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# Local hypothyroidism favours the progression of rat preneoplastic lesions to HCC

Carla Frau<sup>1\*</sup>, Roberto Loi<sup>1\*</sup>, Annalisa Petrelli<sup>2</sup>, Andrea Perra<sup>1</sup>, Silvia Menegon<sup>2</sup>, Marta Anna Kowalik<sup>1</sup>, Silvia Pinna<sup>1</sup>, Vera Piera Leoni<sup>1</sup>, Francesca Fornari<sup>3</sup>, Laura Gramantieri<sup>3</sup>, Giovanna Maria Ledda-Columbano<sup>1</sup>, Silvia Giordano<sup>2§</sup>, Amedeo Columbano<sup>1§</sup>

<sup>1</sup>Department of Biomedical Sciences, Unit of Oncology and Molecular Pathology, University of Cagliari, Cagliari, Italy, <sup>2</sup>Department of Oncology, University of Torino School of Medicine, Candiolo Cancer Institute – FPO, IRCCS, 10060 Candiolo (Torino), Italy; <sup>3</sup>St.Orsola-Malpighi University Hospital, 40138, Bologna, Italy

\* these Authors equally contributed to the work

§ to whom correspondence should be addressed

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## Contact information:

Amedeo Columbano, PhD  
Department of Biomedical Sciences  
Unit of Oncology and Molecular Pathology  
University of Cagliari  
Via Porcell 4, 09124 Cagliari, Italy  
Phone: +39-070-6758345  
Fax: +39-070-666062  
e-mail: [columbano@unica.it](mailto:columbano@unica.it)

Silvia Giordano MD, PhD  
Department of Oncology  
University of Torino, Medical school

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Candiolo Cancer Institute – FPO, IRCCS Strada Provinciale 142  
Candiolo (Torino), 10060, Italy  
Phone + 39 0119933233  
Fax +39 011 9933225  
e-mail [silvia.giordano@unito.it](mailto:silvia.giordano@unito.it)

**List of Abbreviations:** T3, 3,5,3'-L-triiodothyronine; R-H model, resistant-hepatocyte model; TR $\alpha$ , thyroid hormone receptor- $\alpha$ ; TR $\beta$ , thyroid hormone receptor- $\beta$ ; HCC, hepatocellular carcinoma; Krt-19, cytokeratin-19; miRNA, microRNA; DENA, diethylnitrosamine; 2-AAF, 2-acetylaminofluorene; PH, partial hepatectomy; GSTP, placental glutathione S-transferase; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; BrdU, Bromodeoxyuridine; *Dio1*, deiodinase 1; *G6pc*, glucose-6-phosphatase; *Spot14*, thyroid hormone responsive spot14; *App*, amyloid precursor protein; *Dio3*, deiodinase 3; LC, liver cirrhosis; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

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## ABSTRACT

Thyroid hormone receptors (TRs) are ligand-dependent transcription factors that mediate most of the effects elicited by the thyroid hormone 3,5,3'-L-triiodothyronine (T3). TRs have been implicated in tumorigenesis, although it is unclear whether they act as oncogenes or tumor suppressors, and at which stage of tumorigenesis their dysregulation occurs. Using the Resistant-Hepatocyte rat model (R-H model), we found down-regulation of TR $\beta$ 1 and TR $\alpha$ 1 and their target genes in early preneoplastic lesions and HCCs, suggesting that a hypothyroid status favours the onset and progression of preneoplastic lesions to HCC. Notably, TR $\beta$ 1 and, to a lesser extent, TR $\alpha$ 1 down-regulation was observed only in preneoplastic lesions positive for the progenitor cell marker cytokeratin-19 (Krt-19) and characterized by a higher proliferative activity compared to the Krt-19 negative ones. TR $\beta$ 1 down-regulation was observed also in the vast majority of the analyzed human HCCs compared to the matched peritumorous liver or to normal liver. Hyperthyroidism induced by T3 treatment caused up-regulation of TR $\beta$ 1 and of its target genes in Krt-19<sup>+</sup> preneoplastic rat lesions and was associated with nodule regression. In HCC, TR $\beta$ 1 down-regulation was not due to hypermethylation of its promoter, but was associated with an increased expression of TR $\beta$ 1-targeting microRNAs (miR-27a, -181a and -204). An inverse correlation between TR $\beta$ 1 and miR-181a was also found in human cirrhotic peritumoral tissue compared to normal liver.

**Conclusion:** Down-regulation of TRs, especially TR $\beta$ 1, is an early and relevant event in liver cancer development, species- and etiology-independent. The results also suggest that a hypothyroid status of preneoplastic lesions may contribute to their progression to HCC and that the reversion of this condition may represent a possible therapeutic goal to interfere with the development of this tumor.

The thyroid hormones 3,5,3'-triiodo-L-thyronine (T3) and thyroxine (T4) influence a variety of physiological processes (1–2). Most of the effects of T3 are mediated by thyroid hormone nuclear receptors (TRs), which are members of the steroid/thyroid receptor superfamily of nuclear hormone receptors, acting as transcription factors (3-5). Two different TR subtypes, TR $\alpha$  and TR $\beta$ , have been identified as the products of distinct genes (6). Over the past few years a number of studies have highlighted an important role of TRs in cancer development; however, some works have shown that they behave as

oncogenes in several types of human cancer (7-9), while others have provided evidence for their tumor suppressive function (10-13).

As the most abundant TR in hepatic tissue (14), TR $\beta$ 1 has received particular attention for its role in the development of hepatocellular carcinoma (HCC). In this context, TR $\beta$ 1 expression was correlated with increased invasive ability of human HCC cell lines and decreased expression of the anti-metastatic gene *nm23* (15), suggesting its oncogenic role. On the other hand, transfection of TR $\beta$ 1 into human HCC cells reduced xenograft tumor growth in nude mice, promoted partial mesenchymal-to-epithelial transition, and attenuated tumor cell invasiveness (16). Support for a tumor suppressor role of TRs in HCC stems also from the findings that their mutated forms contribute to HCC growth and progression; indeed, mutated TR $\alpha$  (v-ErbA) leads to the development of hypothyroidism and HCC in male mice (17,18) while a high frequency of TRs mutations has been described in human HCC (19). Thus, the role of TRs in HCC is still unclear.

HCC is the third cause of cancer-related deaths worldwide, and is characterized by poor prognosis and few treatment options (20). The development of HCC is a multistep process. HCCs arise most frequently in the setting of chronic liver inflammation and fibrosis due to viral infection, metabolic injury, toxic insults, or autoimmune reactions. These tumors originate from premalignant lesions, ranging from dysplastic foci to dysplastic hepatocyte nodules that are often seen in damaged and cirrhotic livers, and are more proliferative than the surrounding parenchyma (21). Since no effective treatment for HCC exists and, upon diagnosis, most patients with advanced disease have a remaining lifespan of only 4–6 months, it is critical to detect molecular changes taking place in preneoplastic and neoplastic lesions and identify biomarkers and molecular targets useful for an early diagnosis and for therapy.

Unfortunately, the knowledge of molecular events occurring in early stages of HCC development is limited because of difficulties in the histomorphologic distinction between non-malignant nodular lesions (i.e., low-grade and high-grade dysplastic nodules) and early HCCs. Animal models allow the study of different stages of hepatocarcinogenesis, as discrete lesions at different stages of progression can be identified and analyzed; this permits the detection of molecular alterations present in early preneoplastic stages. Although the assessment of the translational value of animal models of human cancer poses a major challenge, the usefulness of the Resistant Hepatocyte (R-H) model (22) has

already been demonstrated; indeed, comparative functional genomics studies on micro-dissected early lesions showed that the gene expression signature associated with the progenitor cell marker cytokeratin-19 (Krt-19) can successfully predict the clinical outcome of human HCC (23). The finding that 78% of genes and 57% of miRNAs dysregulated in rat HCC are similarly altered in human HCC and that 76% of these genes are already dysregulated at very early stages 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 these changes occur (24).

Here we show that down-regulation of TR $\alpha$ 1 and TR $\beta$ 1 is an early event in the tumorigenic process, suggesting that a hypothyroid status of preneoplastic hepatocytes favors their progression to HCC. Our results also indicate that TRs, especially TR $\beta$ 1, act as tumour suppressors in HCC development and that they represent possible therapeutic targets to interfere with the development of this tumor.

