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PRENEOPLASTIC HEPATOCYTE HYPOTHYROIDISM UNDERLIES HEPATOCELLULAR CARCINOMA PROGRESSION

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Abbreviations

- 2-AAF — 2-acetylaminofluorene
- AFB_1 — aflatoxin B_1
- AFP — α-fetoprotein
- APC — adenomatosis polyposis coli
- APP — amyloid β precursor protein
- BMI — body mass index
- BrdU — 5'-bromo-deoxyuridine
- CDKN2A — cyclin-dependent kinase inhibitor 2A
- CSCs — cancer stem cells
- CTNNB1 — β-catenin
- DENA — diethylnitrosamine
- DIO — deiodinase
- DNMTs — DNA methyltransferase enzymes
- EpCAM — epithelial cell adhesion molecule
- ERK — extracellular signal-regulated kinase
- ERα — estrogen receptor α
- FNH — focal nodular hyperplasia
- G6PC — glucose-6-phosphatase
- GSK-3β — glycogen synthase kinase-3β
- GST-P — placental glutathione-S-transferase
- HBV — hepatitis B virus
- HCC — hepatocellular carcinoma
- HCV — hepatitis C virus
- HIF-1α — hypoxia inducible factor-1α
- HIV — human immune-deficiency virus
- ICC — intrahepatic cholangiocarcinoma
- IGF-2 — insulin-like growth factor-2
- IGF-2R — insulin-like growth factor-2 receptor

- KRT-19 cytokeratin-19
- LOH loss of heterozygosity
- MBD methyl-CpG binding domain
- MeCPs methyl-CpG binding proteins
- mTOR mammalian target of rapamycin
- NAFLD nonalcoholic fatty liver disease
- NASH nonalcoholic steatohepatitis
- NCoR nuclear receptor co-repressor
- NHR nuclear hormone receptor
- NOS nitrogen oxygen species
- OCs oral contraceptives
- PH partial hepatectomy
- PI3K phosphoinositide 3-kinase
- PKCα protein kinase Cα
- PLC phospholipase C
- pRB retinoblastoma protein
- PTEN phosphatase and tensin homolog
- RH resistant hepatocyte model
- ROS reactive oxygen species
- RXR retinoid X receptor
- SAM S-adenosylmethionine
- SMRT silencing mediator of retinoic and thyroid receptor
- T_3 triiodothyronine
- T_4 tyroxine
- TGF-β transforming growth factor- β
- TH thyroid hormone
- THR thyroid hormone receptor
- THRSP/SPOT14 thyroid hormone responsive spot14
- TRAP THR-associated proteins
- TREs thyroid Hormone Response Elements
- VDR vitamin D receptors

Introduction

HEPATOCELLULAR CARCINOMA

Epidemiology

Hepatocellular carcinoma (HCC) is the most common primary cancer of the liver and a major health problem worldwide. Incidence is increasing and HCC has risen to became the 5th commonest malignancy worldwide and the third leading cause of cancer-related death *[Parkin DM, 2001]* . Globally, there are approximately 750˙000 new cases of liver cancer per year. Population-based control studies showed that the incidence rate continues to approximate the death rate, indicating that most of the patients who develop HCC die for it *[Jemal A et al, 2011]* . HCC burden is not distributed evenly throughout the world and important differences have been noted between countries. Sub-Saharan Africa and Eastern Asia are considered to have a very high incidence of HCC (over 20 cases / 100˙000 population) . Areas with moderate risk (10-20 cases / 100˙000 population) include Italy, Spain, France, United Kingdom and Latin American countries, and a relative low incidence (5-10 cases / 100˙000 population) is found in the United States, Canada, and in Scandinavia *[Parkin DM, 2002; Montalto G et al, 2002; Ferlay G et al,1998; Bosh FX et al, 2004]* . Although encouraging trends in HCC incidence have been observed in some high-rate areas, in contrast, its incidence is rising in some low-moderate risk areas *[McGlynn KA et al, 2001]* . The main reasons for the observed increase in HCC incidence, are mainly related to the increased occurrence of the hepatitis C (HCV) virus infections and to the increase of immigrants from Asia and Africa to North America and Western Europe countries *[Di Bisceglie AM, 2002; El-Serag HB and Mason AC, 1999; Taylor-Robinson SD et al, 1999; Khan SA et al, 2002]* . In addition to geographical distribution, the global incidence of HCC is also strongly correlated to ethnicity, gender and age. USA are a good example of how the HCC incidence also vary greatly among different populations living in the same region; where, independently of age and sex, HCC rates are two times higher in Asian than in African Americans, whose rates are two time higher than those in whites *[El-Serag HB and Rudolph KL, 2007]* . In almost all populations, males have higher liver cancer rates than females, with male:female ratios usually averaging between 2:1 and 4:1. Reasons for higher rates of

liver cancer in males may relate to sex-specific differences in exposure to risk factors and it is reasonable to think that androgens have an important role in HCC progression. Indeed, experiments show a 2- to 8-fold increase in HCC development in male mice *[Rudolph KL et al, 2000]* . Moreover, studies conducted in Taiwan reported a positive association between increased circulating testosterone levels and HCC in HBV-infected men [*YU MW and Chen CJ, 1993; Yu MW et al, 2001]* . The global age distribution of HCC varies by region, incidence rate, sex and etiology *[Parkin DM, 2002]* . The incidence of HCC increases with age, reaching its highest prevalence among those aged over 65 years and, in almost all areas, female reach the peak 5 years later than males *[El-Serag HB and Rudolph KL, 2007; El-Serag HB, 2007; Parikh S and Hyman D, 2007]* . A shift in incidence towards younger ages has been noted in the last two decades *[Bosch FX et al, 2004]* .

Risk factors

Usually HCC occurs within an established background of chronic liver disease and rarely it develops in a healty liver. Cirrhosis is the most important risk factor, with ~ 70-90% of all detected HCC cases arising in a cirrhotic liver *[Parkin DM et al, 2001]* . Other important risk factors for HCC development include viral infections (hepatitis B and hepatitis C virus), toxic exposures (aflatoxins, pesticides, alcohol intake, tobacco), metabolic disorders (non-alcoholic fatty liver disease, diabetes, obesity) and hormonal factors (oral contraceptives). Less common causes include hereditary hemochromatosis, α_1-antitrypsin deficiency, autoimmune hepatitis, and some porphyrias.

Viral factors

Hepatitis B virus (HBV)

The WHO has reported HBV to be second only to tobacco smoke as a known human carcinogen since about 300 million people have the chronic infection worldwide *[WHO, 2002; El-Serag HB and Rudolph KL, 2007]* . HBV is the leading cause of HCC worldwide, particularly in Asia and Africa, where HBV is endemic. In these areas, it is usually transmitted from mother to newborn (vertical transmission) and 70-90% of HBV-associated HCC develops in the setting of liver cirrhosis; but even in the absence of cirrhosis the infection is an important risk factor *[El-Serag HB and Rudolph KL, 2007]* . This pattern is different in areas with low HCC incidence rates where HBV is acquired in adulthood through sexual and parenteral routes (horizontal transmission), with more than

90% of acute infections resolving spontaneously *[El-Serag HB and Rudolph KL, 2007]* . The mechanisms of HCC development associated to HBV infection have been extensively studied, and both HBV and host hepatocytes may contribute to the final pathogenic outcomes, either individually or synergistically *[Liu CJ and Kao JH, 2007]* . From a global perspective, the burden of chronic HBV infection is expected to decline because of the increasing utilization of HBV immunization, that actually is the most effective measure for the HBV infection prevention *[Kane MA, 2003]* .

Hepatitis C virus (HCV)

HCV is the most important risk factor for HCC in Western European and North American countries *[Montalto G et al, 2002]*. Up to 80% of HCV-infected individuals fail to eliminate the virus acutely and progress to chronic HCV infection *[Suruki RY et al, 2006; Tsai SL et al, 1997; Sarih M et al, 2000; Cucchiarini M et al, 2000; Gruner NH et al, 2000]* . The rate of fibrotic progression following HCV infection is markedly variable, since the natural history of the disease typically extends over several decades. Usually, HCC develops after 10-20 years of HCV infection and the increased risk is largely restricted to patients with cirrhosis or advanced fibrosis *[El-Serag HB and Mason AC, 2000]* . In contrast to HBV, in HCV-infected patients, host and environment factors appear to be more important than viral factors in determining progression to cirrhosis. These factors include older age at the time of infection, male sex, heavy alcohol intake (>50gr/day), diabetes , obesity and co-infection with human immune-deficiency virus (HIV) or HBV *[Cramp ME, 1999]* . Follow-up studies have shown that patients with combined HBV and HCV infection have a higher risk of developing HCC than those with HBV or HCV alone [*Montalto G et al, 2002, Michielsen PP et al, 2005; Sato S et al, 1994*] . The cumulative risk of developing HCC was 23%, 10% and 21%, respectively, after 5 years and 45%, 16% and 28%, respectively, after 10 years *[Montalto G et al, 2002; Chiaramonte et al, 1999]* .

Environmental factors

Aflatoxin B_1 (AFB_1)

Aflatoxin B_1 is a mycotoxin produced by the *Aspergillus flavus* and *Aspergillus parasiticus fungi*. In Asia and sub-Saharan Africa, climatic factors and storage techniques favor the fungus to be a common contaminant of foods, such as grain, corn, peanuts and legumes stored in warm and damp conditions. Animal experiments showed that AFB_1 is a powerful

hepatocarcinogen, leading the International Agency for Research on Cancer to classify it as carcinogenic compound *[IARC, 1987]* . AFB_1 is metabolized to an active intermediate, AFB_1-*exo*-8,9-epoxide, which can bind to DNA and cause damage, including a characteristic mutation in the p53 tumor-suppressor gene (mutation of codon 249, 249^{ser}) *[Garner RC et al, 1972]* . This mutation has been observed in 30-60% of HCCs in aflatoxin-endemic areas *[Bressac B et al, 1991; Turner PC et al, 2002]*. Furthermore, short-term prospective studies in Shanghai, have shown an interaction between AFB_1 exposure and chronic HBV infection. These studies have demonstrated that the urinary excretion of aflatoxin metabolites was associated with a 4-fold increase in HCC risk, whereas HBV infection increased the risk 7-fold. However, HBV carriers who excreted AFB_1 metabolites had a dramatic 60-fold increased risk of HCC *[Qian GS et al, 1994]* . Indeed, it has been observed that areas with a high prevalence of HCC and high aflatoxin intake also correspond to areas with endemic HBV infection *[Montalto G et al, 2002; Groopman JD et al, 1996]* .

Pesticides

Pesticides exposure is one of the environmental factors hypothesized to increase the risk of HCC. Pesticides are believed to exert their carcinogenic potential through one or several mechanisms, such as spontaneous initiation of genetic changes, cytotoxicity with persistent cell proliferation, oxidative stress, inhibition of apoptosis and suppression of intracellular communication *[Rakitsky VN et al, 2000; Ezzat S, et al, 2005]* . A case-control study of HCC in HBV and/or HCV infected patients from Egypt suggested that pesticides had an additive effect on the risk of HCC in rural males, amongst whom the use of carbamate and organophosphate compounds is commonplace *[Ezzat S et al, 2005]* .

Alcohol

Heavy alcohol intake is a well-established HCC risk factor. Depending on the geographical areas analyzed, it can act as a primary cause for the HCC onset or as a cofactor. In USA and in several countries of Western Europe, where there is a high rate of alcohol abuse/addiction, it acts as a primary cause of tumor development. It has been demonstrated that the ingestion of more than 80gr/day of alcohol for more than 10 years increases the risk of developing cancer about 5 times. Instead, alcohol acts as cofactor in developing countries, where its consumption is accompanied by other risk factors, such as

HBV and HCV virus or diabetes. There is also evidence that in the presence of concomitant HCV infection, there is an additional 2-fold increase in HCC risk. Although heavy intake is strongly associated with the development of cirrhosis, there is little evidence of a direct carcinogenic effect of alcohol otherwise. The mechanism by which alcohol causes HCC is still not well known but it is hypothesized that oxidative stress, altered retinoic acid metabolism, DNA methylation, genetic susceptibility and decreased immune surveillance may play an important role in tumor development *[Morgan TR et al, 2004]* .

Tobacco smoke

Several studies conducted in both low- and high-rate HCC areas examined the relationships between cigarette smoke and HCC but of more than 40 studies that examined the association between 1983 and 2002, the number of studies which reported positive associations was almost equal to the number with no associations *[Schottenfeld D et al., 2006]*. Taken together, the available evidence suggest that tobacco alone is not an important risk factor for HCC but the risk is highly increased in the presence of other risk factors, such as HBV, HCV, heavy alcohol intake and other exposure *[El-Serag HB and Rudolph KL, 2007; Evans AA et al, 2002]*.

Metabolic disorders

Several epidemiological studies conducted mainly in the United States, failed to identify a significant association between well-established risk factors and HCC development for more than 50% of cases. These cases seem to be not attributable to the most common risk factors (chronic infection by HBV, HCV, alcohol consumption). Among these patients, a large rate includes patients with metabolic disorders *[Davila JA et al, 2005] ,* such as nonalcoholic fatty liver disease, obesity and diabetes .

Nonalcoholic fatty liver disease (NAFLD)

NAFLD is the most common liver disorder in Western countries. In the United States more than 30% of people are affected and 90% of them are obese (body mass index ≥ 40kg/m^2) *[Browning JD et al, 2004 ; Lazo M, Clark JM, 2008]* . It occurs in the absence of alcohol intake, although liver histology appears consistent with alcoholic hepatitis. The most serious form of NAFLD is represented by nonalcoholic steatohepatitis (NASH) *[Falck-Ytter Y et al, 2001; Angulo P. et al; 1999] ,* that represents the hepatic manifestation of

several metabolic disorders such as obesity and diabetes mellitus type 2 *[Abdelmalek MF, Diehl AM., 2007]* ; indeed NASH manifests nearly universally among diabetic patients who are morbidly obese *[Adams LA et al, 2005]* . Moreover, NASH in association with other components of the metabolic syndrome is thought to increase the risk of developing chronic liver disease, cirrhosis and HCC *[Bugianesi E. et al, 2007]* . A recent study has shown that NAFLD is a principal risk factor in the development of HCC, irrespective of age *[Sanyal AJ et al, 2009]* . This association is alarming, due to the globally high prevalence of these conditions and may contribute to the rising incidence of HCC observed in many industrialized countries.

Obesity

Several large-scale epidemiological studies have associated the increasingly prevalent overweight and obesity with a higher risk of HCC *[Calle EE et al, 2003 ; Bianchini F et al, 2002]* . In a cohort of 900˙000 American adults, the risk of dying from liver cancer was 4.5 times higher in men with a body mass index (BMI) $\geq$35kg/m^2 or above compared to the reference group with a normal BMI (18.5 to 24.9kg/m^2) *[Calle EE et al, 2003]* . A recent meta-analysis concluded that the summary relative risk of liver cancer was 117% for overweight subjects and 189% for obese individuals *[Larsson SC and Wolk A, 2007]* .

Diabetes

Type 2 diabetes is an increasingly common metabolic disorder strongly linked to obesity and characterized by hyperglycemia, insulin resistance, and hyperinsulinemia. It has been associated with increased risk of several cancers. Substantial evidence indicates that diabetes promotes development and progression of HCC *[Giovannucci E et al, 2010 ; El-Serag HB et al, 2006]* . A population-based study that evaluated 14% of the United States population found that diabetes conferred a three-fold risk of HCC *[Davila JA et al, 2005]* . The association between diabetes and HCC has been further demonstrated by studies focused on populations from different geographical locations indicating that in most individuals diabetes may exist for a long time before the diagnosis of HCC and that there are synergistic interactions between diabetes and other HCC risk factors *[Baffy et al, 2012; Hassan MM et al, 2002]* .

Ormonal factors

There is experimental rationale for a possible role of oral contraceptives (OCs) in the development of liver neoplasia. Nuclear estrogen receptors are expressed in hepatocytes and are increased in HCC, suggesting a hormonal responsiveness of hepatic neoplastic tissue *[El-Serag HB and Rudolph KL, 2007]* . Estrogen and progesterone components of OCs have been shown to induce and promote liver tumors in animals *[Yu MC and Yuan JM, 2004]* , probably because estrogens increase proliferation rates, thereby increasing rates of spontaneous mutations *[De BV et al, 1996]* . Several human studies have reported an increased risk of developing benign liver tumors, such as hepatic cell adenoma and focal nodular hyperplasia in women using OCs *[Korula J et al , 1991]* . OC usage also has been linked, although unfrequently, to malignant liver tumors such as HCC, mixed hepatocellular and ductal carcinoma, cholangiocarcinoma and hepatoblastoma *[Rosenberg L , 1991]* .

Natural history of HCC

Hepatocellular carcinoma development is a multistep process and it nearly always develops in the setting of chronic hepatitis or cirrhosis. Retrospective studies focused on HCV patients suggest that HCC manifestation takes about 10 years after diagnosis of cirrhosis and about 30 years after exposure to the virus *[Kiyosawa K. Et al, 1990]* . Conversely, HBV related carcinogenesis is less predictable because HCC development may precede the occurrence of cirrhosis, especially in geographic areas in which the HBV infection is endemic *[McMahon BJ, 2005]* .

The natural history of HCC is fairly long and can be divided into three distinct phases:

1) molecular ; 2) preclinical ; 3) clinical or symptomatic *[Trevisani F et al, 2008]* .

1) *Molecular phase:* The molecular phase includes the sequential genomic alterations leading to cell transformation. It has been postulated that the transformed cell can be either a hepatocyte/biliary epithelial or a liver stem cell *[D' Errico A et al, 1996]* . Mechanisms of cellular transformation are different depending on the cell type. Genetic alterations involving differentiated cells (hepatocytes and cholangiocytes) are thought to confer a growth advantage by promoting proliferation and inhibiting apoptosis, whereas those involving stem cells interfere with the differentiation process *[Trevisani F et al, 2008]* .

2) *Preclinical phase:* is divided in two phases; an initial period, in which the tumor is still too small to be detected by imaging techniques, and a second period, during which the tumor is detectable but still asymptomatic.
3) *Clinical or symptomatic phase:* is characterized by the occurrence of symptoms caused by the tumor burden. In patients with chronic liver disease, HCC usually becomes symptomatic when it reaches a size of 4.5–8cm *[Yuen MF et al, 2000 ; Trevisani F et al, 2002]* .

The complex etiology of HCC affects the possible treatment options offered to patients. Treatments for HCC have been conventionally divided into curative and palliative. Curative treatments, such as resection, liver transplantation, and percutaneous ablation, are the most effective treatments for patients with early-stage disease since they can induce a complete response in a high proportion of patients and are expected to improve survival. Palliative treatments are applied to patients with advanced tumor stages, therefore with poor hepatic and extra-hepatic functions. Palliative treatments include chemotherapy agents and symptomatic drugs, the latter especially applied to end-stage patients *[Josep M Llovet et al, 2003]* .

