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Ceramide and polyunsaturated phospholipids are strongly reduced in human hepatocellular carcinoma

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ABSTRACT

Lipid composition affects membrane function, cell proliferation and cell death and is changed in cancer tissues. Hepatocellular carcinoma (HCC) is an aggressive cancer and this study aimed at a comprehensive characterization of hepatic and serum lipids in human HCC. Cholesteryl ester were higher in tumorous tissues (TT) compared to adjacent non-tumorous tissues (NT). Free cholesterol exerting cytotoxic effects was not changed. Phosphatidylethanolamine, -serine (PS) and -inositol but not phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) were reduced in HCC tissues. Saturated species mostly increased and polyunsaturated species were diminished in all of these phospholipids. Ceramide (Cer) was markedly reduced in HCC tissues and higher levels of sphingomyelin suggest impaired sphingomyelinase activity as one of the underlying mechanisms. Importantly, ceramide in NT increased in HCC stage T3. Ceramide released from hepatocytes attracts immune cells and a positive association of the macrophage specific receptor CD163 with NT ceramide was identified. HCC associated lipid changes did not differ in patients suffering from type 2 diabetes. Protein levels of p53 were induced in TT and negatively correlated with Cer d18:1/16:0 and PS 36:1. Of the lipid species changed in HCC tissues only TT Cer d18:1/16:0, Cer d18:1/24:1, PC 38:6 and LPC 22:6 correlated with the respective serum levels. Our study demonstrates a considerably altered hepatic lipidome in HCC tissues. Ceramide was markedly reduced in HCC tissues, and therefore, raising ceramide levels specifically in the tumor represents a reasonable therapeutic approach for the treatment of this malignancy.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most deadly malignancies and in many cases arises as a consequence of chronic liver diseases caused by viral infections, alcohol abuse and non-alcoholic fatty liver disease (NAFLD) [1]. Thus, disease progresses from hepatitis to cirrhosis to carcinoma. More recently, NAFLD associated HCC in non-cirrhotic livers has been described [1]. Cirrhosis may be absent in up to 49% of these patients but risk factors for hepatocarcinogenesis in these patients have yet to be identified [1].

The liver is the main organ regulating lipid homeostasis, thus circulating and hepatic lipids are affected in chronic liver injury [2–7]. The sphingolipid ceramide stimulates apoptosis and is considered a tumor-suppressor metabolite [8]. Cancer cells have developed mechanisms to avoid accumulation of this lipid [8]. Ceramide is at least two-fold lower in human colon cancer compared to normal colon mucosa. Higher ceramidase activity in the tumor suggests that formation of sphingosine-1-phosphate, which is produced from ceramide by the successive actions of ceramidase and sphingosine kinase and does stimulate cell proliferation, is enhanced [9,10]. Ceramidase inhibition increases cellular ceramide and induces cell death in colon cancer cell lines. Ceramide content and viability of normal hepatocytes is not affected by this inhibitor indicating that this approach may be used to safely treat hepatic colon cancer metastases [9,10]. Acid ceramidase is induced in about 70% of head and neck squamous cell cancers and protects cells from CD95 ligand induced apoptosis [11]. C16-, C24-, and C24:1 ceramides are elevated in the majority of these tumor tissues compared to the corresponding normal tissues while C18 ceramide is significantly decreased. The latter lipid species is related to lymphovascular invasion and nodal disease [12].

Sphingomyelinase hydrolyzes sphingomyelin yielding phosphocholine and ceramide, and sphingomyelin phosphodiesterase 3

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has been identified as a tumor suppressor gene in HCC [13]. Increasing cellular ceramide accumulation is, therefore, a reasonable strategy in tumor therapy [8].

Importantly, higher levels of ceramide species have been described in pancreatic and breast cancer tissues demonstrating that this lipid is not suppressed in all cancers [14,15].

Choline-deficient diets promote hepatocarcinogenesis in rodents especially when combined with carcinogens like diethylnitrosamine [16,17]. Phosphatidylcholine supplemented diets protect from HCC partly by increasing apoptosis [18]. Phosphatidylethanolamine *N*-methyltransferase (PEMT) which synthesizes phosphatidylcholine from phosphatidyl-ethanolamine is reduced in human HCC compared with adjacent normal liver tissue [19]. PEMT is hardly detectable in aflatoxin induced liver cancer and recombinant PEMT2 expressing cells are poorly tumorigenic [20].

Lipidomic profiling has attracted considerable attention in research and has been mostly applied to identify systemic biomarkers for hepatocellular carcinoma. Lysophosphatidylcholine (LPC) species 14:0, 20:3 and 22:6 are reduced in HCC plasma compared to patients with liver cirrhosis. This suggests that low levels of these lipid species are specifically changed in HCC [7]. Long and very long chain ceramides and sphingosine-1-phosphate are significantly elevated in serum of HCC patients when compared to patients with cirrhosis [21].

In the tumor tissue of HCC patients' cholesterol is increased while phosphatidylcholine (PC) is decreased [22]. In PC saturated (16:0, 18:0), omega-6 (18:2, 20:4) and omega-3 (22:5, 22:6) fatty acids are reduced [22]. PC 16:0/16:1 is nevertheless highly abundant in human HCC tissues [23]. Regarding phosphatidylethanolamine the saturated and omega-3 polyunsaturated (22:5, 22:6) fatty acids have been shown to be reduced in HCC [22].

