

The metabolic gene *HAO2* is downregulated in hepatocellular carcinoma and predicts metastasis and poor survival

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Background & Aims: L-2-Hydroxy acid oxidases are flavin mononucleotide-dependent peroxisomal enzymes, responsible for the oxidation of L-2-hydroxy acids to ketoacids, resulting in the formation of hydrogen peroxide. We investigated the role of *HAO2*, a member of this family, in rat, mouse and human hepatocarcinogenesis.

Methods: We evaluated *Hao2* expression by qRT-PCR in the following rodent models of hepatocarcinogenesis: the Resistant-Hepatocyte, the CMD and the chronic DENA rat models, and the TCPOBOP/DENA and TCPOBOP only mouse models. Microarray and qRT-PCR analyses were performed on two cohorts of human hepatocellular carcinoma (HCC) patients. Rat HCC cells were transduced by a *Hao2* encoding lentiviral vector and grafted in mice.

Results: Downregulation of *Hao2* was observed in all investigated rodent models of hepatocarcinogenesis. Interestingly, *Hao2* mRNA levels were also profoundly downregulated in early pre-neoplastic lesions. Moreover, *HAO2* mRNA levels were strongly

downregulated in two distinct series of human HCCs, when compared to both normal and cirrhotic peri-tumoral liver. *HAO2* levels were inversely correlated with grading, overall survival and metastatic ability. Finally, exogenous expression of *Hao2* in rat cells impaired their tumorigenic ability.

Conclusion: Our work identifies for the first time the oncosuppressive role of the metabolic gene *Hao2*. Indeed, its expression is severely decreased in HCC of different species and etiology, and its reintroduction in HCC cells profoundly impairs tumorigenesis. We also demonstrate that dysregulation of *HAO2* is a very early event in the development of HCC and it may represent a useful diagnostic and prognostic marker for human HCC.

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Introduction

L-2-Hydroxy acid oxidases are flavin mononucleotide (FMN)-dependent peroxisomal enzymes, which are members of the flavoenzyme family responsible for the oxidation of L-2-hydroxy acids to ketoacids at the expense of molecular oxygen, resulting in the formation of hydrogen peroxide [1]. In humans, there are three 2-hydroxy acid oxidase genes named *HAO1*, *HAO2*, and *HAO3* (also known as *HAOX1*, *HAOX2* and *HAOX3*), which encode peroxisomal proteins with 2-hydroxy acid oxidase activity [1]. All the members of the hydroxy oxidase family are highly conserved; human *HAO2* shares ~50% identity with human *HAO1* and 72–74% identity with rodent (rat and mouse) *Hao2* [2]. *HAO2* is predominantly expressed in the liver and kidney, and shows greatest enzymatic activity for long chain 2-hydroxy acid substrates. *HAO1* is expressed primarily in the liver and pancreas and shows greatest potency for the two-carbon 2-hydroxy acid substrate glycolic acid, but it is active also on long chain 2-hydroxy fatty acids. *HAO3* is expressed primarily in the pancreas and its preferential substrate is 2-hydroxyoctanoate [1].

Since 2-hydroxy acid oxidases are involved in the oxidation of 2-hydroxy fatty acids, they might also contribute to the general

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Abbreviations: 2-AAF, 2-acetylaminofluorene; CAR, constitutive androstane receptor; CMD, choline devoid-methionine deficient diet; DENA, diethylnitrosamine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSTP, placental glutathione S-transferase; *HAO2*, 2-hydroxy acid oxidase-2; HCC, hepatocellular carcinoma; KRT-19, cytokeratin-19; PH, partial hepatectomy; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; ROS, reactive oxygen species; R-H model, Resistant-Hepatocyte model; R-H, resistant hepatocellular carcinoma cells; TCPOBOP, 1,4-Bis-[2-(3,5-dichloropyridyloxy)] benzene.



