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ROLE OF HYDROXY ACID OXIDASE 2 (HAO2) IN HEPATOCELLULAR CARCINOMA

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LIST OF ABBREVIATIONS

2-AAF	2-acetylaminofluorene
3'-UTR	3'-untraslated region
AFB1	Aflatoxin B1
AFP	α-phetoprotein
aHCC	Advanced hepatocellular carcinoma
AKT/PKB	Thymoma viral protooncogene/Protein kinase B
APC	Adenomatous polyposis coli
BCLC	Barcelona Clinic Liver Cancer
βTrCp	β-transducin repeat-containing protein
CAR	Constitutive androstane receptor
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CK1	Casein kinase 1
Ct	Threshold cycle
DAB	3',3'-diaminobenzidine
DENA	Diethylnitrosamine
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
EGFR	Epidermal growth factor receptor
eHCC	Early hepatocellular carcinoma
EKKB	Ethics Committee of the University Hospital of Basel
ES	Edmondson and Steiner
F-344	Fisher 344
FBS	Fetal bovin serum
FCB2	Flavocytochrome *b*2 or L-lactate dehydrogenase
FFPE	Formalin-fixed paraffin embedded
Fz	Frizlzled
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSH	Glutathione peroxidase

GSK-3β	Glycogen kinase-3β
GSTP	Placental glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HAO1	Hydroxy acid oxidase 1
HAO2	Hydroxy acid oxidase 2
HAO3	Hydroxy acid oxidase 3
HbF	Fetal hemoglobin
HbS	Sickle hemoglobin
HBx	Hepatitis B x protein
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
HEK293T	Human embryonic kidney 293 cells
$HO^{\cdot}$	Hydroxyl
HRP	Horseradish peroxidase
JAKs	Janus kinases
KRT-19	Cytokeratin-19
IACT	Intra-artery chemotherapy
IARC	International Agency for Research on Cancer
IGFR	Insulin growth factor receptor
IHC	Immunohistochemistry
LEF/TCF	Lymphoid enhancer factor/T-cell factor
LMO	Lactate monooxygenase
LOX	Lactate oxidase
MAPK	mitogen-activated protein kinase
MDH	Mandelate dehydrogenase
MEM	Minimum Essential Medium
mTOR	Mammalian target of rapamycin
NO	Nitric oxide
$O_2^{\cdot-}$	Superoxide
OD	Optical density
PAHX	Phytanoil-CoA 2-hydroxylase

PBS	Phosphate buffered saline
PDGFR	Platelet derived growth factor receptor
PH	Partial hepatectomy
PI3K	Phosphatidylinositol-3-kinase
PIP3	Phosphatidyl inositol 3,4,5-triphosphate
PTEN	Phosphatase and tensin homolog
PTS	Peroxisomal targeting signal
qRT-PCR	Quantitative real time- polymerase chain reaction
QTL	Quantitative trait loci
Rb	Retinoblastoma protein
R-H	Resistant-Hepatocyte
RIN	RNA integrity number
RNS	Reactive nitrogen species
ROC	Receiver-operating charactheristic
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SEM	Standard error of the mean
SNP	Nucleotide polymorphism
SOCS	Suppressors of cytokine signaling
SOD	Superoxide dismutase
SPSS	Statistical package for social science
STAT	Signal transducer and activator of transcription
TAE	Transarterial embolization
TACE	Transarterial chemoembolization
TMA	Tissue microarray
TBS-T	Tris-buffered saline with tween 20
TCPOBOP	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
TMA	Tissue macroarray
VEGFR	Vascular endothelial growth factor receptor
VSV	Vesicular stomatitis virus

INTRODUCTION

1. Hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the most common primary liver cancer. It represents the sixth most frequent solid tumor worldwide, with more than half a million new cases per year, and the third cause of cancer-related death (Ferlay J. et al. 2010). Chronic liver disease and cirrhosis are the main causes of HCC in association with major risk factors including hepatitis B (HBV) and hepatitis C (HCV) viruses, alcohol consumption and toxic exposures to aflatoxin B1. Although less frequent, other risk factors include nonalcoholic fatty liver disease (Hashimoto E et al, 2004), diabetes (El-Serag HB et al, 2004; Regimbeau J et al, 2004), hereditary hemochromatosis (Hellerbrand C et al, 2003), obesity (Regimbeau J et al, 2004) and oral contraceptives (Maheshwari S et al, 2007).

1.1. Epidemiology

The heterogenous worldwide incidence of HCC reflects variations in distribution and in the natural history of HBV and HCV infections. More than 80% of cases arise in sub-Saharan Africa and in Eastern Asia; China alone accounts for more than 50% of the world's cases (El-Serag HB. et al. 2007). In these areas HBV is highly prevalent except Japan where HCV is the leading cause (Gao J. et al. 2012). North-America and Europe are considered areas with low incidence rates even if some data indicate a steadily increasing in the West (Parkin DM et al, 2001) where HCV plays a more dominant role.

Moreover, the incidence rate of HCC may vary by ethnicity. In the Unites States, for example, HCC incidence is two times higher in Asians than in African Americans, whose rates are two times higher than in whites (El-Serag HB. et al. 2007).

A higher HCC incidence is also observed in males, with male to female ratio being estimated approximately between 2:1 and 4:1. This divergence can be explained by sex-specific differences in exposure to different risk factors such as HBV and HCV infection and alcohol consumption. In addition, an experimental study has shown in male mice a two to eight fold increase in the development of HCC, supporting the hypothesis that androgens may influence HCC progression. (Rudolph KL et al, 2000). Worldwide, in almost all areas, the peak of higher incidence is observed in males earlier in life than females, with female rates

peaking in the age group 5 years older than the peak age group for males (El-Serag HB. et al. 2007).

1.2. Risk factors

HBV is the most frequent risk factor for HCC, with more than 240 million people with chronic infection worldwide (WHO Media centre, 2014). The relationship between HCC and HBV is well-established from the early eighties even if the role of HBV in the malignant transformation remains unclear.

In this regard, different mechanisms have been proposed. The chronic HBV infection causes a continuous immune-mediated destruction of infected hepatocytes, resulting in hepatocytes proliferation and liver regeneration (Guidotti LG et al, 2006; Seeger C et al, 2007).

The persistent inflammation, the recruitment of immune cells, and the consequent local increase in inflammatory cytokines can produce a locally mutagenic microenvironment; in addition, an excessive amount of reactive oxygen species (ROS) can directly mediate mutagenic processes by damaging DNA. Furthermore, the increase of inflammatory cytokines may induce several signalling pathways involved in gene transcription, cell proliferation, or cell survival (Berasain C et al, 2009).

Moreover, several studies have shown that HBV viral DNA can integrate into the host genome and that it actually occurs in the infected livers since the early stages of natural acute infections. Multiple integrations events have been detected in chronic hepatitis tissues, and integrated HBV sequences have been seen in 80%-90% of HBV-related HCCs (Balsano C et al, 1994). Genome integration of HBV at specific sites has been associated with major genetic alterations, including generalized genomic instability, gene and chromosomal deletions and translocations, amplification of cellular DNA, and generation of fusion transcripts (Reifenberg K et al, 1999; Klein N et al, 1999).

The third mechanism by which HBV contributes to carcinogenesis is by expression of viral proteins, in particular, X protein (HBx). Although HBx does not bind directly to DNA, in the cytoplasm it modulates mitogenic signaling pathways involved in oncogenesis, proliferation, apoptosis, inflammation and immune response. In the nucleus it promotes the interaction with numerous transcription factors modulating gene expression (Bouchard MJ and Navas-Martin S., 2011).

Twenty years after the discovery of HBV, Choo and colleagues in 1989 identified hepatitis C virus (Choo QL et al, 1989), an enveloped, single stranded, positive sense RNA virus (Hoofnagle et al, 1999). HCV is the major risk factor in areas with low prevalence of HBV; the infection is present in about 3% of world's population and 170 million people are chronic carriers (Gao J et al, 2012).

The mechanism by which HCV causes HCC is not well defined. Unlike HBV, it does not integrate into the human genome. The main hypothesis is that in the context of chronic liver damage caused by HCV infection, with a persistent inflammation and hepatocyte regeneration, the resultant cirrhosis can lead to chromosomal damage that can trigger the development of HCC in the modified tissue microenvironment (Gomaa AI et al, 2008). Oxidative stress, steatosis and insulin resistance have been identified as procarcinogenic cofactors in chronic HCV infections (McGivern DR et al, 2009).

A second hypothesis is that interactions between host and viral proteins may have a direct role in HCV-related HCC through the modulation of cellular proliferation, apoptosis and immunological responses (Pang R et al, 2005). In fact HCV core and non-structural proteins can modulate several tumor suppressor genes and their targets and activate different intracellular pathways. HCV core protein has been found to bind to p53 (Ray RB et al, 1997), p73 (Alisi A et al, 2003) and pRb (Cho J et al, 2001). In particular, core and non-structural proteins can modulate cell-cycle control by regulating the cyclin-dependent inhibitor p21/Waf, a target of p53 (Uchida M et al, 2002).

Various studies have shown the role of core proteins in the regulation of three different pathways involved in cell proliferation. Viral proteins can activate the Raf1/MAPK thus promoting cell proliferation (Hayashi J et al, 2000) and the Wnt/β-catenin pathway, which can control DNA synthesis and cell-cycle progression (Fukutomi T et al, 2005). Core proteins modulate also TGFβ signaling, which is involved in cell proliferation, apoptosis, and differentiation (Shin JY et al, 2005).

The association between heavy and prolonged alcohol consumption (more than 50-70 g/day) with both HCC incidence and mortality was found in the mid eighties (Nanji AA et al, 1985). Alcohol ingestion can lead to the development of HCC through several mechanisms: as a carcinogen, leading to the development of cirrhosis, and with a synergistic effect with other risk factors, such as HBV and HCV (Gao J et al, 2012). Some data demonstrated that among consumers of alcohol, the risk of developing HCC increases linearly with a daily intake of more than 60 grams; the simultaneous presence of HCV infection increases about 2-fold

the risk of hepatocellular carcinoma development (Donato F et al, 2002). Other studies confirmed the interaction between alcohol assumption and risk factors like diabetes, obesity and smoking (Wang LY et al, 2003; Yuan JM et al, 2004; Singal AK et al, 2007; Chuang SC et al, 2009).

Aflatoxins are mycotoxins produced by Aspergillus parasiticus and Aspergillus Flavus. Under favourable conditions, like warm and damp environment, these fungi grow and colonize many types of foods such as grain, corn and peanuts. In particular, Aflatoxin B1 (AFB1) was classified as group I carcinogen by the International Agency for Research on Cancer (IARC, 1987). After ingestion, AFB1 requires metabolic conversion to its active intermediate, AFB1-exo-8,9-epoxide, in order to bind DNA and cause damage (Garner RC et al, 1972). In particular, a G>T mutation of the p53 gene at codon 249 has been associated with AFB1 exposure; this mutational inactivation has been observed in 30%-60% of HCC developed in geographical areas that are endemic for AFB1 (Bressac B et al, 1991; Turner PC et al, 2002). Several authors have demonstrated an association between AFB1 exposure and HBV infection (Yeh FS et al, 1989; Qian GS et al, 1994) and that the interaction of both risk factors increased the risk of hepatocarcinogenesis by interfering with the ability of hepatocytes to metabolize aflatoxins (Smela ME et al, 2001; Williams JH. et al. 2004).

The investigation of different mechanisms leading to hepatocarcinogenesis points to common pathogenetic pathways and processes involving, in particular, p53 inactivation or mutation, inflammation, continuous cycles of necrosis and regeneration, and oxidative stress, underlying their essential contribute in HCC development (Farazi PA et al, 2006).

1.3. Molecular mechanisms

As described above, many studies over the years have identified major risk factors for HCC, even if little is known about the molecular mechanisms that contribute to HCC development.

Growing evidence suggests that gradual accumulation of mutations and genetic changes in preneoplastic hepatocytes cause malignant transformation leading to HCC development (El-Serag et al, 2007).

The neoplastic evolution of HCC is a multi-step process (Farazi PA et al, 2006). Different etiologic agents (hepatitis virus infection, toxins, alcohol, etc.) are able to induce hepatic injury followed by necrosis and hepatocyte regeneration; this setting of chronic liver disease can then progress to cirrhosis, a predisposing condition to hepatocarcinogenesis, which

becomes manifest after 20-40 years of latency (El-Serag HB et al, 2007). The cirrhosis process is characterized by the formation of abnormal liver nodules surrounded by collagen deposition and scar tissue. Afterwards, the first step towards HCC is the formation of hyperplastic nodules of regenerating hepatocytes followed by the appearance of dysplastic nodules. The latter pre-malignant lesions consist of abnormal liver architecture with an increase in trabecular thickening and including clear cells, enriched in glycogen or lipids deposits, and nuclear crowding. These dysplastic nodules can develop into HCC and acquire invasive and metastatic ability (Okuda K., 2000). HCC can be graded in well, moderately and poorly differentiate HCC. A tumor can be defined as well differentiated when cell morphology is similar to normal hepatocytes. Conversely, in poorly differentiated tumors, the cells are large and often share common morphological aspects with those of other metastatic tumors; they represent in fact the most malignant type of primary HCC.

Shortening of telomeres is a mechanism involved in chronic liver disease and cirrhosis (Urabe Y et al, 1996; Miura N et al, 1997; Rudolph KL et al, 2001; Kitada T et al, 1995); it has a role in cancer initiation and contributes to the induction of chromosomal instability (Plentz RR et al, 2004 and 2005; Farazi PA et al, 2003). Telomerase reactivation has been associated with malignant progression; it is necessary to restore chromosomal stability to a level compatible with cancer cell viability (Farazi PA et al, 2003 and 2006).

The molecular analysis of human HCC has revealed the accumulation of genetic and epigenetic modifications that cause significant changes in gene expression and in cellular signaling pathways (Aravalli RN et al, 2013). The loss of cell-cycle checkpoints is one of the most frequent alterations in HCC; in particular, p53 and Rb proteins are the most affected (El-Serag HB. et al. 2007).

p53 is a critical tumor suppressor; its pathway prevents cell survival and proliferation during shortening of telomeres (Wright WE et al, 1992), triggers apoptosis in genotoxic stress conditions (Speidel D. et al, 2015), induces cell-cycle arrest in response to oncogene activaction (Di Micco R et al, 2006) and promotes genome integrity (El-Serag HB et al, 2007).

Under normal conditions, p53 cellular levels are low; they are immediately up regulated after intracellular and extracellular stress signals. In half of all human tumors inactivation of the p53 gene is due to a single point mutation, in others, the expression of the p53 protein is not affected but cell signaling pathways involved in cell cycle arrest and apoptosis are defective (Stegh AH, 2012). Several studies have shown a critical role of p53 mutation or inactivation in HCC (Bressac B et al, 1991; Tannapfel A et al, 2001; Jablkowski M

et al, 2005; Hsu IC et al, 1993; Lunn RM et al, 1997). Therefore, disruption of p53 checkpoint function at the cirrhosis stage could provide a selective advantage allowing proliferation of hepatocytes with a critical telomere lenght (El-Serag HB et al, 2007).