## **Experimental Procedures 647**

### ***Animals and treatment***

Male Fischer rats were obtained from Charles River, Milano, Italy. Guidelines for Care and Use of Laboratory Animals were followed during the investigation. All animal procedures were approved by the Ethical Commission of the University of Cagliari and the Italian Ministry of Health. Animals were treated with a single dose of diethylnitrosamine (DENa, 150mg/kg) and, two weeks later, were subjected to the R-H protocol, consisting of a 2 weeks-diet supplemented with 0.02% 2-acetylaminofluorene (2-AAF) and a two-third partial hepatectomy (PH) (25). Rats were then switched to basal diet all throughout the experiment and sacrificed ten weeks and fourteen months after DENa administration. A T3-supplemented diet (4mg/kg of diet) was administered for 4 and 7 days to rats previously subjected to the R-H protocol, starting 5 weeks after 2-AAF withdrawal (For details, see Supp. Material)

### ***Cell cultures and in vitro experiments***

For cell cultures and in vitro experiments see Supp. Material.

### ***Histology and immunohistochemistry***

Liver sections were fixed in 10% formalin and included in paraffin or quickly frozen by immersion in liquid nitrogen and processed for hematoxylin-eosin, or GSTP and Krt-19 immunohistochemistry, as described (24). We considered as Krt-19 positive, all those lesions exhibiting a Krt-19 positive area of at least 5% of the total area of the preneoplastic lesion. The average area occupied by Krt-19 positive hepatocytes was 27% of the total area of the Krt-19 positive nodules microdissected for further analyses.

### ***Nucleic acid extraction, sequencing and qRT-PCR***

For nucleic acid extraction, sequencing and qRT-PCR see Supp. Material

### ***CpG methylation analysis***

The CpG island in the promoter of the TR $\beta$  gene was localized by gathering evidence collected from the UCSC Genome Browser (<http://genome.ucsc.edu/>) and analyzed for cytosine methylation in 15 HCC samples previously subjected to gene expression analysis (Details in the Supp. Material).

### ***Laser Capture Microdissection***

GSTP-positive, Krt-19-positive nodules were identified by immunohistochemical staining of 6 $\mu$ m-thick frozen liver sections. Nodules microdissection was done on 16 $\mu$ m serial sections with a Leica LMD6000, as previously described (24).

### ***Patients***

HCC and cirrhotic tissues were obtained from 52 consecutive patients (males and females, median age  $\pm$  SD: 67.2  $\pm$  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 cases of focal nodular hyperplasia (FNH) and one traumatic liver lesion). Tissues were collected at surgery after obtaining an informed consent, then immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. Histopathological grading was scored according to Edmondson and Steiner criteria (26). No patient received anticancer treatment prior to surgery. The characteristics of patients are detailed in **Supp.Table 2**.

### ***Statistics***

Statistical analyses were performed as reported in the Supporting Material.



## RESULTS

### ***No mutations of TR genes occur in rat HCC***

It was reported that mutations of either TR $\alpha$  or TR $\beta$  occur in 65-75% of human HCCs and that mutant forms of TRs aberrantly interact with either co-repressors or co-activators (19). In contrast, recent deep sequencing analyses of human HCCs did not report any mutation of these receptors (27,28). We thus investigated whether TR mutations were present in rat HCCs. Sequence analysis of exons and the adjacent splicing regions of TR $\alpha$  and TR $\beta$  genes was performed in 10 rat HCCs generated by the R-H protocol. No mutation in any of the TR genes was observed in the analyzed HCCs (**Supp. Table 1**).

### ***TR $\alpha$ and TR $\beta$ are down-regulated in rat HCC***

Alternative to mutational inactivation, dysregulation of TR expression could be a mechanism by which these receptors play a role in the carcinogenic process (29). To establish whether TRs were abnormally expressed in rat HCCs, we analyzed by qRT-PCR the expression levels of the TR $\alpha$ 1 and TR $\beta$ 1 isoforms in 31 HCCs isolated 14 months after DENA treatment, including the previously sequenced ones.

As shown (**Fig. 1A,B**), neoplastic lesions exhibited a significant decrease of the TR $\alpha$ 1 and TR $\beta$ 1 isoforms, compared to normal liver ( $P < 0.01$  for TR $\alpha$ 1;  $P < 0.0001$  for TR $\beta$ 1) and to liver of age-matched rats exposed to AAF-PH in the absence of DENA. Notably, down-regulation of TR $\beta$ 1 was consistently stronger than that of TR $\alpha$ 1. In agreement with mRNA levels, western blot analysis showed a decreased content of the TR $\beta$ 1 protein in HCC compared to normal liver (**Fig. 1C**). Moreover, decreased levels of target genes positively regulated by TR $\beta$ 1, such as *Dio1*, *G6pc* and *Spot14* (30), were observed in HCCs (**Fig. 1D**). Accordingly, *App*, a gene negatively regulated by TR $\beta$ 1 (31), was significantly up-regulated in the same tumors (**Fig. 1D**). Notably, while the expression of *Dio1*, involved in the conversion of tyroxine to T3, was severely down-regulated, the expression levels of *Dio3* (the major physiologic thyroid hormone inactivator, whose re-activation in adult tissues has been correlated with some solid tumors in humans) (32,33), were not modified in HCC (**Fig. 1D**). As expected, *Dio2* was almost undetectable both in normal liver as well as in HCC (data not shown).

### ***Down-regulation of TRs is an early event in hepatocarcinogenesis***

The R-H model allows dissecting the different steps of the carcinogenic process, as phenotypically distinct lesions can be identified at well-defined timings. To determine whether TR down-regulation is an early event during liver carcinogenesis, we laser micro-dissected preneoplastic lesions developed 10 weeks after initiation, identified by their positivity for the placental form of Glutathione S-transferase (GSTP).

As shown in **Fig. 2A**, a significant reduction of expression of TR isoforms was observed in these lesions, compared to the liver of control rats. Notably, similar to HCC, down-regulation of TR $\beta$ 1 was more significant than that of TR $\alpha$ 1. We conclude that down-regulation of TRs matches the onset of hepatic preneoplastic lesions and that continued down-regulation of TRs expression is likely necessary for the maintenance and progression of these lesions.

***TRs are down-regulated and poorly active in a subset of nodules identified by their positivity for Krt-19***

During the carcinogenic process, in the R-H model, a slow but continuous regression of the vast majority of the preneoplastic lesions occurs through remodeling (34). Our previous studies have shown that most HCCs arising in this model are Krt-19<sup>+</sup>, although only a minority of preneoplastic lesions developed at early stages of the process are positive for this marker (24). This suggests that Krt-19<sup>+</sup> lesions have an advantage in the progression to malignancy. Therefore, we wondered whether down-regulation of TRs occurs in all preneoplastic populations or it is specific for the Krt-19<sup>+</sup> one. To this aim, we microdissected both Krt-19 positive and negative nodules and analyzed TRs expression. The results showed that TRs were significantly down-regulated only in Krt-19<sup>+</sup> preneoplastic lesions (**Fig. 2B,C**). In agreement with what observed in HCCs, down-regulation of TR $\beta$ 1 was much more pronounced than that of TR $\alpha$ 1. Therefore, subsequent studies were focused on TR $\beta$ 1.

To assess whether down-regulation of TR $\beta$ 1 also reflects a reduced transcriptional activity of this receptor, we determined the expression of TR $\beta$ 1-target genes by qRT-PCR. Similarly to what observed in HCCs, all the target genes were profoundly down-regulated in KRT-19<sup>+</sup> lesions when compared to control liver or to Krt-19<sup>-</sup> preneoplastic nodules (**Fig. 3A**).

***TR $\beta$ 1 down-regulation is associated with proliferation of normal and preneoplastic hepatocytes***

Since Krt-19<sup>+</sup> preneoplastic lesions are the HCC precursors and display the most significant down-regulation of TRβ1 expression, we sought to determine whether preneoplastic hepatocytes of Krt-19<sup>+</sup> nodules were endowed with enhanced proliferative capacity. As shown in **Fig. 3B** and **Supp. Fig. 1A**, Krt-19<sup>+</sup> lesions had a much higher proliferative activity than Krt-19<sup>-</sup> preneoplastic nodule hepatocytes. Notably, a highly significant down-regulation of TRβ1 expression was detected also in another condition of high hepatocyte proliferation, namely liver regeneration following 2/3 partial hepatectomy (**Fig. 3C** and **Supp. Fig. 1B**). These data suggest the suppressive role of TRβ1 in both normal and preneoplastic proliferating hepatocytes.

