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Increased expression of prostaglandin reductase 1 in hepatocellular carcinomas from clinical cases and experimental tumors in rats

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ABSTRACT

To identify novel tumor-associated proteins, we analyzed the protein expression patterns from experimental hepatocellular carcinoma (HCC) that were induced using hepatocarcinogenesis models in rats. Rats were subjected to two previously described protocols of hepatocarcinogenesis using diethylnitrosamine as a carcinogen: the alternative Solt–Farber (aS&F) protocol, which induces HCC within 9 months, and Schiffer's model, which induces cirrhosis and multifocal HCC within 18 weeks. The patterns of protein expression from tumors and normal liver tissue were examined by SDS-PAGE and the bands identified at 33–34 kDa were analyzed by mass spectrometry. The prostaglandin reductase 1 (PTGR1) showed the highest number of peptides, with a confidence of level >99%. The increased expression of PTGR1 in tumors was confirmed in these two models by Western blotting and by increase in alkenal/one oxidoreductase activity (25-fold higher than normal liver). In addition, the gene expression level of *Ptgr1*, as measured by qRT-PCR, was increased during cancer development in a time-dependent manner (200-fold higher than normal liver). Furthermore, PTGR1 was detected in the cytoplasm of neoplastic cells in rat tumors and in 12 human HCC cases by immunohistochemistry. These analyses were performed by comparing the expression of PTGR1 to that of two well-known markers of hepatocarcinoma, Glutathione *S*-transferase pi 1 (GSTP1) in rats and glypican-3 in humans. The increased expression and activity of PTGR1 in liver carcinogenesis encourage further research aimed at understanding the metabolic role of PTGR1 in HCC and its potential application for human cancer diagnosis and treatment.

1. Introduction

Hepatocellular carcinoma (HCC) is the most common tumor of the liver and the third most common cause of cancer death worldwide. The limited treatment options for intermediate- and advanced-staged cancers (El-Serag et al., 2008) necessitate the search for additional biomarkers to detect liver cancer at early stages. Animal models of hepatocarcinogenesis are commonly used to study the multistep process of human liver carcinogenesis, particularly the preneoplastic stage (Libbrecht et al., 2005; Wu et al., 2009). There are two chemical hepatocarcinogenesis models in the rat that are induced with the carcinogen diethylnitrosamine (DEN) and produce HCC in different contexts of cirrhosis. The Solt and

Abbreviations: HCC, hepatocellular carcinoma; DEN, diethylnitrosamine; S&F, Solt and Farber model; LTB4, leukotriene B4; PGE2, prostaglandin E2; 15d-PGJ2, 15-deoxy-delta-12,14-prostaglandin J2; LXA4, lipoxin A4; 4HNE, 4-hydroxy-2-nonenal; GSTP1, glutathione *S*-transferase pi 1; GGT, γ-glutamyl transferase; PH, 70% partial hepatectomy; ENT, enriched nodular tissue; MS/MS, tandem mass spectra; TFA, trifluoroacetic acid; H&E, hematoxylin-eosin; ANOVA, analysis of variance; rRNA, ribosomal RNA; qRT–PCR, quantitative reverse transcriptase polymerase chain reaction; 2AAF, 2-acetylaminofluorene; mRNA, messenger RNA; cDNA, complementary DNA; NQO1, NAD(P)H dehydrogenase, quinone 1; GSTs, glutathione *S*-transferases; EPHX, epoxide hydrolase; GCL, glutamate-cysteine ligase; UGTs, UDP glucuronosyltransferases.

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Farber (S&F) model induces nodules of resistant hepatocytes that resemble dysplasia, which chronologically progress into HCC without liver cirrhosis within a period of 9 months (Farber and Sarma, 1987; Solt and Farber, 1976). In contrast, Schiffer's model induces cirrhosis and multifocal HCC within 18 weeks (Schiffer et al., 2005).

In this work, we utilized proteomic technology to identify proteins expressed in experimental tumors. Among the proteins identified by mass spectrometry at 33–34 kDa, the top-ranked protein in the tumor samples was PTGR1. This enzyme possesses dual activity, as it is involved in the inactivation of eicosanoids with high biological activity such as leukotriene B4 (LTB4), prostaglandin E2 (PGE2), 15-deoxy-delta-12,14-prostaglandin J2 (15d-PGJ2), and lipoxin A4 (LXA4) (Clish et al., 2000; Hori et al., 2004; Yu et al., 2006), and it also has an antioxidative function through the NADPH-dependent reduction of the carbon–carbon double bond of a variety of unsaturated aldehydes and ketones such as 4-hydroxy-2-nonenal (4HNE), a cytotoxic lipid peroxidation product (Dick and Kensler, 2004). Additionally, PTGR1 reduces fatty acid nitroalkenes to nitroalkanes, inactivating the downstream signaling action of these reactive lipid electrophiles (Vitturi et al., 2013).

PTGR1 has been described as a cytoprotective enzyme that is induced in the rat liver following treatment with cancer chemopreventive agents such as dithiolethiones (Primiano et al., 1998). In this study, we measured PTGR1 expression at both the mRNA and protein levels over the course of liver cancer progression in two models of hepatocarcinogenesis in rats. In addition, PTGR1 was also studied in human HCC cases by immunohistochemistry. The expression of PTGR1 was evaluated as a potential biomarker by comparing its expression level to those of two well-known liver cancer markers, Glutathione *S*-transferase pi 1 (GSTP1) in rat tumors and glypican-3 in human tumors.