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pathway of fatty acid α -oxidation [1], as they can oxidize a broad range of 2-hydroxy acids, ranging from glycolate to long chain 2-hydroxy fatty acids such as 2-hydroxypalmitate [3–5]. These enzymes utilize a flavin cofactor and convert 2-hydroxy acids to 2-keto acids, with the concomitant reduction of molecular oxygen to hydrogen peroxide. By producing hydrogen peroxide, the reaction catalyzed by members of *HAO2* family might contribute to increase the levels of reactive oxygen species (ROS) and, consequently, play a role in cell injury. On the other hand, increased generation of ROS levels might also represent a stimulus for the induction of cell proliferation [6,7]. Unfortunately, no data are available on the role of 2-hydroxy acid oxidases in biological processes involving cell death and cell proliferation, such as cancer.

Our recent microarray analysis showing *Hao2* downregulation in rat HCC [8] prompted us to investigate a possible role of this gene in HCC development. With this aim, we analysed *HAO2* expression in HCCs developed in three different species (mouse, rat and human), and caused by different etiologic agents.

Materials and methods

Animal experiments

Male Fischer (F-344) and Wistar rats, female C3H mice and CD-1 nude mice were obtained from Charles River, Milano, Italy. Guidelines for Care and Use of Laboratory Animals were followed during the investigation. All animal procedures were approved by the Ethical Commissions of the Universities of Cagliari, Bologna, Turin and the Italian Ministry of Health.

Rat models

R-H model: Male F-344 rats were injected intraperitoneally with diethylnitrosamine (DENA, Sigma, St. Louis, MO, USA) at the dose of 150 mg/kg body weight. After a 2-week recovery period, rats were fed a diet containing 0.02% 2-acetylaminofluorene (2-AAF, Sigma) for 1 week, followed by a two-thirds partial hepatectomy (PH), and an additional week of 2-AAF diet [9]. The animals were then returned to the basal diet and euthanized at 10 weeks, 10 months or 14 months. The number of GSTP-positive preneoplastic foci was determined as previously described [8].

CMD model: Male F-344 rats were injected intraperitoneally with a single dose of DENA (150 mg/kg body weight) and fed a choline devoid-methionine deficient diet [10]. Animals were euthanized at 10 weeks, 4 months or 13 months after DENA treatment.

Chronic DENA administration model: DENA was given in the drinking water (100 mg/L) for 8 weeks [11] to male Wistar rats. Two weeks after the end of carcinogen administration, animals were monitored weekly by ultrasound imaging (Esaote) for HCC development and euthanized 1 to 2 months later.

Mouse models

Female C3H mice were injected intraperitoneally with DENA at the dose of 90 mg/kg body weight. After a 1-week recovery period, mice were treated intragastrically with Bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP, 3 mg/kg body weight, Sigma) once/week for 28 weeks. Another group was given only TCPOBOP (once/week) for 42 weeks. The animals were euthanized at 28 and 42 weeks, respectively, time points when HCCs were observed in both the regimen protocols.

Immunohistochemistry

Frozen liver sections were collected and fixed in 10% formalin. Following endogenous peroxidases block (H_2O_2 0.3%, Sigma), aspecific sites were blocked by incubating sections for 30 minutes in 1:10 normal goat serum. Sections were incubated overnight at 4 °C with anti-GSTP antibody (MBL, Nagoya, Japan, MBL, cat# 311, 1:1000) and detected by anti-rabbit HRP (Sigma) and 3,3'-diaminobenzidine (Sigma). Sections were counterstained with Harris hematoxylin. For KRT-19 staining, serial sections were fixed in cold acetone for 20 min. Blocking of endogenous peroxidases and aspecific sites was performed as previously described for GSTP staining. Sections were then incubated with primary mouse

polyclonal KRT-19 antibody (Novocastra, Wetzlar, Germany, cat# NCL-CK19) at 1:100 dilution overnight at 4 °C and then with anti-mouse HRP and 3,3'-diaminobenzidine.