The tumor suppressor retinoblastoma protein (Rb) is a major regulator of cell cycle in response to telomere shortening, DNA damage, and oncogene activaction (Wright WE et al, 1992; Di Micco R et al, 2006). The G1 to S phase transition is regulated by the hypophosphorylated Rb gene which sequesters the E2F family of transcription factors inhibiting the expression of genes required for entry into S phase. The phosphorylation state of Rb is tightly regulated by cyclin-dependent kinases (CDKs) (Goodrich DW. 2006). In human HCC, a deregulation of Rb pathway is observed in more than 80% of cases, with a frequent repression of the CDK inhibitor p16 due to promoter methylation (Azechi H et al, 2001). Besides p53, an impairment of the Rb checkpoint would allow an expansion of hepatocytes with altered telomeres during the cirrhosis stage (El-Serag HB et al, 2007).

Increasing evidence suggests enhanced levels of ROS and RNS (reactive nitrogen species) in many types of cancer cells (Kawanishi S et al, 2006; Marra et M al, 2011; Chuma et al, 2008). ROS are defined as oxygen-containing chemical species with reactive properties. They are produced continuously in cells and derive from metabolic reactions occurring in the mitochondria (Handy DE et al, 2012), peroxisomes (Schrader M et al, 2006) and endoplasmic reticulum (Malhotra JD et al, 2007). Important free radicals in humans, include superoxide ($O_2^{\cdot -}$), hydroxyl ($HO^{\cdot}$) and nitric oxide (NO), as well as non-radical molecules such as hydrogen peroxide (H_2O_2) Cells have developed a complete set of antioxidant mechanisms to counteract and limit damaging effects of free radical formation. These include the enzymes, superoxide dismutase (SOD), glutathione peroxidase (GSH) and catalase. There are further antioxidant defences including Vitamin C and E. At moderate levels, ROS can promote cell proliferation and differentiation (Boonstra J et al, 2004; Schafer FQ et al, 2001). However, the disruption of the redox balance induces high ROS levels that promote cell death and cellular damage (Toyokuni S et al, 1995).

Especially at early stages, cancer cells try to contrast damaging ROS effects, increasing ROS scavengers activity that reduce ROS to levels that are compatible with cellular biological functions, but still higher than in normal cells (Gorrini C et al, 2013).

Some of the more frequently activated oncogenic pathways found in human HCC include the Wnt/β-catenin, MAPK AKT/mTOR and JAK/STAT (Aravalli et al, 2013).

β-catenin is the critical downstream effector in Wnt signaling pathway where it mediates a nuclear response after the binding of Wnt proteins to Frizzled receptors at the plasma membrane. In unstimulated adult cells, when the Wnt pathway is inactive, β-catenin is recruited into a destruction complex with Axin, adenomatous polyposis coli (APC), casein kinase 1 (CK1) and glycogen synthase kinase-3β (GSK-3β). CK1 and GSK-3β phosphorylate β-catenin at specific residues (Amit S et al, 2002) which results in β-catenin recognition by the β-transducin repeat-containing protein (βTrCP) for ubiquitination and subsequent proteasomal degradation (Monga SP. 2014).

Binding of Wnt proteins to the Frizzled (Fz) receptor induces the disruption of the complex resulting in cytoplasmic accumulation of β-catenin and nuclear translocation. In the nucleus, β-catenin binds to the LEF/TCF (lymphoid enhancer factor or T-cell factor), exerting its function through the transcriptional regulation of its target genes (Logan CY et al, 2004). β-catenin target genes are involved in embryonic and tissue development, cellular differentiation, survival, regeneration, self-renewal and in oncogenic transformation of several cell types, including hepatocytes (Cadigan KM et al, 1997; Nusse R et al, 2005; Peifer M et al, 2000; Willert K et al, 2004.). The aberrant activation of this pathway has been found, in about 20-40% of HCC cases, linked to activating β-catenin mutations (De la Coste A et al, 1998; Zucman-Rossi J et al, 2007).

MAPK (mitogen-activated protein kinase) cascade transduces signals from tyrosine kinase receptors, such as EGFR (Epidermal growth factor receptor), IGFR (Insulin growth factor receptor), PDGFR (Platelet derived growth factor receptor), MET, VEGFR (Vascular endothelial growth factor receptor), upon the binding to their ligands. In this cascade, active Ras (GTP-Ras) causes the sequential activation of Raf, Mek and Erk. The latter transactivates numerous growth-related genes such c-Jun, c-Fos, c-Myc. MAPK signaling is involved in cell survival, differentiation, adhesion and proliferation (Roberts PJ et al, 2007; Schmidt CM et al, 1997). Moreover, it is often found to be deregulated in various types of human tumors, including HCC (Huang P et al, 2010; Wagner EF et al, 2009).

The PI3K/Akt/mTOR pathway also plays an important role in cell growth, survival regulation, metabolism, and inhibition of apoptosis (Kudo M. 2012). It acts through phosphatidylinositol-3-kinase (PI3K) and generation of phosphatidyl inositol 3,4,5-triphosphate (PIP3), which binds to and activates the serine/threonine kinase Thymoma viral protooncogene (Akt/PkB) (Woodgett JR. 2005). Activation of Akt signaling and impaired expression of phosphatase and tensin homolog (PTEN) (a negative regulator of Akt) have

been reported in 40%–60% of human HCC (Hu TH et al, 2003). The serine/threonine kinase mTOR (mammalian target of rapamycin) is an important mediator in the PI3K/Akt pathway, that regulates protein synthesis mediating cell growth (Engelman JA et al, 2009).

Signal transducers and activators of transcription (STATs), which consist of a family of transcription factors, are activated by a large number of cytokines, hormones, and growth factors (Harrison DA. 2012) through tyrosine phosphorylation by Janus kinases (JAKs). Activated STATs stimulate the transcription of suppressors of cytokine signaling (SOCS) genes and their proteins that act to negatively regulate receptor complexes by inactivating JAKs or blocking recruitment sites for STATs. The JAK/STAT pathway activates cell proliferation, migration, differentiation, and apoptosis (Yoshikawa H et al, 2001). The activation of JAK/STAT pathway as consequence of a deregulation of SOCS has been reported in HCC (Calvisi DF. et al. 2006)

1.4. Clinical aspects

The knowledge of the main risk factors, and the mechanisms through which these act, could help to reduce the incidence of HCC, through appropriate prevention that aims to limit viral transmission and alcoholism. The spread of HBV infection can be effectively reduced by vaccination. In Italy a mandatory vaccination policy was introduce in 1991 for all new borns and 12 year old adolescents, and a screening for women in the third quarter of pregnancy (Zanetti AR et al, 2008). To date, unfortunately, there is no vaccine against HCV and prevention of infection should focus on proper health care conditions. Moreover, public awareness regarding the risks of excessive alcohol consumption might contribute to the reduction of HCC risk (Bruix J et al, 2004).

If effective primary prevention is not possible, other approaches aimed at preventing the spread of these viruses are necessary in order to remove risk factors and their ability to induce chronic liver damage and cirrhosis. The effect of antiviral therapy in patients with chronic hepatitis B or C on the prevention of the development of HCC is controversial.

Therefore, the only strategy is the early detection that can be achieved with a surveillance program (Bruix J et al, 2004). A liver ultrasonography every 6 months is recommended only for patients with cirrhosis of any etiology or for those without cirrhosis but with chronic HBV infection (Bruix J et al, 2011). It is well tolerated and widely available, and capable of achieving sensitivity of 60–80% and specificity beyond 90%. (Singal A et al, 2009). The serological test of α-fetoprotein (AFP) is no longer recommended for effective

surveillance because of its low diagnostic sensitivity and specificity (Singal A et al, 2009; Lok AS et al, 2010).

Moreover, the histological grade and the staging of the tumor are important prognostic factors in the management of patients, in assessment of prognosis and in treatment assignment.

The histologic grading of HCC is usually evaluated by the Edmondson and Steiner (ES) grading system, which was first described in 1954 (Edmondson HA et al, 1954). The diagnostic criteria used by the authors to determine the degree of cell differentiation are the quality of the cytoplasm (quantity, granularity and acidophilia), the nuclear aspect (size and degree of hyperchromatism) and the nuclear/cytoplasmic ratio. They also included the cohesive quality, the function and the architecture of tumor cells. Using these criteria the authors classified tumors into four grades.

Grade I belong to the best differentiated tumors. Its diagnosis is possible only after the demonstration of more aggressive growths in other parts of the carcinoma with grade II.

Cells in grade II are similar to normal hepatic cells with hyperchromatic and larger nuclei than normal with an acidophilic, abundant cytoplasm. Acini are frequent and their lumina of various sizes are often filled with bile or protein precipitate.

In grade III nuclei of tumor cells are progressively larger and more hyperchromatic but with less cytoplasm; this is acidophilic and with segregation of granular material toward the cell border. Some breakup or distortion of the usual trabecular pattern is present. Tumor giant cells are numerous.

Grade IV is characterised by the presence of intensely hyperchromatic nuclei that occupy a greater portion of the cell with a scanty and less granular cytoplasm. Another characteristic of these tumors is the loss of trabecular pattern and the lack of cohesiveness of the cells that would predispose to early and widespread metastases.

Importantly, grading heterogeneity inside a tumor is frequently observed, making difficult the classification of the tumor (Pawlik TM et al, 2007). Accordingly, and compared to other carcinomas, HCCs are usually classified in well-, moderately and poorly differentiated (Paradis V. 2013).

The Barcelona Clinic Liver Cancer (BCLC) staging classification was first published in 1999 (Llovet JM et al, 1999) and has been refined and further developed in 2003 (Llovet JM et al, 2003). This system is widely accepted to stratify individual tumors according to the

different available treatments. In agreement to this staging system, patients can be classified into five groups: very early, early, intermediate, advanced and end-stage.

The very early stage includes patients with a well compensated cirrhosis, single and well differentiated HCC, less than 2 cm in size. The diagnosis of tumor is possible only after surgery and these patients can benefit of curative treatments.

Asymptomatic patients with single HCC smaller than 5 cm in diameter or three nodules equal or less than 3 cm compose the early stage (Mazzaferro V et al, 1996). These patients with preserved liver function are candidate to curative treatments.

The intermediate stage is formed by patients with large, multifocal HCCs, who are asymptomatic and without an invasive pattern, defined as macrovascular invasion or extrahepatic spread. In these patients the transarterial chemoembolization (TACE) is considered the elective treatment.

The advanced stage is characterized by patients with mild cancer-related symptoms and an invasive pattern. Until very recently, there was no effective treatment and patients were therefore candidates for therapeutic trials.

The end-stage is characterized by an extensive tumor involvement and an impaired physical status. It also includes patients with severe impairment of liver function and unsuitable for transplantation. The expected survival is shorter than 3–6 months and these patients should be treated only with palliative therapies.

In according with BCLC system, patients can therefore be assigned to different therapeutic strategies. Patients diagnosed at an early stage can undergo curative treatments such as surgical resection, transplantation or percutaneous ablation; they offer a high rate of complete responses and, thus, potential for cure. Treatment options for HCC include also palliative care, which is not aimed to cure, but in some cases can obtain good response rate and even improve survival (Llovet JM et al, 2003). Unfortunately, only 30%-40% of patients benefit of these treatments in western countries and hence the vast majority receive palliative therapy (Bruix J et al, 2002).

Surgical resection is reserved to patients with very early and early stage HCC and without portal hypertension (Bruix J et al, 1996). This patients are the best candidates for the resection of a hepatic segment, with survival rates exceeding 60%-70% at 5 years; the major pitfall is the high rate of tumor recurrence being more than 50% at 3 years during follow-up (Llovet JM et al, 2003).

Liver transplantation is the treatment option for patients with early HCC in decompensated cirrhosis; the 5-year survival rate is over 70% and the risk of tumor recurrence is less than with surgery (Llovet JM et al, 2003). The main obstacle of this treatment is the paucity of donor livers; it increases the waiting time for transplantation and leads to patients drop out from the waiting list as a consequence of HCC progression. The monthly drop-out rate is around 4% (Sarasin FP et al, 2001; Llovet JM et al, 2005)

Percutaneous ablation is the treatment of choice for early, unresectable HCC (Livraghi T et al, 1995; Lencioni R et al, 1997; Rossi S et al, 1996; Buscarini L et al, 2001; Okada S et al, 1999). Destruction of tumor tissue can be obtained by direct injection of chemical substances (ethanol, acetic acid, boiling saline) or by physical modification of the cancerous tissue temperature (radiofrequency, microwave, laser, cryotherapy) (Okada S et al, 1999). This treatment modality has the advantage of a very short hospitalization, low cost and rare complications (Livraghi T et al, 1995). Efficacy of percutaneous ablation, defined as absence of contrast uptake on computerized tomography one month after the procedure (Bruix J et al, 2001), greatly depends on tumor size. It decreases from 90-100% in HCCs less than 2 cm, with a survival rate a 5-years of 71%, to 70% in HCCs sized 2–3 cm and 50% in HCCs sized 3–5 cm (Okada S et al, 1999; Mazzanti R et al, 2004a,b).

Palliative treatments can be proposed to patients in intermediate and advanced tumor stage that do not benefit from curative interventions. They include transarterial chemoembolization (TACE), transarterial embolization alone (TAE), intra-artery chemotherapy (IACT) and radiation therapy (Mazzanti R et al, 2007). Systemic chemotherapy with several kinds of drugs as well as hormonal therapy have been shown to be ineffective in the treatment of HCC, toxic, and without survival benefits (Bruix J and Sherman M. 2005). Among palliative treatments only TACE with cisplatin or doxorubicin has been proven to improve survival (Llovet JM and Bruix J, 2003); it is indicated in patients with intermediate stage HCC or early tumors in which curative treatments are unfeasible (Mazzanti R et al, 2008). Chemoembolization combines selective injection of chemotherapeutic drugs into the hepatic artery with obstruction of the vessels feeding the tumor. Despite the large use of TACE in treatment of unresectable HCCs, an universally standard protocol is currently not available. Patients with portal thrombosis or advanced disease in which TACE treatment is not recommended could ameliorate their survival by IACT (Mazzanti R et al, 2008); a pilot study has shown a benefical effect of 5-fluorouracil (5-FU) infusion on response rate and survival (Mazzanti R et al, 2004a).

The advent of targeted therapies and the approval of the systemic treatment of advanced HCC with the kinase inhibitor sorafenib have provided some hope for the future. Currently, only sorafenib is approved for HCC treatment; however, it improves survival by only a couple of months. Consequently, HCC remains one of the few cancer types with an increasing cancer-related mortality. Although no definite breakthrough in treatment is currently in sight, new therapeutic targets have recently come to the spotlight. One of these targets is the tyrosine kinase receptor for the Hepatocyte Growth Factor (HGF), encoded by the MET gene, known to promote tumor growth and metastasis in many human organs.

1.5. Animal model of hepatocarcinogenesis

Due to the multistage nature of HCC, the molecular pathogenesis of this cancer cannot be properly understood without more information on the molecular alterations characterizing its early development. Unfortunately, the study of the early steps of HCC development in humans is complicated due to the late stage in which the tumor is diagnosed and to the heterogeneity inside a tumor that makes difficult a clinical classification. Therefore, animals model that enable dissection of the several steps of hepatocarcinogenesis are required.

In my thesis we used two different rodent models of hepatocarcinogenesis to investigate which alterations found in HCC could take place at early stages of the process, suggesting therefore their possible critical role.