Two decades ago, HCC prognosis was dismal because diagnosing at advanced stages precluded the optimum use of radical treatments and most patients died within 1 year, irrespective of treatment *[Okuda K et al, 1985]* . In developed countries this *scenario* has completely changed, since 30-40% of patients are now being diagnosed at initial stages when curative treatments can be optimally applied *[Bruix J and Llovet JM, 2002]* . Indeed, the short-term prognosis of patients with HCC has improved, but long term prognosis remains unsatisfactory and survival at 5-10 years after curative treatments remains dismal *[Masuzaki R et al, 2008; Shiina S et al, 2012]* . One of the most important reasons that contribute to the poor prognosis is that patients with HCC usually show important liver dysfunctions, generally as a result of cirrhosis, and consequently are ineligible for surgical and non-surgical treatments. Given the established links between liver cirrhosis and development of HCC, there is a strong rationale for surveillance of patients with cirrhosis, and guidelines support observation of this group, regardless of etiology *[Arun J. Sanyal, 2010]* . Prevention of HCC is also an important goal, and opportunities exist to advance the development of preventive measures. The success of preventive intervention was first demonstrated by the vaccination program against HBV which was implemented in the 1980s and successfully reduced both the number of HBV carriers and the incidence of

HCC in children. Moreover, studies performed in patients with HCV, with and without cirrhosis, have shown that treatment with interferon therapy is associated with a lower risk for developing HCC *[Chang MH, 1997; Ni YH et al, 2001; Ni YH et al, 2007; Chang et al, 2009]* . The serum marker α-fetoprotein (AFP) and ultrasounds represent the most relevant diagnostic tests for HCC, however levels of sensitivity and specificity of these screenings are very limited when used alone; combining ultrasonography and AFP appears to improve detection rates *[Ryder SD, 2003]* .

Molecular mechanisms

Advances in molecular profiling studies using DNA microarray-based gene-expression experiments have provided increased awareness about the regulatory networks altered in human HCC and have also provided useful gene expression-based signatures that can distinguish tumor subtypes, assist clinical staging and predict patient outcomes. As mentioned above, more than 90% of HCC cases develop in the setting of chronic hepatitis or cirrhosis suggesting that chronic inflammation plays a key role in altering the liver microenvironment, increasing the risk of carcinogenesis *[Grisham JV, 2001; Bosh FX et al, 1999; Buendia MA, 2000]* . Therefore, there is a strong need to elucidate the molecular mechanisms that predispose to tumor development, especially those involved in the inflammatory pre-neoplastic phase. To explain hepatic carcinogenesis, two models are currently proposed: the first one is the clonal evolution model that implies a multi-step process of tumor development from precancerous lesions to metastatic carcinoma ; the second model, recently proposed, is the cancer stem cells model *[Kumar M et al, 2011]* .

The clonal evolution model of hepatocarcinogenesis

The longstanding clonal evolution model for HCC development is a multistep event, which may take as long as 30 years to unfold. The various etiological factors, particularly inflammation and viral hepatitis, seem to contribute significantly to approximately 90% of HCC cases by creating phenotypically altered hepatocytes. The main causes of HCC (viral hepatitis, alcoholic hepatitis and non-alcoholic steatohepatitis) typically give rise to hepatic alterations culminating in a chronic inflammation of the liver *[El-Serag HB, Rudolph KL, 2007]* . In this conditions many hepatocytes die, inflammatory cells invade the liver and connective tissue is deposited *[Thorgeirsson SS & Grisham JW, 2002]* . For example, in chronic viral hepatitis, the host immune responses to HBV or HCV are often unable to

completely clear the infection, resulting in chronic stimulation of an antigen-specific immune response *[Budhu A and Wang XW, 2006]* . Virus-infected hepatocytes are killed by host immune cells and by the intrinsic cytopathic effects of the hepatitis viruses, triggering the production of various cytokines and growth factors and subsequently inducing compensatory hepatocyte regeneration. This persistent cycle of necroinflammation and hepatocyte regeneration is thought to increase the risk of genetic mutation in hepatocytes, and furthermore, to promote survival and expansion of initiated cells *[Levrero M, 2006; Maeda S, 2010; He G and Karin M, 2011]* . Additionally, reactive oxygen species (ROS) and nitrogen oxygen species (NOS), generated by both initiated cells and inflammatory cells, could accelerate hepatocarcinogenesis through several mechanisms, such as the induction of oxidative DNA damage, DNA methylation, and hepatocyte injury *[He G and Karin M, 2011]* . The result of these conditions is the production of phenotypically altered hepatocytes that are considered monoclonal in origin *[Yeh SH et al, 2001; Aihara T et al, 1994]* . These aberrant hepatocytes, through a multistep process, initially give rise to development of preneoplastic foci, which with further accumulation of mutational events and aberrant growth, can transform into primary HCC and finally metastatic HCC *[Sakamoto M et al, 1991]* . Progression of hepatocarcinogenesis is associated with multiple molecular mechanisms which together lead to the loss of tumor suppressor genes and to the gain of oncogenes as a consequence of genetic and epigenetic alterations that represent the basis for the alteration of important signaling pathways *[Kumar M et al, 2011]* .

Genomic alterations

HCCs are deeply heterogeneous and characterized by a multitude of chromosomal alterations that gradually accumulate during the carcinogenic process *[Wilkens L et al, 2002]*. Previous genome-wide gene expression studies have revealed altered gene expression patterns that correlate with clinical outcome *[Woo HG et al, 2011]* . Moreover, comparative genomic studies and SNP-arrays, through a comparison between HCC samples and non tumoral tissue, have been necessary to highlight a wide mixture of genomic aberrations that can be used as fingerprints to clarify different tumor features and to distinguish between different tumor subtypes *[Wilkens L et al, 2000; Chen YJ et al, 2000]* . Several studies reported that human HCCs are frequently characterized by chromosomal amplifications at arms 1q, 6p, 8q and 17q *[Thorgeirsson SS and Grisham JW 2002; Homayounfar K et al, 2009]* that may result in activation of oncogenes, such as the activating mutations of the β-catenin

(CTNNB1) gene *[Wong CM et al, 2001; Miyoshi Y et al, 1998]* , whereas chromosomal deletions and loss of heterozygosity (LOH) were found respectively in the chromosomal arms 1p, 1q, 4q, 5q, 6q, 8p, 9p, 13q, 16p,16q and 17p and 1p, 4q, 6q, 8 p, 9p, 13q, 16p, 16q, 17p *[Thorgeirsson SS and Grisham JW 2002; Homayounfar K et al, 2009; Laurent-Puig P et al, 2001; Nagai H et al, 1997]* . The loss of these regions has been associated with loss of tumor suppressor genes such as TP53 (p53), retinoblastoma RB1 (pRb) *[Edamoto Y et al, 2003; Murakami Y et al, 1991]* , CDKN2A (p16INK4A) *[Laurent-Puig P et al, 2001; Liew CT et al, 1999]* and insulin-like growth factor-2 receptor (IGF-2R) *[De Souza AT et al,1995; Oka Y Et al, 2002]* .

Cellular signaling pathways involved in hepatocarcinogenesis

Wnt/β-catenin. The Wnt signaling pathway was originally identified in *Drosophila melanogaster* and is evolutionary conserved in nematodes, insects and vertebrates *[Cox RT & Peifer M, 1998]* . The most critical and studied Wnt pathway is the canonical Wnt pathway, which functions by regulating the amount of the transcriptional coactivator β-catenin that controls key developmental gene expression programs involved in the control of embryonic development and adult homeostasis. In the absence of Wnt signaling, cytoplasmic β-catenin complexes with the tumor suppressor adenomatosis polyposis coli (APC), with Axin1, and with glycogen synthase kinase-3β (GSK-3β). In this complex, GSK-3β phosphorilates β-catenin, targeting it for ubiquitination and subsequent degradation. When Wnt signaling receptors are engaged, conformational changes in the Axin complex cause the release of β-catenin, which then localizes to the nucleus and activates transcription of target genes such us Myc, Cyclin D1 and COX2 *[Chiba T et al, 2007; Clevers H 2006; Kikuchi A, 2000]* . In HCC, several transcriptomic and proteomic studies have indicated an increase in Wnt signaling, possibly as a result of an accumulation of Axin1 mutations at sites that bind β-catenin and/or CTNNB1 mutations along sites marked for phosphorylation by GSK-3β *[Yamashita T et al, 2009; Cavard C et al, 2008]*.

p53. TP53 gene encodes the p53 protein which plays a pivotal role in the DNA-damage response network, including cell cycle arrest, apoptosis, DNA repair and cellular senescence. Therefore, is not surprising that TP53 loss of function mutations or allelic deletions in chromosome 17p are commonly associated with human carcinogenesis *[Hussain SP and Harris CC, 2006]* . Aflatoxin B_1 is a mutagen for the TP53 gene, causing G:C to T:A transvertions at the third base pair in codon 249 (converting arginine to serin),

and the rate of TP53 R249S mutation may be accelerated in the presence of viral infection *[Aguilar F et al, 1993; Kirk GD et al, 2005]* . Furthermore, HBV encodes a viral protein, HBx, which can specifically bind to p53 and suppress p53-induced apoptosis *[Wang XW et al, 1994]*.

pRB. The tumor suppressor retinoblastoma (pRb1) is a nuclear protein encoded by the RB gene, located on chromosome 17 *[Dynlacht BD et al, 1994]* . pRb is a major cellular barrier to cancer development and it controls cell cycle progression at the Restriction Point G1/S via repression of the E2F transcription factor family of proteins. In human HCC, the pRB signaling pathway is altered in more than 80% of cases.

Myc. Myc is a potent oncogene, which appears to be constitutively up-regulated in many human cancers, representing a phenomenon of "oncogene addiction". Its increased expression in HCC is commonly caused by the activation of its locus through chromosome amplification *[Wilkens L et al, 2004]* . One possible mechanism by which Myc contributes to hepatocarcinogenesis is through the induction of telomerase, thereby bypassing cellular senescence *[Wu KJ et al, 1999]*.

PI3K/PTEN/Akt. Akt is involved in a number of biological processes, such as cell survival, cell growth, apoptosis and differentiation. The activation of the Akt pathway is mediated by either an activated tyrosine kinase receptor, or more rarely by the constitutive activation of PI3K or the loss of *phosphatase and tensin homolog* (PTEN), a negative regulator of Akt. The loss of PTEN expression via a loss of heterozigosity in chromosome 10q along with an activation of Akt has been reported in 40%-60% of HCC cases *[Hu TH et al, 2003; Blanco-Aparicio C et al, 2007]* .

TGF-β/ IGF-2. TGF- β is an inflammatory cytokine implicated in an array of functions such as cell growth, differentiation, migration, apoptosis, adhesion, survival and immunity *[Derynck R et al, 2001]* . IGF-2R, a tumor suppressor gene, promotes the degradation of IGF-2 and also the simultaneous activation of transforming growth factor- β (TGF- β) signaling, thereby halting cell proliferation and carcinogenesis *[Dennis PA and Rifkin DB, 1991]* . Inflammation and subsequent genomic mutations in IGF-2R result in IGF-2 over-expression and a reduction in the inhibitory effects of TGF-β signaling, a feature commonly observed in the development of HCC *[Breuhahn K et al, 2004; El-Serag HB and Rudolph KL, 2007]* .

The cancer stem cells (CSCs) model

This model, recently proposed, may provide a more personalized approach to address diagnostic and therapeutic strategies in the clinic. This model hypothesizes that HCC could be derived from progenitor cells or de-differentiated transformed cells, thereby explaining the heterogeneity in HCC morphology, clinical, behavior, and molecular profiling *[Yamashita T et al, 2009; Yang XR et al, 2010]* . Since the liver is an organ with regenerative capability, it has bi-potential progenitor cells that can give rise to hepatocytes or cholangiocytes, which could possibly develop into HCC or ICC (intrahepatic cholangiocellular carcinoma), respectively *[Haruna Y et al, 1996; Wu PC et al, 1999]* . The cancer stem cell model is not intended to be contradictory to the step-wise model, but merely complementary in explaining the origin of a more comprehensive group of HCC cases and the arising issues in diagnosing and treatment. For example, mature hepatocytes, cholangiocytes or bi-potential progenitor cells that acquire mutations through random genetic or epigenetic events can introduce a genetic imbalance in the primary tissue, resulting in the de-differentiation of mature cells and the loss of cell cycle control and/or the ability to continuously self-renew. Depending on the extent of genetic alterations, the tumor cells may remain benign or progress to malignancy. Therefore, events initiated by the multistep carcinogenesis model can also result in heterogeneous tumors with stem cell capability *[Kumar M et al, 2011; Reya T et al, 2001]* . "Liver-specific CSCs" have been isolated in HCC by several cell surface antigens including CD133, CD90, CD44, OV6, CD24, and the epithelial cell adhesion molecule (EpCAM) *[Lee TK et al, 2009]* . The capacity of CSCs for self-renewal and tumorigenesis is thought to involve various cancer-related signaling pathways that may serve as molecular targets for novel cancer treatment strategies. These pathways and potential targets include self-renewal (e.g. Wnt/β-catenin, Bmi-1, Notch and Sonic Hedgehog), cell growth (e.g. PTEN and IL-6), survival (e.g. ABC multidrug efflux transporters) and differentiation (e.g. hepatocyte nuclear factor–4α) *[Lee TK et al, 2009; Wang B and Jacob ST, 2011]* .

EPIGENETICS

The basis of epigenetics

The term "epigenetics" was originally coined by Conrad Waddington to describe heritable changes in a cellular phenotype that were indipendent of alterations in the DNA sequence. Despite decades of debate and research, a consensus definition of epigenetics remains both contentious and ambiguous *[Berger SL et al., 2009]* . Today, according to the most accepted definition, epigenetics is the study of alterations in gene expression without changes in DNA sequence, hence the name epi (Greek: επι- over, above, outer) –genetics *[Wu Ct, Morris JR, 2001]* . Epigenetic mechanisms are involved in regulating many cellular processes including embryonic development, chromatin structure, X-chromosome inactivation, genomic imprinting, and chromosome stability *[Kierszenbaum AL, 2002; Okamoto I et al, 2004 and Robertson KD, 2005]* . These phenomena are mediated by several epigenetic modifications, such as DNA methylation at cytosine residues in CpG dinucleotides, histone tail methylation, acetylation, phosphorilation, ubiquitynation, sumoylation that result in alteration of chromatin structure *[Pons D and Jukema JW, 2008]* . Generally, CpG methylation is associated with heterochromatic gene silencing and with repression of transcription in euchromatic regions when occurring at CpG islands of gene promoters, while histone modifications can result both in activation or repression of transcription based on the specific modification and on the target aminoacid residue in the histone tail *[Dillon N 2004; Grunstein M, 1997]* . DNA and histone modifications influence gene expression by making DNA inaccessible (e.g. by adding a methyl group to the DNA) or accessible (by removing it) for transcription factors and other proteins involved in gene transcription regulation.

DNA methylation

DNA methylation is one of the most common epigenetic mechanisms of gene silencing. The mechanism refers to the binding of a methyl group to the 5th carbon atom of the cytosine ring to form a new base, 5-methyl-cytosine (5mC) *[Reik W et al., 2001; Goll MC et al., 2004]* . Patterns of DNA methylation are conserved through DNA replication and are inherited by daughter cells; addition of methyl groups takes place immediately after DNA replication, and is completed within 1 minute *[Reik W et al, 2003; Okuwaki M et al, 2004]*.

The result of such modification prevents the formation of the transcriptional initiation complex, or the elongation of those already initiated *[Singal R and Ginder DG, 1999]* . The CpG dinucleotide is under-represented throughout the genome, but short regions of 0.5-4 kb in length, known as CpG islands, are enriched in CpG content *[Bird A, 2002; Takai D & Jones PA, 2002]* . Most CpG islands are found at the promoters of 50-60% of all human genes and are, generally, unmethylated in normal cells *[Jones PA and Baylin SB, 2007]* . In mammals the methylation mechanism is catalyzed by DNA methyltransferase enzymes (DNMTs) that transfer a methyl group to DNA by using S-adenosylmethionine (SAM) as the methyl donor. DNA methylation is performed by "de novo" methyltransferases that methylate previously unmethylated cytosines, while maintenance of methylation is performed by "maintenance methyltransferases", that maintain the methylation pattern once it is established *[Giannino D et al, 2003; Okano M et al, 1999]* . Four isoforms of DNMT are known to be involved in DNA methylation: DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L *[Roberts RJ et al, 2003; Kuo HC et al, 2005]* . DNMT1 is a "maintenance methyltransferase" that is responsible for copying of the already established methylation pattern by recognizing the hemimethylated sites in DNA helix, and it is present always in replication forks of the cells undergoing division *[Easwaran HP et al, 2004; Pradan S and Esteve PO, 2003]* . Inactivation of this enzyme in mice brings about the global loss of methylation and abnormal biallelic expression of imprinted genes *[Beard LE and Jaenisch R, 1993; Howell CY et al, 2001]* . In contrast to DNMT1, the biological activity of DNMT2 does not demonstrate affinity towards CpG sequences, and knock-out mice for this gene do not show recognizable abnormalities *[Dong A et al., 2001]* . It is assumed that this enzyme plays some role in DNA methylation, but it appears not to have any DNA methyltransferase activity *[Okano M et al, 1998]* . Members of DNMT3 subfamily (e.g. DNMT3a and DNMT3b) are highly expressed in the developing mouse embryo and participate in the processes of de novo methylation that set up DNA methylation patterns early in development. Dnmt3L, a protein that by itself has no DNMT activity, colocalizes with DNMT3a and DNMT3b and is essential for establishing methylation imprints in the female germ line. Genetic analysis of the various DNMTs has established that DNA methylation is essential for vertebrate development. Loss of methylation causes apoptosis in embryos and fibroblasts, but not in embryonic stem cells or human cancer cells, and leads to both widespread de-repression of ectopic gene expression and transcriptional activation of transposable elements *[Jaenisch R and Bird A, 2003]* . DNA methylation can

influence gene expression in two ways: (1) Directly - the methylation of CpG sequences changes the recognition site for a particular transcription factor and (2) indirectly – by binding of specific proteins which have affinity for methylated CpG sequences *[Meehan RR, 2003]* . These are proteins which bind to methylated CpG groups (methyl-CpG binding proteins, MeCPs) via a domain responsible for binding to CpG sequences (methyl-CpG binding domain, MBD), and are consequently called MBD1; MBD2, MBD3, MeCP1 and MeCP2 *[Bird A 2002; Ballestar E 2001]* . An altered pattern of DNA methylation can be associated to several diseases, including cancer. Indeed, it has been shown that epigenetic silencing as the consequence of aberrant methylation of CpG islands promoter regions, results in the loss of function of tumor suppressor genes *[Feltus FA et al, 2003; Miyoshi HH et al, 2004]* . Silencing of the THRβ gene by promoter hypermethylation has been found in human cancer including breast, lung, and thyroid carcinoma *[Li Z et al, 2002; Iwasaki Y et al, 2010; Ling Y et al, 2010; Joseph B et al, 2007; Dunwell TL et al, 2009]*.