### ***TRβ silencing enhanced proliferation of HCC cells in vitro***

To directly prove the suppressive role of TRβ1 on cell proliferation, we moved to *in vitro* experiments. TRβ1 expression was determined in human HCC cell lines. (**Supp. Fig. 2**). For further studies we selected Mahlavu and HepG2 cells displaying different levels of TRβ. TRβ silencing enhanced cell growth in Mahlavu cells (**Fig. 4A**, **Supp. Fig. 3A**) and boosted cell migration of HepG2 cells (**Fig. 4B**, **Supp. Fig. 3B**). These data further support the notion that TRβ1 is a negative regulator of cell proliferation.

### ***TRβ1 down-regulation is not due to CpG island methylation***

Gene promoter methylation is a well-known mechanism responsible for the silencing of many genes (35). Therefore, we determined whether changes in the methylation status of the CpG island of the TRβ1 promoter could be responsible for its down-regulation in the R-H model. However, very low levels of methylation of the TRβ1 CpG island were detected in the analyzed HCCs, with no significant differences compared to controls (**Supp. Table 3**). Thus, it is very unlikely that TRβ1 down-regulation is the consequence of a hypermethylated status.

### ***TRβ1-targeting miR-27a, miR-181a and miR-204 are highly expressed in rat HCC***

Since miRNAs play a fundamental role in the control of gene expression (36), we investigated whether their increased expression could be responsible for TRβ1 down-regulation in HCC. Initially, in rat HCCs, we analyzed the expression of miRNAs (miR-21, miR-27a, miR-181a, miR-221, miR-146a and miR-204) demonstrated to target TRβ1 in different cell types (37-39). As shown in **Fig. 4C**, miR-27a, miR-146a, miR-181a and miR-204 were up-regulated in rat HCCs displaying TRβ1 down-regulation. We then analyzed

the expression of TR $\beta$  and miR-27a - the miRNA statistically most dysregulated in rat HCCs ( $P = 0.0036$ ) - in five HCC cell lines (**Fig. 4D**). Interestingly, an inverse relationship between the expression of miR-27a and TR $\beta$  was observed in the HCC cell lines examined ( $R^2 = 0.67$ ) (**Fig. 4D**), suggesting that this miRNA might negatively regulate TR $\beta$  expression in HCC cells. Indeed, when we transfected HuH7, HepG2 and Mahlavu cells with a miR-27a mimic, we observed a decrease of TR $\beta$  expression (**Fig. 4E**).

#### ***TR $\beta$ 1 is down-regulated in human HCC and in precancerous peritumoral tissue***

Next, we determined whether the results found in the R-H model could be of translational value for human HCC. Therefore, TR $\beta$ 1 mRNA levels were determined in a cohort of 52 human HCCs (the characteristics of the study population are described in **Supp.Table 2**) and the corresponding peritumoural liver tissues. Similarly to what observed in rats, human HCCs showed down-regulation of TR $\beta$ 1 compared to matched cirrhotic tissues (LC) (**Fig. 5A**). Mean expression in HCCs vs. LCs vs. normal livers (n. 12) was:  $1.65 \pm 1.10$  vs.  $2.34 \pm 1.19$  ( $P < 0.01$ ) vs.  $3.59 \pm 1.76$  ( $P < 0.0001$ ) (**Fig. 5B**). Overall, TR $\beta$ 1 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. No significant difference in TR $\alpha$  mRNA levels was found between HCCs and cirrhotic as well as normal liver tissues (data not shown). No difference was found between patients displaying TR $\beta$ 1 downregulation and the others in term of clinical characteristics such as age, etiology, tumor focality, tumor size, AFP serum levels or grading.

Cirrhotic tissue surrounding HCC might be considered as a precancerous tissue, on the basis of the “field effect”. Therefore, we investigated whether the expression of TR $\beta$ 1 and its target genes *DIO1* and *G6PC* was dysregulated in precancerous tissue vs. control liver. The results (**Fig. 5C**) showed a significant down-regulation of *DIO1* and *G6PC* in peritumoral cirrhotic tissue compared to control liver ( $P < 0.001$  and  $P < 0.0001$ , respectively), suggesting that dysregulation of TR $\beta$ 1 signaling is an early event also in human hepatocarcinogenesis.

#### ***MiR-181a is up-regulated in human cirrhotic peritumoral tissue***

Based on the findings of up-regulation of TR $\beta$ 1-targeting miRNA in rat HCC we assessed the expression of miR-181a, -27a and -204 in liver cirrhotic tissue. As shown in **Fig. 5D**, miR-181a was significantly up-regulated in liver cirrhosis vs. control liver ( $P = 0.04$ ).

Although statistical significance was not reached for miR-27a and miR-204, both of them showed a trend towards an increase of expression (fold change vs. control liver was 1.6 and 1.5, respectively). Therefore, dysregulation of these miRNAs might contribute to the decrease of TR $\beta$ 1 expression at early stages of human HCC development.

### ***Treatment with T3 resulted in up-regulation of TR $\beta$ 1 and of its target genes***

The results obtained in the R-H model suggest that the local hypothyroid status of preneoplastic lesions may favor their progression to HCC. To test this hypothesis, hyperthyroidism was induced in nodule-bearing rats by treatment with T3 and the expression of TR $\beta$ 1 and of its target genes was determined in Krt-19<sup>+</sup> preneoplastic lesions. As shown in **Fig. 6A**, a 4-day treatment with T3 resulted in increased expression of TR $\beta$ 1 and of its target genes. In agreement with previous studies (40), the switch from hypo- to hyperthyroid status caused by T3 treatment resulted in the regression of the preneoplastic lesions 7 days after treatment (**Fig. 6B**). Regression involved both Krt-19 negative as well as Krt-19 positive nodules, but preferentially the latter type of lesions (**Supp. Table 4**). Notably, an increased expression of TR $\beta$  after treatment with T3 was also observed in HCC cell lines (**Fig. 6C**). No effect of T3 was observed when TR $\beta$  was silenced, indicating the TR $\beta$ -dependency of this effect.

## **Discussion**

The role of TR genes in tumor development and progression is ambiguous. Indeed, while TRs act as oncogenes in several types of human cancers (7-9), other evidences point to a tumor suppressive role (10-13). The main findings stemming from our work indicate that down-regulation of TRs, especially TR $\beta$ 1, i) is a very early event in the multistage process of rat hepatocarcinogenesis and in human HCC development, ii) is statistically significant in Krt-19<sup>+</sup> preneoplastic lesions (considered the precursors of HCC in the R-H model) but not in Krt-19<sup>-</sup> (undergoing spontaneous regression), iii) occurs in 84% of rat HCCs and 77% or 71% of human HCCs, when compared to normal livers or to peritumoral tissues, respectively.

Altogether, these results demonstrate that TR $\beta$ 1 down-regulation is associated to HCC onset and progression, in a species- and aetiology-independent fashion, and suggest that it acts as a tumor suppressor gene. Indeed, TR $\beta$ 1 silencing in HCC cells resulted in promotion of cell growth and migration. In the same line, down-regulation of TR $\beta$ 1 was

observed in Krt-19<sup>+</sup> lesions, endowed with a higher proliferative capacity, and in the post-surgery regenerating liver. These data strongly suggest that this receptor acts as a negative regulator of cell proliferation.

The observation that the inhibition of the TR $\beta$ 1-dependent pathway occurs in the most aggressive preneoplastic lesions (Krt-19<sup>+</sup>), suggests that the “hypothyroid status” of these lesions may favor their evolution and progression to HCC. The relationship between hypothyroidism and cancer is unclear and it is still a matter of debate (for a review, see 41); indeed, odds ratio for ovarian and pancreatic cancer almost doubled in patients with a history of hyperthyroidism (42,43). Moreover, hyperthyroidism was associated with more advanced clinical stage and higher risk of recurrence in prostate cancer patients (44). 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 (45) and hypothyroidism was significantly more prevalent in patients with HCC of unknown etiology (46). The latter studies suggest that hypothyroidism can be a permissive factor for HCC development. Our present data showing that a local hypothyroid condition characterizes the cirrhotic peritumoral tissue, considered to be a pre-cancerous stage, further support the role of hypothyroidism in human HCC.