The first model employed was the rat Resistant-Hepatocyte (R-H). The R-H or Solt-Farber rat model (Solt DB et al, 1977) allows the identification of phenotypically distinct lesions along the various steps of liver carcinogenesis in the rat (early nodules, adenomas, early HCCs, and advanced HCCs).

This experimental model constists of a classical initiation-promotion procedure: initiation of hepatocytes is determined by a single necrogenic dose of diethylnitrosamine (DENA); promotion is achieved by 2 weeks of dietary exposure to 2-acetylaminofluorene (2-AAF), that selectively inhibits the proliferation of normal hepatocytes and 2/3 partial hepatectomy (PH) as a growth stimulus for DENA-initiated hepatocytes.

Initiated cells, unlike normal hepatocytes, are indeed capable to clonally expand and rapidly give rise to early preneoplastic lesions identifiable by their positivity to the placental form of the enzyme glutathione-S-transferase (GSTP). During the carcinogenic process, the vast majority of the preneoplastic lesions re-differentiate througha process named

remodeling (Enomoto K et al, 1982) with a gradual loss of the GSTP and other preneoplastic markers (Perra A et al, 2009). Only persistent nodules progress, to adenomas and early HCCs (eHCCs) clearly visible at about 10 months after DENA initiation. Fourteen months after initiation, all rats present advanced HCCs (aHCCs).

Previous studies allowed to distinguish within $GSTP^+$ preneoplastic lesions, , a subpopulation of persistent nodules that express the intermediate filament protein cytokeratin 19 (KRT-19) (Andersen JB et al, 2010).

KRT-19 is expressed in normal adult liver only in epithelial cells of the bile ducts, but not in hepatocytes. Its abnormal expression is found in several pathological conditions including remodeling of the parenchyma in livers with chronic hepatitis or cirrhosis, capillarization of hepatic sinusoids and ductular oval cell proliferation (Su Q et al, 2003).

A comparative analysis of the gene expression profiles of early preneoplastic lesions and of early and advanced HCCs, has revealed a common signature, thus demonstrating their ability to progress to HCC, giving rise to almost all HCCs generated in the Resistant hepatocyte model (Andersen JB et al, 2010). Furthermore, the gene expression signature associated with the rat lesions positive for the stem/progenitor cell marker KRT-19 predicts the clinical outcome of human HCC, giving a translational value to this model (Petrelli A et al, 2014).

In the second animal model, female C3H/HeNCrl mice were treated chronically with repeated doses of the CAR (Constitutive androstane receptor) ligand TCPOBOP (1,4-bis[2-(3,5-dichloropyridyloxy)]benzene), with or withouth a single administration of DENA.

TCPOBOP is the most potent nuclear receptor CAR agonist, that similar to phenobarbital, induces liver hyperplasia and hypertrophy in mice (Dragani TA et al, 1985; Manenti G et al, 1987) by accelerating the entry of hepatocytes into the S phase of cell cycle (Ledda-Columbano et al, 2000). Moreover, TCPOBOP is both a nongenotoxic carcinogen on its own and a potent tumor promoter when combined with genotoxic agents (Diwan BA et al, 1992; Columbano A et al, 2003; Ledda-Columbano GM et al, 2000; Locker J et al, 2003). In our study TCPOBOP induces the development of HCCs in mice treated with or without DENA after 28 and 42 weeks, respectively.

2. Hydroxy acid oxidases

L-2 Hydroxy acid oxidases are flavin mononucleotide (FMN)-dependent peroxisomal enzymes. Although their role is still unknown, these enzymes are members of the

flavoenzyme family capable of oxidizing several 2-hydroxy acids, ranging from glycolate to long chain 2-hydroxy fatty acids such as 2-hydroxypalmitate (Angermüller S et al, 1989; Fry DW et al, 1979; Schwam H et al, 1979), to 2-keto acids. This reaction results in hydrogen peroxide (H_2O_2) formation at the expense of molecular oxygen (Jones JM et al, 2000) (**Fig. 1**).

Fig. 1. Schematic representation of the catalytic reaction of hydroxy acid oxidases

Other enzymes belonging to this family have been identified in different organisms: flavocytochrome *b*2 or L-lactate dehydrogenase (FCB2) from yeast (Lederer F. 1991), lactate monooxygenase (LMO) from Mycobacterium (Ghisla S et al, 1991), lactate oxidase (LOX) from Aerococcus Viridans (Maeda-Yorita, K et al, 1995), mandelate dehydrogenase (MDH) from Pseudomonas putida (Lehoux IE et al, 1999) and glycolate oxidase from plants (Volokita M et al, 1987).

In mammals, 2-hydroxy acid activity was first described in rat, both in kidney and liver (Blanchard M et al, 1944; 1945; 1946); it was also reported in mouse (Duley J et al, 1974) and hog renal cortex (Robinson JC et al, 1962). In both prokaryotes and eukaryotes, all family members share a highly conserved nucleotide and amino acid sequences (Barawkar DA et al, 2012).

Only at the beginning of the 21st century, Jones and collaborators identified for the first time three human 2-hydroxy acid oxidases named HAO1, HAO2, and HAO3 (also known as HaoX1, HaoX2 and HaoX3), homologs of plant glycolate oxidase, which encode peroxisomal proteins with 2-hydroxy acid oxidase activity (Jones JM et al, 2000). In particular, the human HAO1 cDNA is 1743 bp in length and codes a protein of 370 amino acids with a predicted molecular weight of 41 kDa. The HAO2 cDNA is 1417 bp long encoding a protein of 351 amino acids with an estimated molecular weight of 39 kDa. The HAO3 cDNA sequence is 1821 bp which encodes a predicted protein of 353 amino acids and a molecular mass of 39 kDa. HAO2 and HAO3 share 70.4% sequence identity and only about 45% with HAO1. As described by Jones and colleagues, all three gene products show substrate preference and distinct tissue expression patterns. HAO1 is expressed at high levels in liver and pancreas and to a much lesser extent in kidney and placenta, displaying the highest activity with glycolate but also toward glyoxylate, 2-hydroxyoctanoate, and 2-

hydroxypalmitate. High expression levels of HAO2 were detected both in liver and kidney with small amounts also in thymus. It is preferentially active against long chain 2-hydroxy acids, in particular 2-hydroxyoctanoate or 2-hydroxypalmitate. HAO3 is found to be expressed primarily in pancreas with 2-hydroxyoctanoate as substrate.

Very few data are available in the literature about human 2-hydroxy acid oxidases; to date, their functional role remains to be identified.

Some studies focused on Hao2 have been conducted in rat models of genetic hypertension, in order to investigate genetic determinants of blood pressure regulation. In fact, Hao2 has been identified as a candidate gene for the systolic blood pressure quantitative trait loci (QLT) in rats (Lee SJ et al, 2003); another genome-wide linkage study seemed to confirm the potential relation between Hao2 and hypertension in humans (Rico-Sanz J et al, 2004). To establish the involvement of Hao2 in blood pressure regulation, several authors, availing themselves of the rat Hao2 crystal structure (Cunane LM et al, 2005) developed potent and selective rat Hao2 inhibitors (Barawkar DA et al, 2011; 2012) that seem to inhibit Hao2 leading to a reduction of blood pressure in the rat hypertension models analysed.

Another study (Ma Q et al, 2007) showed the possible role of Hao2 in the treatment of patients with sickle cell anemia in response to hydroxyurea, a drug that increases concentration of fetal hemoglobin (HbF) inhibiting the polymeration of sickle hemoglobin (HbS). This study suggests that single nucleotide polymorphisms (SNPs) of several genes, including Hao2, are associated with the HbF response to treatment with hydroxyurea.

In their work on the identification of the three human hydroxy acid oxidases (Jones JM et al, 2000), Jones and coworkers hypothesized their possible contribution in α-oxidation of fatty acids. Their oxidative metabolism can proceed by distinct pathways, including α-, β- and ω-oxidation (Wanders RJ et al, 2011).

The discovery of fatty acid α-oxidation is closely related to the finding of high amounts of phytanic acid in kidney, liver and brain of patients affected with Refsum disease, a rare genetic disease characterized by retinitis pigmentosa polyneuropathy, deafness, ataxia, ichthyosis and cardiac manifestations.

α-oxidation in humans is a process in which 3-methyl branched fatty acids, such as phytanic acid, are shortened to 2-methyl branched fatty acids, which can then be further degraded by peroxisomal and mitochondrial β-oxidation (**Fig. 2**). Despite several studies some of the individual enzymatic steps remain to be identified. Research focused on the

definition of this pathway reveals that this mechanism is predominantly, if not exclusively, peroxisomal (Jansen GA et al, 2006).

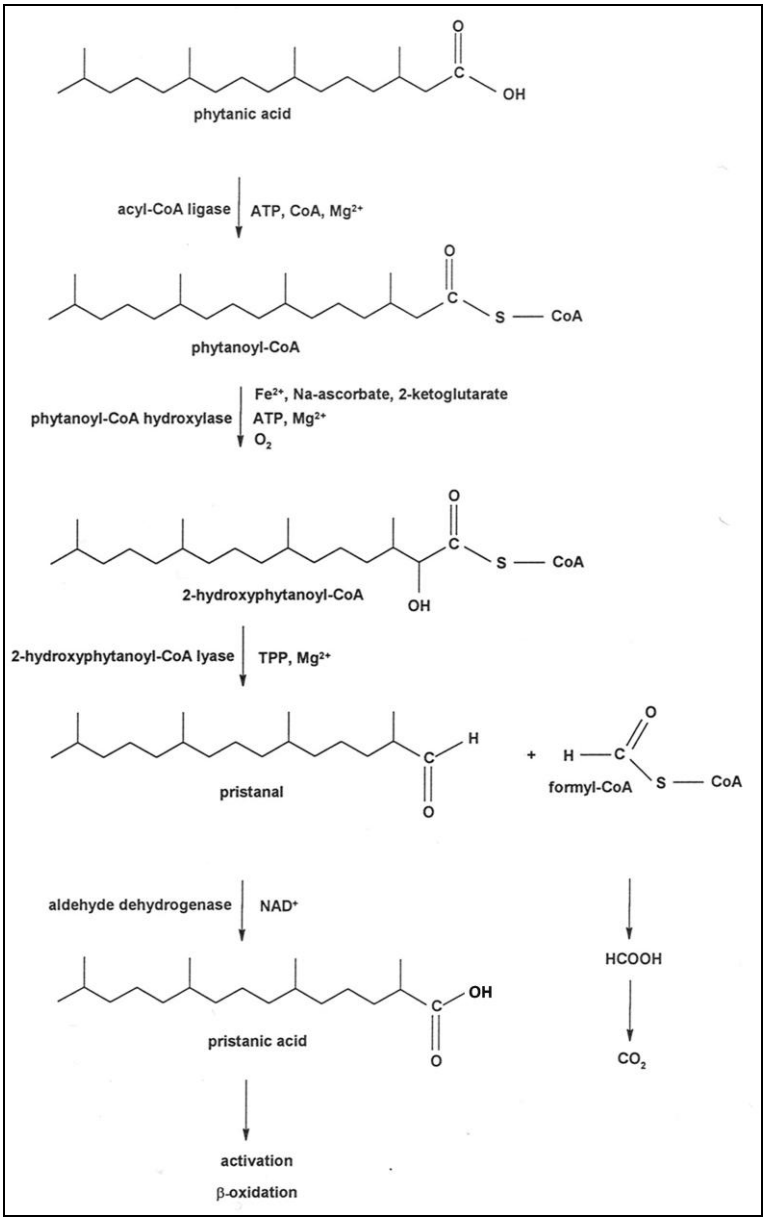

Fig. 2. The phytanic acid alpha-oxidation pathway. Adapted from Foulon V et al, 2005.

The first step in α-oxidation involved the activation of phytanic acid to phytanoil CoA by acyl-CoA synthetase (Watkins PA et al, 1994), which can occur in mitochondria, endoplasmic reticulum and peroxisomes (Wanders RJ et a., 2011). In the second step, phytanoyl-CoA 2-hydroxylase (PAHX), a PTS2 (peroxisomal targeting signal) containing peroxisomal matrix protein, catalyzes the hydroxylation of phytanoil CoA to form 2-hydroxyphytanoyl-CoA (Mihalik SJ et al, 1995; Jansen GA et al, 1999a) which is subsequently

decarboxylated to pristanal and formyl-CoA by 2-hydroxyphytanoyl-CoA (Foulon V et al, 1999; Jansen GA et al, 1999b). In the last step, pristanal is converted into pristanic acid in a reaction catalized by aldehyde dehydrogenase (Jansen GA et al, 2001) which is then further degraded by peroxisomal β-oxidation.

Alternatively to this model, some reports indicate a second pathway in which fatty acid α-oxidation may involve the formation of 2-ketophytanic acids intermediate from 2-hydroxyphytanic acids, both in rat and human liver (**Fig. 3**) (Wanders RJ et al, 1995; Vamecq, J. et al, 1988; Verhoeven, NM et al, 1997; Wanders RJ et al, 1994).

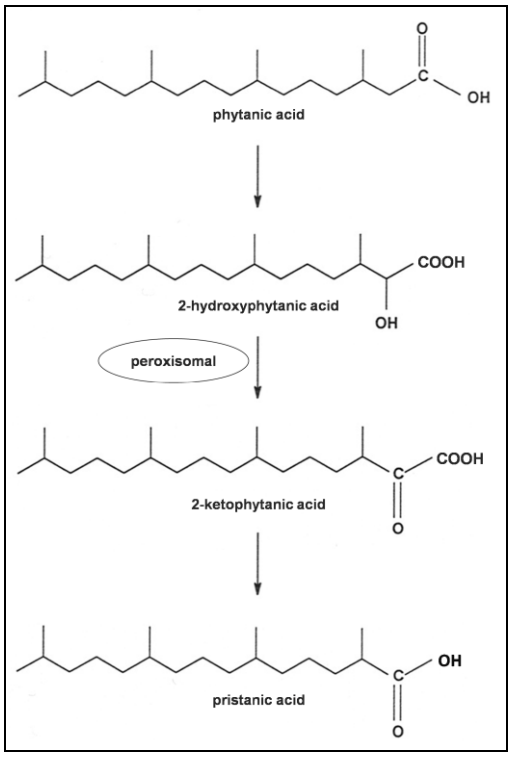

Fig. 3. Formation of pristanic acid from 2-hydroxyphytanic acid. Adapted from Verhoeven NM et al, 1997.

These studies suggest that, at least in rat liver, this conversion is catalyzed by an enzyme localized in peroxisomes which uses molecular oxygen as second substrate. The subsequent finding that the formation of 2-ketophytanic acid is strongly reduced if molecular oxygen is substituted by nitrogen, and it is also associated with H_2O_2 formation, further supports the involvement of an oxidase in the reaction.

Unfortunately, this mechanism is still under debate and to date the role of hydroxy acid oxidases still needs to be clarified.

As previously described, hydroxy acids oxidase are peroxisomal enzymes and produce H_2O_2 as part of their normal catalytic cycle. In 2003, Recalcati and colleagues showed in rat a

down-regulation of Hao1 in response to oxidative stress, thus providing that a decrease of this ROS producing enzyme may prevent excessive H_2O_2 formation in liver peroxisomes (Recalcalti S et al, 2003).