microRNAs

miRNAs biogenesis and functions

microRNAs (miRNAs or miRs) are non-coding, single-stranded RNAs of ~22 nucleotides (nt) and constitute a novel class of gene expression regulators that are found in both plant and animals *[Bartel DP, 2004]* . Their discovery dates from 1993 and since then thousands of miRNAs have been identified *[Lee RC, Feinbaum RL and Ambros V 1993]* . Many miRNAs are evolutionarily conserved from worms to humans, which implies that these miRNAs direct essential process both during development and in the adult organism, controlling a wide range of biological functions such as cellular proliferation, differentiation, apoptosis and stress response. miRNAs negatively regulate the expression of protein-coding genes at the post-transcriptional level, by binding to specific, complementary sequences located in the 3'-untranslated (3'-UTR) region of target mRNAs in one or two ways depending on the degree of complementarity between themselves and the target mRNAs. miRNAs that bind with perfect complementarity to mRNA sequences induce the RNA-mediated interference (RNAi) pathway, in which mRNA transcripts are cleaved by the miRISC complex. This mechanism is mainly observed in plants but miRNA-directed mRNA cleavage has also been shown to occur in mammals *[Llave C et al, 2002; Palatnik J et al, 2003; Tang G et al, 2003; Yekta S et al, 2004]*. However, most animal miRNAs are thought to bind with imperfect complementary sites within the 3'-UTRs of their mRNA targets and to inhibit the initiation of translation via the miRISC complex , without affecting mRNA levels. Recent findings indicate that miRNAs that share only partial complementarity with their targets can also induce mRNA degradation, but it is unclear if translational inhibition precedes destabilization of target genes [*Esquela-Kerscher A and Slack FJ, 2006]* . Almost 50% of miRNA genes are located in the introns of protein-coding genes or long non-coding RNA transcripts, whereas the remainder are independent transcription units. Biogenesis of a miRNA begins with the synthesis by Pol II of a long hairpin transcript known as pri-miRNA (primary miRNA) which are processed within the nucleus by the RNase III Drosha and its cofactor Pasha, in a pre-miRNA (miRNA precursor) of ~ 70 nt *[Lee Y et al, 2002; Zeng Y, Yi R and Cullen BR, 2003; Lee Y et al, 2003]* . The nuclear export protein Exportin 5 carries the pre-miRNA from the nucleus to the cytoplasm where it is subsequently converted to a mature duplex miRNA by another RNAse III enzyme,

DICER1 to generates a duplex (miRNA:miRNA* duplex) of about 22 nt containing two strands, termed miRNA and miRNA*, corresponding to the two sides of the base of the stem*[Yi R et al., 2003; Lund E et al., 2004]*. After binding with the Argonaute protein (Ago), the miRNA:miRNA* duplex is incorporated into the miRISC complex (RNA-induced silencing complex). One strand of the duplex remains in Ago as mature miRNA (the guide strand or miRNA), whereas the other strand (the passenger strand or miRNA*) is degraded *[Bartel DP, 2004]* . Nucleotides 2-7 of the mature miRNA sequence create the "seed region" that primarily specifies the target mRNA that the miRNA will bind to *[Lewis BP et al, 2003; Grimson A, 2007]* .

Computational analysis has shown that a single miRNA can control the expression of about one-third of all human mRNAs. Target mRNAs can be diverse in their functions; they include transcription factors, secreted factors, receptors and transporters. On the other hand, each mRNA can be regulated by different miRNAs *[Lim LP et al., 2005; Lewis BP et al, 2003]* . Several studies have provided evidence that dysregulated miRNA expression contributes to initiation and progression of human cancers *[Esquela-Kerscher and Slack, 2006; Melo SA, 2011]* . miRNAs regulate molecular pathways in cancer by targeting various oncogenes and tumour suppressors*[Zhang B et al, 2007]* , and have a role in cancer-stem-cell biology, angiogenesis, epithelial-mesenchymal transition, metastasis, and drug resistance. Loss of miRNA expression can have substantial effects on multiple cellular functions and their dysregulation can cause tumorigenesis *[Kong Yi W, 2012]* . miRNAs can be regulated at various levels, including stability, processing, sequence identity and binding to target mRNAs. Therefore, these regulatory pathways are susceptible of being altered in cancer cells *[Kong Yi W, 2012]* . Recent studies have reported the involvement of both genetic and epigenetic mechanisms in miRNA deregulation that can potentially lead to cancer development *[Ventura A and Jack T, 2009]* . In addition, approximately 50% of all annotated human miRNA genes are located at fragile sites or areas of the genome that are associated with cancer which are prone to breakage and rearrangement in cancer cells [*Calin GA et al., 2004]* . Since the control of gene expression by miRNAs is strongly dependent on their mRNA targets, they can behave as oncogenes and/or tumor suppressor genes, depending on the function performed by their targets *[Ventura A and Jack T, 2009; Plasterk RH, 2006]* . Those microRNAs whose expression is increased in tumors may be considered as oncogenes. These miRs, called "oncomirs", usually promote tumor development by negatively inhibiting tumor suppressor genes and/or genes that control cell differentiation

or apoptosis *[Zhang B et al, 2007]* . An excellent example of "oncomir" is provided by miR-21, one of the most frequently up-regulated miRNAs in cancer *[Chan JA et al 2005; Iorio MV et al, 2005]* . One possible mechanism by which miR-21 acts as an oncogene is suggested by the evidence demonstrating that it targets several tumor suppressor genes, including PTEN, thus increasing cell proliferation and inhibiting apoptosis *[Gabriely G et al, 2008; Darido C et al, 2011]* . A similar behavior is described for miRNAs 15a e 16-1 that negatively regulate expression of BCL2, an anti-apoptotic gene frequently over-expressed in several human cancers, including leukemias and lymphomas *[Cimmino A et al., 2005]* . Thus, the deletion or the down-regulation of these miRNAs would be responsible for the increased expression of BCL2, potentially leading to evasion of apoptosis. Instead, miRNAs whose expression is decreased in tumor cells are considered tumor suppressors. Tumor suppressor miRNAs usually prevent tumor development by negatively regulating and inhibiting oncogenes and/or genes that control cell differentiation or apoptosis *[Zhang B et al, 2007]* . Currently, several miRNAs are considered to be tumor suppressor genes: miRNA let-7a is one of the founding member of this family. The human let-7 family comprises 12 closely related members of miRNAs encoded by a chromosome region that is usually deleted in human cancer, especially lung cancers, where a strong correlation between low levels of the miRNA and poor prognosis has been demonstrated *[Calin GA et al, 2004; Takamizawa J et al, 2004]*. Johnson et al. reported that let-7 is down-regulated in lung cancer and is associated with elevates RAS expression.*[Johnson SM et al, 2005]* .

The role of miRNAs in HCC

During the past decade, it has been well established that specific miRNAs modulate various cellular processes in the liver and several studies revealed that the expression of miRNAs is deregulated in human HCC in comparison with matched non-neoplastic tissue and that their aberrant expression correlates with severity and poor prognosis of HCC *[Murakami Y et al, 2006; Gramantieri L et al, 2008; Ura S et al, 2009; Calin GA et al, 2004; Huang XH et al, 2009]* . For example, in one study, the expression of miR-199a, miR-92, miR-106a, miR-222, miR-17-5p, miR-18 and miR-20 correlated with the degree of tumor differentiation *[Murakami Y et al, 2006]* . In addition, it was also found that the dysregulation of miRNAs not only is involved in tumor progression, but it is also associated with the role of risk factors directly involved in tumor development, as demonstrated by the discovery that HBV and HCV induce different sets of miRNAs during infection *[Nordenstedt H et al, 2010;*

Ura S et al, 2009] . Although changes in the expression of microRNAs between tumor specimens and the normal corresponding tissue have been investigated in HCC as well, the obtained results are often discordant and do not allow the identification of the miRNAs critical for development and progression of HCC. This can be due to discordance between the different technical platforms employed and to the intrinsic heterogeneity of the different tumor populations analyzed. Furthermore, among the microRNAs whose expression has changed, several are probably altered not as cause but as consequence of the tumorigenic status. Nevertheless, several microRNAs were identified as aberrantly expressed by more than one study, thus indicating that irrespective of the technical platform or set of samples used, these microRNAs were most likely involved in liver tumorigenesis. Consistent deregulation of miR-122, miR-199, miR-221, and miR-21 appears to be particularly important in HCC; among these miRNAs both miR-122 and miR-199a are among the miRs most highly expressed in normal liver *[Hou J et al, 2011]* . miR-122 is a hepato-specific miRNA, accounting for more than 70% of the total liver miRNA population and it acts as a key regulator of fatty acid and cholesterol metabolism *[Jopling C, 2012]* and as a regulator of the differentiation of adult hepatocytes via repression of genes not specific to the liver *[Xu H et al, 2010; Esau C et al, 2006; Krutzfeldt J et al, 2006]*. The loss of its expression was observed in more than 70% of HCC and one of the mechanisms through which it plays an active role in tumorigenesis appears to be the activation of cyclin G1 *[Gramantieri L et al, 2007]* . Other studies, showed that miRNAs associated with cell cycle inhibition (miR-34a, miR-101, miR-199-a-5p and miR-223) are down-regulated in HCC *[Aravalli RN, 2013; Li N et al, 2009; Wong QW et al, 2008]* , and miRs involved in cell proliferation and inhibition of apoptosis (miR-17-92 polycistron, miR-21, miR-96, miR-221 and miR-224) are up-regulated *[Connolly E et al, 2008; Fornari F et al, 2008; Meng F et al, 2007; Wang Y et al, 2008]* . miR-221 has received much attention for its suggested tumor-promoting activity. It is up-regulated in 70%-80% of HCC samples and HCC cells overexpressing miR-221 show increased growth, proliferation, migration, and invasion capability *[Garofalo M et al, 2009; Pineau P et al, 2010]* . miR-21 has been shown to be overexpressed in HCC as well as in other several human malignancies, including breast, colon, lung, pancreas, prostate, and stomach cancer *[Volinia S et al, 2006*] . Overexpression of miR-21 in cultured human cells can determine evasion from apoptosis and increase tumor cell proliferation and migration *[Chan GA et al, 2005; Si ML et al, 2007]* . In vivo miR-21 inhibition suppressed cell proliferation and increased apoptosis in a cancer

xenograft model *[Si ML et al, 2007]*. Discovery of aberrantly expressed miRNAs in HCC has helped to reveal novel mechanisms of liver tumorigenesis . Furthermore, since the profiling of miRNA expression levels in HCC could be associated with bio-pathological and clinical features, miRNA expression can be a potential useful tool for HCC classification and for improving prognostic stratification, in particular in early HCC, where the availability of potentially curative treatments requires a more sophisticated diagnostic approach. An emerging area of investigation is the potential use of miRNAs as circulating biomarkers. Indeed, they are present at different levels in the serum or plasma of patients affected by a range of disease in comparison with healthy subjects. Their stability in formalin-fixed and paraffin-embedded samples as well as in body fluids is an important property that enables their detection and quantification in biological samples. Finally, inhibition of oncomiRs or restoration of oncosuppressive microRNAs represent a new potential therapeutic approach for HCC treatment.

ANIMAL MODELS OF HEPATOCARCINOGENESIS

The Resistant Hepatocyte model (RH)

Unfortunately, knowledge about molecular events in early stage HCC development is limited because of clinical difficulties in the histopathologic distinction between non malignant nodular lesions (low grade and high grade dysplastic nodules) from early HCC. Animal models facilitate the study of different stages of hepatocarcinogenesis in that discrete lesions at different stage of progression can be identified and analyzed, thus helping to detect molecular alterations already present in early preneoplastic stages. Among the most widely used experimental models for the characterization of the process of liver carcinogenesis, in our studies we applied the resistant hepatocyte model, also known as Solt & Farber model, which allowed us to carry out the analysis of the different sequential steps leading to HCC development using the rat as animal model *[Solt DB et al, 1977]* . The system consists of three components: an initiator , diethylnitrosamine (DENA); 2-acetylaminofluorene (2-AAF), a selective inhibitor of normal hepatocyte proliferation that it is ineffective against hepatocyte previously initiated by DENA, and a generalized potent growth stimulus represented by 70% partial hepatectomy (PH). Initiated cells undergo to clonal expansion to give rise to early preneoplastic lesions, identified by their immunohistochemistry positivity for the placental form of the enzyme glutathione-S-transferase (GST-P). In the following weeks, nodules become macroscopically visible and they occupy most part of liver. During the carcinogenic process, a slow but continuous regression of the vast majority of the preneoplastic lesions occurs through a remodeling process in which hepatocytes gradually lose the staining for GST-P, and reacquire a differentiated phenotype *[Enomoto K et al, 1982]* . Conversely, only a smaller fraction of preneoplastic nodules progresses to develop adenomas, and after 10-14 months after DENA, to HCCs. GST-P+ preneoplastic nodules can be further divided into Cytokeratin-19 (KRT-19) positive or negative lesions, depending on the expression of this protein. KRT-19 is a protein of the cytoskeleton intermediate filaments, normally expressed in adult liver by the bile duct epithelial cells, but not by normal hepatocytes *[Enomoto K et al, 1982]*. Our previous studies showed that all the HCCs arising in this model are KRT-19+, although only a minority of preneoplastic lesions developed at early stages of the process are positive for this marker. Furthermore, HCCs and KRT-19+ nodules share a common

gene expression profile, suggesting that rat HCC derived from this sub-population of KRT-19+ preneoplastic lesions *[Andersen JB, Loi R et al 2010]* .

The translational value of the Resistant Hepatocyte model has already been demonstrated; indeed comparative genomic studies on laser capture-microdissected early lesions showed that the HCCs generated with the RH model have a gene expression signature very similar to a specific human HCC subtype that expresses KRT-19 and which is characterized by the worst prognosis *[Andersen JB, Loi R et al, 2010]* . Moreover, the finding that 78% of genes and 57% of miRNAs deregulated in rat HCC are similarly altered in human HCC and that 76% of these genes were already deregulated at very early stage of the process, further supports the translational value of this model in predicting not only the molecular changes relevant to human HCC, but also the stage at which this changes occur *[Petrelli A et al, 2014]* .

THYROID HORMONE RECEPTORS (THRs)

Thyroid hormones actions

Thyroid hormones tyroxine (T_4) and triiodothyronine (T_3) synthesis and secretion is regulated by a negative-feedback system that involves the hypothalamus, pituitary, and thyroid gland [hypothalamic/pituitary/thyroid (HPT) axis] *[Shupnik MA et al, 1989]* . Thyroid hormones are produced by the follicular cells of the thyroid gland, through iodination of the tyrosine residues *[Zimmermann MB, 2009; Rubio IG and Medeiros-Neto G, 2009]* . Under physiological conditions, T_4 is the main hormone secreted into the bloodstream by the thyroid gland but the major active form is represented by T_3. Metabolism of thyroid hormones (THs) and conversion of T_4 to the more active form T_3, is regulated in extra-thyroidal tissues through deiodination reactions catalyzed by the deiodinases enzyme (DIO1, DIO2, DIO3). Type I deioidinase is responsible for the conversion of the majority of T_4 to T_3 in liver and kidney through 5'-deiodination of the outer ring of T_4. Type II deiodinase is found in brain, pituitary, and brown adipose tissue and primarily converts T_4 to T_3 for intracellular use. 5-deiodination by type I and type III deioidinase, which is found primarily in placenta, brain, and skin, leads to the generation of rT_3, the key step for thyroid hormones inactivation. rT_3 and T_3 can be further deiodinated in the liver and sulfo- and glucuronide-conjugated before excretion in the bile *[Engler D and Burger AG, 1984; Dayan CM and Panicker V, 2009]* . There is also an enterohepatic circulation of TH mediated by intestinal flora that deconjugates some of these compounds and promotes the reuptake of THs *[Yen PM, 2001]* . Both T_3 and T_4 act via THRs. However, the THR binding affinity of T_4 is considerably lower than that for T_3. The physiological actions of THs affect almost every organ and system. Thyroid hormones, particularly T_3, are potent regulators of multiple physiological activities, including cellular metabolic rate, heart and digestive functions, muscle function, brain development, and bone maintenance *[YH Huang et al, 2008; Pilo A et al, 1990]* . The liver has been shown to be a major target of thyroid hormone action *[Feng X et al, 2000]* . We, and other authors, demonstrated that T_3 stimulate hepatocyte proliferation and DNA synthesis in animal models of liver regeneration and hyperplasia by induction of Cyclin D1 expression *[Francavilla A et al, 1994; Bockhorn M, et al 2007; Columbano A et al, 2008; Pibiri M et al, 2001]*. Although THs may exert their effects on a number of intracellular functions, their primary

effect is exerted on the transcriptional regulation of target genes via interaction with the thyroid hormone nuclear receptors (THRs) *[Yen PM, 2001]* . In addition, non-genomic actions of thyroid hormones, not involving direct regulation of transcription by THRs, have been recently described *[Davis PJ et al, 2010]* .

Thyroid hormone receptors structure and isoforms

THRs belong to the nuclear hormone receptor superfamily (NHR-superfamily) and act as T_3-inducible transcription factors. The NHR-superfamily includes the steroid, vitamin D and retinoic acid receptors as well as "orphan" receptors for which there are no known ligands or function *[Beato M et al, 1995; Lazar MA, 1999]* . Mutational analyses of THRs and comparison with other members of the NHR-superfamily have yielded much information on the structural organization of THRs *[Lazar MA, 1993; Yen PM and Chin WW, 1994]* . All THRs exhibit a modular structure organized in distinct protein domains: an amino-terminal domain, a central DNA-binding domain (DBD), a hinge region and a carboxy-terminal ligand-binding domain (LBD) *[Yen PM, 2001]* . **[Fig.1]**

The amino-terminal Domain. The amino-terminal region has variable lengths and divergent sequences among the THR isoforms and among different species *[Koenig RJ et al., 1988]* . This domain is involved in modulation of cell-specific and promoter-specific transcription *[Tora L et al, 1988; Tora L et al,1989]* . Moreover, the "A/B" domain of THRs recruits an assortment of co-regulatory proteins that can participate in ligand-independent transcriptional regulation *[Meghan D Rosen and Martin L Privalsky, 2011]* .