Searching for possible mechanisms responsible for TR $\beta$ 1 down-regulation, we ruled out hypermethylation of TR $\beta$ 1 promoter in rat HCC. Moreover, we could not find any evidence of mutations of TRs, in spite of a high percentage of mutations of TR $\alpha$  and TR $\beta$  previously reported in human HCC (19); in this context, our results are in agreement with two very recent studies based on whole exon deep-sequencing analysis, where no mutation of TRs has been detected in human HCC from different geographic areas (27,28). We also investigated whether TR $\beta$ 1 down-regulation could be due to up-regulation of TR $\beta$ 1-targeting miRNAs. In this respect, in rat HCC, we observed an inverse relationship between TR $\beta$ 1 expression and four miRNAs (miR-27a, miR-146a, miR-181a and miR-204), known to directly regulate TR $\beta$  expression in other cell types (37-39). Notably, a similar inverse relationship between TR $\beta$ 1 and miR-181a expression was observed also in human cirrhotic peritumoral tissue. Moreover, transduction of human HCC cells with miR-27a, the most significantly up-regulated miRNA in rat HCC, inhibited TR $\beta$ 1 expression, suggesting that an increase of specific miRNAs may contribute to TR $\beta$ 1 down-regulation in HCCs.

Irrespectively of the mechanism(s) responsible for TR $\beta$ 1 down-regulation, our

results showed a strongly decreased expression of TR $\beta$ 1 and of its classical target gene *DIO1* at early stages of hepatocarcinogenesis; in turns, *DIO1* inhibition leads to reduced T4 to T3 conversion and to a negative loop causing local hypothyroidism. Our data - suggesting the relevance of the hypothyroid status in cancer development - are in accordance to a report showing that miRNAs targeting *DIO1* in clear cell renal carcinoma influence intratumoral thyroid hormone levels (39). Indeed, these authors demonstrate that tumor-specific changes in intracellular T3 concentration correlated with changes of the *DIO1*-targeting miR-224 (47). Notably, miR-224 is one of the most up-regulated miRNAs in Krt-19+ preneoplastic lesions and HCCs (24). Collectively, these and 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 TR $\beta$ 1. In agreement with the possible critical role of local hypothyroidism in HCC development, T3 administration rapidly induces a switch from hypothyroid to hyperthyroid status of preneoplastic lesions and is associated with disappearance of preneoplastic lesions and with inhibition of HCC development and lung metastases (40).

In conclusion, the present work presents evidence that TR $\beta$ 1 acts as a tumor suppressor in very early steps of HCC development and poses the challenging question as to whether reversion of a local hypothyroid condition could offer a possible therapeutic tool to interfere with the progression of this tumor.

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## LEGEND TO FIGURES

**FIGURE 1. TR $\alpha$ 1 and TR $\beta$ 1 are down-regulated in rat HCCs. A, B)** Left panels: TR $\alpha$ 1 and TR $\beta$ 1 expression was assessed by quantitative TaqMan RT–PCR in control liver, in the liver of rats exposed to AAF/PH and in 31 HCCs. Right panels: the average levels of TR $\alpha$ 1 and TR $\beta$ 1 were calculated as fold change difference compared to control livers.  $\beta$ -actin was used as endogenous control. Error bars represent SEM. Significantly different from control for \*\*  $P < 0.01$ ; \*\*\*\*  $P < 0.0001$ . NS: not significant. **C)** Western blot analysis of TR $\beta$ 1 in rat HCC.  $\beta$ -actin was used as housekeeping gene. **D)** Expression of TR $\beta$ 1 target genes and *Dio3* in HCC vs. control. Significantly different from control for \*\*\*\*  $P < 0.0001$ ; \*\*\*  $P < 0.001$ ; \*\*  $P < 0.01$ . NS: not significant.

**FIGURE 2. TR $\alpha$ 1 and TR $\beta$ 1 are down-regulated in early preneoplastic nodules. A)** TR $\alpha$ 1 and TR $\beta$ 1 expression was assessed as indicated in Legend to Fig. 1 in preneoplastic nodules developed 10 weeks after DENA treatment. The mRNA average levels of TRs were calculated as fold change difference between the expression in 37 GSTP-positive nodules and 6 controls.  $\beta$ -actin was used as endogenous control. Error bars represent SEM. \*\*\*  $P < 0.001$ ; \*\*\*\* $P < 0.0001$ . **B,C)** Left panels: TR $\alpha$ 1 and TR $\beta$ 1 expression was assessed by quantitative TaqMan RT-PCR in control livers and in Krt-19<sup>-</sup> and Krt-19<sup>+</sup> preneoplastic lesions. Right panels: average levels of TR $\alpha$ 1 and TR $\beta$ 1 calculated as fold change difference compared to controls. \*\*\*\*  $P < 0.0001$ ; \*\*\*  $P < 0.001$ ; \*  $P < 0.05$ .

**Figure 3. TR $\beta$ 1 is down-regulated in preneoplastic and normal proliferating hepatocytes. A)** The expression of three TR $\beta$ 1 target genes, *Dio1*, *Spot14* and *G6pc*, was evaluated in 37 laser-microdissected rat nodules positive or negative for Krt-19 and compared to normal liver (control). A striking down-regulation of TR $\beta$ 1-positively regulated target genes was detected in the Krt-19<sup>+</sup> preneoplastic lesions. **B)** Top: Representative microphotographs illustrating BrdU incorporation in GSTP<sup>+</sup> preneoplastic nodules positive and negative for Krt-19. Dashed area demarcates a Krt-19-negative nodule (original magnification 20X); Bottom: microphotographs (original magnification 40X) showing higher power views of nodules depicted in the top panel. Stainings were performed on serial sections. **C)** TR $\beta$ 1 expression in the liver of rats subjected to 2/3 PH and sacrificed at different time intervals. TR $\beta$ 1 expression was assessed by quantitative TaqMan RT-PCR. The levels were calculated as fold change difference between the expression in operated rats and controls.  $\beta$ -actin was used as endogenous control. Error bars represent SEM of technical triplicates. \*\*\*\*  $P < 0.0001$ ; \*\*\*  $P < 0.001$ ; \*\*  $P < 0.01$ ; \*  $P < 0.05$ ; NS, not significant.

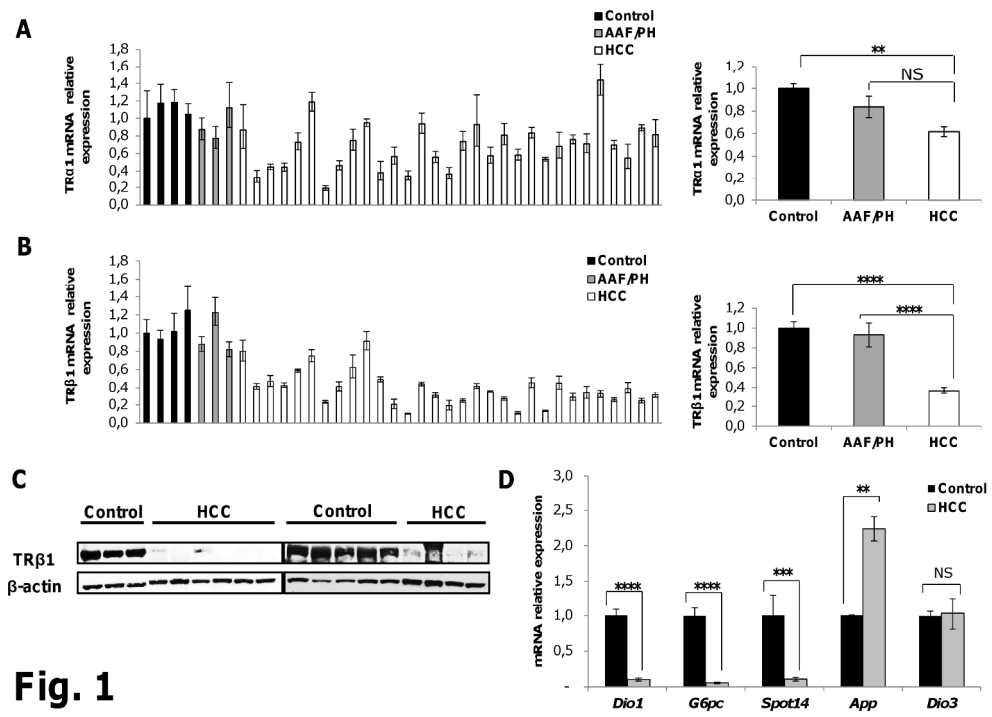
**FIGURE 4. TR $\beta$  is down-regulated by microRNAs.** Effect of TR $\beta$  silencing on **A)** cell viability of Mahlavu cells and **B)** migration ability of HepG2 cells, transfected with a control siRNA (siC) or with a TR $\beta$  siRNA (siTR $\beta$ ). **C)** Expression of selected miRNAs in rat HCC. MiRNA expression was evaluated by quantitative TaqMan RT-PCR and reported as fold change difference between HCCs and age-matched control livers. 4.5S was used as endogenous control. Error bars represent SEM of 20 rat HCCs and 8 age-matched

controls. **D)** Left panels: expression of miR-27a and of TR $\beta$  in 5 different HCC cell lines. Scatter plot indicating an inverse correlation between miR-27a and TR $\beta$ . **E)** HCC cell lines (HuH7, Mahlavu, HepG2) were transfected with a miR-27a mimic or a control siRNA (siC). TR $\beta$  expression was determined by Taqman RT-PCR. \*\*\*  $P < 0.001$ ; \*\*  $P < 0.01$ ; \*  $P < 0.05$ .

