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An integrative analysis of chemically-induced cirrhosis-associated hepatocarcinogenesis: Histological, biochemical and molecular features

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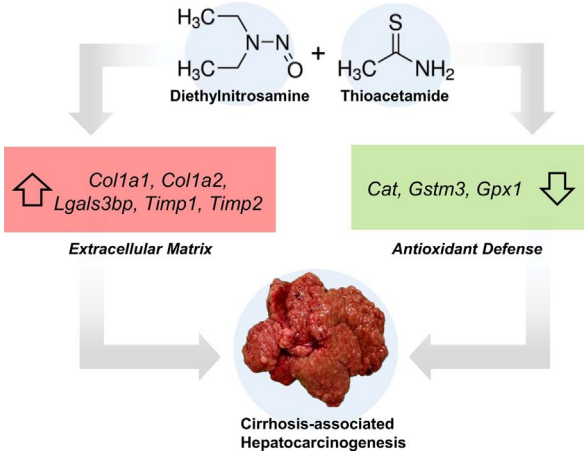
GRAPHICAL ABSTRACT



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ABSTRACT

This study aimed the integrative characterization of morphological, biochemical and molecular features of chemically-induced cirrhosis-associated hepatocarcinogenesis. Thus, male Wistar rats were submitted to a diethylnitrosamine (DEN)/thioacetamide (TAA)-induced model. Liver tissue was processed for global gene expression, histopathological and collagen evaluations; as well as immunohistochemical and oxidative stress analysis. Gene Ontology and functional analysis showed the upregulation of extracellular matrix deposition genes, such as collagen type I alpha 1 and 2 (*Col1a1* and *Col1a2)* and tissue inhibitor of metalloproteinase 1 and 2 genes (*Timp1* and *Timp2*). In agreement these findings, animals presented extensive liver cirrhosis with increased collagen deposition (Sirius red). Besides, the animals developed many glutathione S-transferase pi (GST-P)-positive preneoplastic lesions showing high cell proliferation (Ki-67), in keeping with the *Gstp1* and *Gstp2*

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increased gene expression. DEN/TAA-treated rats also showed the upregulation of tumorigenesis-related annexin A2 gene (*Anxa2*) and few neoplastic lesions (hepatocellular adenomas, carcinomas, and cholangiocarcinoma). In contrast, gene expression and activity of antioxidant enzymes were decreased (glutathione peroxidase, total glutathione-S-transferase, and catalase). The model featured remarkable similarities to human hepatocarcinogenesis. Our findings could bring up new molecular insights into cirrhosis-associated hepatocarcinogenesis, and provide a suitable animal model for the establishment of further diagnostic, preventive and therapeutic approaches.

1. Introduction

The hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and is considered a complex, multistep and multifactorial disease (Sanyal et al., 2010). The HCC is responsible for, approximately, 800.000 newly diagnosed cases and deaths *per* year worldwide (GLOBOCAN, 2013). This cancer usually emerges in a background of cirrhotic tissue (~70–90% of cases), mainly caused by chronic liver diseases linked to chronic hepatitis B and C virus infections and chronic ethanol intake (Sanyal et al., 2010; Yang et al., 2011). Cirrhosis, the end-stage of liver disease, is associated with high morbidity and mortality indexes worldwide (Mokdad et al., 2014; Scaglione et al., 2015), and was responsible for more than one million of deaths around the world in 2010 (Mokdad et al., 2014). The drastically altered microenvironment in the cirrhotic liver predisposes to several genetic and epigenetic alterations that can promote the development of preneoplastic or neoplastic lesions, mainly HCC (Zhou, 2014).

Considering the importance of chronic inflammatory microenvironment in the pathogenesis of HCC, different chemically-induced rodent models of fibrosis/cirrhosis and/or hepatocarcinogenesis are widely applied in translational research since they mimic some of the human disease features, including histopathology and clinical outcomes (Bakiri and Wagner, 2013; De Minicis et al., 2013; Yanguas et al., 2015). Diethylnitrosamine (DEN) is mainly used as an initiating agent of rodent hepatocarcinogenesis while the thioacetamide (TAA) is frequently applied for inducing liver fibrosis/cirrhosis, establishing a promoting environment to DEN-induced lesions (Ito et al., 2000; Wallace et al., 2015). The hepatic bioactivation of DEN generates reactive metabolites and reactive oxygen species (ROS), which lead to the formation of mutagenic DNA adducts (Qi et al., 2008). In addition, the bioactivation of TAA produces reactive metabolites which bind proteins and lipids thereby causing oxidative stress, centrilobular necrosis and, ultimately, hepatic stellate cell (HSC) activation and collagen deposition (Wallace et al., 2015). This altered hepatic microenvironment of increased oxidative stress, genome instability and HSC activation in rodents resembles human cirrhosis-associated hepatocarcinogenesis (Cichoż-Lach and Michalak, 2014).

Previous studies provided data on the global proteomic profile of cirrhotic microenvironment in TAA-treated rats, as well as on the global gene expression of DEN-induced cirrhosis-associated hepatocarcinogenesis in rats, in order to explain the molecular events involved in the progression of preneoplastic liver nodules into HCC (Low et al., 2004; Liu et al., 2009). Nonetheless, most of these studies lack on associating the molecular alteration profile to the subsequent biochemical and morphological alterations during cirrhosis-associated hepatocarcinogenesis.

Therefore, we aimed the integrative characterization of the molecular, morphological, and biochemical aspects of chemically-induced cirrhosis-associated hepatocarcinogenesis, featuring its many similarities to the human disease. The characterization of this model could also bring up new molecular insights into cirrhosis-associated hepatocarcinogenesis, as well as provide a standard rodent model for the establishment of diagnostic, preventive and therapeutic approaches for hepatocarcinogenesis in humans.

2. Materials and methods

2.1. Animals, treatments and sampling

Four-week-old male Wistar rats were purchased from the Multidisciplinary Center for Biological Investigation (CEMIB, UNICAMP, Campinas, SP, Brazil). After a 2-week acclimatization period, the animals were randomly allocated into two groups (n = 10 rats/group): a vehicle-treated group (untreated) and a group submitted to a classical chemically-induced cirrhosis-associated hepatocarcinogenesis model. This group received a single intraperitoneal (i.p.) injection of DEN [200 mg/kg body weight (b.wt.), in 0.9% saline] (Sigma-Aldrich, USA) (Ito et al., 2000), and two weeks later, the animals were submitted to six cycles of TAA (Furtado et al., 2012). During these cycles, animals received TAA twice a week (200 mg/kg b.wt., i.p., in 0.9% saline) (Sigma-Aldrich, USA) during three weeks, and each cycle was followed by one week without treatment (Supplementary data 1). Animals were euthanized 24 h after the last TAA administration by exsanguination under ketamine/xylazine anesthesia (91 and 9.1 mg/kg b.wt., respectively). In terms of experimental design, considering that 70% to 90% of human HCC cases emerge in a cirrhotic background, the repeated administration of a fibrogenic agent instead of a non-fibrogenic promoting drug is recommended to induce hepatocellular lesions that resemble human pathology (Sanyal et al., 2010).

Body weight and food intake were measured weekly throughout the experimental period. The animals received a commercial chow (LABINA, Purina, Brazil) and water *ad libitum* and were housed in polypropylene cages in a room maintained at 22 ± 2 °C, 55 ± 10% humidity and with a 12-h light/dark cycle (light between 07:00 a.m. and 07:00 p.m.). The experiments were carried out under protocols approved by Institute of Biosciences/UNESP Ethics Committee on Use of Animals (CEUA) (Protocol number 1073/2014) and all animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 2011).

2.2. Tissue processing, histological and immunohistochemical procedures

At necropsy, the liver was removed, washed with a saline solution (0.9% NaCl), macroscopically examined for the occurrence of gross abnormalities and weighted. Representative liver samples were either fixed in 4% phosphate-buffered formalin during 24 h for paraffin embedding or frozen at −80 °C for posterior molecular analyses. Paraffin-embedded liver samples were sliced into 5-μm thick sections and stained with hematoxylin–eosin (HE) for histopathological analysis according to well-established criteria (Thoolen et al., 2010). Other sections were stained by Sirius red for morphometric analysis of collagen content (Furtado et al., 2012). Lastly, the immunoexpression of glutathione S-transferase pi (GST-P, preneoplastic and neoplastic lesions marker), and Ki-67 (cell proliferation marker) was immunohistochemically detected in liver sections. Briefly, deparaffinated 5-μm liver sections on silanized slides were treated sequentially with citrate buffer (120 °C, 5 min) in a Pascal Pressure Chamber (Dako Cytomation, Denmark), 3% H_2O_2 in phosphate-buffered saline (PBS) (10 min) and nonfat milk (60 min). After, the slides were incubated with anti-Ki-67 (ab16667, 1:100 dilution, Abcam, UK) or anti-GST-P