The DNA binding domain (DBD). The DNA binding domain (DBD) is located in the central portion of THR and contains two "zinc-fingers". This critical region has been shown to be important in sequence-specific recognition of hormone response elements of DNA and it is also responsible for dimerization of THRs *[Rastinejad F et al, 1995]* .

The hinge region. The "hinge region" bridges the DNA-binding domain and the hormone binding domain and contains an amino acid sequence that is associated with the nuclear localization of THRs *[Evans RM, 1988]* .

The ligand-binding domain (LBD). The LBD is necessary for TH binding and also plays critical roles for dimerization, transactivation, and basal repression by unliganded THRs *[Yen PM, 2001]* . This domain consists of 12-helices which are arranged to create a pocket for ligand binding and plays a crucial role because it is essential for the switch of the receptor from a silencer to an activator. In the unliganded conformation, helix 12 protrudes away from the

core structure of the LBD and allows co-repressor binding. Hormone binding induces helix 12 to contact to the LBD core, resulting in co-repressor release and co-activator association *[Apriletti et al, 1998]* .

THRs are encoded by two genes, THRα (NR1A1) and THRβ (NR1A2) located at separate loci, on human chromosomal regions 17q11.2 and 3p24.3 , and in 10q31 and 15p16 rat chromosomal regions.

Rat THRα1, THRβ1, THRβ2, and THRβ3 are the major functional receptors, while several isoforms, THRα2, THRα3, THRΔα1, THRΔα2, and THRΔβ3 are dominant-negative antagonists *[Cheng SY et al, 2010; Williams GR 2000]* **[Fig.1A]** . THRα2 and THRα3 result from alternative splicing of THRα1 mRNA, whereas the truncated variants, THRΔα1 and THRΔα2, are transcribed from an internal promoter located within intron 7. The THRβ2 isoform is generated by alternative promoter usage and mRNA splicing. While the expression of THRα is ubiquitous and variable, THRβ is developmentally regulated *[Yen PM, 2001]* . Both THRα1 and THRβ1 are expressed in almost all rat tissues. THRβ1 mRNA is highly expressed in brain, liver and kidney, whereas THRβ2 is transcribed in a tissue-specific manner in the anterior pituitary, hypothalamus, developing brain, and inner ear. Rat THRβ3 and THRΔβ3, generated by alternative splicing, are widely expressed *[Williams GR, 2000]* . The truncated variant, THRΔβ3, lacks the DNA-binding domain that inhibits transcriptional activation mediated by T_3-liganded THRs.

The human receptors identified to date include THRα1 and THRα2, generated from the THRα gene via alternative splicing, as well as THRβ1 and THRβ2 from the THRβ gene *[Shahrara S et al., 1999; Sakurai A et al., 1989; Nakai A et al., 1988; Yen PM et al, 1992]* **[Fig.2B]** . Human THRα1, THRβ1, and THRβ2 are the main ligand-binding receptors. Both human THRα1 and THRα2 mRNA are highly expressed in brain, prostate, and thyroid, and to a significantly lower extent in other tissues. The relative amounts of the three human THR mRNAs (THRα1, THRα2, and THRβ) are roughly comparable in each tissue type *[Wu SM et al, 2012]* . A novel human carboxy-terminal spliced variant of THRβ1 that lacks T_3-binding ability and acts as a dominant negative isoform, designated THRβ4, has recently been cloned. THRβ4 is widely expressed in all human tissues, but it is particularly abundant in testis and skeletal muscle *[Tagami T et al, 2010]* . Interestingly, whereas human THRα1, THRα2, and THRβ1 are abundant in different tissues, none of them is highly expressed in the liver, the major TH target organ *[Mitsuhashi T et al,1988]* .

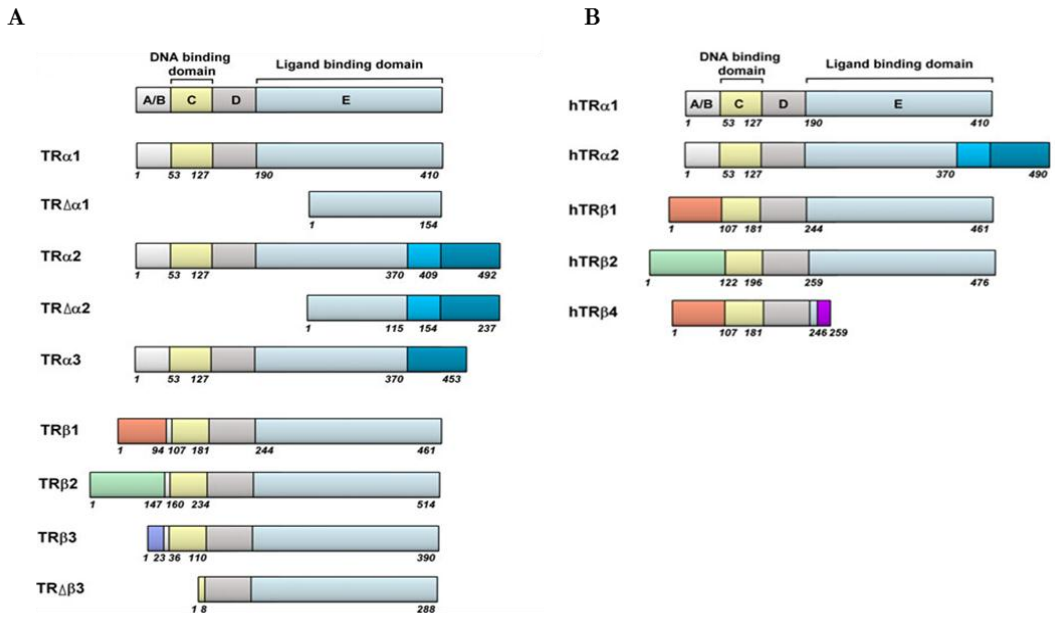

Fig.1: Schematic representation of rat **(A)** and human **(B)** THRα and THRβ isoforms. *Wu S. et al. Cell. Mol. Life Sci., 2012.*

Transcriptional activities of THRs

THRs exert their transcriptional activities as a homodimers or as a heterodimers with other nuclear receptors, including retinoid X receptor (RXR) and other retinoic acid receptor subtypes, and vitamin D receptors (VDR). TRs generally heterodimerize with RXR; this heterodimer exhibits the highest T_3 binding affinity and remains stable during ligand binding *[Zhang XK and Pfahl M, 1993; Kliewer SA et al, 1992]* . THRs mediate their genomic effects on eukaryotic transcription by binding to specific DNA elements in the promoter, enhancer and silencer regions of target genes. These DNA elements are known as Thyroid Hormone Response Elements (TREs). TREs within promoter regions of T_3 target genes generally contain a core consensus "half-site" with the sequence (A/G)GGT(C/A/G)A.

A typical TRE contains two half-site sequences in palindromic, direct-repeat, or inverted-repeat arrangements that are recognized by THRs *[Yen PM, 2001]* **[Fig.2]**.

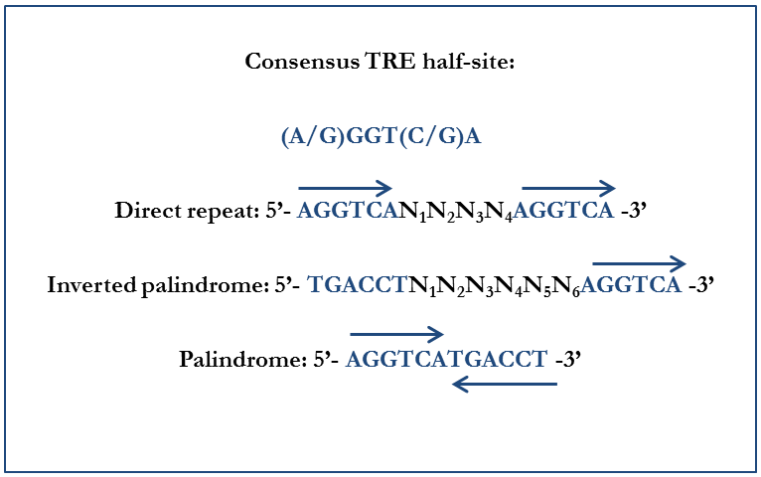

Fig.2: Thyroid hormone response element (TRE).
Half-site orientation and optimal nucleotide spacing between half-sites. N refers to nucleotides, and arrows show direction of half-sites on the sense strand.

In contrast to steroid hormone receptors that are transcriptionally inactive in the absence of ligand, unliganded THRs bind to TREs and may modulate transcription of target genes. Based on the effect of the availability of the ligand on the transcriptional activity of THRs, these TREs can be classified as positive (pTRE) or negative (nTRE) **[Fig.3]**. Liganded receptor leads to repression of target gene transcription in the case of nTREs and to activation of transcription for pTREs. In contrast, the absence of hormone, hence the binding of unliganded receptor leads to transcriptional activation in the case of nTREs, and to transcriptional repression in the case of pTREs *[Eckey M et al, 2003]* .

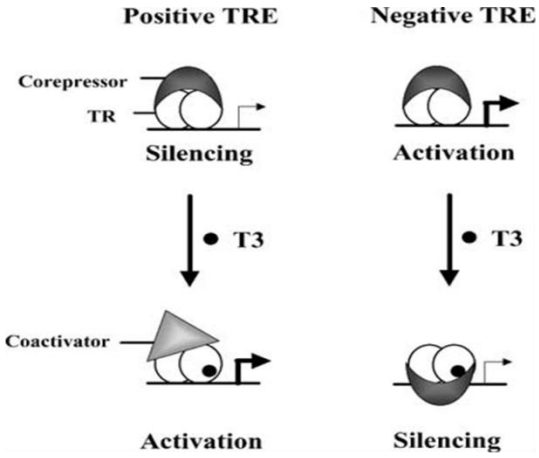

Fig.3: Comparison between positive and negative thyroid hormone response elements (TREs). *Eckey M. et al. Molecular and Cellular Endocrinology, 2003.*

The mechanism of transcriptional regulation by nTREs is poorly known, while pTREs have been thoroughly studied. The process of transcriptional regulation for pTREs can be described as follow **[Fig.4]** : in the absence of T_3, THRs usually act as transcriptional repressors by their association with co-repressor molecules, such as NCoR (nuclear receptor co-repressor) or SMRT (silencing mediator of retinoic and thyroid receptor) *[Cheng SY et al, 2010]* . NCoR and SMRT proteins serve as platform for the repressor complexes that contain histone deacetylases and trigger tight nucleosome remodeling reactions *[Yoon HG, 2003]* . Binding of T_3 induces conformational changes in THRs that facilitate the recruitment of multiple co-activator complexes to the promoters of target genes, leading to chromatin loosening and transcriptional activation. Liganded THR interacts with the co-activator complex including the steroid hormone receptor CoA (SRC) and members of the p160 family *[McKenna NJ, O'Malley BW, 2002]* . SRC-1 functions as a histone acetyltransferase to modify chromatin structures for promoting nuclear receptor transactivation. The transcriptional activities of THRs are regulated by SRC/p300 and other large complexes, namely, THR-associated proteins (TRAPs) *[Fondell JD, 1996]* . These sequential or combinatorial complexes associated with ligand-dependent THR may contribute to differential responses for the appropriate expression of target genes *[Glass CK, Rosenfeld MG , 2000]* . Moreover, THRs and their co-regulators are targets of various post-translational modifications in response to exterior stimuli that manipulate their activity and alter THR-mediated gene expression *[Yen PM, 2001; Lazar MA, 1993]* .

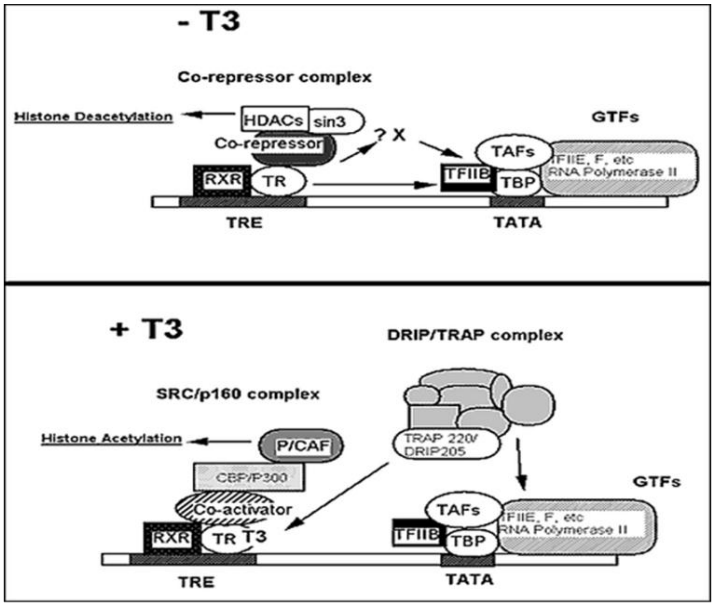

Fig.4: Molecular model of THRs transcriptional regulation.*Yen, Paul M. Physiological Reviews, 2001.*

Non-genomic effects of thyroid hormones

A few years ago, it became evident that THs could also act through a different mechanism. Actions of thyroid hormones that are not triggered by liganding to intranuclear THRs are termed "non-genomic". These processes may be initiated in the cytoplasm or in the plasma membrane by binding of T_4 and T_3 to a plasma membrane protein, the integrin αvβ3 *[Cheng SY 2010; Bergh et al, 2005]* . αvβ3 contains two binding sites, denoted as S1 and S2, which translate the TH signal differently *[Davis et al, 2011; Freindorf et al, 2012]* . S1 exclusively binds T_3 at physiological concentrations and leads to PI3K activation. S2 binds T_4, and to a lesser extent T_3, and ultimately activates the ERK1/2 pathway *[Lin HY et al, 2009]* . T_4 stimulates activation of extracellular signal-regulated kinase 1/2 (ERK1/2) through phospholipase C (PLC) and protein kinase Cα (PKCα). T_4-activated ERK1/2 also modulates intracellular protein trafficking of estrogen receptor α (ERα) and THRβ1 from the cytoplasm to the nucleus and acts locally at the plasma membrane to activate the sodium proton exchanger (NHE). Complex cellular events induced from the cell surface receptor include angiogenesis and tumor cell proliferation *[Lin HY et al 2009; Wu S et al, 2012]* . T3-induced PI3K activity may be initiated by binding to membrane-bound integrin αvβ3 or via a mechanism that is cytoplasmic in origin *[Bhargava M et al, 2009]* . Binding of T_3 to αvβ3 activates the PI3K signal pathway via Src kinase activation leading to translocation of THRα1 from the cytoplasm to the nucleus and increased target gene expression. In the cytoplasm, T_3 rapidly activates the PI3K pathway and initiates the downstream transcription of target genes. The T_3-liganded THRβ1 in the cytoplasm interacts with the PI3K regulatory subunit, p85a, and induces phosphorylation of Akt (also known as protein kinase B, PKB) . Activated Akt translocates to the nucleus and subsequently phosphorylates nuclear mammalian target of rapamycin (mTOR). Consequently, activation of mTOR triggers the expression of ZAKI-4a (also termed DSCR1L, Down syndrome critical region gene 1-like) and HIF-1α. Ultimately, the pathway may lead to alterations in numbers of pumps inserted in the membrane and increased activity of the sodium pump (Na,K-ATPase) in the plasma membrane. In addition, THRα1 interacts with the p85a subunit of PI3K in a T_3-dependent manner, resulting in the activation of Akt and endothelial nitric oxide synthase (eNOS). Moreover, binding of T_3/T_4 to the recognition site of integrin αvβ3 can activate PI3K, stimulating shuttling of THRβ1 from the cytoplasm to the nucleus and increasing expression of target genes such as hypoxia inducible factor-1α (HIF-1α) *[Lin HY et al, 2009]* .

Many studies have indicated that THRs alterations could be involved in development and progression of several types of human diseases, including metabolic [*Rosen MD and Privalsky ML, 2009]* , autoimmune *[Tassi V et al, 1995]* , neurological *[Kripke DF et al, 2009]* , cardiovascular diseases *[Goumidi L et al, 2011b]* and cancers .

Roles of THRs in tumorigenesis

Several studies support the involvement of THRs in tumorigenesis. The first evidence came from the discovery that THRα1 is the cellular counterpart of the retroviral v-erbA that is involved in the neoplastic transformation leading to acute erytroleukemia and sarcomas *[Sap et al, 1986; Thormeyer and Baniahmad, 1999]* . v-erbA is an aberrant chicken THRα1 that does not bind T_3 and has lost the ability to activate gene transcription. V-erbA competes with THR for binding to TREs and interferes with the normal transcription activity of liganded-THR on several promoters *[Yen PM et al, 1994; Chen HW and Privalsky ML, 1993]* . Thus the v-erbA oncoprotein is thought to repress constitutively, through its dominant negative activity, a certain set of genes that prevent cellular transformation. Indeed, male transgenic mice overexpressing v-erbA develop hepatocellular carcinomas, thereby providing evidence that v-erbA oncoprotein can promote neoplasia in mammals through its dominant negative activity *[Barlow C et al, 1994]* . Evidence of aberrant expression or mutations of THRs in various human cancers further supports the involvement of thyroid hormone receptors in oncogenesis. Loss of expression of the THRβ gene associated to truncation/deletion of chromosome 3p was reported in several malignancies including lung, melanoma, breast, head and neck, renal cell, uterine cervical, ovarian, and testicular tumors *[Leduc F et al, 1989; Sisley K et al, 1993; Chen LC et al, 1994; JM Gonzales-Sancho et al, 2003; Huber-Gieseke T et al, 1997; IU Ali et al, 1989]* . Decreased expression due to silencing of the THRβ gene by promoter hypermethylation has been described in several human cancers including breast, lung, and thyroid carcinoma, supporting the possible tumor suppressive role of this receptor. [*Li Z et al, 2002; Iwasaki Y et al, 2010; Ling Y et al, 2010; Joseph B et al, 2007; Dunwell TL et al, 2009]* . Recent studies provide evidence that THRβ expression could also be repressed via regulatory mechanism mediated by microRNAs *[Jazdzewski K et al, 2011]* . The THRα locus undergoes frequent loss of heterozigosity (LOH) in sporadic breast cancer, and rearrangement of the THRα gene has also been reported in leukemia, breast, and stomach cancer *[Futreal PA et al,1992; Yokota J et al,1988; Dayton AI et al,1984]* . These findings collectively suggest that THRs can

act as tumor suppressors in human cancer. This hypothesis is also supported by experiments showing that transgenic mice ($THR\beta^{pv/pv}$) harboring a PV mutation (the PV mutation is a C-insertion at codon 448 of the THRB gene and leads to a frame-shift of the carboxyl-terminal 14 amino acids of THRβ1, resulting in total loss of T_3 binding and transcriptional activation) originally identified in a patient with thyroid hormone resistence (RTH), spontaneously developed thyroid cancer *[Suzuki H et al, 2002]* . The THRβpv mutation seems also to promote breast cancer development *[Guigon C et al, 2011]* . Moreover, Zhu et al. recently showed that mice devoid of functional THRs ($THR\alpha1^{-/-}$ $THR\beta^{-/-}$) spontaneously develop follicular thyroid cancer and lung metastasis *[Zhu XG et al, 2010]* . Conversely, other studies suggest that THRs behave as oncogenes stimulating proliferation in several cancer cell types, including pituitary *[Barrera-Hernandez G et al, 1999]* , glioma *[Davis FB et al, 2006]* , breast *[Hall LC et al, 2008]* , and prostate cancer *[Tsui KH et al, 2008]* . It has been demonstrated that THRα1 through cooperation with the WNT/β-catenin signaling pathway enhances epithelial cell proliferation and promotes intestinal tumorigenesis *[Plateroti M et al, 2006; Kress E et al, 2009]* . Despite several studies focused on the role of THRs in several human cancer, their role in tumor development is still unclear. Since actions of THRs are complex and tissue type-dependent, aberrant activity of various isoforms might have different effects in promoting different tumor types.