**FIGURE 5. TR $\beta$ 1 is down-regulated in human HCCs.** **A)** TR $\beta$ 1 expression in human HCCs. The levels were calculated as fold change difference between the expression in HCCs and peritumoural livers. Error bars represent the standard deviation of technical triplicates.  $\beta$ -actin was used as endogenous control. LC: liver cirrhosis. **B)** Expression of TR $\beta$ 1 in human HCC, peritumoral tissue and normal liver. TR $\beta$ 1 mRNA levels were evaluated by quantitative RT-PCR. **C)** *DIO1* and *G6PC* expression in human LC. The levels were calculated as fold change difference between the expression in peritumoral LC vs. normal liver. **D)** Expression of miR-181a, miR-27a and miR-204 in LC and normal control liver. The levels were reported as fold change difference between LC and normal control liver. RNU6B was used as endogenous control. Error bars represent SD of 52 cirrhotic liver samples and 12 patients devoid of cirrhosis. Significantly different from control for \*\*\*\*  $P < 0.0001$ ; \*\*\*  $P < 0.001$ ; \*\*  $P < 0.01$ .

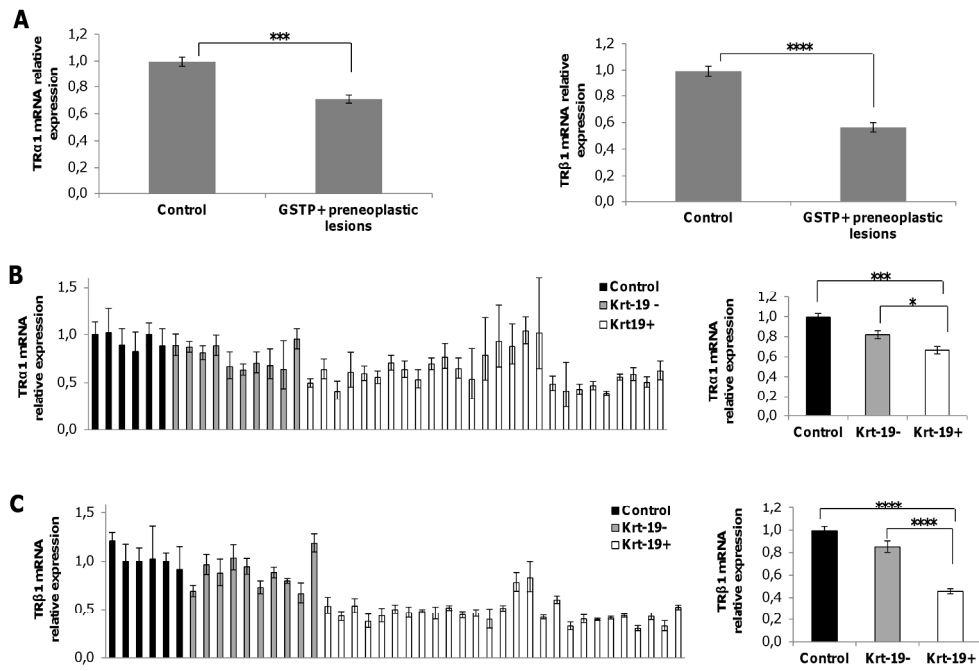
**FIGURE 6. T3 treatment results in up-regulation of TR $\beta$ 1 and of its target genes.**

**A)** Effect of T3 treatment on TR $\beta$ 1 (left panel) and on its target genes (right panel) in Krt-19<sup>+</sup>-preneoplastic lesions. Nodule-bearing rats 10 weeks after DENA treatment were fed a T3-supplemented diet (4 mg/kg) for 4 days. TR $\beta$ 1, *Dio1*, *G6pc* and *Spot14* mRNA levels were evaluated by quantitative TaqMan RT-PCR.  $\beta$ -actin was used as endogenous control. Error bars represent SEM of 4-5 nodules/group. **B)** Macroscopic view of livers from rats with or without T3 treatment for 7 days. Note the translucent spots on the surface of untreated rats and their almost total disappearance in rats receiving T3 for 7 days. **C)** Effect of T3 on TR $\beta$  expression in HuH7 and Mahlavu cells transfected with a control siRNA (siC) or a TR $\beta$  siRNA (siTR $\beta$ ). Cells were treated with 100 nM or 1 $\mu$ M T3 for 72 hours.



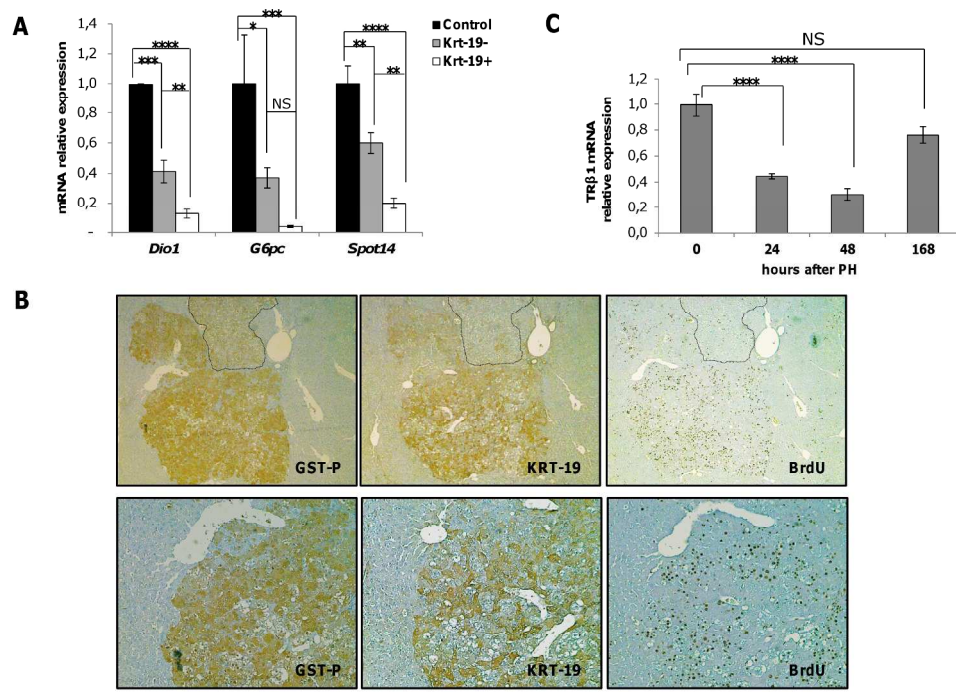
**Fig. 1**

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**Fig. 2**

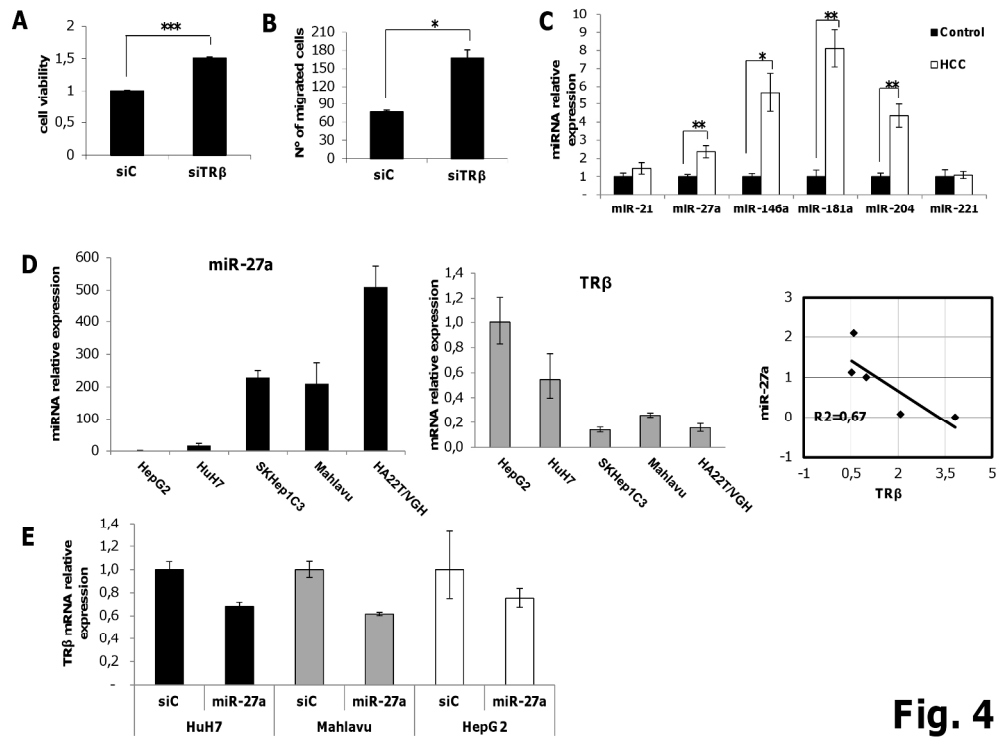
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**Fig. 3**

