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Dynamic metabolic change is indicative of inflammation-induced transformation of hepatic cells☆

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ABSTRACT

The observation that prolonged inflammation plays a causative role in cancer development has been well documented. However, an incremental process that leads from healthy to malignant phenotypes has not yet been described. Experimentally induced hepatocellular carcinoma is considered one of the representative laboratory models for studying this process. Hepatic exposure to viral infection or toxic reagents leads to chronic inflammation and gradual transformation into hepatocellular carcinoma. Here we present metabolomic profiles of hepatic cells at different stages during inflammation-induced cellular transformation by N-nitrosodiethylamine. Using gas chromatography–mass spectrometry, we quantitatively assessed the changes in cellular metabolites during the transformation process in hepatitis and liver cirrhosis. Further pathway analysis of the differentially expressed metabolites showed that carbohydrate metabolism and lipid metabolism were greatly altered in hepatitis and liver cirrhosis, respectively. Additionally, the enhanced inflammation in cirrhosis was associated with a shift from carbohydrate metabolism to lipid and amino acid metabolism. Among the differentially expressed metabolites found in diseased mouse livers, D-glucose and D-mannitol showed the most significant changes, highlighting them as potential early-diagnostic biomarkers of hepatocellular carcinoma development. Taken together, these investigations into the dynamic metabolic changes that occur during the precancerous stages of hepatocellular carcinoma add to and refine understanding of how chronic inflammation ultimately leads to cancer. Furthermore, the findings set the stage for identifying metabolites that may serve as early-diagnostic indicators of these unfolding events.

1. Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide (Siegel et al., 2013; Schütte et al., 2009). The genesis of HCC comprises complex processes involving multiple factors and stages. Epidemiological studies have shown that chronic inflammation is a major cause of HCC (Allavena et al., 2008). Chronic hepatitis causes DNA damage, leading to increased chance of genetic alteration. In the case of HCC, hepatitis gradually progresses to liver cirrhosis, the final stage of liver fibrosis, which ultimately develops into HCC; it is a chronic liver disease that usually takes 20–30 years to develop in humans, and approximately 8 months in mouse models. Elucidation of the mechanisms by which the entire process unfolds would significantly aid in the development of targeted therapies. More importantly, the formulation of preventive treatments targeting the transition from hepatitis to cirrhosis will greatly reduce the risk of HCC development.

Functional genomics and transcriptomics have been used to identify specific genes, proteins, and signaling pathways during hepatocarcinogenesis (Marquardt and Andersen, 2012; Nakagawa and Maeda, 2012; Shiraha et al., 2013). Functional genomics analysis is a powerful tool in identifying aberrantly expressed genes,

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but the inconsistency between observed mRNA levels and protein levels leaves unanswered questions concerning post-translational regulation. Proteomic approaches are an alternative tool for obtaining essential information on post-translational events. However, proteomic studies still have limitations in deciphering mechanisms of biological processes due to their complexity. In contrast, changes in many cellular processes are better tracked through metabolic pathways. Metabolic profiling is more convenient and allows for higher throughput than genomic and proteomic profiling. We therefore focused on metabolomics to analyze these changes.

Previous efforts on HCC metabolomics have primarily focused on the identification of metabolite markers in blood and urine samples. Such samples can be contaminated by mesenchymal cells and epithelial cells that surround lesions of liver cells (Tan et al., 2012). Therefore, direct analysis of cell or tissue samples should be more accurate.

In this study, we utilized a mouse hepatitis–cirrhosis model induced by N-nitrosodiethylamine (DEN) – a widely used carcinogen that causes HCC through DNA damage and oxidative stress. The DEN-induced hepatocarcinogenesis is in many ways very similar to human HCC (Verna et al., 1996), e.g., it was recently shown that hepatocyte proliferation is required for DEN-induced carcinogenesis (Kalinichenko et al., 2004). With the goal of identifying reliable and sensitive diagnostic biomarkers in HCC, we used gas chromatography–mass spectrometry system (GC/MS) to quantitatively detect changes of metabolites at different periods during the inflammation-induced malignant transformation of hepatic cells. The differentially expressed metabolites may serve as important indicators for the pathological change of hepatic cells, and thus be used as markers for clinical diagnosis of precancerous stages. In addition, the altered metabolites may also participate in inflammation-induced hepatocarcinogenesis (Sakamoto, 2009).