Thyroid hormone levels and cancer

As mentioned above, an aberrant activity of THRs is associated with several human cancers. Many reports suggest that also changes in thyroid hormone levels exert a similar effect but results obtained to date are ambiguous. Population-based case-control studies of risk factors associated to development of ovarian and pancreatic cancers found that a history of hyperthyroidism is associated with a twofold increase of cancer risk *[Ness et al, 2000; Ko et al, 2007]* . Moreover, hyperthyroidism was associated with more advanced clinical stage and higher risk of recurrence in prostate cancer *[Lehrer et al, 2002]* . Consistent with hypothyroidism being beneficial, clinical studies showed that hypothyroidism correlates with a lower risk of primary mammary carcinoma and a reduction in progression to invasive disease *[Cristofanilli M et al, 2005]* . Similarly, farmacologically induced hypothyroidism improves survival in glioblastoma when used together with tamoxifen *[Hercbergs AA et al, 2003]* . In contrast, however, other studies indicate that low thyroid hormone levels increase the risk of HCC in humans, and high T_3/T_4 is therapeutic. A case

control study showed that women with a history of hypothyroidism had a 2.8 fold higher risk of HCC *[Hassan et al, 2009]* . Reddy et al. demonstrated that hypothyroidism was significantly more prevalent in patients with HCC of unknown etiology than in HCC patients with alcoholic liver disease or HCV, suggesting that hypothyroidism may be a permissive factor for the development of HCC *[Reddy et al, 2007]* .

THRs in HCC

Several studies have been performed to investigate the role of THRs in HCC development and progression and many reports suggest THRs as tumor suppressors in HCC. The first evidence was represented by the demonstration that mutated THRα (v-ErbA) leads to the development of hypothyroidism and HCC in male mice *[Barlow C et al, 1994]*. Moreover, several experiments have shown that mutated or truncated forms of THRs are expressed at high frequencies in human HCCs and in human hepatoma cell lines. These mutant forms of THRs display loss of transcriptional activities, defects in the release and binding of ligand-driven co-repressors, and act as dominant-negative forms *[Lin KH et al, 1999; Chan IH and Privalsky ML, 2006; IH Chan and ML Privalsky, 2009; Lin KH et al, 1996; Lin KH et al, 2001]*. Nevertheless, in spite of the previously described unusual high percentage of mutations of THRα and THRβ genes, we did not find any mutation of both genes in rat HCCs *[Manuscript in preparation]* . Our results are in agreement with two very recent studies based on whole genome deep-sequencing analysis, where no mutations of THRs have been detected in human HCCs of different etiology *[Guichard C et al, 2012; Cleary SP et al, 2013; Joseph B et al, 2007]*. Studies conducted in hepatoma cell lines and in human HCCs have indicated a role for TH receptors in the induction of the tumor suppressor DKK4 via WNT pathway and in the repression of proto-oncogenes Sp1 and PTTG1 *[Chen RN et al, 2008; Liao CH et al, 2012]* . Particular attention has been given to the THRβ1 isoform, representing the most abundant THR in hepatic tissue. A recent study showed that transfection of THRβ1 into human SKHep1 cells reduced HCC xenograft tumor growth in nude mice, promoted mesenchimal-to-epithelial transition and attenuated tumor cell invasiveness *[Martinez-Iglesias O et al, 2009]*. On the other hand, a less recent study showed that expression of THRβ1 is highly correlated with increased invasiveness in human HCC cell lines and decreased expression of the anti-metastatic gene nm23 *[Lin KH et al, 1995]*. The role of hypothyroidism in HCC development has also been investigated. Recent epidemiological studies suggested a significant association between hypothyroidism and

incidence of HCC, independent of other HCC risk factors *[Reddy A et al, 2007; Hassan MM et al, 2009]*. Supporting the tumor suppressor role of THRs in HCC, especially THRβ1, our previous studies performed using a carcinogen-induced rat HCC model, demonstrated that treatment of animals with T_3 or the THRβ1-agonist GC1, reduced the number of preneoplastic nodules, HCCs and lung metastasis *[Ledda-Columbano, GM et al, 2000; Perra A et al, 2009]* . Notably, mitogenic effect of T_3 in hepatocytes, is accompanied by an antitumoral effect probably related to activation of a differentiation program in preneoplastic hepatocytes [*László V et al, 2008; Perra A et al, 2009]* .

Overall, despite the strong evidence suggesting a key role of THRs in HCC development, it is still unclear if they act as oncogenes or tumor suppressor genes.

Aim of the work

Several studies have focused on studying alterations of thyroid hormone receptors in HCC, but so far it remains unclear whether they act as oncogenes or tumor suppressors. Previous studies performed in our laboratory on the Resistant Hepatocyte model of rat hepatocarcinogenesis showed that hyperthyroidism induced in rats by T_3 administration exerts a strong antitumoral effect, inducing a rapid disappearance of preneoplastic lesions and the inhibition of HCC development and lung metastasis. This findings suggest that THRs act as tumor suppressors in rat HCC development. Therefore, the purpose of the present study was to investigate the role of THRs, particularly the THRβ1 isoform, in the hepatocarcinogenesis process. Our research has been done through the use of the Resistant Hepatocyte model, which allows to analyze the different stages of tumor development and to identify molecular alterations already present in the early stages of the process. In addition, to assess whether data obtained in the animal model could have a translational value for human hepatocarcinogenesis, we extended our studies, performing in vitro experiments with human hepatoma cell lines and finally evaluating THRs expression levels in human HCC samples.

Materials and methods

Cell lines

To perform in vitro experiments five human hepatoma cell lines were used, namely HepG2, Mahlhavu, HuH7, SKHep1C3 69.2 and HA22T/VGH, kindly provided by Dr. S. Giordano, IRCC Institute of Candiolo (Turin, Italy). Cells were cultured as follows:

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Hyclone), 2mM glutamine (Life Technologies) and 1% Penicillin-Streptomycin (Life Technologies). Mahlavu and HuH7 cells were cultured in DMEM supplemented with 10% inactivated FBS, 2mM glutamine and 1% penicillin-streptomycin. SKHep 1C3 69.2 cells were maintained in Minimal Essential Medium (MEM, Life Technologies), 10% FBS, 1% sodium pyruvate (Life Technologies), 1% non-essential amino-acids (NEAA, Life Technologies), and 1% antibiotics. HA22T/VGH were cultured in RPMI with 10% inactivated FBS, 1% sodium pyruvate and 1% penicillin-streptomycin. Serum has been inactivated in water bath at 56°C for 1 hour. Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere.

Cell transfection

HuH7 cells in 10cm plate were transiently transfected with 300 pmol of miRNA precursors: pre-miR-27a, pre-miR-181a and pre-miR-204 (Pre-miR™ miRNA Precursor Molecule, Life Technologies, Carlsbad, USA) or microRNA Inhibitor (Anti-miRNA miRNA Inhibitor molecule, Life Technologies, Carlsbad, USA) using Lipofectamine 2000 (Life Technologies, Carlsbad, USA) following manufacturer instructions.

For silencing of THRB expression, HuH7 cells were transfected using 300 pmol of anti-THRB siRNA (siTHRB) and Lipofectamine 2000 (Life Technologies, Carlsbad, USA). Control cells were transfected using a scrambled siRNA (Silencer Negative Control, Life Technologies, Carlsbad, USA). One day before transfection, for each condition to be tested, approximately 550,000 cells were seeded in 10 cm plates to have a 30-50% confluence at the time of transfection. Transfection was performed as follows:

a. 300 pmol of oligomer were diluted in 1500µL Opti-MEM I Reduced Serum Medium without serum.

b. 12 μL /10 cm plate of Lipofectamine 2000 were diluted in 1500 μL Opti-MEM and the mix incubated 5 min at RT.
c. After the 5-minute incubation, the diluted oligomer was combined with the diluted Lipofectamine 2000. After 20 min of incubation at RT, the oligomer-Lipofectamine 2000 complex was added to each plate containing cells and Opti-MEM.
   Cells were then incubated at 37°C in a CO_2 incubator and 24 hours after transfection, cells were seeded with the appropriate density for subsequent biochemical and biological assays. RNA was extracted 72 hours after transfection and used for RT- PCR experiments.

T_3 treatment

3,3',5 Triiodo-L-thyronine sodium salt (Sigma-Aldrich, St. Louis, MO, USA) has been dissolved in NaOH 1M and in an appropriate volume of medium to final concentrations of 100nM and 1μM. One day after transfection, 100˙000 HuH7 cells transfected with siC (scramble siRNA) and THRβ siRNA were seeded in 6 cm plates and cultured in DMEM supplemented with 5% FBS and treated with T_3 100nM or, T3 1μM T_3 and vehicle as a negative control.

Animals

Male Fisher F-344 rats (Charles River, Milano, Italy) weighing 100-125 gr have been used for the study. Animals have been fed a standard diet (Ditta Mucedola, Milano, Italy) and maintained at 25ºC temperature and 12 hours light/dark daily cycle, with food and water *ad libitum*. Guidelines for the 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.

Experimental Protocol

Rats belonging to the experimental group were treated in accordance with the Resistant Hepatocyte protocol **[Fig.5]**. Animals were injected i.p. with a single dose of diethylnitrosamine (DENA) (Sigma Aldrich, St. Louis, MO, USA), dissolved in saline, at the dose of 150 mg/kg body weight. After a 2-week recovery period, rats were placed on a diet containing 0.02% 2-acetylaminofluorene (2-AAF) (Sigma Aldrich, St. Louis, MO, USA) for 1 week, then subjected to a standard two-thirds partial hepatectomy (PH) *[Higgins*

GM, Anderson RM, 1931] and kept for an additional week on the 2-AAF-containing diet. Rats were then switched to basal diet all throughout the experiment.

Two additional groups were used as controls: one group was subjected to the promoting regimen (2-AAF+PH) in the absence of carcinogen, and in a second group both the initiating and the promoting regimens have been omitted.

Each experimental group was divided into two subgroups; the first of them was sacrificed at ten weeks from DENA administration, at a time when GSTP-positive preneoplastic lesions are present; the second one has been sacrificed at fourteen months to evaluate the presence of fully developed HCCs. HCCs were selected on the basis of the criteria proposed in *"Histologic typing of liver tumors of the rat" [Stewart HLW, 1980]* .

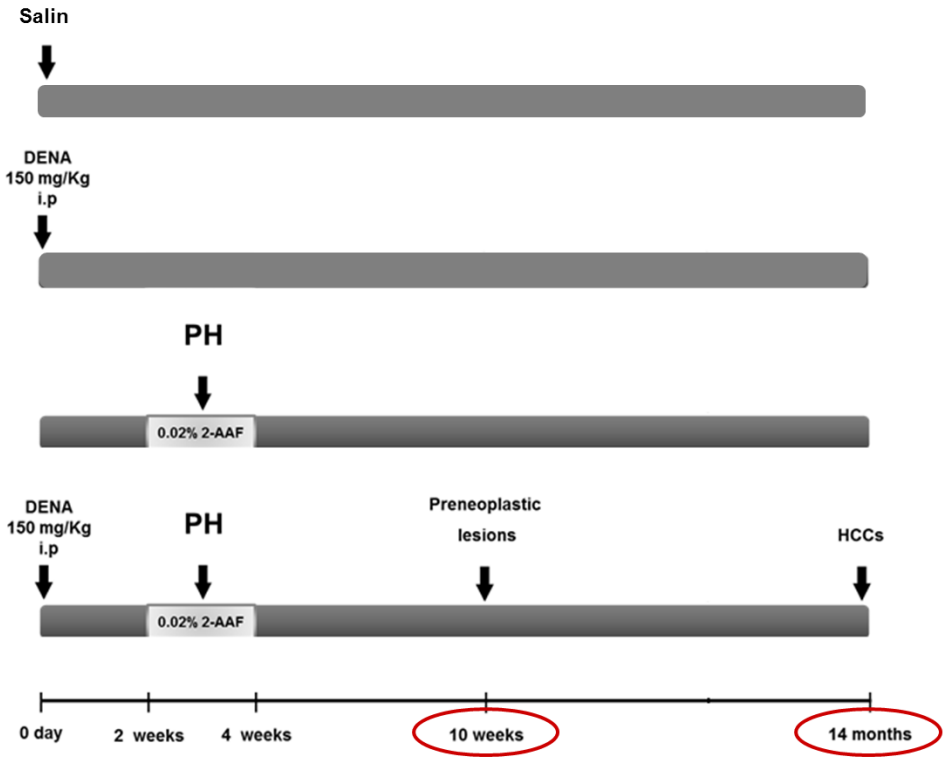

Fig.5: Experimental protocol.

For assessment of THRs expression during liver regeneration, rats were subjected to a standard 2/3 PH according to the method described by Higgins and Anderson *[Higgins GM, Anderson RM, 1931]*, and sacrificed after 24, 48 hours and one week after PH. Livers collected at the time of the surgery were used as controls .

Treatment with 5'-bromo-deoxyuridine (BrdU)

In order to assess the proliferative activity of hepatocytes, rats subjected to the analysis of KRT 19- and KRT-19+ preneoplastic lesions and animals subjected to PH were treated with 5-bromo-2'-deoxyuridine(BrdU) (Sigma Chemical Co., St Louis, MO, USA) **.**

For assessment of proliferative activity in preneoplastic lesions, BrdU was dissolved in drinking water (1 mg/mL) and given *ad libitum* for 7 days before sacrifice. For assessment of hepatocyte proliferation during liver regeneration associated to PH, BrdU was administered intra-peritoneally (i.p.) 2 hours prior to sacrifice at a dose of 50 mg/100g body weight.

Histology and immunohistochemistry

Tissue preparation

Immediately after sacrifice, livers were cut into several pieces and subjected to different analyses. For immunohistochemistry analysis, liver sections were fixed in 10% formalin, embedded in paraffin and stored at RT. Other sections were frozen in cold isopentane or quickly frozen by immersion in liquid nitrogen and stored at -80°C.

Hematoxylin and Eosin staining

4 µm paraffin-embedded liver sections were deparaffinized in xylene and then hydrated in a decreasing series of alcohol. Sections were then stained with Carazzi Hematoxylin for 15 min and in 1% acidified alcoholic eosin for 30 seconds, then dehydrated in ascending series of alcohol.

GST-P and KRT-19 immunohistochemistry

6 µm isopentane-frozen liver sections were fixed in acetone at -20°C for 20 minutes. Endogenous peroxidases were blocked with Peroxidase Block Reagent (DAKO, Milano, Italy) for 10 minutes**.** Blocking of aspecific sites was performed in normal goat serum 1:10 in PBS buffer for 1 hour at RT. Anti-GSTP antibody (rabbit polyclonal antibody MBL, Germany) was applied overnight at 4 °C at 1:1000 dilution. Sections were then incubated with anti-rabbit HRP secondary antibody at 1:200 dilution for 1 hour at RT. Sections were then stained by a brief incubation with 3-3' diaminobenzidine tetrahydrochloride hydrate (DAB) (Dako Envision, Denmark). KRT-19 protein was detected by applying anti-KRT19 primary antibody (mouse monoclonal antibody Novocastra, Leica Biosystems, Milano)

diluted 1:50 for 2 hours at RT and by incubating sections with anti-mouse HRP secondary antibody 1:200 in PBS at RT for 1 hour. Staining was revealed by a solution containing the chromogenic DAB. Counter staining was performed with Harris Hematoxylin Solution (Sigma-Aldrich, St. Louis, MO, USA). Finally, sections were dehydrated in ascending series of alcohol and mounted with coverslip.

BrdU staining

Four-micrometer-thick sections were deparaffinized, treated with 2 N HCl for 1h, then immersed in 0.1% Trypsin Type II-S from Porcine Pancreas (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at 37°C, and incubated sequentially with 1:10 Normal Goat Serum (Dako Denmark A/S, Glostrup, Denmark) for 30 minutes, 1:50 Mouse Monoclonal Anti-BrdU Antibody (Becton Dickinson, San Jose, CA, USA) for 1,5 hours and with Dako EnVision+ System Labeled Polymer-HRP Anti-Mouse (DakoCytomation, Carpinteria, CA, USA) for 30 minutes. The sites of peroxidase binding were detected by incubation with DAB. Counter staining was performed with Harris Hematoxylin Solution (Sigma-Aldrich, St. Louis, MO, USA). A segment of duodenum, an organ with a high rate of cell proliferation, was included from each animal to confirm delivery of BrdU. Labelling index was expressed as number of BrdU-positive hepatocyte nuclei per 100 nuclei. At least 2500 hepatocyte nuclei for each liver were scored.

Laser Capture Microdissection

Pre-neoplastic lesions of animals sacrificed ten weeks after initiation were identified by immunohistochemical staining for GST-P and KRT-19 proteins. Fourteen-µm-thick serial frozen sections were attached to 2-µm PEN-membrane slides (Leica, Bannockburn, IL). Immediately before performing dissection, each section was rapidly stained with a 3½ minute H&E procedure. This step was performed in order to identify the localization of the lesions of our interest, which were previously identified, as mentioned above, on serial sections subjected to GST-P and KRT-19 IHC. Micro-dissection was performed using a Leica laser microdissection apparatus (LMD6000). Handling time per slide did not exceeded 15 minutes to avoid RNA degradation.