Peroxisomes are subcellular organelles bounded by a single membrane, which are present in the majority of eukaryotic cells. They were isolated and characterized by de Duve that defined them as a cell organelle which contains at least one H_2O_2-producing oxidase together with catalase, a H_2O_2-degrading enzyme (de Duve C. 1965). Mammalian peroxisomes play a pivotal role in metabolic pathways, including fatty acids α- and β-oxidation, ether-phospholipid biosynthesis, glyoxylate metabolism, amino acid catabolism, polyamine oxidation, and the oxidative part of the pentose phosphate pathway (Wanders RJ et al, 2006). Many of the enzymes participating in these pathways produce ROS and RNS as byproducts of their catalytic reactions (Antonenkov VD et al, 2010). It has been estimated that peroxisomes contribute to 35% of the H_2O_2 production in rat liver (de Duve C et al, 1966), inducing oxidative stress; this is also confirmed by the long-term administration of peroxisome proliferators in rodent liver cells (Kasai H et al, 1989).

Numerous observations indicate also that peroxisomes contain various ROS-metabolizing enzymes protecting cells from oxidative stress and accounting for 20% of the oxigen consumption in rat liver (Boveris A et al, 1972). Indeed, an abnormal functioning of peroxisomes causes increase apoptosis in the development of mouse cerebellum (Krysko O et al, 2007); in humans, an inherited deficiency of catalase, the most abundant peroxisomal ROS-metabolizing enzyme, induces an increased risk of developing age-related diseases such as diabetes, atherosclerosis, and cancer (Góth L et al, 2000).

These findings collectively suggest the idea that peroxisomal metabolism and cellular oxidative stress are closely interconnected (Fransen M et al, 2012; Bonekamp NA et al, 2009; Schrader M et al, 2006).

Overall, very few data are present in literature about hydroxy acid oxidases and their role is still unclear.

The role of Hao2 in cancer is still unknown. A study of gene expression profiles for human intrahepatic cholangiocarcinoma showed among down-regulated genes also Hao2 (Wang AG et al, 2006), although no hypothesis about its role have been proposed by the authors.

AIM OF THE WORK

Previous microarray analysis done in our laboratory has shown that Hao2 was one of the most down-regulated genes in advanced HCCs developed with the R-H model of hepatocarcinogenesis. Since its role is still unclear and no studies are reported in the literature about Hao2 and cancer, the aim of my PhD thesis was to shed light on the possible role of Hao2 in HCC development, in two different rodent models and in two distinct cohorts of human patients. Furthermore, using a multistage model of rat hepatocarcinogenesis, I also wished to determine whether alterations of the expression of Hao2 could take place at early stages of the tumorigenic process.

MATERIALS AND METHODS

1. Animals and Treatments

Male Fischer 344 (F-344) rats and female C3H/HeNCrl mice were purchased from Charles River Laboratories (Milano, Italy). Before starting experimental procedures, animals were housed at constant room temperature (25°C) and 12 hours light/dark cycles and fed with rodent standard diet (Mucedola, Milano, Italy) and *ad libitum* access to water. Guide for Care and Use of Laboratory Animals were followed during the investigation. All animal procedures were approved by the Ethical Commission of the University of Cagliari and the Italian Ministry of Health.

Rat Model of hepatocarcinogenesis: Rats were subjected to the Resistant-Hepatocyte (R-H) model (Solt DB et al, 1977, **Fig. 4**), which consists of a single intraperitoneal injection of 150 mg/kg body weight of diethylnitrosamine (DENA, Sigma Aldrich, Milano, Italy) dissolved in saline. Following a 2-week recovery period, rats were fed a diet containing 0.02% 2-acetylaminofluorene (2-AAF, Sigma Aldrich, Milano, Italy) for two weeks, that induces a cytostatic effect on normal hepatocytes. To trigger the rapid growth of DENA-initiated hepatocytes which are resistant to the mitoinhibitory effect of 2-AAF, one week after the exposure to 2-AAF, rats underwent a standard two-thirds partial hepatectomy (PH) (Higgins GM et al, 1931). Rats were then maintained on basal diet all throughout the experiment and sacrificed 10 weeks and 14 months after DENA administration.

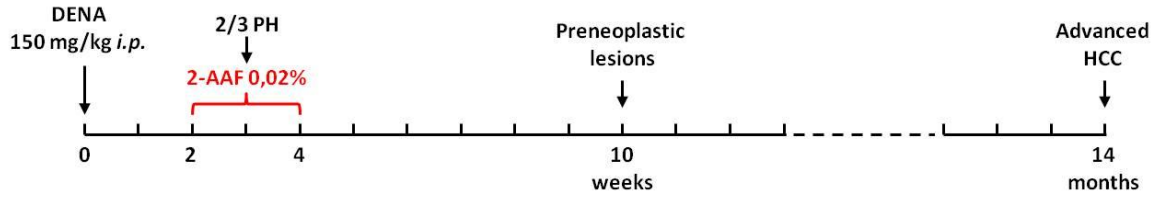

Fig. 4. The Solt-Farber Resistant-Hepatocyte rat model.

To assess the expression levels of Hao2 during liver regeneration, rats were subjected to a standard 2/3 PH (Higgins GM et al, 1931), and sacrificed after 24, 48 and 168 hours. Livers collected at the time of the surgery were used as controls.

Mouse model of hepatocarcinogenesis: Mice were randomized into two experimental protocols. The first group (**Fig. 5A**) was injected intraperitoneally with DENA, dissolved in saline, at a dose of 90 mg/kg body weight. After one week of recovery period, mice were treated once a week with the Constitutive Androstane Receptor (CAR) ligand TCPOBOP (3mg/kg body weight) dissolved in dimethyl sulfoxide (DMSO) and given intragastrically in a corn-oil solutionl. Matched-aged mice treated only with TCPOBOP were used as controls.

The second group (**Fig. 5B**) was given weekly intragastric doses of TCPOBOP (3 mg/kg body weight), in the absence of DENA administration. Matched-aged mice treated with corn oil were used as control. Mice were sacrificed after 42 weeks of treatment, a time point when HCCs were observed. Mice treated weekly with corn oil were used as controls.

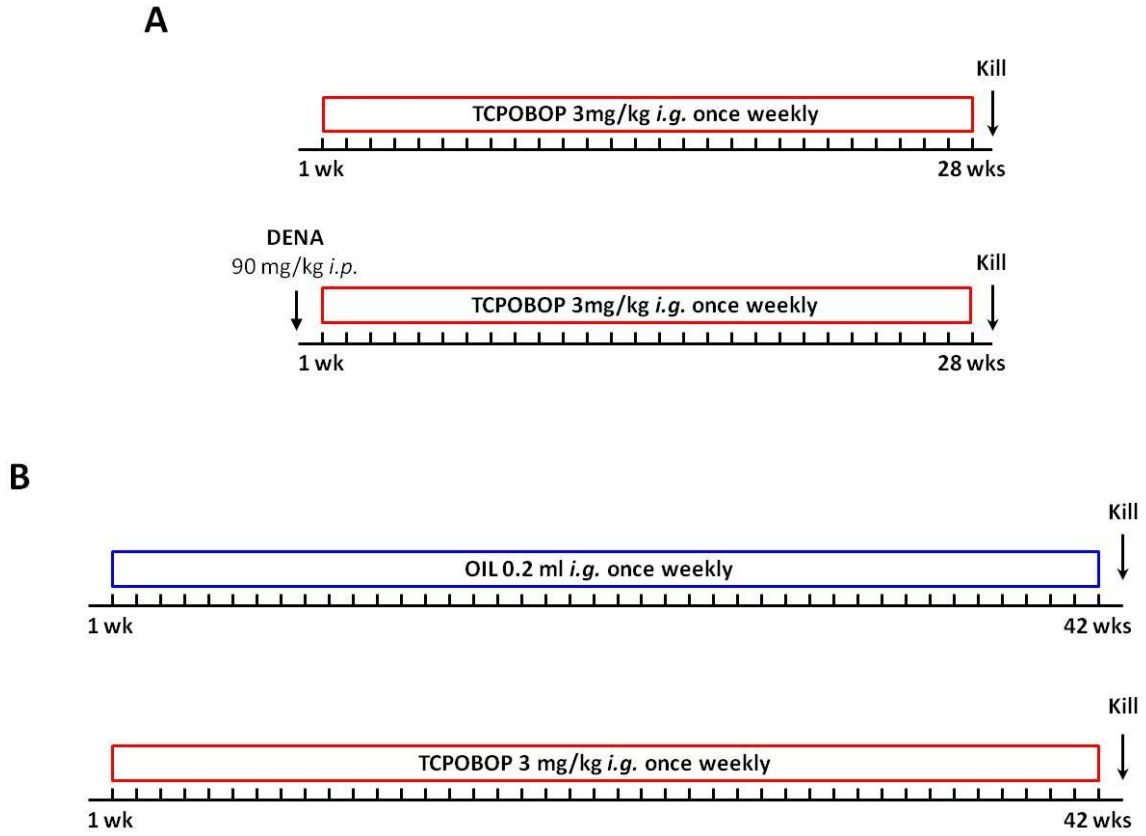

Fig. 5. Schematic representation of experimental mouse models. HCC was generated by **A)** a single injection of DENA followed by treatment with the CAR ligand TCPOBOP (once/week/28 weeks), **B)** repeated doses of the non-genotoxic agent TCPOBOP (once/week/42 weeks), in the absence of DENA pre-treatment.

In both rat and mouse protocols, immediately after the sacrifice, livers were divided into several sections which were stored according to three different methods. Sections were fixed in 10% buffer formalin, embedded in paraffin and stored at room temperature for

immunohistochemistry staining. Other sections of the liver were snap-frozen in isopentane (2-Methylbutane) and liquid nitrogen, and immediately kept at -80°C for criosectioning. The remaining liver was snap-frozen in liquid nitrogen and stored at -80°C for DNA, RNA and protein extraction.

2. Immunohistochemistry

Isopentane-frozen rat liver sections were serially sliced at 6 µm thickness using a Leica CM 1950 cryostat, mounted directly on super frost slides (Fisher Scientific, Pittsburgh PA), and air dried for 5 minutes before immunohistochemical staining.

2.1. Hematoxilin and eosin staining

Liver sections were fixed in acetone at -20°C for 5 minutes and stained for 3 minutes with Mayer's haematoxylin and 1% aqueous eosin for 20 seconds. Sections were then dehydrated through ascending alcohol series, cleared with xylene, air-dried and then mounted using synthetic mounting and coverslipped.

2.2. Glutathione S-transferase staining

Frozen liver section were fixed in 10% buffer formalin for 6 hours. After several washes in water and in phosphate buffered saline (PBS), endogenous peroxidase activity was blocked by 0,3% hydrogen peroxide (Sigma-Aldrich, Milano, Italy) in distilled water for 10 minutes. Unspecific binding sites were then removed by incubating section for 30 minutes in 1:10 normal goat serum in PBS. Slides were then incubated overnight at 4°C with 1:1000 anti-GSTP antibody (MBL, 311, Nagoya, Japan) and with anti-rabbit Horseradish Peroxidase (HRP) conjugated antibody (Sigma Aldrich, Milano, Italy) at a dilution of 1:300 for 30 minutes at room temperature. Positive reaction was visualized by 3, 3'-diaminobenzidine (DAB, Sigma Aldrich, Milano, Italy) for 6 minutes at room temperature. Sections were counterstained with Harris hematoxylin, dehydrated through graded alcohols, cleared and mounted in synthetic mounting media.

2.3. Cytokeratin-19 staining

Frozen liver sections were fixed in cold acetone for 20 minutes. Block of endogenous peroxidases and aspecific sites were performed as described previously for GSTP staining. Slides were then incubated with primary mouse polyclonal anti-Krt-19 antibody (Novocastra,

NCL-CK19, Leica Biosystems, Milano, Italy) at a dilution of 1:100 overnight a 4°C and then with 1:200 anti-mouse Horseradish Peroxidase (HRP, Sigma-Aldrich, Milano, Italy) conjugated antibody at room temperature for 30 minutes. Staining was developed with 3,3'-diaminobenzidine (DAB) for 6 min at room temperature, and then tissue sections were counterstained with Harris hematoxylin dehydrated in graded alcohols, and mounted in synthetic mounting media.

3. Laser capture microdissection

Sixteen-µm-thick serial frozen sections were cut and attached to 2-µm RNase free PEN-membrane slides (Leica, Wetzlar, Germany). Immediately before micro-dissection, frozen sections were stained by a 2.45 minutes H&E procedure. Briefly, sections were rapidly hydrated (30 seconds in Ethanol 100 and 95%), stained in Mayer's hematoxilin for 90 seconds, washed in water for 20 seconds, stained in 0.5% alcoholic Eosin for 10 seconds and dehydrated by Ethanol 100% for 30 seconds. Then, sections were microdissected by Leica LMD6000 (Leica Microsystems Inc., Buffalo Grove, IL); the whole procedure was performed within 20 minutes to prevent RNA degradation. Microdissected lesions were collected into caps of 0.5 ml microcentrifuge tubes filled with 100 µl of Extraction Buffer (XB) and incubated for 30 minutes at 42°C. To collect tissue extracts into the microcentrifuge tubes, samples were centrifugated at 800 x *g* for two minutes and then frozen at -80°C until extraction with PicoPure RNA isolation kit (Arcturus, Life Technologies, Monza, Italy).

4. RNA extraction

4.1. RNA isolation using PicoPure RNA isolation kit

Total RNA was isolated from rat micro-dissected lesions (controls, preneoplastic lesions and HCCs) with PicoPure RNA isolation kit according to manifacturer's instructions. Briefly, after pre-condition of the RNA Purification Column with Conditioning Buffer (CB), 100 µl of 70% ethanol was added to the tissue extract, transferred to RNA purification column and centrifuged at 100 x g for 2 minutes for RNA binding, followed by a quick spin at 16000 x g for 30 seconds to remove flowthrough. RNA Purification Column was then washed three times by Washing Buffer 1 and 2 and then transferred to a new 0.5 ml microcentrifuge tube provided by the kit. Sixteen µl of DNase/RNase free distilled water (Gibco, Life technologies, Monza, Italy) was added; the tube assembly was left to incubate at room

temperature for 1 minute and centrifuged at 1000 x g for 1 minute to distribute elution buffer in the column, followed by spinning at 16000 x g for 1 minute to elute RNA. The eluted RNA was then stored at -80°C.

4.2. RNA isolation using TRIzol® Reagent

Total RNA from frozen mouse, human tissue and human HCC cell lines was extracted using TRIzol® Reagent (Invitrogen, Life Technologies, Monza, Italy), according to manifacturer's instructions.

Briefly, 1 ml of TRIzol was added to 50-100 mg of hepatic tissue. Samples were homogenized with a Polytron homogenizer and incubated 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complex. Then, 0.2 ml chloroform/ml of TRIzol, were added, shaked by hand for 15 seconds and incubated for 3 minutes at room temperature. After centrifugation (15 minutes at 12000 x g at 4°C) the mixture was separated into three phases: a lower red phenol-chloroform phase containing proteins, a white interphase containing DNA, and a colorless upper aqueous phase containing RNA. Aqueous phase was transferred into a new tube and RNA was precipitated by addition of 500 ml 100% isopropanol followed by 10 minutes incubation at room temperature and centrifugation at +4°C at 12000 x g for 10 minutes. The resulting RNA pellet was washed in 75% ethanol and dissolved DNase/RNase free distilled water in at heat block set at 60°C for 10 minutes.

5. Quantitative and qualitative analysis of nucleic acids

Total RNA concentrations and purity ratios (260/280 and 260/230) were measured using NanoDrop 1000 Spectrophotometer (Thermo Scientific, France). RNA integrity was assessed by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) by evaluating the RNA Integrity Number (RIN). All procedures were performed according to manufacturer's protocol. Only RNA samples with a RIN equal to or higher than 7 were further used in the study.

6. RT-PCR (Reverse Transcription Polymerase Chain Reaction)

To investigate by quantitative real time-PCR (qRT-PCR) mRNA expression levels of Hao2, total RNA was retro-transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Life Technologies, Italy).

Different amounts of total RNA were diluted in 10 µl of DNase/RNase free distilled water. The 20 µl reaction mixture contained: 2 µl of RT buffer (10X), 2 µl of Random Primers (10X), 0.8 µl of dNTP mix (100 mM), 1 µl of MultiScribe Reverse Transcriptase, 1 µl of RNase Inhibitor and 3.2 µl of DNase/RNase free distilled water and 10 µl of the appropriate total RNA at the desired concentration. Thermo cycle condition was: 25° C for 10 minutes, 37° C for 120 minutes and 85° C for 5 minutes, followed by a 4° C hold. Samples were then stored at -20° C until next use.

7. Quantitative Real Time PCR

Retro-transcribed cDNAs were used for gene expression analysis performed by qRT-PCR. Reaction mixtures were prepared to a final volume of 10 µl containing: 4 µl of cDNA template (2.5 ng/µl), 5 µl of 2X TaqMan Gene Expression Master Mix (Applied Biosystem, Life Technologies, Italy), 0.5 µl of 20X TaqMan assay (Applied Biosystem, Life Technologies, Italy) and 0.5 µl of RNase free water.

The following TaqMan probes were used for Hao2 gene expression: Rn00583454_m1 for rat, Mm00469507_m1 for mouse and Hs00213002_m1 for human.

Reactions were performed in a ABI PRISM 7300HT thermocycler (Applied Biosystem, Life Technologies, Italy); the cycle conditions were set as follows: 50° C for 2 minutes and 95° C for 10 minutes, followed by 40 cycles at 95° C for 15 seconds and at 60° C for 1 minute.

Each sample was analysed in triplicate; the housekeeping genes rat and mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and human β-actin were used for normalization. To determine the relative expression levels of Hao2, the $2^{-\Delta\Delta CT}$ method was used. The threshold cycle (Ct) value of the target gene was normalized to that of the endogenous reference and compared with a calibrator.

8. Protein isolation

Rat liver samples were homogenized in RIPA Buffer (1X PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS) containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich, Milano, Italy) with a Polytron homogenizer and centrifuged two times at 12000 rpm for 15 minutes at 4 °C. The supernatant was collected in a new tube and stored at -80 °C.

Proteins from human HCC cell lines were directly extracted using Laemmli buffer (Tris-HCl 0.5M, pH 6.8, SDS 10%), pre-warmed at 95°C, which was dispensed on cells on a culture

dish (after removal of media and washing with PBS). Cells were then gently scraped from the culture dish and transferred into a 1.5 ml tube. Samples were incubated in an heating block at 95°C for 3 minutes and then stored at -80°C.

Protein concentrations of both tissue and cell lysates was evaluated with the BCA Protein Assay kit (Pierce Biotechnology, Rockford, USA) using bovine serum albumin (BSA, Sigma-Aldrich, Milano, Italy) as standard.

9. Western blotting

Aliquotes of 100 µg of protein were denatured by heating for 10 minutes at 70°C in NuPAGE® LDS Sample Buffer (4X) (Invitrogen), separated by NuPage Novex Bis-Tris 10% gels (Invitrogen, Life Technologies, Monza, Italy), and transferred onto nitrocellulose membranes (Invitrogen, Life Technologies, Monza, Italy) by electroblotting. Membranes were blocked in 5% BSA in Tris-buffered saline (NaCl 5M, Tris-HCl 1M pH 7.4) containing 0.1% Tween 20 (TBS-T) for 40 minutes and probed with 1:200 goat polyclonal anti-Hao2 antibody (sc-242990, Santa Cruz Biotechnology, Santa Cruz, CA) in 5% BSA, overnight at 4°C. Membranes were then incubated with 1:5000 anti-goat horseradish peroxidase (HRP) -secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 5% BSA for 90 minutes at room temperature. After three washes in TBST, proteins were revealed with the Super Signal West Pico (Pierce Chemical Co., New York, NY). Levels of Hao2 were normalized to the housekeeping gene anti-actin (A4700, Sigma-Aldrich, Milano, Italy) that was used also as loading control.

10. Cell lines

For all in vitro experiments 9 HCC human cell lines and cells isolated by a HCC bearing rat (R-H cells, Petrelli A et al, 2014) were used.

HA22T/VGH, Hep3B, HepG2, HuH7, Mahlavu and Sk-Hep-1 were kindly provided by Dr. Silvia Giordano, IRCC Institute of Candiolo (Turin, Italy). In addition, SNU 182, SNU 398 and SNU 475 cells were kindly provided by Dr. Laura Gramantieri, St. Orsola-Malpighi University Hospital (Bologna, Italy). Cells were maintained at 37°C in a 5% CO2 humidified atmosphere. All culture medium and their constituents were purchased from Gibco (Life Technologies, Monza, Italy).

HA22T/VGH cells were cultured in RPMI (Roswell Park Memorial Institute)-1640 medium with 10% inactivated fetal bovine serum (FBS, Lonza, Basel, Switzerland), 1%

sodium pyruvate, and 1% penicillin-streptomycin. Serum was inactivated in water bath at 56°C for 1 hour.

Hep3B and Sk-Hep-1 were cultured in Minimum Essential Medium (MEM) with 10% FBS, 1% sodium pyruvate, 1% non-essential amino acids and 1% penicillin-streptomycin.

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% glutamine and 1% Penicillin-Streptomycin.

HuH7 and Mahlavu cells were cultured in DMEM supplemented with 10% inactivated FBS, 1% glutamine and 1% penicillin-streptomycin.

SNU 182, SNU 398 and SNU 475 cells were cultured in RPMI-1640 medium with 10% FBS, 1% glutamine, and 1% penicillin-streptomycin.

The R-H cells were obtained by classical collagenase perfusion techniques from HCC-bearing rats subjected to the R-H protocol and sacrificed at 14 months. Cells were cultured in collagen-coated plates, in RPMI-1640 medium with 10% FBS (Petrelli A et al, 2014).

10.1. Stable transduction with Hao2

Human Embryonic Kidney 293 cells (HEK293T, ATCC, Manassas, VA, USA) were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Gibco, Life Technologies, Monza, Italy) with 10% FBS in a 5% CO_2 atmosphere. The HAO2 construct (HsCD00439253) was purchased from DNASU plasmid repository of the Biodesign Institute, Arizona State University and cloned into pLX304 (Gateway V5-tagged lentiviral expression vector). Lentiviral vector for the constitutive expression of HAO2 was produced by transient transfection of the bidirectional transfer plasmid, the packaging plasmids pMDLg/pRRE and pRSV.REV, and the vesicular stomatitis virus (VSV) envelope plasmid pMD2.VSV-G (15, 6.5, 2.5, and 3.5 μg, respectively, for 10-cm dishes) in HEK293T cells using the calcium phospate method (Chen CA et al, 1988). The virus containing medium was collected at 48h from transfection and used to transduce Mahlavu and R-H cells. Cells were transduced in six-well plates (10^5 per well) in 2 ml total medium in the presence of polybrene, an attachment factor to enhance virus-cell fusion.

10.2. Crystal violet cell growth assay

Both Mahlavu (500 cells/well) and R-H cells (1000 cells/well) and their Hao2 transduced counterpart were seeded in 96-well plates. Cell growth was evaluated by crystal violet staining 96 hours, 6 days and 8 days after seeding. Cells were fixed with a solution of

22% glutaraldehyde in PBS at room temperature on a rotary shaker for 20 minutes and then washed with PBS. Cells were stained with a crystal violet solution at room temperature on a rotary shaker for 20 minutes, washed two times in PBS and in distilled water. After air-drying at room temperature, cells were dissolved in 10% acetic acid in distilled water for 20 minutes at room temperature on a rotary shaker. One-hundred µl of each sample were than transferred in a 96-well plate and optical density (OD) was measured at 590 nm (PerkinElmer 2030 Software).

10.3. Soft agar colony formation assay

Anchorage-independent growth was determined by the colony formation assay in soft agar in wild type and Hao2 transduced, Mahlavu and R-H cells. It was performed in a 24 well plate embedding $3x10^3$ cells/well in 0.5% SeaPlaque low melting agarose (BioWhittaker Molecular Applications-BMA, 50100) on top of a layer of growth medium containing 1% agar. The cell suspension was incubated in a humidified atmosphere in the presence of 5% CO2 at 37° C and, after 2 weeks, colonies were stained with crystal violet and quantified by counting all visible colonies.

10.4. Rat Tumorigenicity Assay

Wild type and stably transduced Hao2 R-H cells (10^6/rat) in 20% Matrigel Matrix (BD Biosciences, Milano, Italy) were injected subcutaneously in the right flank of F-344 syngeneic male rats (n=7 rats for wild-type and n=6 rats for transduced Hao2 R-H cells). Rats were monitored twice/week for monitoring tumor formation.

11. Human samples

Two cohorts of patients carrying HCC were examined. The first consisted of HCCs and matched non-neoplastic liver parenchyma obtained from 59 consecutive patients undergoing liver resection for HCC and 5 liver healthy donors. Specimens and clinico-pathological data were obtained from the Institute of Pathology, University Hospital of Basel, Switzerland. All patients gave written informed content 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 anti-cancer 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 have been used in this study. The clinico-pathologic features of patients are described in **Table 1**.

Factors		Frequency	%
Gender			
	Female	5	8.5
	Male	47	79.7
	No data	7	11.9
Age			
	<65	20	33.9
	>65	32	54.2
	No data	7	11.9
Mean (range)	70.5 (26-89)		
Foci			
	Multifocal	30	50.8
	Unifocal	20	33.9
	No data	9	15.3
AFP (>250 ng/ml)			
	Negative	36	61.0
	Positive	13	22.0
	No data	10	16.9
Fibrosis grade			
	F0	1	1.7
	F1	3	5.1
	F2	2	3.4
	F3	2	3.4
	F4	43	72.9
	No data	8	13.6
Edmonson grade			
	2	37	62.7
	3	13	22.0
	4	2	3.4
	No data	7	11.9
Etiology			
	No Virus	30	50.8
	Virus	22	37.3
	No data	7	11.9
Metastasis			
	No	43	72.9
	Yes	16	27.1

Table 1. Clinicopathological Data of the HCC studied cohort from Institute of Pathology, University Hospital of Basel, Switzerland.

As to the second set of patients, it consisted of HCCs and matched cirrothic tissues obtained from 59 consecutive patients (45 males and 14 females) undergoing liver resection for HCC at the Policlinico S. Orsola-Malpighi, Bologna, Italy. Eight normal liver tissues were obtained from patients undergoing liver surgery. No patient received anticancer treatment prior to surgery. The characteristics of patients are described in **Table 2**.

Factors		Frequency	%
Gender			
	Female	14	23.7
	Male	45	76.3
Age			
	≤65	23	39
	>65	35	59.3
	No data	1	1.7
Mean (range)	67,84 ± 7.73		
Foci			
	Multifocal	27	45.8
	Unifocal	32	54.2
AFP			
	< 20 ng/mL	19	32.2
	> 20 ng/mL	36	61.0
	No data	4	6.8
Edmonson grade			
	1	1	1.7
	2	13	22.0
	3	32	54.2
	4	13	22.0
Nodule size			
	<3 cm	8	13.6
	3</>5 cm	32	54.2
	> 5 cm	19	32.2
Etiology			
	HBV	10	16.9
	HCV	35	59.3
	HBV + HCV	2	3.4
	HBV + Ethanol	1	1.7
	HCV + Ethanol	2	3.4
	Ethanol	1	1.7
	No data	8	13.6

Table 2. Clinicopathological Data of the HCC studied cohort from the Policlinico S. Orsola-Malpighi, Bologna.

11.1. Microarray analysis

Human microarray analysis was performed in collaboration with the University Hospital of Basel. RNA for the microarray was isolated for Transcriptomic profiling from 59 HCC needle biopsies matched with their corresponding non-neoplastic liver parenchyma and 5 normal liver donors with Direct-Zol RNA MiniPrep Kit (Zymo Research) including on-column DNAse treatment. RNA concentration was assessed using NanoDrop ND2000 (Nanodrop) and RNA integrity was monitored on Bioanalyzer 2100 using RNA6000 Chip (Agilent). 270 ng

of DNAse-treated total RNA was subjected to target synthesis using the WT Expression kit (Ambion) following standard recommendations. Fragmentation and labeling of amplified cDNA were performed using the WT Terminal Labeling Kit (Affymetrix). Synthesis reactions were carried out using a PCR machine (TProfessionnalTrio, Biometra) in 0.2ml tubes (Starlab). Eighty-five µl cocktail (23.4ng/µl labeled DNA) were loaded on GeneChip® Human Gene 1.0ST arrays (Affymetrix) and hybridized for 17 hours (45°C, 60rpm) in Hybridization oven 645 (Affymetrix). The arrays were washed and stained on Fluidics Stations 450 (Affymetrix) by using the Hybridization Wash and Stain Kit (Affymetrix) under FS450_0002 protocol. The GeneChips were scanned with an Affymetrix GeneChip Scanner 3000 7G. DAT images and CEL files of the microarrays were generated using Affymetrix GeneChip Command Control (version 4.0). Afterwards, CEL files were imported into Qlucore software and Robust Multichip Average (RMA) normalized. Subsequently, principal component analysis to discriminate between engineered and control cells will be performed. Quantile normalization and data processing were performed using the GeneSpringGXv11.5.1 software package (Agilent, USA). The gene signature value was assessed using the BRB-ArrayTool (v4.3.2, NIH).

11.2. Tissue microarray (TMA) and immunohistochemistry

For immunohistochemical analysis of HAO2, in collaboration with the University Hospital of Basel, we analysed formalin-fixed paraffin embedded (FFPE) tissue samples organized into a tissue microarray (TMA), as previously described (Baumhoer D et al, 2008). Briefly, this array contains 434 tissue specimens from both HCC and non-neoplastic liver tissue samples. Roughly 60% of used specimens used for the TMA construction were obtained from patients that underwent surgical resection without prior treatment for HCC, while the remaining were collected from autopsy cases. Histologic grading and classification of used liver samples was performed by 2 experienced pathologists according to the World Health Organization classification and Edmondson & Steiner grading system.

Sections of 4 µm of paraffin embedded tissue were immunostained using a primary antibody against HAO2 (NBP2-32037 Novus Biologicals, Littleton, CO, USA). Automated IHC were carried out in the Ventana BenchMark (Ventana Medical Systems, Tucson, AZ, USA) platform by using the primary antibody against HAO2 diluted at 1:10.

12. Statistical analysis

For qRT-PCR analysis, data are expressed as mean ± standard error (SEM). Analysis of significance was done by One-Way ANOVA, followed by Tukey-Kramer multiple-comparison post-hoc test and t Student's test using the GraphPad software (La Jolla, California).