2. Experimental procedures

2.1. Chemicals

All chemical compounds, including DEN, phenobarbital (PB), pyrrolidine dithiocarbamate (PDTC), indomethacin (INN) and lipopolysaccharide (LPS, from *Escherichia coli* 0127:B8, Sigma #L3129) were purchased from Sigma (Sigma, USA). The antiepileptic drug PB can increase the occurrence of cancer by accelerating clonal expansion of cells that were transformed during tumor initiation. It is frequently used as a tumor promoter in rodent liver (Stahl et al., 2005). PDTC is a selective NF-κB inhibitor and an antioxidant; INN is a non-steroidal anti-inflammatory drug; and LPS can induce Toll-like receptor 4 (TLR4)-dependent systemic inflammation.

2.2. Establishment of the hepatitis–cirrhosis mouse model

Mice treated with DEN and PB develop chronic liver inflammation, cirrhosis and hepatocellular carcinoma (Maeda et al., 2005; Allavena et al., 2008; Sarma et al., 1986). Six-week old male C57BL/6 mice were randomly divided into five groups, and administered with different chemicals through intraperitoneal injections (i.p.) once per week for up to 24 weeks. Mice were treated with PBS (control group, $n = 12$), DEN (DEN group, $n = 24$), a combination of DEN and PDTC (DEN + PDTC group, $n = 24$), DEN and INN (DEN + INN group, $n = 24$), or a combination of DEN and LPS (DEN + LPS, $n = 24$). The concentrations of the chemicals administered were based on the body weight of the mice: DEN, 80 mg/kg; PDTC, 50 mg/kg; INN, 10 mg/kg; LPS, 1.25 mg/kg. During the treatments (24 weeks), body weights of the mice were checked weekly. To enhance DEN-induced tumor formation, water containing 0.05% phenobarbital (PB) was given starting at the tenth week after the first DEN injection. After 12 weeks of treatment, four mice from each treatment group and two mice from the control group were sacrificed each month. The body and liver weights were measured each time. All animal handling procedures have been approved by the Institutional Review Board of Xiamen University (IRBXU).

2.3. Collection of serum and liver tissue samples

To prepare serum samples, blood was collected from the orbital vein and kept at 4 °C for 3–4 h for coagulation. The serum was separated from the blood by centrifugation at 4000 × *g* for 10 min. Isolated serum sample was either used immediately for experiments or flash frozen in liquid nitrogen.

Mouse livers were reversely perfused from the inferior vena cava to the portal vein with PBS after sacrificing mice by cervical dislocation. Liver tissues were cut into 50–100 mg individual pieces and used immediately for experiments, fixed in 4% paraformaldehyde for histological studies, or kept at −80 °C for future use.

2.4. Hematoxylin and eosin staining

To perform histological analysis, liver tissues were fixed in 4% paraformaldehyde for 12–24 h and embedded in paraffin. Sections of the tissues (~5 μm) were stained by hematoxylin and eosin according to standard protocols. The specimens were examined under light microscope (Motic, BA 400).

2.5. Real-time quantitative RT-PCR and Western blot

Total RNA was isolated from liver tissues using RNAiso Plus kit (TaKaRa). A two-step reverse transcription-PCR procedure was performed with PrimeScript™ RT reagent Kit (TaKaRa) according to manufacturer's instructions. Purified cDNA was quantified by Rotor Gene 6000 with SYBR® Premix Ex Taq™ (TaKaRa). The expression levels of different genes were normalized to beta-actin mRNA. All PCRs were performed in triplicate.

For Western blot analysis, tissues were homogenized and lysed at 4 °C in 1% Triton X-100 lysis buffer containing protease inhibitors. After centrifugation at 12,000 × *g* for 10 min at 4 °C, the protein concentration was determined by Bradford assay. Equal amounts of total protein were resolved by SDS-PAGE, transferred to membranes, immunoblotted with specific primary and secondary antibodies, and the signals were detected by chemiluminescence (Pierce). Primary antibodies for HIF-1α (Proteintech, China) and NOS2 (Abclonal, China) were used according to the manufacturer's instructions.

2.6. Sample preparation for GC–MS

100 mg pieces of liver tissue were homogenized and dissolved in 500 μL methanol for 30 s at 0 °C. The homogenates were centrifuged at 12,000 × *g* for 10 min at 4 °C. The resulting supernatant (300 μL) was transferred to a GC sampling vial containing 10 μL internal standard ribitol (0.1 mg/mL) and dried in a vacuum centrifuge concentrator before subsequent derivatization. Samples were derivatized and treated by methoximation through a 90 min 37 °C reaction with 40 μL of 20 mg/mL methoxyamine hydrochloride in pyridine to protect carbonyl moieties. This was followed by derivatization of protons through a 30 min 37 °C reaction with an addition 80 μL N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA).