RNA and lipids components (r_i, $i = 2,3,4$ with $C_i = 1$, $i = 2,3,4$).

The LCM algorithm was coded in Matlab environment and includes pre-processing procedures subtracting the background, Savitzky–Golay smoothing (2nd