Differences in patient survival were assessed using the Kaplan–Meier method and analysed using the log-rank test in univariate analysis. Cut-off scores were selected by evaluating the receiver-operating characteristic (ROC) curves. The point on the curve with the shortest distance to the coordinate (0, 1) was selected as the threshold value to classify cases as "positive/overexpressing" or "negative/down-regulated". Analyses were performed using the SPSS (Statistical Package for Social Science) software (IBM, Armonk, NY).

P-value were considered significant at $p< 0.05$.

RESULTS

1. Analysis of Hao2 in rat preneoplastic lesions and HCCs

Previous gene expression profiling performed in advanced HCCs generated by the Resistant Hepatocyte rat model, has shown that Hao2 was among the five mostly down-regulated genes (**Table 3,** Petrelli A et al, 2014)

Gene	Full name	Fold change aHCC/Control
Cyp2c	cytochrome P450, subfamily 2	- 69.57
Obp3	alpha-2u globulin PGCL4	- 49.07
Ca3	carbonic anhydrase 3	- 29.79
Dhrs7	dehydrogenase/reductase (SDR family) member 7	- 22.80
Hao2	**hydroxyacid oxidase 2**	**- 10.86**
Cdh17	cadherin 17	- 10.80
Olr59	olfactory receptor 59	- 9.86
Avpr1a	arginine vasopressin receptor 1A	- 8.75

Table 3. Most down-regulated genes in microdissected advanced HCCs (fold change versus controls <-5). Adapted from Petrelli A et al, 2014.

Based on these preliminary data, we performed real time PCR to validate these results and, in particular, to investigate whether Hao2 down-regulation occurs also during early stages of liver carcinogenesis. To this aim, we laser-microdissected preneoplastic lesions identified, by their positivity to GSTP, and HCCs, generated at 10 weeks and 14 months after DENA treatment, respectively.

As shown in **Fig. 6**, a significant reduction of the expression of Hao2 was observed in all $GSTP^+$ preneoplastic nodules and HCCs, compared to normal liver samples. Hao2 mean expression in HCCs vs. preneoplastic lesions vs. control livers was: 0.06 ± 0.01 vs. 0.25 ± 0.06 ($p<0.05$) vs. 1 ± 0.05 ($p<0.001$).

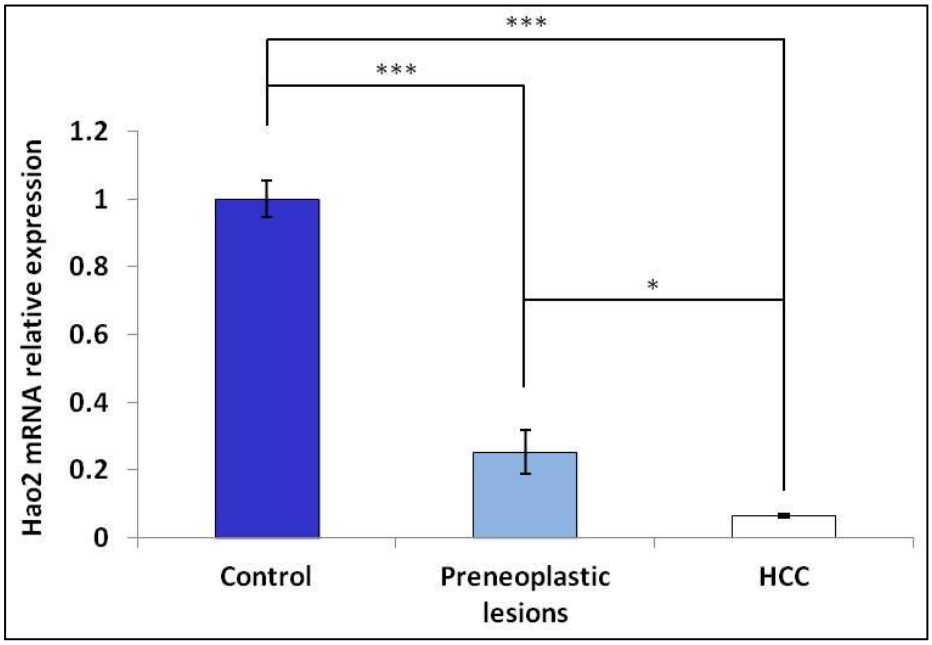

Fig. 6. Hao2 average mRNA expression in rat control livers (n=4), preneoplastic lesions (n=19) and HCCs (n=9), as assessed by quantitative real-time PCR using the 2^{-ddCt} method and rat GAPDH as endogenous control. Each bar represents mean ± standard error (SEM), calculated as fold-change difference. Tukey-Kramer test: *** $p<0.001$; * $p<0.05$.

In agreement with mRNA levels, western blot analysis showed a very low level of Hao2 protein content in HCCs compared to normal livers (**Fig.7**).

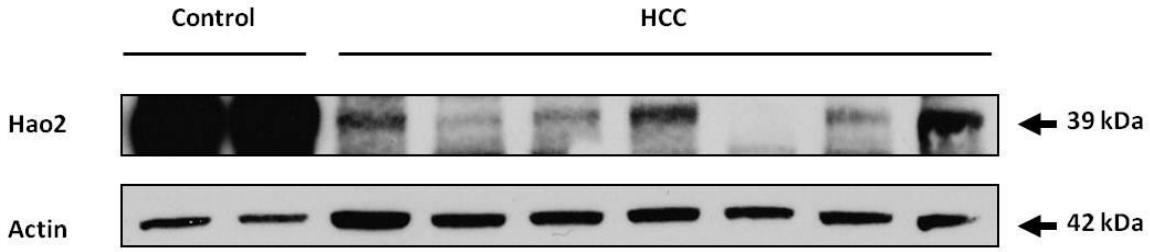

Fig. 7. Total liver tissue lysate of controls (n=2) and HCCs (n=7) were analysed by Western Blot. Protein loading for each sample was verified using anti-actin antibody.

These results demonstrated that down-regulation of Hao2 occurs in early stages of carcinogenesis and is maintained all along the tumorigenic process.

2. Hao2 is mostly down-regulated in the subset of nodules expressing the putative progenitor cell marker Krt-19

The well-characterized Resistant Hepatocyte (RH) rat model allows to identify phenotypically distinct lesions along the various steps of carcinogenesis (early nodules,

adenomas, early HCC, and advanced HCC). These lesions can be classified according to their positivity to two well-known tumor markers, GSTP and Krt-19. At early stages (10 weeks after DENA initiation), two types of preneoplastic lesions are identified: persistent and remodeling nodules. While the former progress to HCC, the latter undergo re-differentiation to a mature phenotype that precedes their disappearance (Enomoto K et al, 1982). Our previous findings showed that remodelling nodules are characterized by a positive staining for GSTP, but not for Krt-19; on the opposite, persistent GSTP+/Krt-19+ nodules have been identified as the progenitors of HCC (Andersen JB et al, 2010).

To investigate whether down-regulation of Hao2 is specific for the subset of Krt-19 positive nodules or is common for all preneoplastic lesions, we laser-microdissected both Krt-19 positive and negative nodules and analysed Hao2 mRNA levels by qRT-PCR. The results showed that although a down-regulation of Hao2 occurred in both types of preneoplastic lesions ($p<0.001$), a significantly stronger decrease characterized Krt-19+ lesions (**Fig. 8 A,B**). Hao2 mean expression in Krt-19 positive vs. Krt-19 negative vs. control liver was: 0.08 ± 0.02 vs. 0.48 ± 0.09 vs. 1 ± 0.05.

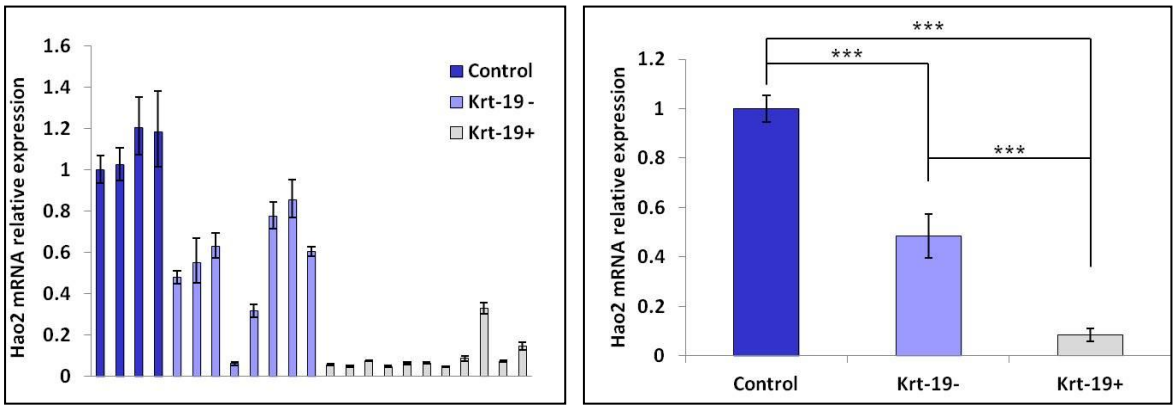

Fig. 8. QRT-PCR analysis of Hao2 mRNA expression levels in rat control livers (n=4), Krt-19- nodules (n=8) and Krt-19+nodules (n=11). (A) The levels of Hao2 were calculated as relative mRNA expression using the 2^{-ddCt} method and rat GAPDH as endogenous control. (B) Average expression of Hao2 mRNA levels was reported as fold change differences between samples in panel A. Each bar represents mean ± standard error (SEM). Tukey-Kramer test: *** $p<0.001$.

These data demonstrate that a sustained down-regulation of Hao2 characterized a subset of nodules expressing the progenitor cell marker Krt-19 and considered to be the precursor cell population of HCC.

3. Is Hao2 down-regulation a species-dependent event or a common feature of HCC?

To determine whether down-regulation of Hao2 is species-dependent or a common event in hepatocarcinogenesis, we analysed by qRT-PCR the expression of Hao2 in a mouse model of liver carcinogenesis, consisting of a single injection of DENA followed by treatment with the CAR ligand TCPOBOP (once/week/28 weeks). TCPOBOP induces liver hyperplasia and hypertrophy (Dragani et al, 1985; Manenti et al, 1987), and promotes HCC development 28 weeks after DENA administration (Kowalik MA et al, 2011). These results, similarly to those obtained in rats, showed a significantly down-regulation of Hao2 in mouse HCCs as compared to control liver (**Fig. 9A,B**). Hao2 mean expression in HCCs vs. control liver was: 0.14 ± 0.01 vs. 1 ± 0.16 ($p < 0.001$).

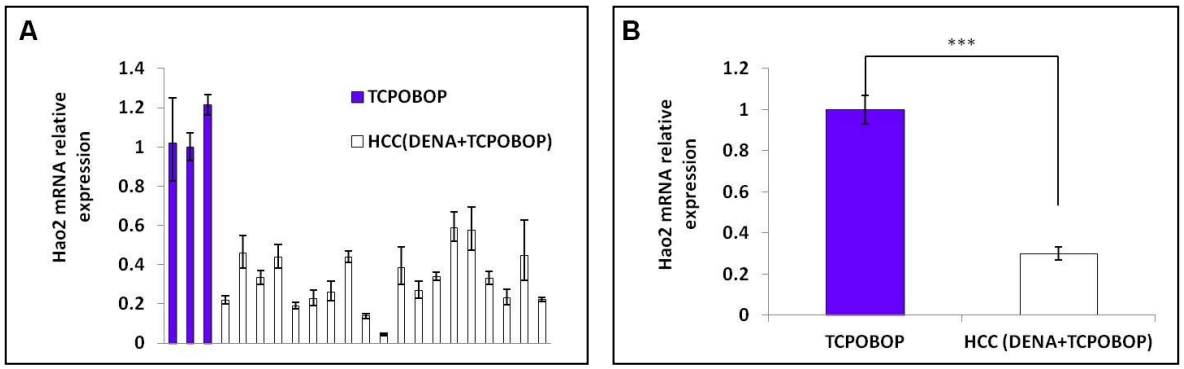

Fig. 9. QRT-PCR analysis of Hao2 mRNA expression in in matched-aged mice treated weekly only with TCPOBOP (n=3) and HCC (n=19) developed after 28 weeks of repeated doses of TCPOBOP following a single injection of DENA (A) The levels of Hao2 were calculated as relative mRNA expression using the 2^{-ddCt} method and mouse GAPDH as endogenous control. (B) Average expression of Hao2 mRNA levels was calculated as fold change of samples in panel A. Each bar represents mean ± standard error (SEM). Unpaired t-test: *** $p<0.001$.

4. Down-regulation of Hao2 takes place also in HCCs generated in the absence of administration of genotoxic agents

In both the previous models, initiation of the carcinogenic process is triggered by the genotoxic agent DENA. To explore the possibility that down-regulation of Hao2 could be the consequence of a direct interaction and DNA damage caused by DENA-derived metabolites, we used a mouse model in which HCC was generated following repeated doses of the non-genotoxic agent TCPOBOP, in the absence of DENA pre-treatment.

The results showed that HCCs developed in 100% of mice, although much later than in the DENA+TCPOBOP group (42 vs. 28 weeks, data not shown). As shown in **Fig. 10 A,B**, a

strong down-regulation of Hao2 was observed also in TCPOBOP-induced HCCs (Hao2 mean expression in HCCs vs. control liver was: 0.23 ± 0.04 vs. 1 ± 0.16; p<0.001).

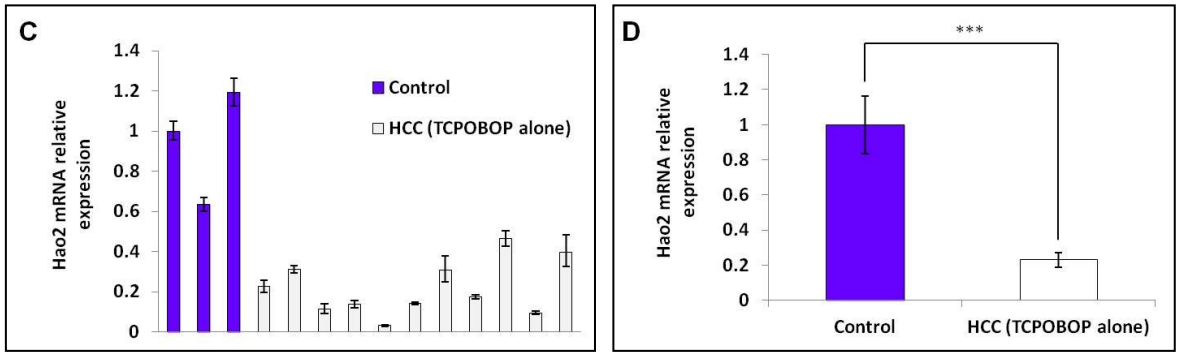

Fig. 10. QRT-PCR analysis of Hao2 mRNA expression levels in matched-aged mouse control livers (n=3) and HCC (n=11) generated by repeated injections of TCPOBOP for 42 weeks. (A) The levels of Hao2 were calculated as relative mRNA expression using the 2^{-ddCt} method and mouse GAPDH as endogenous control. (B) Average expression of Hao2 mRNA levels was calculated as fold change of samples in panel A. Each bar represents mean ± standard error (SEM). Unpaired t-test: *** p<0.001.