In the present study a comprehensive analysis of lipids in HCC tissues and adjacent non-tumorous tissues was performed. Serum lipids of the respective patients were analyzed to evaluate whether hepatic changes translate into altered circulating lipids. The characterization of HCC related lipid signatures is essential for the identification of molecular pathways contributing to hepatocarcinogenesis, the establishment of biomarkers and the development of therapies.

2. Materials and methods

2.1. Human liver tissues

HCC tissue and adjacent non-tumorous tissue of 21 male patients was obtained. These tissues have been partly used in a previous study [24]. Details of the study group are summarized in Table 1. TNM staging was done as described [25]. Experimental procedures were performed according to the guidelines of the charitable state controlled foundation Human Tissue and Cell Research (HTCR), with the written informed patient's consent approved by the local ethical committee of the University Hospital of Regensburg.

Table 1
Characteristics of the study population. HBV and HCV infections were excluded. Median values and range are listed. When data of less than 21 patients had been documented the respective number is shown in superscript. Abbreviations used: Alanine aminotransferase, ALT; aspartate aminotransferase, AST; body mass index, BMI; Non-alcoholic steatohepatitis, NASH; not documented, n.d.

Number	21
Gender	Male
Type 2 diabetes	11
No fatty liver/fatty liver/NASH	11/6/4
Age (years)	63 (47–84)
BMI (kg/m^2)	27.7 (19.7–44.6)
AST (U/l)	31.5 (14–145)^{20}
ALT (U/l)	42 (23–378)^{20}
Bilirubin (mg/dl)	0.6 (0.2–2.5)^{20}
Liver fibrosis	13
Grading G1/G2/n.d.	3/14/4
Staging T1/T2/T3/n.d.	9/6/5/1
Lymphatic invasion: no/yes	20/1
Vascular invasion: no/microvascular/macrovascular/n.d.	12/6/2/1

2.2. Quantification of lipids

Lipids were quantified by direct flow injection electrospray ionization tandem mass spectrometry (ESI–MS/MS) in positive ion mode using the analytical setup and strategy described previously [26]. In brief, plasma samples and liver homogenates were prepared according to the method of Bligh and Dyer [27] in the presence of non-naturally occurring lipid species as internal standards and analyzed on a Quattro Ultima triple-quadrupole mass spectrometer (Micromass, Manchester, UK) using an autosampler (HTS PAL, Zwingen, Switzerland) and a binary pump (Model 1100, Agilent, Waldbronn, Germany) with a solvent mixture of methanol containing 10 mM ammonium acetate and chloroform (3:1, v/v). A precursor ion of *m/z* 184 was used for phosphatidylcholine (PC), sphingomyelin [26] and LPC [28]. A neutral loss of 141 and 277 Da was used for phosphatidylethanolamine (PE) and phosphatidylinositol (PI) [29], respectively. Neutral loss scan of *m/z* 185 was used for phosphatidylserine (PS). Sphingosine based ceramides (Cer d18:1) and hexosylceramide (HexCer d18:1) were analyzed using a fragment ion of *m/z* 264 [30,31]. Free cholesterol (FC) and cholesteryl ester (CE) were quantified using a fragment ion of *m/z* 369 after selective derivatization of free cholesterol (FC) [32]. Correction of isotopic overlap of lipid species and data analysis for all lipid classes were performed by self programmed Excel Macros. Lipid species were annotated according to the recently published proposal for shorthand notation of lipid structures that are derived from mass spectrometry [33]. Glycerophospholipid annotation is based on the assumption of even numbered carbon chains only. Sphingomyelin species annotation is based on the assumption that a sphingoid base d18:1 is present. In case the fatty acid composition was not determined, annotation represents the total number of carbons and double bonds. For example, PC 36:4 comprises species like PC 16:0/20:4 or 18:2/18:2. Liver lipids are given as nmol/mg wet weight.

2.3. SDS-PAGE and immunoblotting

Proteins (20 μg of non-tumorous and/or tumorous liver tissue) were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad, Munich, Germany). Incubations with antibodies were performed in 1.5% BSA in TBS, 0.1% Tween. Detection of the immune complexes was carried out with the ECL Western blot detection system (Amersham Pharmacia, Deisenhofen, Germany). Quantification was done using ImageJ software [34]. Monoclonal anti CD163 antibody was from Morphosys AbD (Düsseldorf, Germany), p53 antibody was from Santa Cruz Biotechnology (Heidelberg, Germany) and GAPDH antibody was from New England Biolabs GmbH (Frankfurt, Germany).

2.4. Mutation analysis by direct sequencing

Mutations in TP53 were analyzed by direct sequencing of exons including exon/intron boundaries. The analysis of the TP53 gene was performed using oligonucleotides already described (http://www-p53.iarc.fr/Download/TP53_DirectSequencing_IARC.pdf). Primer combinations used in the current study are listed in Table 2. The PCR reaction mix (Qiagen Taq PCR Core Kit, Qiagen GmbH, Hilden, Germany) contains 5 μl 10× PCR buffer (contains 15 mM $MgCl_2$), 10 μl 5× Q-Solution, 1 μl dNTPs (10 mM each), 0.5 μl Taq polymerase (5 u/μl) and 0.5 μl of each PCR-primer (25 μM) in a final volume of 50 μl. Approximately 40–270 ng genomic DNA isolated from affected and non-affected liver biopsies was used for amplification. PCR products were purified with QIAquick PCR purification Kit according to the manufactures' protocol (Qiagen GmbH, Hilden, Germany) and visualized on a