RNA extraction

RNA extraction from preneoplastic lesions

Total RNA was extracted from preneoplastic lesions and from respective control livers with mirVana miRNA Isolation kit (mirVana, Ambion, Life Technologies, Monza) according to manufacturer's instructions. Briefly, dissected lesions were dissolved in 300 μL of Lysis Buffer (LB) and 30 μL of miRNA Homogenate Additive. After a 10 minutes incubation on ice, 300 μL of acid-phenol:chloroform: isoamyl alcohol (125:24:1) were added to the samples. Samples were then centrifuged for 5 minutes at maximum speed to separate the aqueous and organic phases. After recovery of the aqueous phase, 1.25 volumes of 100% ethanol were added and the mixture was transferred to a Filter Cartridge. Samples were centrifuged and after three washing steps with Wash Buffers, RNA was eluted in 100 μL of DEPC pre-heated (95°C) water.

RNA purification from rat HCCs and control livers

Total RNA from advanced HCCs (14 months after DENA) and control livers was isolated using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA). 1 mL of Trizol was added to 80-100mg of hepatic tissue and samples were homogenized with a power homogenizer. After a centrifugation step of 10 min at 12000 x g at 4 °C, samples were incubated 5 min at RT to permit the complete dissociation of nucleoprotein complexes. 0.2 mL chloroform for each mL of Trizol used were added and samples were centrifuged 15 min at 12000 x g. After centrifugation the mixture separates into a lower red phenol-chloroform phase containing proteins, a white interphase containing DNA, and a colorless upper aqueous phase containing RNA. RNA was then precipitated by addition of 500 μL isopropanol (Fisher Scientific, Thermo Fisher Scientific, France) and subsequently with 1 mL of 100% ethanol. Finally, the pellet RNA was dissolved in RNase-free water (Gibco, Life Technologies, Milan). In order to completely eliminate proteoglycans and polysaccharides (glycogen), the eluted RNA was subjected to a further purification process conducted using 3M sodium acetate at pH 5.2. After a second precipitation in 75% ethanol, the RNA pellet was air dried and then resuspended in RNase-free water.

RNA purification from human hepatoma cell lines

Total RNA of human hepatoma cell lines was extracted with miRNeasy Mini Kit (QIAGEN, Valencia, CA). Cells were lysed in 600 μL QIAzol Lysis Reagent and incubated

5 minutes at RT (15-25 °C) to promote the dissociation of the nucleoprotein complexes. After addition of 140 µL chloroform and after centrifugation, the mixture separated in three phases. To the upper aqueous phase, containing the RNA, 500 µL of 100% ethanol were added and the samples were then transferred to RNeasy spin columns and centrifuged at 8000 x g for 15 sec at RT. Before proceeding with the purification, RNA was subjected to digestion with DNase in order to avoid contamination by genomic DNA. After DNA digestion, the RNA adsorbed to the resin of the column, was washed two times using 500 µL of washing buffer and finally eluted with 30 µL of RNease free water by centrifugation for 1 min at 8000 x g.

Quantitative and qualitative analysis of nucleic acids

The quantity of RNA was measured by NanoDrop ND1000 Spectrophotometer (Thermo Scientific, France), while RNA integrity was assessed by Agilent Bioanalyzer 2100. Only RNA samples with a RIN (RNA Integrity Number) equal to 7 or higher were included in the study.

Analysis of mRNA and miRNAs expression levels

RT-PCR (Reverse Transcription Polymerase Chain Reaction)

To perform the analysis of mRNA and microRNA expression, the RNA has been retrotranscribed to cDNA using two different protocols:

- High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Life Technologies, Monza, Italy) for used for the mRNA analysis. 1.5 µg of RNA were diluted in 10 µL of RNase free H_2O to a final concentration of 150 ng/µL. For each sample 2 µL of RT buffer (10X), 2 µL of Random Primers (10X), 0.8 µL of dNTPs (100 mM), 1 µL of MultiScribe Reverse Transcriptase enzyme, 1 µL of RNase Inhibitor and water until 20 µL were added. Samples were then incubated at 25 °C for 10 minutes, at 37 °C for 120 minutes and at 85 °C for 5 minutes. Samples were then stored at -20 °C until next use.
- TaqMan®MicroRNA Reverse Transcription Kit (Applied Biosystem, Life Technologies, Monza, Italy) was used for the retrotranscription of miRNAs. 10 ng of total RNA were diluted to reach a final concentration of 2 ng/µL. For each sample, the retro-transcription mixture was composed of: 4.16 µL of H_2O, 1.5 µL of Reverse Transcription Buffer (10X), 0.15 µL of dNTPs (100 mM), 0.19 µL of RNase Inhibitor and 1 µL of MultiScribe Reverse

Transcriptase. For each miRNA 3 μL of specific primer were used. Subsequently the samples were incubated at 16 °C for 30 min, then at 42 °C for 30 min and at 85 °C for 5 min. Samples were then stored at -20 °C until next use.

Real Time PCR

Retro-transcribed cDNAs were used for the assessment of mRNA and micro RNA expression levels by Real Time PCR.

For gene expression analysis, the amplification reaction was performed in a final volume of 10 μL containing: 4 μL of cDNA (2.5 ng/μL), 5 μL of TaqMan Gene Expression Master Mix (Applied Biosystems, Life Technologies, Monza, Italy) and 1 μL of TaqMan probe (Applied Biosystems, Life Technologies, California, USA). The following TaqMan probes were used:

- Rn 01464144_m1 for the analysis of rat THRα1 isoform (thyroid hormone receptor alpha1)
- Rn 01464143_m1 for the analysis of rat THRα2 (thyroid hormone receptor alpha2)
- Rn 01537799_m1 for the analysis of rat THRβ1 (thyroid hormone receptor beta1)
- Rn 00562044_m1 targeting both rat isoforms THRβ1-β2 (a probe that recognize both thyroid hormone receptor beta1 and beta2 isoforms)
- Rn 00689876_m1 for the analysis of rat G6PC (Glucose-6-phosphatase)
- Rn 01511034_m1 for the analysis of rat THRSP/SPOT14 (Thyroid hormone responsive spot14)
- Rn 00572183_m1 for the analysis of rat DIO1 (Deiodinase 1)
- Rn 00570673 for the analysis of APP (Amyloid β precursor protein)
- Hs 00230861_m1 for the analysis of human THRβ (thyroid hormone receptor beta)
-Hs00174944_m1for the analysis of human DIO1 (Deiodinase 1)
- Hs00609178_m1for the analysis of human G6PC (Glucose-6-phosphatase)

Reactions were performed in a ABI PRISM 7300HT thermocycler (Applied Biosystems) with the following cycling conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec each and a final step at 60 °C for 1 min.

For the analysis of microRNAs expression, the RT product was diluted in 52 μL of H_2O. 4.5 μL of the diluted cDNA was amplified with 14.5 μL of TaqMan Universal Master Mix II no UNG (Applied Biosystems, Life Technologies, Monza, Italy) and 1 μL of a specific

TaqMan microRNA Assays (Applied Biosystems, Life Technologies, California, USA). The TaqMan miRNA assays used are:

- 000397 for the analysis of miR-21,
- 000408 for the analysis of miR-27a,
- 000468 for the analysis of miR-146a,
- 000480 for the analysis of miR-181a,
- 000524 for the analysis of miR-221,
- 000508 for the analysis of miR-204

Parameters used to perform the reaction are: 10 min at 95°C followed by 40 cycles at 95 °C for 15 sec each and a final step at 60 °C for 1 min.

For both mRNA and miRNAs expression, each sample was analyzed in triplicate. The housekeeping gene β-actin for gene expression and 4.5S (rat) and RNU48 (human) for miRNAs expression were used for normalization. Expression levels were evaluated with the $2^{-\Delta\Delta Ct}$ method and represented as relative expression compared to a calibrator control.

Protein isolation

Rat liver samples were homogenized in RIPA Buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) containing protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA), incubated for 2 h at 4 °C with shaking and centrifuged at 12000 rpm for 20 min at 4 °C. The supernatant was collected and stored at -80 °C.

Protein concentration of whole-cell lysates was evaluated with the BCA Protein Assay kit (Pierce Biotechnology, Rockford, USA) and equal amounts of total proteins were used to perform Western blotting experiments.

Western blotting

100μg of protein were resolved in NuPAGE Tris-Acetate Mini Gel 7% (Life Technologies, Carlsbad, USA) and transferred to nitrocellulose membrane. Membranes were blocked for 1 hour at RT in 5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA) diluted in TBS-T buffer (NaCl 5M, Tris-HCl 1M pH 7.4, 0.1% Tween-20), washed three times in TBS-T for 10 min, and incubated overnight at 4 °C with anti-TRβ1 primary antibody (Thyroid Hormone Receptor beta-1 Monoclonal Antibody J52, Pierce

Biotechnology, Rockford, IL, USA) 1:500 in 5% BSA. After washing 3 times for 10 min in PBS, membranes were incubated for 1 h at RT with anti-mouse horseradish peroxidase-conjugated IgG secondary antibody at 1:10000 dilution (sc-2005, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then washed 3 times for 10 min in PBS. Proteins were detected using Supersignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, USA). Subsequently, membranes were stripped and incubated 1 hour with anti-β-actin antibody diluted 1:500 (Monoclonal Anti-Actin, Clone AC-40, Sigma-Aldrich, St. Louis, MO, USA), washed three times with PBS and incubated for 1 h at RT with HRP-conjugated anti-mouse secondary antibody at 1:1500 dilution, washed three times and then subjected to the final detection step**.** Levels of TRβ1 proteins were normalized to Expression of the housekeeping gene β-actin was used as loading control.

DNA extraction and CpG methylation analysis

Genomic DNA was extracted from HCCs isolated from livers of rats subjected to the Resistant Hepatocyte model and from normal control livers with QIAamp DNA Mini Kit (QIAGEN, Valencia, CA), according with the manufacturer's instructions and the amount of extracted DNA was measured using a NanoDrop1000 spectrophotometer.

CpG islands in the promoters of TRα and TRβ genes were localized by gathering evidence collected from the UCSC Genome Browser (http://genome.ucsc.edu/) *[Kent WJ et al, 2002]* and analyzed for cytosine methylation in 15 HCC samples previously subjected to gene expression analysis.

Promoter CpG methylation was assessed by pyrosequencing.

2μg of DNA were bisulfite-converted with Epitect Bisulfite kit (Qiagen). Briefly, after addition of Bisulfite Mix (85μL), DNA protect Buffer (35μL) and water to a final volume of 140μL, the bisulfite reaction was performed in a thermal cycler (9700 Applied Biosystems) following manufacturer's recommendations. The converted DNA was then purified with Epitect spin columns using appropriate buffers in accordance with manufacturer's instructions. 50 μg of converted DNA were PCR amplified in a reaction mix consisting of PCR-buffer 1X, 1.5mM of $MgSO_4$, dNTPs 0.2mM each, 1 unit of Platinum –Taq DNA polymerase (Invitrogen) with the following forward and reverse primers, respectively: 5'-GGGATTGGGATGTTAGTTT-3', 5'-ATCACACCCCAACCCTCTT-3'. Pyrosequencing was performed according to the

manufacturer's instructions using the following sequencing primer: 5'-GGGATTGGGATGTTAGTTT-3'. 10 consecutive CpGs were assessed for methylation.

Patients

HCC and cirrothic tissues were obtained from 52 consecutive patients (males and females, median age ± SD: 67.2 ± 7.9, range 49-82 years) undergoing liver resection for HCC. Twelve normal liver tissues were obtained at surgery for large benign liver masses (5 haemangiomas, 6 FNH (Focal nodular hyperplasia) and one traumatic liver lesion). All tissues were obtained by Policlinico S.Orsola-Malpighi, Bologna, Italy (Dr. L. Gramantieri). Tissues were collected at surgery after obtaining an informed consent, immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Histopathological grading was scored according to Edmondson and Steiner criteria *[Rozen S. & H.J. Skaletsky, 2000]* . No patient received anticancer treatment prior to surgery. The characteristics of patients are detailed in **Table 1**.

Characteristics of HCC patients	
Gender (M/F)	42/10
Age (median±SD)	67.2±7.9
Etiology of CLD	
HBV (%)	11/52 (21.1%)
HCV (%)	31/52 (59.6%)
None (%)	6/52 (11.5%)
HCV + Ethanol abuse (%)	4/52 (7.6%)
Focality	
Uni-focal (%)	30/52 (57.6%)
Multi-focal (%)	22/52 (42.3%)
AFP	
< 20 ng/mL (%)	22/52 (42.3%)
> 20 ng/mL (%)	30/52 (57.6%)
Nodule size	
< 3 cm (%)	21/52 (40.3%)
<3/ >5 cm (%)	19/52 (36.5%)
> 5 cm (%)	12/52 (23,1%)
Grading	
G1 (%)	2/52 (3.8%)
G2 (%)	13/52 (25%)
G3 (%)	27/52 (51.9%)
G4 (%)	10/52 (19.2%)

Table 1: Characteristics of patients and hepatic tissue samples used for the analysis of THRs expression.

Statistics

Data are expressed as mean ± standard deviation (SD). Analysis of significance was done by t Student's test and by One-Way ANOVA using the GraphPad software (La Jolla, California).

Results

Analysis of THRs expression in rat HCCs

Deregulation of THRs expression represents a mechanism by which THRs play a role in the carcinogenic process in several human cancers. To establish whether THRs were abnormally expressed in rat HCCs, we analyzed the expression levels by Real-Time PCR of the THRα1 and THRβ1 isoforms in 31 HCCs isolated at 14 months after DENA treatment. As shown **[Fig.6]** , neoplastic lesions exhibited a significant decrease of the THRα1 **[Fig.6A,B]** and THRβ1 **[Fig.6C,D]** isoforms, compared to normal liver and to livers of age-matched rats exposed to AAF-PH in the absence of DENA. Notably, the THRβ1 isoform expression was reduced by 63% **[Fig.6D]** compared to normal liver, whereas the THRα1 isoform was reduced by 38% **[Fig.6B]** ($P<0.01$ for THRα1 and $P<0.0001$ for THRβ1).

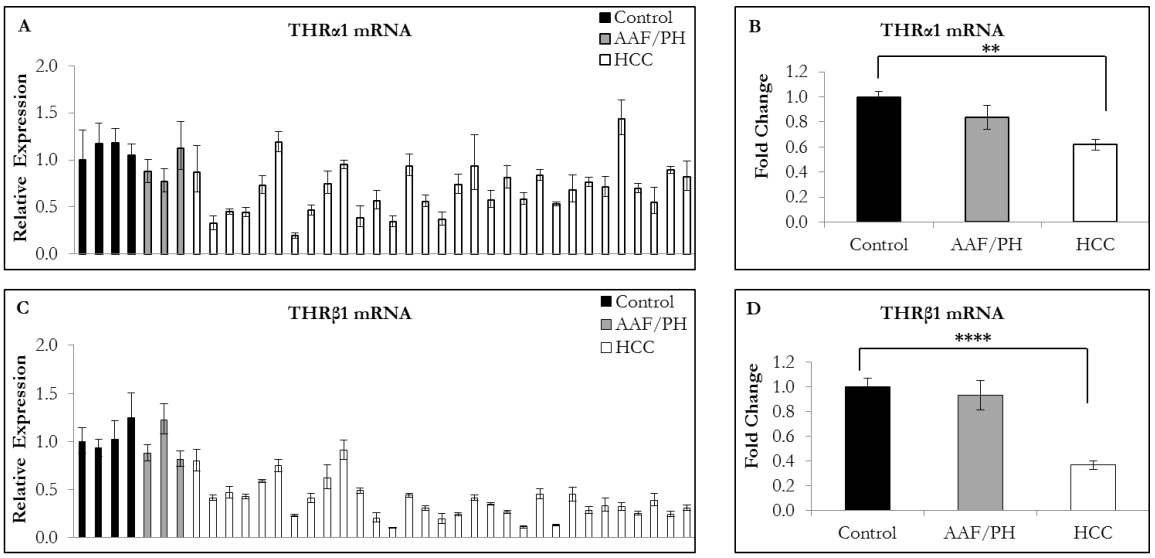

Fig.6: THRα1 **(A,B)** and THRβ1 **(C,D)** mRNA expression assessed by RT-PCR in 4 control liver samples, in 3 liver of rats exposed to AAF/PH and in 31 rat HCCs generated with the Resistant Hepatocyte model .
(A,C) The levels of THRα1 and THRβ1 were calculated as Relative mRNA expression and normalized using rat β-actin as endogenous control.
(B,D) Average expression of THRα1 and THRβ1 mRNA of samples included in panels A and C, respectively, for the three experimental groups.
** $P<0.01$; **** $P<0.0001$

In agreement with mRNA levels, western blot analysis showed a decreased content of THRβ1 protein compared to normal liver **[Fig.7]**

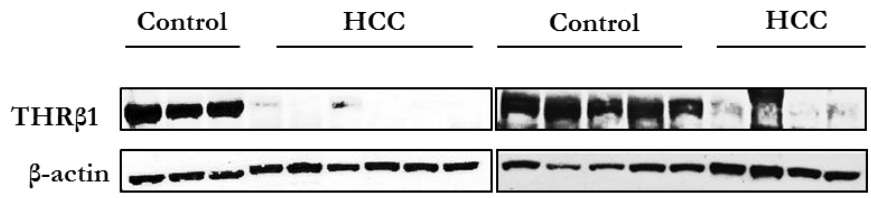

Fig.7: Western blot analysis of THRβ1 in rat HCC and control livers. β-actin was used as housekeeping protein.

To confirm the loss of expression of the receptor, we assessed its transcriptional activity. To this aim, we evaluated the levels of expression of THRβ1-regulated target genes. Decreased levels of target genes positively regulated by T_3 such as DIO1, G6PC and SPOT14 *[Feng X et al, 2000]* were observed in HCCs compared to normal liver. Conversely, amyloid-β precursor protein (APP), a gene that is negatively regulated by THRβ *[O'Barr SA et al, 2006]*, was significantly up-regulated in the same tumors. **[Fig.8]**.