The finding that a strongly down-regulation of Hao2 is observed in both mouse models, with or without the genotoxic agent DENA, demonstrates that the dysregulation of this gene is not only species- but also etiology-independent.

5. The role of Hao2 in proliferation of normal hepatocytes

Since preneoplastic and neoplastic lesions exhibit a higher proliferation rate compared with adult quiescent liver, we wished to investigate whether the expression of Hao2 is linked to the proliferative status of hepatocytes. To this aim, we determined Hao2 expression in normal rat hepatocytes during liver regeneration following 2/3 partial hepatectomy. QRT-PCR analysis showed a highly significant down-regulation of Hao2 after 24 and 48 hours of surgery, a time corresponding to the peak of S phase, with a trend towards control levels at 168 hours, a time when organ regeneration is almost completed (**Fig. 11 A,B**). (Hao2 mean expression 168h vs. 48h vs.24h after PH vs. control liver was: 0.37 ± 0.11 vs. 0.11 ± 0.02 vs. 0.14 ± 0.02 vs. 1 ± 0.06).

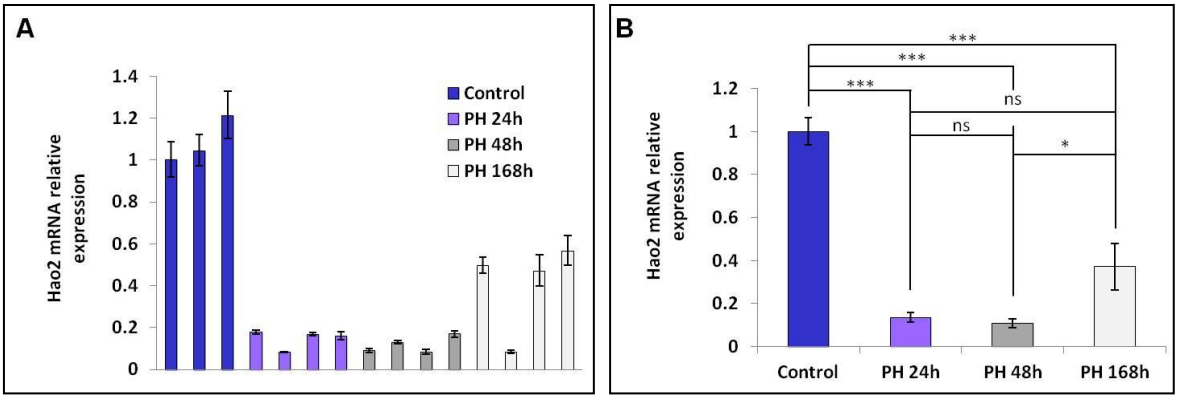

Fig. 11. QRT-PCR analysis of Hao2 mRNA expression levels in rat control livers (n=3) and in animals subjected to 2/3 PH and sacrificed at 24 (n=4), 48 (n=4), and 168 hours (n=4) afterwards. (A) The levels of Hao2 were calculated as relative mRNA expression using the 2^{-ddCt} method and rat GAPDH as endogenous control. (B) The average level of samples in panel A was calculated as fold change. Each bar represents mean ± standard (SEM). Tukey-Kramer test: *** $p<0.001$, * $p<0.05$, ns: not significant.

These data show that Hao2 is down-regulated also during the active proliferation of normal hepatocytes and suggest a possible involvement of Hao2 in the entry of hepatocytes into the cell cycle.

6. HAO2 expression is strongly down-regulated in human HCC

In order to investigate whether the results obtained in rat and mouse experimental protocols of hepatocarcinogenesis could be of translational value for human HCC, we performed microarray analysis in 59 patients carrying HCC and subjected to liver biopsy matched with their corresponding non-neoplastic liver parenchyma and in 5 healthy liver donors (Clinicopathological data are summarized in Table 1). As shown in **Fig. 12**, down-regulation of HAO2 occurred in all HCCs compared to healthy liver donors. Additionally, samples matched analysis (tumor vs. non cancerous cirrhotic tissue) revealed a down-regulation of HAO2 in 84.7% of HCCs (50 out of 59 tumors).

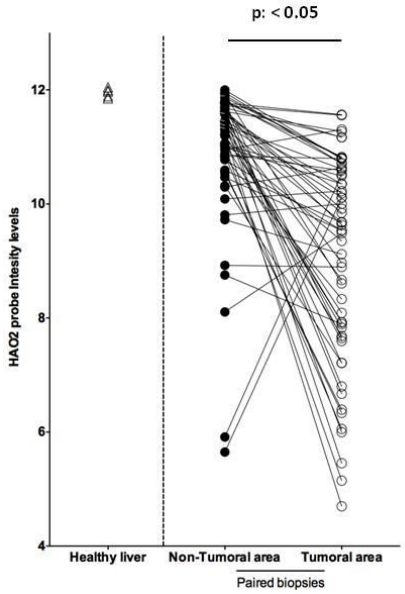

Fig. 12 HAO2 expression levels in non neoplastic liver samples (n=5) and HCC specimens (n=59) compared to their matched non tumoral counterpart. HAO2 mRNA expression is assessed by Affimetrix microarray and measured as probe intensity levels. Paired Student t-test: tumoral vs. non tumoral area p<0.05.

Furthermore, we analysed expression levels of HAO2 in patients classified according to the Edmondson and Steiner (ES) grading system and their aetiology. Low expression of Hao2 was significantly observed in high-grade (III-IV) vs. low-grade (I-II) HCCs ,**Fig. 13A**, but not in association with different aetiological agents (**Fig. 13B**).

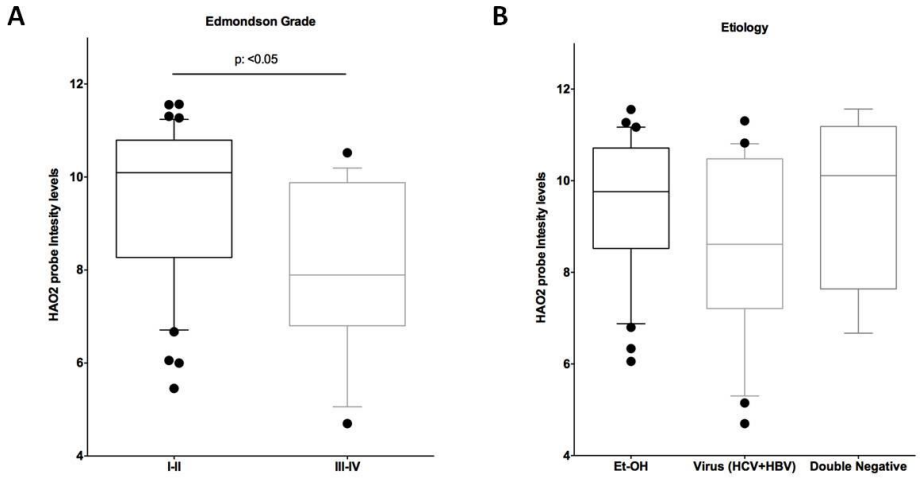

Fig.13 Stratification of patients in according to (A) the Edmondson and Steiner (ES) grading system and (B) the aetiological related agent. HAO2 mRNA expression is assessed by Affimetrix microarray and measured as probe intensity levels. Unpaired Student t-test: low (I-II) vs. high grade (III-IV) p<0.05.

Finally, to explore whether HAO2 expression is associated with clinical progression and outcome of HCC patients, we examined the incidence of metastases and patient overall survival (OS) rates using Kaplan-Meier analysis. The metastatic status was defined as either regional lymph node invasion and/or distant organ involvement. Patients with low HAO2 expression showed an increased metastatic activity (**Fig. 14A**) and a decreased OS (median of 10 versus 31 months in low vs. high HAO2, respectively; **Fig. 14B**).

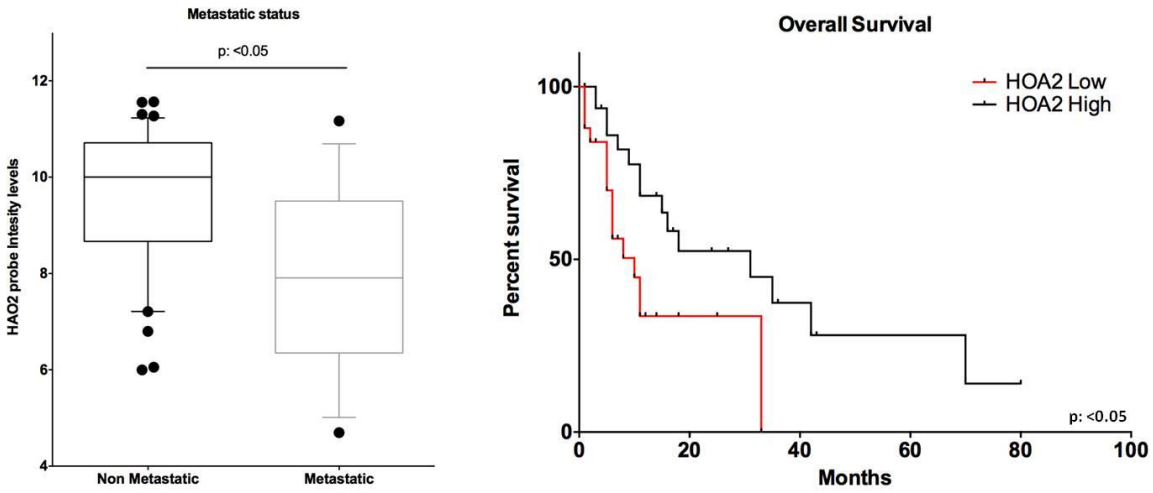

Fig. 14 HAO2 expression levels, analysed by Affimetrix microarray and measured as probe intensity levels, in association with (**A**) metastasis formation and (**B**) predicts poor pantients survival. Survival plot was analysed using the Kaplan-Meier method. ROC analysis was used to discriminate between high and low expressing levels of HAO2. Unpaired Student t-test: all P values <0.05.

Next, we investigated the expression levels of Hao2 in another distinct series of 59 human HCCs and their corresponding peritumoural tissues provided by the Policlinico S. Orsola-Malpighi of Bologna. The characteristics of the study population are described in Table 2. Similarly to what observed in the previous cohort, HCC down-regulation was confirmed in all samples compared to normal liver donors (**Fig. 15A,B**) and in 88.1 % of samples compared to matched cirrhotic tissues (52 out of 59 tumors ,**Fig. 15A,B**).

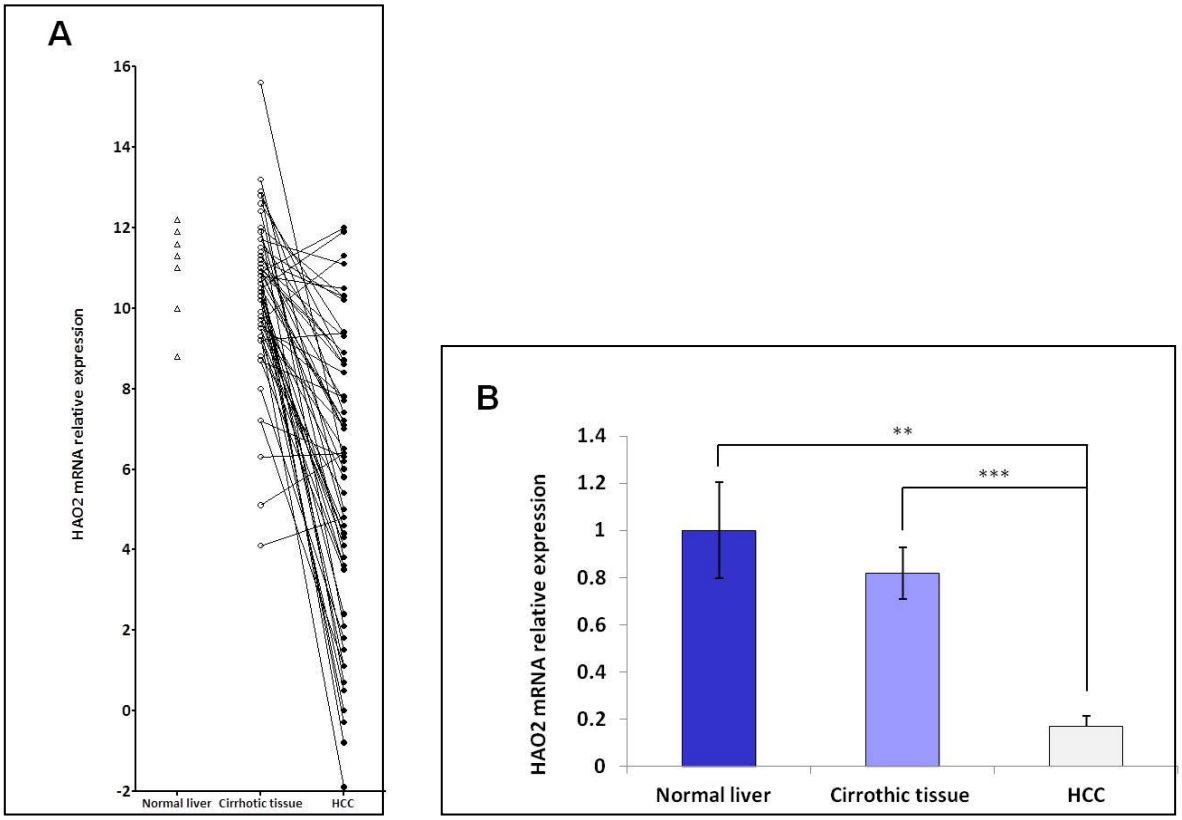

Fig. 15 QRT-PCR analysis of Hao2 mRNA expression levels in human HCCs and matched cirrhotic tissues (n=59) and in normal liver (n=8). (A) The levels of Hao2 were calculated as relative mRNA expression using the 2^{-ddCt} method and represented as log 2. Human β-actin was used as endogenous control. (**B**) The average levels of samples in panel A were calculated as fold change. Each bar represents mean ± standard error (SEM). Tukey-Kramer test: ** p<0.01,*** p<0.001.

Altogether these data show that in humans, as well as in rodents, HAO2 mRNA levels are significantly down-regulated in HCC development.

7. HAO2 protein content is decresed in human HCC

We next evaluated the content of HAO2 protein in human HCC. Initially, we performed immunohistochemical staining (IHC) for HAO2 in tissue specimens from both HCC and non-neoplastic liver tissue samples from patients of the Basel cohort. Initial studies were performed to find the most appropriate antibody for IHC staining. From our search, while antibodies for western blot were provided by different companies, only the Novus Biologicals antibody was recommended for HAO2 staining in human tissues. Using this antibody, we found positivity for HAO2 in human cirrhotic areas (**Fig. 16A**) which was reduced in most of human HCCs (**Fig. 16B,C**). In addition tissue microarray (TMA) staining showed (**Fig. 16D**) that approximately 60% of HCC analysed exhibited a reduced HAO2 staining when compared to surrounding liver (**Fig. 16D,E**).