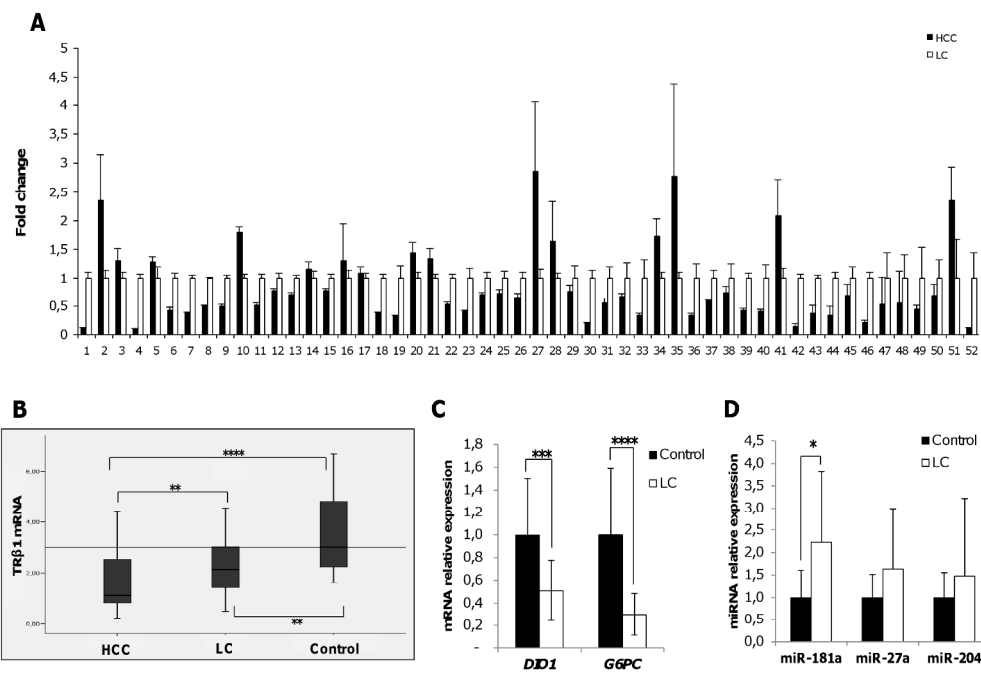
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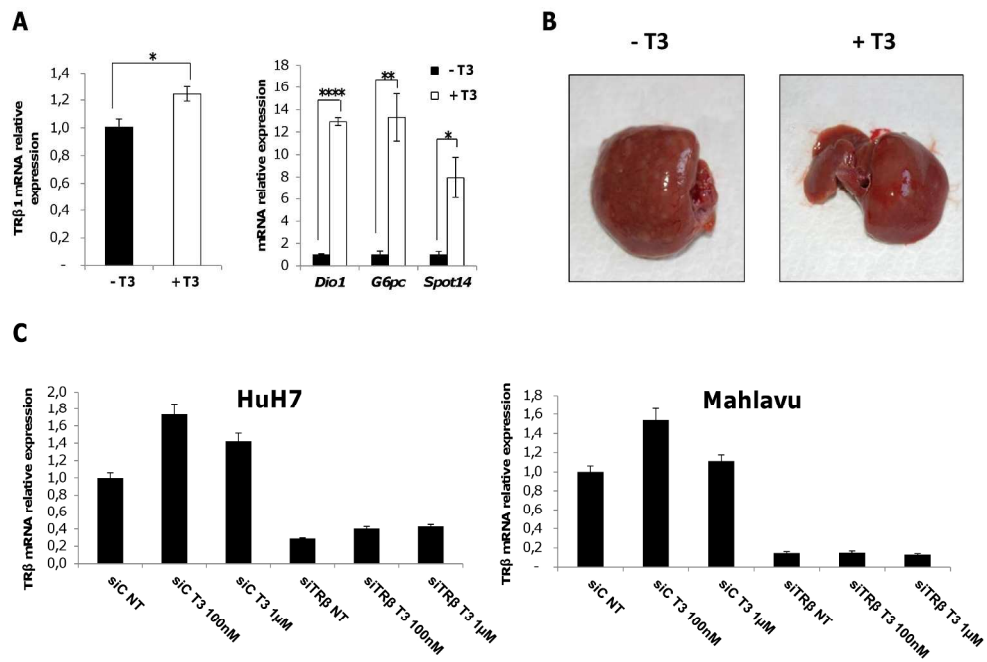
**Fig. 4**

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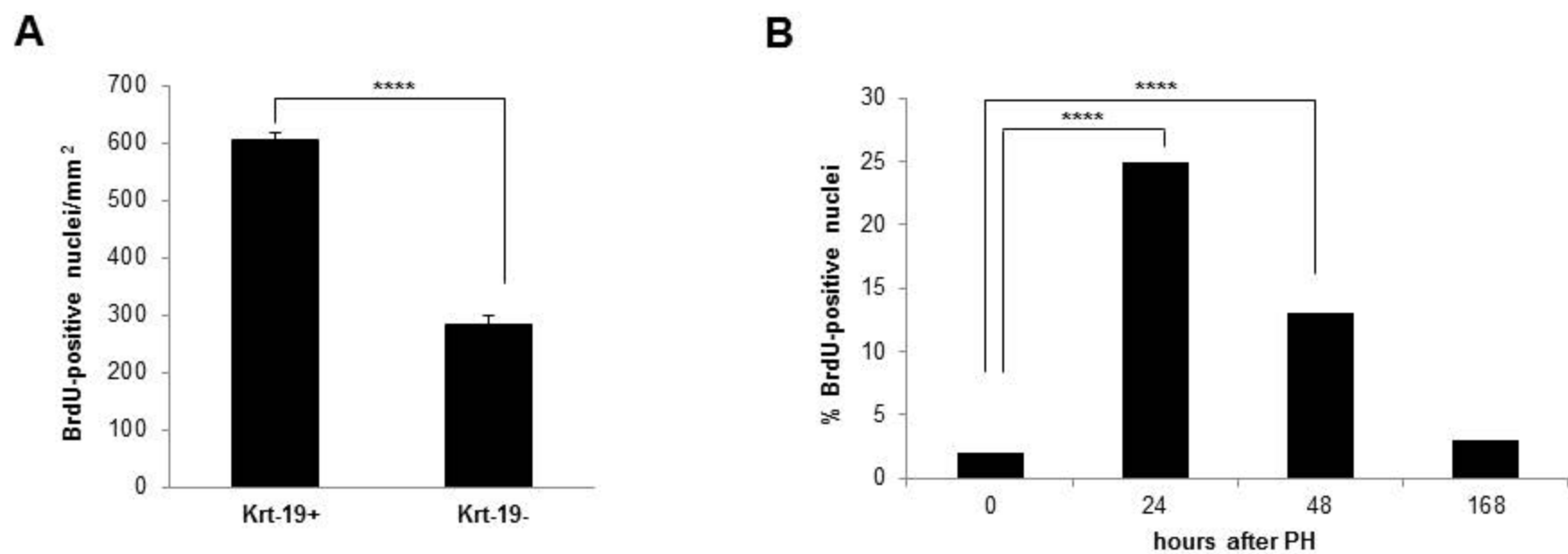
**Fig. 5**