2. Material and methods

2.1. Animal procedures

Forty male F344 rats weighing 200 g were used for the two models of hepatocarcinogenesis: the alternative Solt and Farber (aS&F) protocol (Carrasco-Legleu et al., 2004; Marche-Cova et al., 1995) and the DEN-induced hepatocarcinogenesis model described by Schiffer et al. (2005) (Schiffer's protocol). Animals were obtained from the Animal Production and Experimentation Unit (UPEAL-Cinvestav, Mexico, DF, Mexico). All experiments followed committee guidelines and the institutional protocols for animal care. Groups of 20 rats for each protocol were treated as indicated in Fig. 1. Normal livers from a group of five adult rats (weighing 200–300 g) were used as controls. Animals were sacrificed by exsanguination under ether anesthesia, and then the livers were excised, washed in physiological saline solution, and frozen in 2-methylbutane with liquid nitrogen and stored at −70 °C. Nodular lesions were identified in histological sections according to gamma-glutamyltransferase (GGT) activity and then dissected from the frozen liver with a stainless steel cork borer (internal diameter, 1 mm) (Perez-Carreon et al., 2006). As a result, a collection of 20 nodules per liver was designated as enriched nodular tissue (ENT).

2.2. Protein extraction and mass spectrometry

The tissue samples (50 mg) were homogenized in 1 ml of lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 0.25% Na-deoxycholate, 1 mM EDTA, and 1× of protease inhibitor cocktail (ProteoBlock, Fermentas). Equivalent amounts of protein (30 μg) were separated by 10% SDS-PAGE. Gels were stained with colloidal Coomassie blue. Unique bands were excised from the gels and processed for protein identification at INMEGEN's proteomics facility as described (Perez et al., 2010). Identification of the proteins from the peptides was performed using a 4800 Plus MALDI-TOF/TOF mass spectrometer (Applied Biosystems, USA). Tandem mass spectra (MS/MS) were analyzed using the Protein pilot software (Applied Biosystems), and the peak list of tandem mass spectra was submitted for a search against rat species in the UniProtKB/Swiss-Prot database using the Paragon algorithm.

2.3. Western blot analysis

Proteins (30 μg) were separated by 10% SDS-PAGE and transferred onto PVDF membranes (Millipore) by electroblotting. Membranes were blocked with 5% non-fat milk and then incubated overnight at 4 °C with mouse polyclonal anti-LTB4DH (alternative name for PTGR1) antibody (Abnova) at a 1:500 dilution. After washing, the membranes were incubated for 1 h with an anti-mouse peroxidase-conjugated secondary antibody. Chemiluminescence was obtained by adding the Immobilon Western Chemiluminescent HRP Substrate (Millipore), and images were obtained with a digital image VersaDoc™ MP 5000 System (BioRad). The membranes were reprobed with an anti-GSTP1 antibody at a 1:1000 dilution (Sigma Aldrich) followed by a mouse monoclonal anti-actin antibody (Chemicon International).

2.4. Alkenal/one oxidoreductase activity of PTGR1

The NADPH-dependent alkenal/one oxidoreductase activity of PTGR1 was measured according to the continuous spectrophotometric rate determination of NADPH oxidation using trans 2-nonenal as a substrate, according to the method described by Dick et al. (2001). The final reaction consisted of a solution of 0.1 mM trans 2-nonenal, 0.1 mM of NADPH, and 0.2 mg/ml of the protein extract in 50 mM potassium/sodium phosphate buffer, pH 7.0 at 30 °C. The liver protein extracts were obtained in RIPA buffer with a proteinase-inhibitor cocktail as described previously. The oxidation rate of NADPH at 340 nm was monitored for 10 min in two consecutive conditions: without the substrate *trans*-2-nonenal and after the addition of the substrate for another 10 min. The difference in absorbance by minute was obtained, and the enzymatic activity was calculated from the molar extinction coefficient for NADPH (6.2 $(mM\,cm)^{-1}$) and expressed as nmol of NADPH/min/mg of protein.

2.5. Quantitative RT-PCR analysis

Total RNA was obtained from 30 mg of tissue by column-based extraction (RNeasy Mini kit, Qiagen). The cDNA reactions were prepared from 750 ng of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and a 1/10 dilution was used for quantitative PCR. The reactions were carried out using TaqMan gene expression assays in an 7900 HT Fast Real Time PCR system (Applied Biosystem, Mexico). FAM dye-labeled probes (exon–exon boundary) for rat *Gstp1* (Rn00561378_gH), *Ptgr1* (Rn00593950_m1), and 18S rRNA (Rn03928990) transcripts were obtained from Applied Biosystems. The *Gstp1* and *Ptgr1* data were normalized against the 18S rRNA gene expression using the comparative Ct method.

2.6. Clinical samples

Paraffin-embedded hepatic biopsies and resection samples from 12 cases of HCC (six females, six males: mean age 54.4 years, range 17–70 years) were obtained from the archive (years: 1993–2008) at the Centro de Especialidades Médicas del Estado de Veracruz, CEMEV, Mexico. The Scientific and Ethical Hospital Committee of CEMEV approved this study (permission number: 005/2011). All