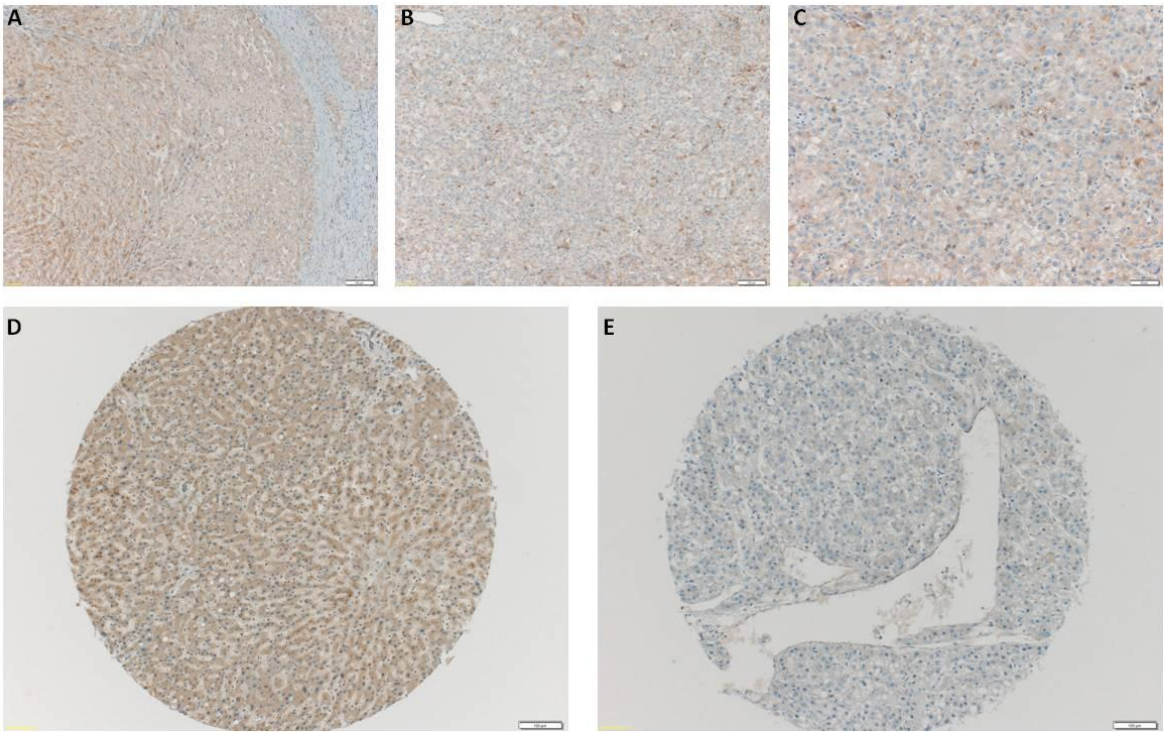

Fig. 16. Representative pictures of HAO2 immunohistochemical in HCC and non-neoplastic liver tissue samples. HAO2 protein levels analised by IHC staining in (**A**) cirrhotic liver at 100x magnification and in HCC samples (**B**) at 100x (**C**) and 200x magnification. TMA staining in non-neoplastic liver tissue (**D**) and HCC punches (**E**) at 100x magnification.

Although the results of IHC were encouraging, we were not completely satisfied by the immunostaining of liver samples. Therefore, we performed western blot analysis in 7 human HCCs and their cirrhotic counterparts. As shown in **Fig. 17**, HAO2 protein content was strongly decreased in 5/7 HCCs, when compared to their corresponding cirrhotic peritumorural livers.

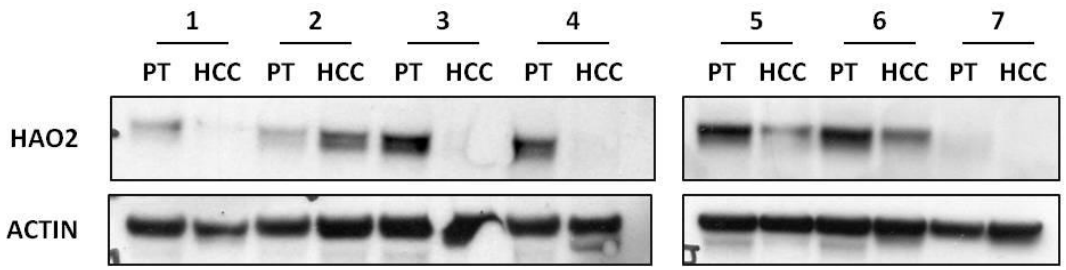

Fig. 17. HAO2 expression levels analysed by western blot in 7 HCCs and their cirrhotic peritumoral livers (PT). Actin antibody was used as an internal control.

These results clearly show that HAO2 is down-regulated at both mRNA and protein levels in human HCC.

8. HAO2 *in vitro* and *in vivo*

To better understand the biological role of HAO2, we decided to investigate the expression levels of Hao2 in 7 HCC human cell lines: HA22T, HepG2, HuH7, Mahlavu, SNU 182, SNU 398, SNU 475. QRT-PCR showed that Hao2 was expressed at very low levels in all cell lines analysed (ΔCt ranging from 32-34 in HuH7 and HepG2, to 37-39 in all other cell lines, **Fig. 18**). The results were quantified as Ct (threshold cycle) values, defined as the cycle number of PCR at which the amplified product is first detected; Ct value is inversely related to the starting amount of target. Similarly, low Hao2 mRNA expression levels was found in R-H rat liver cells, obtained from HCC bearing rats (Petrelli A et al, 2014).

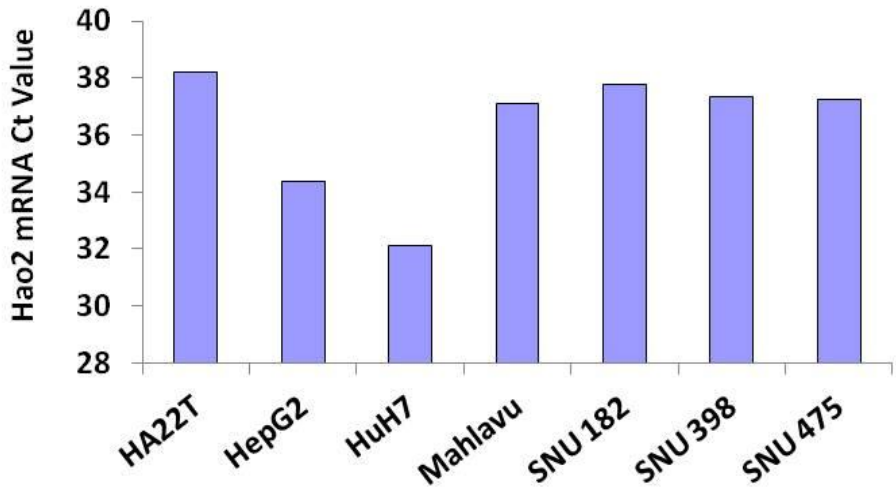

Fig. 18. QRT-PCR analysis of HAO2 mRNA expression levels in 7 human HCC cell lines, HA22T, HepG2, HuH7, Mahlavu, SNU-182, SNU-398, SNU-475. The levels of Hao2 were indicated as Ct value using human beta-actin as endogenous control.

Accordingly, no detectable signal was detected by western blot analysis in all 7 HCC cell lines (**Fig. 19**).

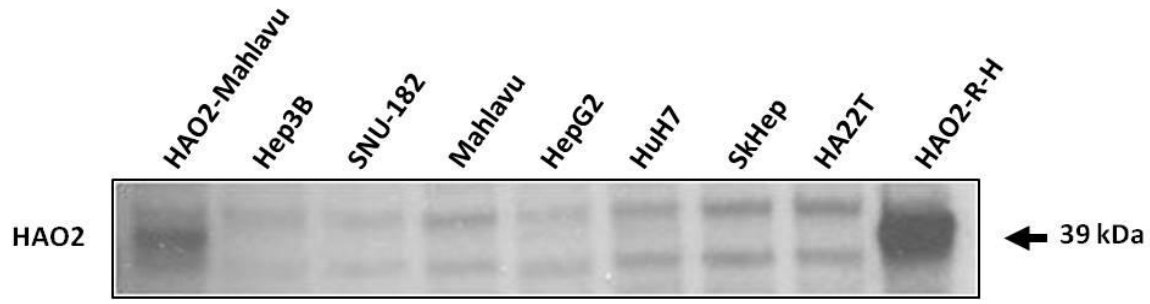

Fig. 19. Western blot analysis of HAO2 in human and R-H cell lines. HAO2-transduced Mahlavu (HAO2-Mahlavu) and R-H cell lines (HAO2-R-H) were used as positive controls.

Therefore, the down-regulation of Hao2 was confirmed also in human HCC cell lines at both mRNA and protein levels.

In order to investigate whether an increase in HAO2 expression could hamper cell growth, we performed soft agar and cell growth assays in human Mahlavu and rat R-H cell lines transduced with a lentiviral construct containing HAO2; we did not observe significant alteration in anchorage-dependent or independent growth ability between human and rat cell lines compared to their transduced counterparts (data not shown).

Moreover, we tested *in vivo* if the increase of Hao2 expression could impact the tumorigenic ability of Hao2-transduced cells. Therefore, parental and Hao2-transduced R-H cell lines were subcutaneously grafted into syngeneic F-344 rats. We found that while parental R-H cells were able to form tumors in 3/7 animals, no tumors was observed in all 6 rats grafted with Hao2-transduced cell lines within 40 days post injection (**Fig. 20**).

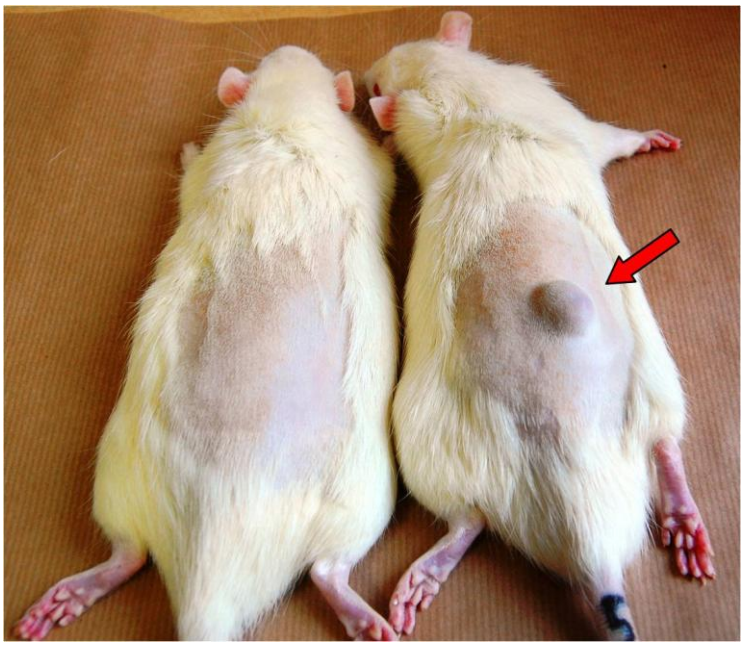

Fig. 20. Photograph of rats 40 days after the injection of wild type (right) or Hao2-transduced (left) R-H cancer cells. Cells were subcutaneously injected into the right posterior flanks of syngeneic male Fischer F-344 rats. Tumor is shown by the arrow.

This data suggest that down-regulation of Hao2 could effectively support tumor growth.

DISCUSSION

L-2 Hydroxy acid oxidases are flavin mononucleotide (FMN)-dependent peroxisomal enzymes which are able to oxidize a number of 2-hydroxy acids to 2-keto acids resulting in hydrogen peroxide formation at the expense of molecular oxygen (Angermüller S. 1989; Fry DW et al, 1979; Schwam H et al, 1979).

Currently, very few data are available in literature about hydroxy acid oxidases and their functional role remains to be defined. Some authors reported an involvement of these enzymes in fatty acid α-oxidation (Jones JM et al, 2000) or in regulation in blood pressure (Lee SJ et al, 2003; Rico-Sanz J et al, 2004), albeit the mechanisms are not fully explained. By producing hydrogen peroxide, during their enzymatic activity, hydroxy acid oxidases might contribute to generate oxidative stress, by increasing ROS levels. High ROS levels are associated with cell death and cellular damage and with an abnormal cancer cell growth (Toyokuni S et al, 1995).

Unfortunately, the role of Hao2, if any, in cancer is still unknown. Indeed, the only study associating Hao2 and cancer was a study of gene expression profile in human intrahepatic cholangiocarcinomas. This study identified Hao2 among down-regulated genes in these tumors (Wand AG et al, 2006), but no comments or hypotheses about its role were proposed by the authors.

To our knowledge, the results obtained in my thesis are the first to show that Hao2 is profoundly down-regulated in HCCs. The main findings stemming from our study can be summarised as follows: i) Hao2 is down-regulated in 100% of rat and mouse HCCs and in about 80% of human HCCs; ii) Hao2 down-regulation is species and etiology independent; iii) Hao2 down-regulation is a very early event in multistage hepatocarcinogenesis; iv) it is more pronounced in the most aggressive preneoplastic lesions; v) its levels are inversely correlated with overall survival and metastasis; vi) transduction of Hao2 inhibits the tumorigenic potency of HCC cells in vivo.

At the present, no data are available to explain the reduced expression of Hao2 observed in HCC. Several epigenetic or genetic alterations or post-transcriptional regulation, such as that caused by miRNA could be involved. Searching for Hao2-targeting miRs, we found that miR-183 is predicted to bind to complementary sequences located in the 3'-untranslated (3'-UTR) region of Hao2 mRNA. Previous work done in our laboratory has

shown that this miR is up-regulated in Krt-19 positive preneoplastic lesions (75-fold) and in HCCs (15-fold) developed with the R-H model of hepatocarcinogenesis (Petrelli A et al, 2014). Notably, the same lesions exhibited Hao2 down-regulation, when compared to control liver, suggesting that increased levels of miR-183 could be responsible for Hao2 down-regulation. Further studies will be performed to establish if this or other miRs are indeed responsible for Hao2 down-regulation in the carcinogenic process.

Although the mechanisms underlying Hao2 decrease in pre- and neoplastic lesions are unclear, it is possible to speculate that this enzyme plays a role in hepatocyte proliferation and survival. Indeed, the findings that i) the expression levels of Hao2 decreased during liver regeneration after PH, and that, ii) the maximal Hao2 down-regulation occurs in the most aggressive rat preneoplastic lesions (Krt-19^{+}), which proliferate at a higher rate than those negative for this marker (Petrelli A et al, 2014), suggest that Hao2 is inversely correlated to cell cycle, in a way resembling tumor suppressor genes.

Another possible explanation for the biological effect driven by decreased levels of Hao2 could be linked to the ability of this enzyme to produce hydrogen peroxide. Loss of this enzyme may represent a cytoprotective mechanism by which genetically-altered cells can escape excessive ROS generation and cell damage/death.

Independently upon the mechanism through which Hao2 regulates hepatocyte behaviour, our findings can have a relevant translational value in terms of both prognosis and diagnosis. Indeed, the novel finding that Hao2 expression is virtually absent in preneoplastic lesions, suggests its possible use as a diagnostic marker. This possibility is supported by our preliminary findings showing immunohistochemical positivity for HAO2 in human cirrhotic areas, but not in most of human HCC. It is possible to hypothesize that HAO2 staining might allow early identification of dysplastic nodules and their discrimination from cirrhotic regenerative nodules, a diagnosis that is presently problematic in most circumstances.

HAO2 can also be useful for prognostic purposes. Indeed, as shown in my thesis, the levels of HAO2 in HCC inversely correlate with increased metastatic ability of the tumor and overall survival of the patients.

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