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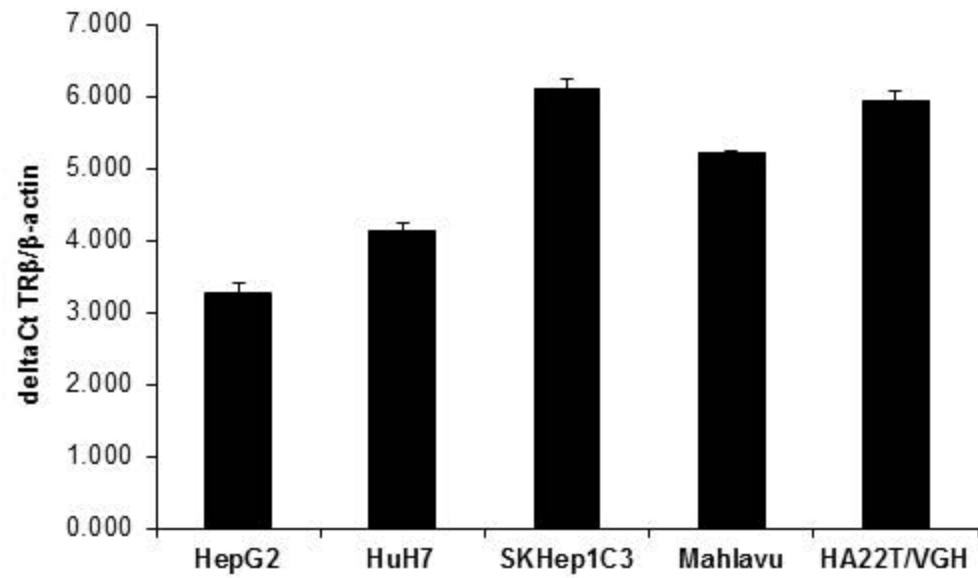


**Fig. 6**

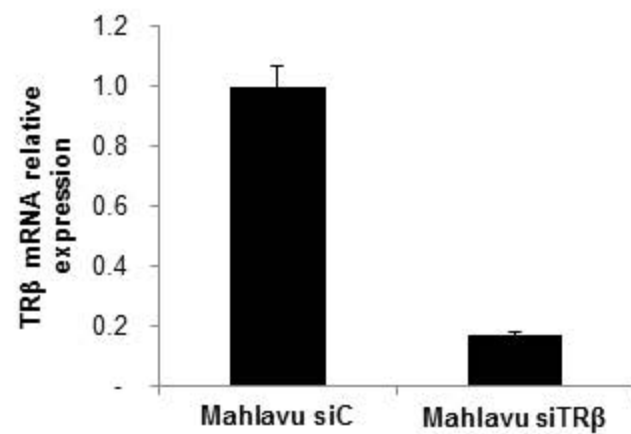
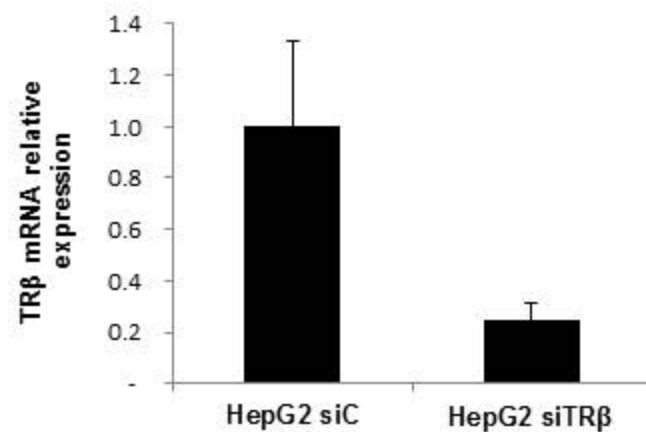
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**Fig. S1**



**Fig. S2**

**A****B****Fig. S3**

## SUPPLEMENTAL MATERIAL

### ***DNA extraction and sequencing***

Genomic DNA was extracted from HCCs isolated from livers of rats subjected to the R-H model and from normal control livers with QIAamp DNA Mini Kit, (QIAGEN, Valencia, CA), according to the manufacturer's instructions. DNA sequences corresponding to coding exons and adjacent intronic sequences of TR $\alpha$ 1, TR $\alpha$ 2, TR $\beta$ 1 and TR $\beta$ 2 were amplified by PCR on a GENEAMP PCR System 9700 (Applied Biosystems) using HF Platinum Taq DNA Polymerase (Life Technologies). Oligos were designed with the software "Primer 3". (**Supp.Table 1** and **Supp. Material**). After an initial denaturation cycle of 5 minutes at 94°C, 35 cycles were performed according to the following scheme: denaturation at 94°C for 30 seconds, annealing at 61°C for 30 seconds, extension at 68°C for 1 minute. The reaction was concluded after a further extension step at 68°C for 7 minutes. PCR products were separated by 2% agarose gel electrophoresis. DNA bands corresponding to the amplified products were revealed by ethidium bromide staining. The size of the amplified bands was determined by comparison with molecular weight markers (100bp and 1kb, Promega, Madison, WI). The bands corresponding to the amplified DNA, each corresponding to an exon plus the adjacent intronic sequence, were removed from the gel and then subjected to DNA extraction (MinElute Gel Extraction Kit, Qiagen). DNA was sequenced with the Dye Terminator method. High resolution vertical electrophoresis was performed at BMR Genomics (Padua, Italy). DNA sequences were analyzed with the software Chromas Pro. (Technelysium, South Brisbane, Australia).

### ***RNA extraction and qRT-PCR***

Total RNA was extracted from preneoplastic lesions with the MirVana kit (Life Technologies, Monza, Italy) and stored at -80°C until needed. RNA was quantified by NanoDrop ND1000 (Thermo Scientific, Waltham, MA), while RNA integrity was assessed by Agilent Bioanalyzer 2100 (Milano, Italy). Only RNA samples with a RIN (RNA Integrity Number)  $\geq 7$  were included in the study. For HCCs and human HCC cell lines, RNA was extracted with RNeasy Plus Mini Kit (QIAGEN) or with TRizol RNA isolation reagent (Life Technologies). RNA was retrotranscribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Life Technologies) using random primers. The levels of expression of the thyroid hormone receptors were evaluated by real time PCR analysis with an ABI PRISM 7300HT thermocycler (Applied Biosystems) on 31 samples of advanced HCC and on 37 samples of GSTP-positive preneoplastic nodules.

The following TaqMan probes were used for the analysis: Rn 01464144\_m1 for the TR $\alpha$ 1 isoform, Rn 01537799\_m1 for TR $\beta$ 1, Rn 00689876\_m1 for *G6pc*, Rn 01511034\_m1 for *Spot14*, Rn 00572183\_m1 for *Dio1*, Rn 00570673 for *App*, Rn 00568002\_s1 for *Dio3*, hs00230861\_m1 for human TR $\beta$ 1. The housekeeping gene  $\beta$ -actin was used for normalization of gene expression. For human studies, total RNA was extracted by using Trizol (Life Technologies, Grand Island, NY) according to the manufacturers' instructions. Real Time PCR and RT-PCR quantification (IQ SYBR Green Supermix, Bio-Rad Laboratories, Hercules, CA) of *THRA1* (NM\_001190918), *THRB1* (NM\_000461), *DIO1* (NM\_000792) and *G6PC* (NM\_000151) genes was performed by using the following primers: **(THRA)**: forward TGGACAAAGACGAGCAGTG; reverse AGGAATAGGTGGGATGGAGG; **(THRB)**: forward CATCAAACTGTCACCGAAGC; reverse TCCAAGTCAACCTTTCCACC; **(DIO1)**: forward ACACCATGCAGAACCAGAG reverse AGAACAGCACGAACTTCCTC. **(G6PC)**: forward AGGGAAAGATAAAGCCGACC; reverse AGAACAGCACGAACTTCCTC. **(G6PC)**: forward AGGGAAAGATAAAGCCGACC; reverse AGCAAGGTAGATTCGTGACAG. A Prime Time qPCR Assays was used for  $\beta$ -actin (ACTB) determination (primeTime qPCR assay, IDT, Coralville, IA). (b-actin): forward ACCTTCTACAATGAGCTGCG; reverse CCTGGATAGCAACGTACATGG; probe: 5'-/56-FAM-ATCTGGGTC/ZEN/ATCTTCTCGCGGTTG/3IABkfq/3'. Comparative CT method was used to calculate the mRNA expression level in all samples. Real Time experiments were performed in triplicate. To evaluate the expression levels of the microRNAs the following TaqMan MicroRNA Assays were used: hsa-miR-21 ID: 000397, hsa-miR-27a ID: 000408, hsa-miR-146a ID: 000468, hsa-miR-181a ID: 000480, hsa-miR-221 ID: 000524, hsa-miR-204 ID: 000508. Each sample was analyzed in triplicate. 4.5S (rat), RNU48 and RNU6B (human) were used for normalization of miRNA expression. Expression levels of the different isoforms were evaluated with the  $\Delta\Delta Ct$  method and represented as relative expression compared to a calibrator control.