Laser-capture Microdissection (LMD)

Sixteen μ m thick serial frozen sections of rat livers were attached to 2 μ m RNase free PEN-membrane slides (Leica, Wetzlar, Germany). Microdissection (Leica, LMD6000) was followed by H&E staining, as described [12]. RNA was extracted from microdissected samples using the PicoPure RNA Isolation Kit (Arcturus, Life Technologies, Carlsbad, CA, USA), according to manufacturer instructions.

Protein extraction and Western blot

For protein analysis, cells and rat liver samples were lysed in 2% SDS, 0.5 M Tris-HCl. Western blots were performed according to standard methods. The following antibodies were used: anti-*HAO2* (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti- β -actin (I-9, Santa Cruz Biotechnology). Final detection was carried out with the ECL system (Amersham, Uppsala, Sweden).

Cell lines

Human HCC cell lines were cultured in complete medium with 10% fetal bovine serum (Gibco-Life Technologies, Carlsbad, CA) in a 5% CO_2 atmosphere. HepG2 were purchased from ATCC. HA22T/VGH and SKHep1C3.69.2 cells were generously supplied by Dr. G. De Petro [13]; Hep3B-TR, HuH7 and Mahlavu were kindly provided by Dr. N. Atabey; cells were authenticated by genetic sequencing. SNU 182, SNU 398 and SNU 475 were from ATCC (LGC Standards, Milano, Italy). Rat HCC cells (R-H) were isolated by a HCC bearing rat, as described [8].

Evaluation of ROS levels and lipid peroxidation

ROS levels were evaluated using CellRox Green (Thermo Fisher Scientific) following manufacturer's instructions. Fluorescence values were normalized to protein content. Lipid peroxidation was evaluated using TBARS Assay Kit (Cayman Chemical) following manufacturer's instructions. Absorbance values (532 nm) were normalized to protein content. For the detection of total protein redox state, N-(biotinoyl)-N'-(iodoacetyl) ethylene diamine (BIAM) labelling was performed as follows: cells were rapidly rinsed in liquid nitrogen, exposed to RIPA buffer containing 100 μ M of BIAM diamine and incubated for 15 min at room temperature. Lysates were then clarified by centrifugation and 500 μ g of each sample were immunoprecipitated using 20 μ l of Streptavidin-agarose beads (Sigma). Immunocomplexes labelled with BIAM were separated by SDS-PAGE and the biotinylated/reduced fraction was visualised with conjugated HRP-streptavidin.

Stable transduction with *HAO2* and in vivo tumorigenesis experiment

In order to obtain a stable expression, rat R-H cells were transduced either with a lentiviral vector containing the *hHAO2* cDNA (pLX304/*HAO2*, HsCD00439253, DNASU Plasmid Repository Arizona State University, Tempe, AZ) or an empty lentiviral vector as a control (mock). Stably mock and *HAO2*-transduced R-H cells (1,000,000/mouse) were injected subcutaneously in the flank of CD-1 nude mice (5 mice per group). Tumor size was measured twice a week by caliper. Subcutaneous tumor volume was calculated using the formula $4/3 \pi (D/2) (d/2)^2$, where d is the minor tumor axis and D is the major tumor axis. Mice were sacrificed 19 days after injection, and tumors were excised.

Patients

Two cohorts of patients carrying HCC were examined. In the first, patients' specimens and clinico-pathological data were obtained from the Institute of Pathology, University Hospital of Basel, Switzerland. All patients gave written informed consent to the study, which was approved by the Ethics Committee of the University Hospital of Basel (EKKB). HCC diagnosis was verified by pathological examination; no anticancer treatments were given before biopsy collection. Tumor differentiation was defined according to Edmondson's grading system. Only biopsies containing at least 50% of tumor cells and no necrotic area were used in this study. The clinico-pathologic features of these samples are described in [Supplementary Table 1](#). The second cohort consisted of HCC and cirrhotic tissues obtained from 59 consecutive patients (45 males and 14 females, median age $\pm$ SD: 65.2 $\pm$ 7.9, range 49–80 years) undergoing liver resection for HCC at the Department of Sur-

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