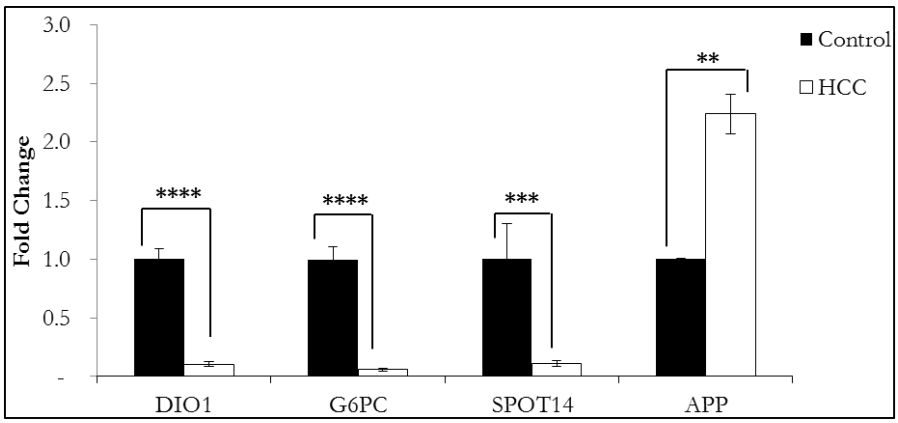

Fig.8: Average expression of THRβ1 target genes assessed by RT-PCR in 3 control liver samples and in 9 rat HCCs. Rat β-actin was used as endogenous control.
** P<0.01 , *** P<0.001 , **** P<0.0001

Analysis of THRs expression in preneoplastic lesions

The RH model allows to dissect the different steps of the carcinogenic process as it is possible to isolate phenotypically distinct lesions at well-defined stages, including very early preneoplastic lesions. This allows to decipher molecular alterations which occur during early stages of the tumoral process. Therefore, to determine whether THRs down-regulation is an early event during liver carcinogenesis, we isolated preneoplastic lesions

developed 10 weeks after initiation and identified by their immunohistochemical positivity for the placental form of Glutathione-S-transferase (GST-P) *[Satoh K et al, 1985]* and analyzed THR expression levels. As shown **[Fig.9]** , a significant reduction of expression of THR isoforms was observed in these lesions compared to the liver of control rats ($P<0.001$ for THRα1; $P<0.0001$ for THRβ1).

Notably, similar to HCC, a higher decrease of the expression of THRβ1 **[Fig.9B]** (more than 50% compared to normal liver samples) was observed when compared to that of THRα1 **[Fig.9A]** (30% reduction of expression).

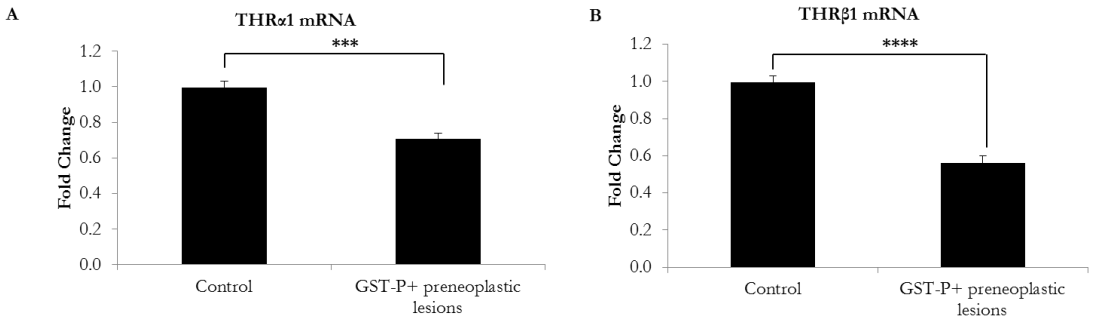

Fig.9: THRα1 **(A)** and THRβ1 **(B)** average mRNA expression assessed by RT-PCR in preneoplastic lesions. The levels of expression were calculated as fold change between 37 GST-P+ preneoplastic lesions and 6 control liver samples. Rat β-actin was used as endogenous control.
*** $P<0.001$, **** $P<0.0001$

We conclude that down-regulation of THRs matches the onset of hepatic preneoplastic lesions, and that continued down-regulation of THRs expression is probably necessary for the maintenance and progression of these lesions.

THRs expression levels in rat KRT-19-/KRT-19+ preneoplastic lesions

It is well established that during the carcinogenic process, a slow but continuous regression of the vast majority of the pre-neoplastic lesions occurs, that is characterized by a progressive loss of the less-differentiated phenotype and by the return to a normal one (remodeling) *[Enomoto K et al, 1982]*. Our previous studies have shown that the vast majority of HCCs arising in the RH model of hepatocarcinogenesis are KRT-19 positive, despite the fact that they represent a minority of the preneoplastic lesions developed at early stages of the process *[Andersen JB, Loi R et al 2010]*. This suggests that the subset of KRT-19+ lesions has an intrinsic advantage in the progression to malignancy. Therefore, it is critical to understand whether down-regulation of THRs occurs in all preneoplastic

populations or it discriminates between KRT-19+ or KRT-19- subpopulations. To this aim, we microdissected both KRT-19+ and KRT-19- nodules and analyzed THRs expression in both preneoplastic nodule populations. The results showed a significant down-regulation of both THRα1 and THRβ1 only in KRT-19+ preneoplastic lesions, but not in their negative counterpart [**Fig.10**] (mean expression for THRα1 in KRT-19+ vs KRT-19- vs normal liver: 0.67 ± 0.04 vs 0.82 ± 0.04 vs 1.00 ± 0.03; mean expression for THRβ1: 0.46 ± 0.02 vs 0.85 ± 0.05 vs 1.00 ± 0.04). In agreement with results obtained from HCC, down-regulation of THRβ1 [**Fig.10C,D**] was much more pronounced than that of THRα1 [**Fig.10A,B**]. Therefore, subsequent studies are focused on THRβ1.

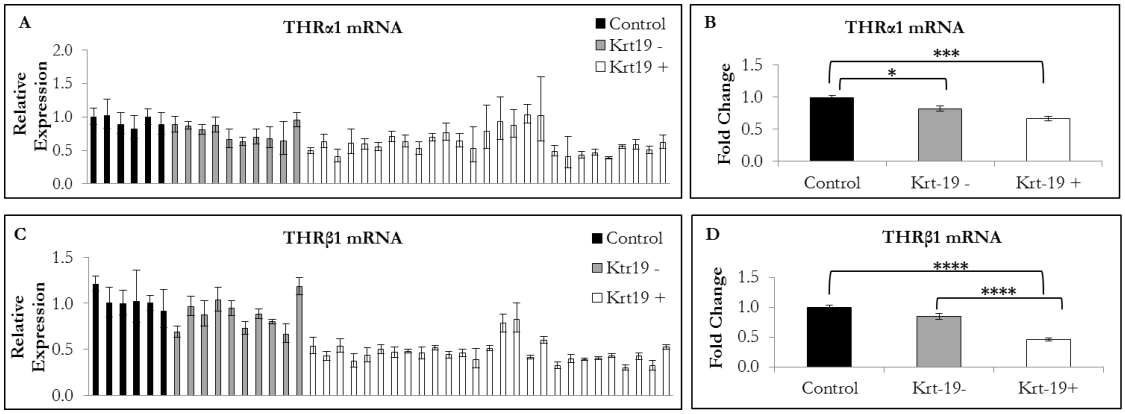

Fig.10: THRα1 **(A,B)** and THRβ1 **(C,D)** mRNA expression assessed by RT-PCR in 6 control liver samples, 10 KRT-19- nodules and 27 KRT-19+ nodules.
(A,C) The levels of THRα1 and THRβ1 were calculated as relative mRNA expression and normalized using rat β-actin as endogenous control.
(B,D) Average expression of THRα1 and THRβ1 mRNA of samples included in panels A and C, respectively., for the three experimental groups.
* p<0.1 , *** P<0.001 , **** P<0.0001

To confirm down-regulation of THRβ1 in preneoplastic lesions, we determined expression levels of the already characterized target genes DIO1, G6PC and SPOT14. Similarly to what observed in HCCs, all these target genes were profoundly down-regulated in KRT-19+ lesions when compared to control liver or to KRT-19- preneoplastic nodules **[Fig.11]** confirming that THRβ1 mRNA reduction also reflects a reduced transcriptional activity of this receptor.

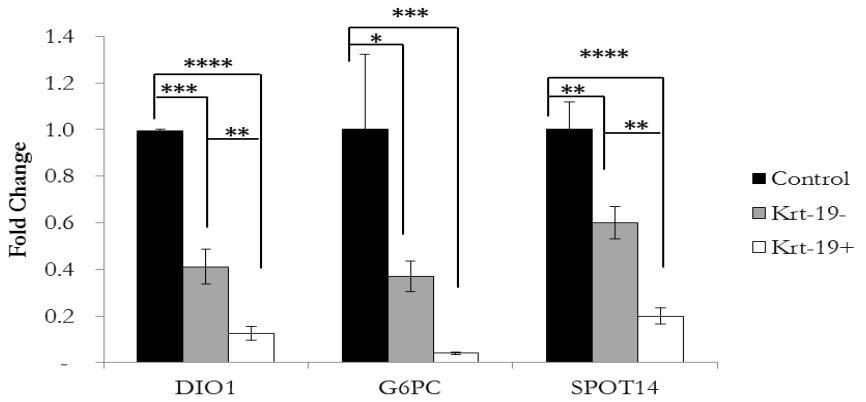

Fig.11: Average expression of THRβ1 target genes assessed by RT-PCR in 3 control liver samples and in 6 KRT-19- and 6 KRT-19+ preneoplastic lesions**.** Rat β-actin was used as endogenous control.
* P<0.1 ; ** P<0.01 ; *** P<0.001 ; **** P<0.0001

Our results suggest that the downregulation of THRβ1 results in decreased expression of its target gene DIO1 that leads to the reduction of T_4 to T_3 conversion and to a negative loop causing local hypothyroidism. In order to verify this hypothesis we exposed human hepatoma cells to increasing concentrations of T_3 and assessed THRβ levels of expression. To this aim we analyzed the levels of expression of THRβ in 5 human hepatoma cells, namely HepG2, HuH7, SKHep1C3, Mahlavu and HA22T/VGH. THRβ levels in these cells were generally low, with the exception of HepG2 and HuH7, as demonstrated by the cycle threshold (Ct) for each cell line examined, ranging from about 26.7 to 30.8 for SKHep, Mahlavu and HA22T cells (data not shown). Interestingly, an inverse relationship was observed between THRβ expression levels and the degree of cell differentiation **[Fig.17F]**. Indeed, more differentiated hepatoma cells (HepG2, HuH7), exhibited much higher levels of the receptor than less differentiated cell lines (SKHep1C3, Mahlavu and HA22T/VGH)*[Yuzugullu H et al, 2009]*. Among the human hepatoma cell lines, we selected for further experiments the HuH7 cells since they showed high levels of expression of THRβ. HuH7 cells were transfected with a siRNA targeting THRβ mRNA in order to down-regulate THRβ expression and with a control siRNA. This resulted in about a 70% decrease of THRβ expression in siRNA transfected cells compared to cells transfected with a control siRNA **[Fig.12]** . Furthermore, THRβ downregulation resulted in decreased expression levels of its direct target genes DIO1 and G6PC (about a 40% and 80% reduction compared to controls, respectively) **[Fig.12]** .

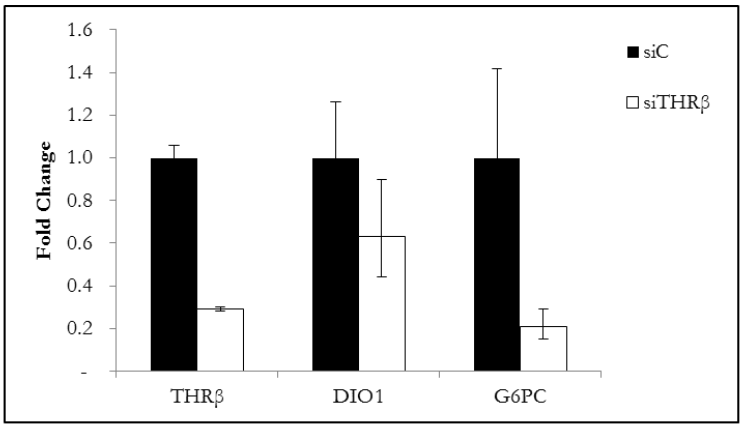

Fig.12: THRβ, DIO1 and G6PC expression was assessed by qRT-PCR in HuH7 cells transfected with a THRβ siRNA or a control siRNA.

In order to address whether exposure of HuH7 cells to increasing concentrations of T_3 could result in the restoration of THRβ levels, we treated the transfected HuH7 cells with T_3, 100nM and 1μM and assessed THRβ mRNA expression. In cells transfected with the siRNA that targets THRβ the expression of the receptor was slightly increased compared to control (relative expression for THRβ in HuH7+T_3 1μM vs HuH7+T_3 100nM vs HuH7 non treated (NT): 0.43 vs 0.40 vs 0.29); while in cells transfected with irrelevant siRNA we observed a much higher increase of THRβ levels (fold change for THRβ in HuH7+T_3 1μM vs HuH7+T_3 100nM vs HuH7 NT: 1.42 vs 1.74 vs 1.0) **[Fig.13]** .

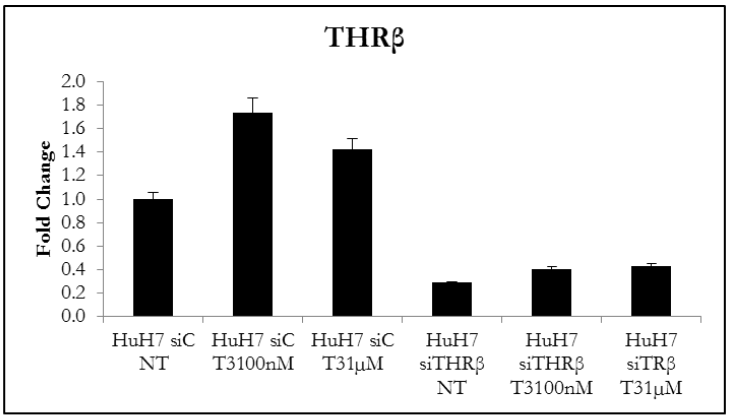

Fig.13: THRβ mRNA expression levels after T_3 treatment in HuH7 cells. Cells were transfected with a THRβ siRNA and a control siRNA and treated with two different concentration of T_3 (100nM and 1μM) or vehicle.

Therefore in HuH7 cells cultured in T_3 supplemented conditions, higher levels of THRβ receptor were observed, suggesting that THRβ expression may be affected by the availability of its ligand T_3.

THRβ1 expression is down-regulated in proliferating normal and preneoplastic hepatocytes.

Since KRT-19+ preneoplastic lesions are the precursor cell population of HCC and display the highest down-regulation of THRβ1 expression, we sought to determine whether the striking decrease of THRβ1 mRNA levels could be associated to an enhanced proliferative capacity of KRT-19+ preneoplastic hepatocytes. For this purpose, we determined BrdU incorporation as an index of proliferative activity in preneoplastic lesions. We treated animals with BrdU dissolved in drinking water for seven days and until two hours before sacrifice. Thus, we assessed nuclear BrdU incorporation in GST-P+, KRT-19- and KRT-19+ preneoplastic nodules **[Fig.14]** .

As shown **[Fig.14G]** KRT-19+ lesions had, indeed, a much higher proliferative activity than KRT-19 negative preneoplastic nodule hepatocytes The number of BrdU-positive nuclei/mm^2 in KRT-19+ vs KRT-19- was 606.04±10.42 vs 283.23±16.82. (P<0.0001).

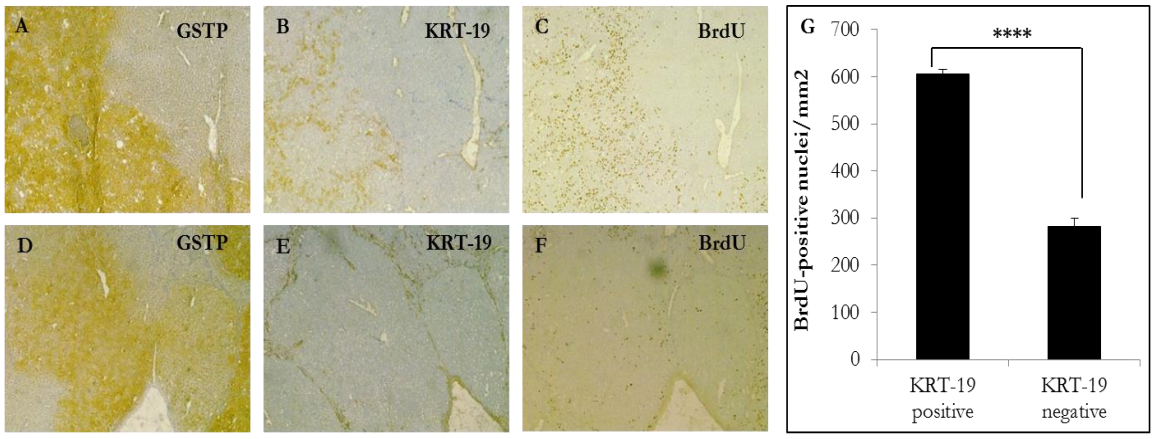

Fig.14: Representative microphotographs illustrating BrdU incorporation in GST-P+ preneoplastic nodules positive for KRT-19 and in KRT-19- lesions. GST-P staining **(A,D)** ; IHC staining for KRT-19 **(B,E)** ; BrdU staining **(C,F)** . Labelling Index (L.I.) of KRT-19+ and KRT-19- hepatocytes in preneoplastic nodules **(G)**. BrdU dissolved in drinking water (1mg/mL) was administered for 7 days and removed 2 hours prior to sacrifice. L.I. is expressed as number of BrdU-positive hepatocyte nuclei/100 nuclei. Results are expressed as means ± S.E. of 5 rats per group. At least 20˙000 hepatocyte nuclei per group were scored. (Original magnification 40x) **** P<0.0001

Notably, when we performed experiments on regenerating livers of animals subjected only to 2/3 PH and sacrificed at different timings after surgery, we demonstrated a significant down-regulation of THRβ1 expression during hepatic proliferation (mean expression 48h vs 24h after PH vs control: 0.30±0.04 vs 0.44±0.02 vs 1.0±0.09) ; THRβ1 expression returned to basal levels at the end of the regenerative process (mean expression 168h after PH vs control: 0.76±0.06 vs 1.0±0.09) **[Fig.15]** , further supporting the suppressive role of THRβ1 in both normal and preneoplastic hepatocyte proliferation.

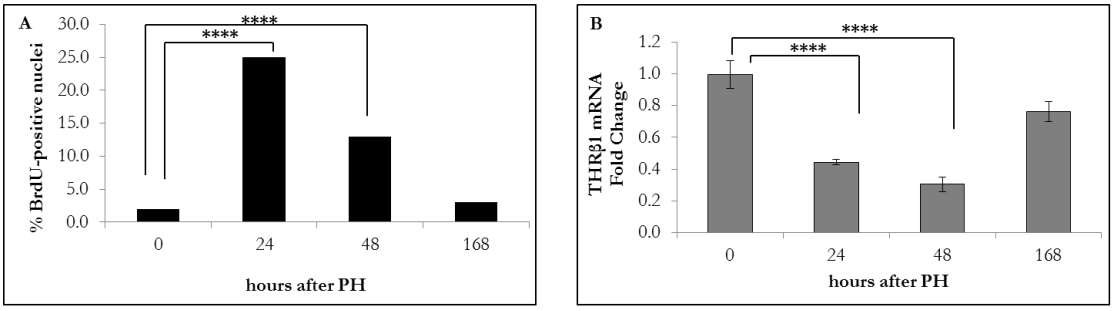

Fig.15: (A) Labeling Index (L.I.) in rats subjected to 2/3 PH. BrdU (50 mg/100gr body weight) was injected intraperitoneally 2 hour prior to sacrifice. L.I. is expressed as number of BrdU-positive hepatocyte nuclei/100 nuclei. 5 rats per group were assessed. At least 20˙000 hepatocyte nuclei per group were scored. **** P<0.0001
(B) THRβ1 expression was assessed by quantitative TaqMan RT-PCR. The levels were calculated as fold change difference between the expression in rats subjected to PH and controls. β-actin was used as endogenous control. **** P<0.0001

Analysis of CpG island methylation status of THRβ gene

Hypermethylation of gene promoter is a well-known mechanism responsible for the silencing of many genes, including tumor suppressors in human cancers *[Baylin SB and Jones PA, 2011]*. Silencing of the THRβ expression by hypermethylation of the promoter region of the THRβ gene is a frequent event in breast, lung, colon, thyroid cancers and acute lymphoblastic leukemia [*Li Z et al, 2002; Iwasaki Y et al, 2010; Ling Y et al, 2010; Joseph B et al, 2007; Dunwell TL et al, 2009]*. Therefore, we wished to determine whether changes in the methylation status of the CpG islands of the THRβ gene could be responsible for down-regulation of this receptor. To this aim, we performed pyrosequencing experiments on 15 rat HCCs. Very low levels of methylation (average rate of methylation in HCC vs controls was 4.02%±0.14 vs 4.53±0.65) of the CpG islands of THRβ were detected in the HCCs analyzed, with no significant differences compared to controls (P value=0.23) **[Table2]** . Thus, results make very unlikely the possibility that down-regulation of THRs is the consequence of a hypermethylated status of the THRβ gene promoter.

	METHYLATION FREQUENCY (%) AT 10 INDIVIDUAL CpG SITES										
	1	2	3	4	5	6	7	8	9	10	Ave.
Control liver 1	7	0	6	6	6	9	8	6	7	0	5.5
Control liver 2	8	0	0	5	6	8	8	7	0	6	4.8
Control liver 3	4	2	2	3	5	4	5	3	5	0	3.3
HCC 1	5	2	3	4	3	6	4	5	4	0	3.6
HCC 2	6	0	3	6	5	5	5	6	5	0	4.1
HCC 3	4	5	3	3	4	5	5	5	3	0	3.7
HCC 4	8	0	5	7	5	4	8	5	7	0	4.9
HCC 5	8	3	3	4	5	0	5	4	5	4	4.1
HCC 6	9	0	0	6	4	7	6	5	5	3	4.5
HCC 7	4	3	0	5	6	5	5	6	9	5	4.8
HCC 8	6	0	0	4	4	0	8	7	0	6	3.5
HCC 9	5	0	3	4	3	6	5	4	3	0	3.3
HCC 10	4	2	3	3	2	5	4	5	5	3	3.6
HCC 11	4	3	3	5	6	4	3	4	0	4	3.6
HCC12	8	0	0	8	6	0	6	0	8	0	3.6
HCC 13	3	3	4	5	3	3	4	7	4	0	3.6
HCC 14	6	0	5	7	4	6	5	4	5	4	4.6
HCC 15	7	4	0	8	6	5	5	7	6	0	4.8

Table 2: Methylation frequency at specific CpG sites in the promoter of the THRβ gene. 10 CpG sites were scored for methylation by pyrosequencing. 3 age-matched control normal livers and 15 HCCs were analyzed. Average rates of methylation for each sample are indicated in blue color.

Investigation of the role of microRNAs in THRβ1 down-regulation

miRNAs have been shown to play a fundamental role in the control of gene expression *[Calin GA et al, 2006]* and recent studies have provided evidence that expression of THRβ can be repressed by the action of microRNAs *[Jazdzewski K et al, 2011]*. Therefore, we investigated whether upregulation of miRNAs could be the cause of THRβ downregulation in rat HCC. Initially, we selected six miRNAs shown to target THRs in different cell types, namely miR-21, miR-27a, miR-181a, miR-221, miR-146a and miR-204 *[Jazdzewski K et al, 2011; Nishi H et al,2011; Master A et al, 2010]*. and we measured by Real-Time PCR the expression levels of these miRNAs in rat HCCs. As shown **[Fig.16]** miR-27a, miR146a, miR-181a and miR-204 (miRNA vs control: 13.93±3.71 vs 1.00±0.53; 5.65±1.08 vs 1.00±0.17; 8.12±1.03 vs 1.00±0.36; 4.34±0.65 vs 1.00±0.18, respectively) were found to be up-regulated in rat HCC displaying THRβ1 down-regulation.

To investigate if up-regulation of miRNA in rat HCC could be of translational value and to better understand the role of miRNAs in the control of THRβ expression, we moved to *in vitro* experiments. The expression levels of the same miRNAs were assessed in five human hepatoma cell lines, namely HepG2, HuH7, Skep1C3, Mahlavu, HA22T. Resulting expression levels of THRβ mRNA and selected miRNAs are shown in **[Fig.17]** .

Interestingly, an inverse relationship between the expression of miR-27a and miR-181a versus THRβ was observed in the five HCC cell lines examined (r=-0.848 for miR-27a and r=-0.816 for miR-181a) suggesting that this two miRNAs might negatively regulate THRβ1 expression in HCC cells **[Fig.18]** .

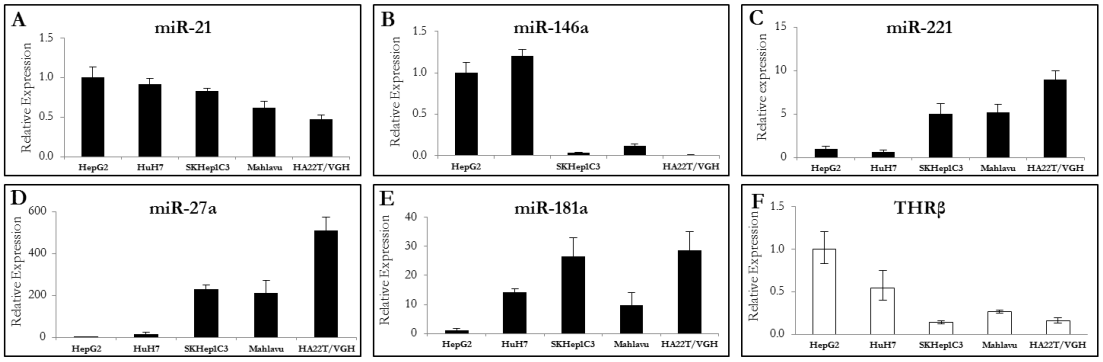

Fig.17: Expression of THRβ **(F)** and selected miRNAs **(A-E)** in 5 different human hepatoma cell lines assessed by Real-Time PCR. RNU48 was used as endogenous control for miRNAs analysis.

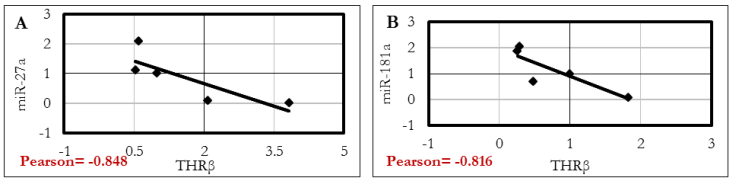

Fig.18: Scatter plot correlating THRβ and microRNA expression for miR-27a **(A)** and miR-181a **(B)** respectively. The Pearson product-moment correlation coefficient (also indicated as r) is a measure of the strength of the linear relationship between the two variables THRβ and microRNA . Pearson's r can range from -1 to 1. An r of -1 indicates a perfect negative linear relationship between variables, an r of 0 indicates no linear relationship between variables, and an r of 1 indicates a perfect positive linear relationship between variables.

In order to investigate whether miRNAs affect THRβ1 expression in human hepatoma cell lines, we transfected HuH7 cells with precursors of the most up-regulated miRNAs in rat HCC, namely miR-27a, miR-181a and miR-204 and we verified that high expression levels were achieved **[Fig.19]** .

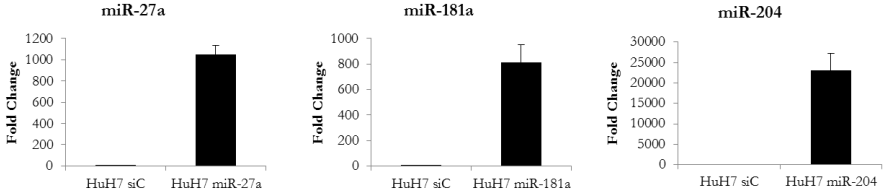

Fig.19: Transfection of Pre-miR miRNA precursors (miR-27a, miR-181a and miR-204) in HuH7 cells was assessed by Real-Time PCR and demonstrated by comparison with HuH7 cells transfected with a control siRNA (siC).

Evaluation of THRβ expression levels in HuH7 cells transfected with pre-miRs respect to HuH7 transfected with a control siRNA (siC) or with antagomiRs revealed a significant decrease of the expression of THRβ mRNA **[Fig.20]** .

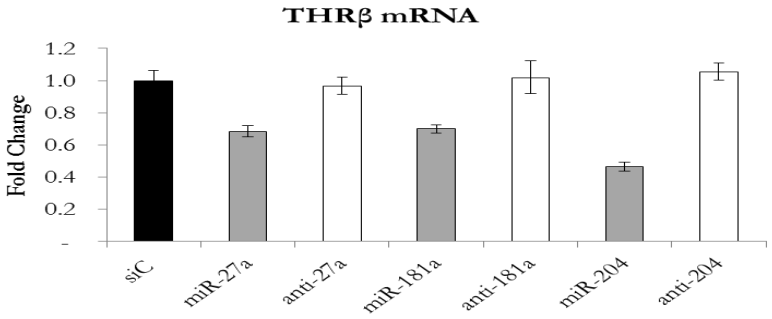

Fig.20: THRβ mRNA expression levels were evaluated by Real-Time PCR in HuH7 cells transfected with miRNAs, antagomiRs and control siRNA (siC).

Analysis of TRs expression in human HCC

Next, we sought to determine whether the results obtained in the RH model could be of translational value for human HCC. Therefore, THRβ1 mRNA levels were determined in a cohort of 52 HCC patients (the characteristic of the tissue samples are described in Table 1). THRβ1 expression levels in HCCs were compared to THRβ1 expression in matched cirrhotic tissues (LC) **[Fig.21]** and in 12 normal liver samples **[Fig.22]** . Similarly to what observed in rats, human HCCs showed down-regulation of THRβ1 compared to matched cirrhotic tissues (LC) and to normal livers (mean expression in HCC vs LC vs normal liver: 1.65 ± 1,10 vs 2,34 ± 1,19 vs 3.59 ± 1.76) (Fig.B), ANOVA $p<0.0001$. Overall, THRβ1 was down-regulated in 71% of HCCs, when compared to matched non-cancerous cirrhotic tissues and in 77% of cases when compared to normal liver tissues. Regarding THRα1 expression, no significant difference was found across HCCs, cirrhotic tissue and normal liver (data not shown).

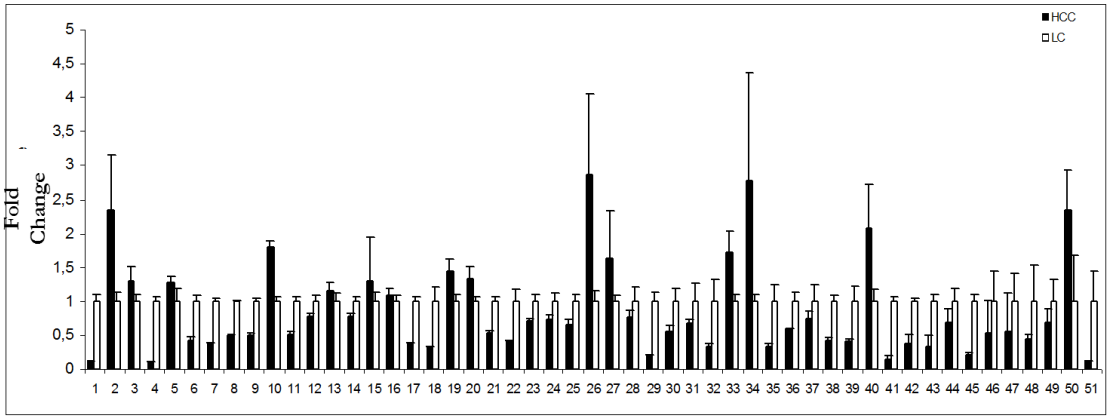

Fig.21: THRβ mRNA expression in human HCCs assessed by qRT-PCR. The levels were calculated as fold change difference between the expression in HCCs and peritumoural tissues.

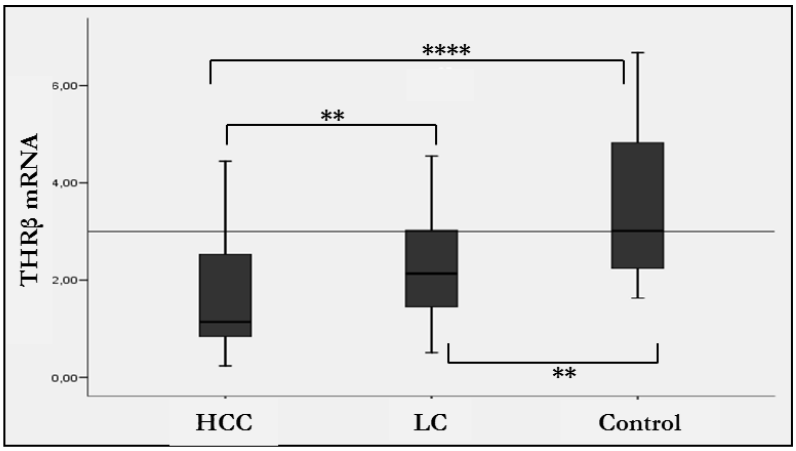

Fig.22: Expression of THRβ in human HCC, peritumoural tissue and normal liver evaluated by qRT-PCR.

Discussion

The role of THR genes in tumor development and progression is ambiguous. Indeed, while THRs act as oncogenes in several types of human cancers *[Barrera-Hernandez G et al, 1999; Hall LC et al, 2008; Plateroti M et al, 2006; Kress E et al, 2009]* , other reports have provided evidence that they play a tumor suppressive role *[Zhu XG et al, 2010; C Barlow et al, 1994]* . Notably, no information is available on the stage of the tumorigenic process at which deregulation of THRs may occur. The present study highlights the possible tumor suppressive role of THRs in HCC development. The main findings stemming from our work indicate that down-regulation of THRs, especially THRβ1, i) is a very early event in the multistage process of hepatocarcinogenesis, ii) is statistically significant in KRT-19+ preneoplastic lesions, which are considered the precursors of HCC in the R-H model, but not in those undergoing regression (KRT-19-), iii) occurs in about 80% of rat HCCs and iv) is observed in 77% or 71% of human HCCs when compared to normal livers or to peritumoral tissues, respectively.

Altogether, these results demonstrate that THR down-regulation is associated to HCC onset and progression, in a species- and aetiology-independent fashion, and suggest that they act as tumor suppressor genes. Indeed, the findings that down-regulation of THRβ1 is associated with a higher proliferative capacity of KRT-19+ lesions and that a significant decrease of THRβ1 also occurs in post-surgery liver regeneration, strongly support the concept that this receptor acts as a negative regulator of cell proliferation.

The observation that inhibition of the THRβ1-dependent pathway occurs in the most aggressive preneoplastic lesions (KRT-19+), suggests that their "hypothyroid status" may favour their evolution and progression to HCC. The relationship between hypothyroidism and cancer is unclear and it is still a matter of debate. Population-based case-control studies of risk factors associated to development of ovarian and pancreatic cancers found that a history of hyperthyroidism is associated with a twofold increase of cancer risk *[Ness et al, 2000; Ko et al, 2007]* . Moreover, hyperthyroidism was associated with more advanced clinical stage and higher risk of recurrence in prostate cancer *[Lehrer et al, 2002]* . Opposite results were obtained in two case-control studies where women with a history of hypothyroidism had a 2.8-fold higher risk of HCC *[Hassan et al, 2009]* and hypothyroidism was significantly more prevalent in patients with HCC of unknown etiology *[Reddy et al,*

2007] . These studies suggest that hypothyroidism can be a permissive factor for HCC development.

Searching for possible mechanisms responsible for THRβ1 down-regulation we ruled out hypermethylation of THRβ1 promoter in rat HCC. Moreover, we could not find any evidence of mutations of THRs, in spite of an unusual high percentage of mutations of THRα1 and THRβ1 (65% and 76%, respectively) previously reported in human HCC *[Lin KH et al., 1996; Lin KH et al, 1999]* .

However, our results are in agreement with two very recent studies based on whole exon deep-sequencing analysis, where no mutations of THRs have been detected in human HCC of different etiology *[Guichard C et al, 2012; Cleary SP et al, 2013; Joseph B et al, 2007]* . On the other hand, in rat HCC we observed an inverse relationship between THRβ1 expression and three miRNAs (miR-27a, miR-181a and miR-204), known to be direct regulators of TRs expression in other cell types *[Jazdzewski K et al, 2011; Nishi H et al, 2011; Master A et al, 2010]* . Notably, transduction of human HCC cells with these miRNAs inhibited THRβ1 expression, suggesting that up-regulation of these miRNAs may contribute to THRβ1 down-regulation also in HCCs.

The strong down-regulation of THRβ1 results in decreased expression of its classical target gene DIO1; in turns, DIO1 inhibition leads to the reduction of T_4 to T_3 conversion and to a negative loop causing local hypothyroidism. In this context, a recent report suggests that miRNAs targeting DIO1 in clear cell renal carcinoma influence intratumoral thyroid hormone levels *[Boguslawska J, 2014]*.

Indeed, these Authors demonstrate that tumor-specific changes in intracellular T_3 concentration correlate with changes of the DIO1-targeting miR-224 *[Boguslawska J, 2014]* . Notably, miR-224 is one of the most up-regulated miRNAs in KRT-19 positive preneoplastic lesions and HCCs *[Petrelli A et al, 2014]*. Collectively, these results, together with our present findings, provide evidence that specific miRNAs may contribute to local hypothyroidism in pre- and neoplastic lesions and to loss of the oncosuppressive activity of THRβ1. In agreement with this hypothesis, hyperthyroidism induced by T_3 administration is able to induce a rapid disappearance of preneoplastic lesions and to inhibit HCC development and lung metastasis *[Ledda-Columbano GM et al, 2000; Perra A et al, 2009]* , further supporting the concept that THRs act, indeed, as tumor suppressors in HCC development.

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