**Western blot analysis:** western blot analysis was performed as described (24). Anti-TR $\beta$ 1 primary antibody (J52, Pierce Biotechnology, Rockford, IL) was used. B-actin (Sigma-Aldrich) was used as a loading control.

### **CpG methylation analysis**

Promoter CpG methylation was assessed by pyrosequencing. 2 $\mu$ g of DNA were bisulfite-converted with Epiect Bisulfite kit (Qiagen). Briefly, after addition of Bisulfite Mix (85ml), DNA protect Buffer (35 $\mu$ l) and water to a final volume of 140 $\mu$ l, the bisulfite reaction was performed in a thermal cycler (9700 Applied Biosystems) following manufacturer's



recommendations. The converted DNA was then purified with Epiect spin columns using appropriate buffers in accordance with manufacturer's instructions. 50 µg of converted DNA was PCR-amplified with PCR-buffer 1X, 1.5mM of MgSO<sub>4</sub>, dNTPs 0.2mM each, 1 unit of Platinum Taq DNA polymerase (Life Technologies) 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.

### ***Cell culture and in vitro experiments***

HepG2, HuH7 (ATCC, Manassas, VA, USA), Mahlavu (kindly provided by Dr. N. Atabey) human HCC cells were cultured in DMEM complete medium with 10% fetal bovine serum (Lonza, Basel, Switzerland) in a 5% CO<sub>2</sub> atmosphere.

Cells were transiently transfected with 100 pmol of a miR-27a mimic, or of TRβ or control siRNAs (Ambion) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). For viability assay, 1500 cells/well were seeded in 96well plates and cell viability was evaluated after 72 hours by Cell TiterGlo (Promega). For the migration assay, 7x10<sup>4</sup> cells were seeded in Boyden chambers (Corning) in DMEM 5%FBS. After 48 hours, cells that did not migrate were removed; migrated cells were stained with crystal violet and counted. Treatment with T3 (Sigma) was performed for 72 hours at 100nM and 1mM.

### ***Statistical Analysis***

Data are expressed as mean ± standard deviation (SD) or mean ± standard error (SEM). Analysis of significance was done by t Student's test and by One-Way ANOVA using the GraphPad software (La Jolla, California). Correlations between TRb1 and its transcriptional target genes were assayed by Pearson's test. Chi-square test was used to explore relationships between up- or down-regulation of TRb1 and clinicopathological variables, including serum α-fetoprotein (AFP), tumour size, grade, aetiology and tumor focality. *P*-values were considered significant at < 0.05. Statistical calculations were executed using SPSS version 15.0 (SPSS, Chicago, IL, USA).

## **SUPPLEMENTARY FIGURES**

**Figure S1: A)** LI of hepatocytes of Krt-19<sup>+</sup> and Krt-19<sup>-</sup> preneoplastic nodules. BrdU

dissolved in drinking water (1 mg/ml) was administered for five days and removed 2 hours prior to sacrifice. LI is expressed as number of BrdU-positive hepatocyte nuclei/100 nuclei. Results are expressed as mean  $\pm$  SEM of 5 rats per group. At least 20,000 hepatocyte nuclei per group were scored. \*\*\*\*Statistically significant from Krt-19-negative nodules;  $P < 0.0001$ . **B)** Labeling Index (LI) in rats subjected to 2/3 PH. Bromodeoxyuridine (BrdU) (50 mg/100 g b.wt.) was injected intraperitoneally 1 hour prior to sacrifice. Mean  $\pm$  SEM of 5 rats per time point. \*\*\*\*Significantly different from time 0 for at least  $P < 0.0001$ .

**Figure S2:** TRb1 expression in HCC cell lines shown as DCt to endogenous control (b-actin).

**Figure S3:** TRb expression in Mahlavu (left panel) and HepG2 (right panel) cells transfected with a control siRNA (siC) or a TRb siRNA (siTRb).

**Table S1.** Summary of target exons, oligonucleotide primers used for amplification and number of detected mutations (N° MUT) for the sequencing study of TRa and TRb genes. Exon-intron boundaries were included in the analysis. Because of its length, exon 1 of the TR $\beta$ 2 isoform was split in two portions, each amplified and sequenced by a separate primer pair, as indicated.

TARGET EXON	FORWARD PRIMER	REVERSE PRIMER	N° MUT.
alpha 1-2 ex. 1	ccagtctcttggcgtgct	gaccagctctctttaccaga	0
alpha 1-2 ex. 2	gggggtggaagtctcttagg	cttgaggaggccaagtatg	0
alpha 1-2 ex. 3	aaggtagagttgggtgggtaca	aaggggaatcaggcaacag	0
alpha 1-2 ex. 4	agctggggacggaacc	ttgcaatttgggaccaagac	0
alpha 1-2 ex. 5	atcagggcgagtgattctg	ttgggctggactaaaccaag	0
alpha 1-2 ex. 6	tgagcggtaaaaggggtct	accagtgaggctctgtgac	0
alpha 1-2 ex. 7	ctcggggaggatgtactgac	agagagaggcaagcaagagc	0
alpha 1-2 ex 8	tcctctggctgtcctagacc	tgtcccttctctcaagctc	0
alpha 2 ex 9	ccagaggctcatcttggaaat	ccctggagaaggagtagcat	0
beta 1 ex. 1	ctgcccacatcacacggtt	tcaggctgtaagaccccagt	0
beta 1 ex. 2	aacgaccggccttctaaaat	tggtagcgggagagagagac	0
beta 2 ex. 1 (1/2)	tgaagtgaggcgaactaggc	gtccaggcctgttccagata	0
beta 2 ex. 1 (2/2)	ggaagacagtccggcttaca	gtgctccgatgcaaagattc	0
beta 1 ex. 3 (beta 2 ex. 2)	tcctgtctggatgagaaacctt	ctgagttccgatggctgtct	0
beta 1 ex. 4 (beta 2 ex. 3)	aggctaagggccagaacaat	ccacagaggttcctgctttc	0
beta 1 ex. 5 (beta 2 ex. 4)	catgtgactccaggctcaga	actgcccatcacaaaaatgg	0
beta 1 ex. 6 (beta 2 ex. 5)	cacgggttctttcagcttt	tcattttgcatggactctgc	0
beta 1 ex. 7 (beta 2 ex. 6)	cagtggttgctttccaaca	acccacgaagctctcttct	0
beta 1 ex. 8 (beta 2 ex. 7)	tcactcctccgcctctaaa	gaaacgaagaaacgacaacaga	0

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**Table S2.** Characteristics of HCC patients

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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%)

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**Table S3.** Methylation frequency at specific CpG sites in the promoter of the TR $\beta$  gene. 10 CpG sites were scored for methylation by pyrosequencing. Three age-matched control normal livers and 15 HCCs were analyzed. Average levels (Ave) of methylation for each sample are indicated in bold.

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

**Table S4. Percentage of Krt-19 positive nodules persisting after 7 days of treatment with T3**

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Treatment	n. GSTP <sup>+</sup> nodules/cm <sup>2</sup>	n. Krt-19 <sup>+</sup> Nodules/cm <sup>2</sup>	% Krt-19 <sup>+</sup> nodules/GSTP nodules
DENA + AAF + PH + BD	59,5 ± 23,6	16,5 ± 8,6	27%
DENA + AAF + PH + T3	22,4 ± 4,7*	1,9 ± 0,9*	9%

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Rats injected with a single dose of DENA were exposed to the R-H protocol as described in Materials and Methods. Five weeks after 2-AAF withdrawal, animals were split into two groups. The first was kept on a basal diet while the second group was fed a T3-supplemented diet. All animals were sacrificed 7 days thereafter. Percentages represent the number of Krt-19<sup>+</sup> nodules out of 100 GSTP<sup>+</sup> nodules. Results are expressed as mean ± SEM of 5 rats per group. \*P<0,001