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Expression of NKG2D ligands is downregulated by beta-catenin signaling and associates with HCC aggressiveness

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Abstract (word count 247)

Background/Aims: The NKG2D system is a potent immunosurveillance mechanism in cancer based on recognition of the NKG2D ligands expressed on tumour cells by activating receptor natural killer group 2, member D (NKG2D) on immune cells. Here, we evaluated the expression of NKG2D ligands in HCC, in both humans and mice, taking the genomic features of HCC tumours into account.

Methods: The expression of NKG2D ligands (*MICA*, *MICB*, *ULBP1* and *ULBP2*) was analysed in large human HCC datasets by Fluidigm TaqMan and RNAseq methods, and in two mouse models (mRNA and protein levels) reproducing the features of both major groups of human tumours.

Results: We provide compelling evidence that expression of the *MICA* and *MICB* ligands in human HCC is associated with tumour aggressiveness and poor patient outcome. We also found that the expression of *ULBP1* and *ULBP2* was associated with poor patient outcome, and was downregulated in *CTNNB1*-mutated HCCs displaying low levels of inflammation and associated with a better prognosis. We also found an inverse correlation between *ULBP1/2* expression levels and the expression of β -catenin target genes in HCC patients, suggesting a role for β -catenin signalling in inhibiting expression. We showed in HCC mouse models that β -catenin signalling downregulated the expression of Rae-1 NKG2D ligands, orthologs of *ULBPs*, through TCF4 binding.

Conclusions: We demonstrate that the expression of NKG2D ligands is associated with aggressive liver tumorigenesis and that the downregulation of these ligands by β -catenin signalling may account for the less aggressive phenotype of *CTNNB1*-mutated HCC tumours.

Lay summary

The NKG2D system is a potent immunosurveillance mechanism in cancer. The expression of NKG2D ligands has been little investigated in HCC. We show here, in a large cohort of HCC patients and dedicated HCC mouse model, that the expression of NKG2D ligands is associated with aggressive tumorigenesis and downregulated in *CTNNB1*-mutated HCC. We also show that β -catenin signalling downregulates NKG2D ligands in mice.

Highlights

- The expression of *MICA* and *MICB* is associated with HCC tumour aggressiveness and poor patient outcome.
- The expression of *ULBP1* and *ULBP2* is associated with poor patient outcome and is downregulated in *CTNNB1*-mutated HCC and inversely correlated with the level of β -catenin target gene expression.
- Expression of the mouse *Rae-1* NKG2D ligand is regulated by β -catenin signalling via TCF4 in hepatocytes.
- The expression of *KLRK1* (NKG2D) and *ULBP1* is associated with immune cell signatures in HCC.
- Low levels of NKG2D ligand expression in *CTNNB1*-mutated HCC may account for the less inflamed and less aggressive phenotype of these tumours.

Introduction

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer-related death and the fifth most common cancer worldwide (1). It is responsible for approximately 800,000 deaths each year. About 90% of HCCs develop in a background of chronic liver disease of various degrees of severity, the induction of fibrosis and/or subsequent cirrhosis (2). Nevertheless, in <10% of cases, HCC develops in a healthy liver (3,4). The most common risk factors for HCC are infections with hepatotropic viruses (HBV, HCV), alcohol consumption, aflatoxin B1 exposure, haemochromatosis and metabolic syndrome, which is frequently seen in patients with diabetes and/or obesity (4). Cirrhosis, the final stage of chronic liver disease, remains the main risk factor for HCC development in Western countries (5). Our understanding of HCC pathogenesis has increased steadily over the last 20 years. Functional genomics studies have recently provided an accurate picture of the landscape of genetic alterations and identified the core pathways deregulated in HCC (6). The most prevalent mutations after those of the *TERT* promoter concern *TP53* and *CTNNB1* (5,7) defining two major groups of tumours with specific features. *TP53*-mutated HCCs are aggressive proliferating tumours with high levels of inflammation (hot tumours) and genomic instability. Conversely, HCCs with activating mutations of *CTNNB1* (*CTNNB1*-mutated HCCs) have a less severe phenotype associated with a favourable prognosis and low levels of proliferation and inflammation (cold tumours). Treatment options for HCC remain very limited. Most involve multi-targeted tyrosine kinase inhibitors, providing little survival benefit over best supportive care (8). The major role of the immune system in controlling tumour growth and metastasis provides the basis for most cancer immunotherapies. Immune checkpoint blockade, based on inhibitors of PD-1 and/or CTLA-4, is considered an exciting perspective for

HCC treatment (for review(9)). However, only 25% of HCCs are eligible for immunotherapy, as they express markers of an inflammatory response (10). Recent findings suggest that *CTNNB1*-mutated HCCs displaying β -catenin activation are associated with immune escape and resistance to anti-PD-1 therapy (11,12). New therapeutic strategies based on our understanding of the tumour and its relationship with its immune microenvironment are, therefore, urgently required to improve HCC treatment.

With regards to cancer surveillance, the NKG2D-ligand/NKG2D pathway has been the subject of intense research in cancer, infection and autoimmunity (13). The activating receptor NKG2D, which was discovered more than two decades ago, initially on the surface of NK cells, has proved to be a critical player in the immune surveillance of cancer. The NKG2D-dependent elimination of tumour cells expressing at least one cognate ligand is well documented in both *in vitro* (14–17) and *in vivo* models of transplanted tumours (18–21). The induction and upregulation of NKG2D ligands result from various stress signals, including infection, DNA damage, heat shock and hyperproliferation (22,23).

The expression of NKG2D ligands has been observed in various human cancers. These ligands are cell surface proteins structurally related to the major histocompatibility complex (MHC). In humans, the two most studied NKG2D ligands are MHC class-I-related chain A and B, the MICA and MICB proteins, and the six, less studied, unique long 16 (UL-16)-binding proteins (ULBP1-6) (24). Their counterparts in mice are the retinoic acid early inducible gene-1 *Rae-1* (orthologous to ULBPs), minor histocompatibility H60a-c and murine UL16-binding protein-like transcript (MULT1) proteins. In humans, variable levels of *MICA*, *MICB* and *ULBP1-3* ligand expression have been observed in haematopoietic

malignancies, including acute and chronic leukaemias (of lymphoid and myeloid origin) (25), and in solid tumours (26–30). Ligand expression is heterogeneous between cancer types and individuals. Several studies have highlighted the paradoxical relationship between ligand expression and patient outcome. Briefly, high levels of cell-surface ligand expression are associated with better disease-free survival in colorectal (31), cervical (32) and nasopharyngeal carcinomas (33), but with poor prognosis in breast (34), lung (35) and ovarian cancers (36). The expression of NKG2D ligands in HCC remains insufficiently explored, as conflicting data have been obtained concerning the potentially beneficial or deleterious role of NKG2D in HCC(13).

In this study, we investigated the expression of the NKG2D ligand genes *MICA/B* and *ULBP1/2* in a large human HCC dataset and in two mouse models reproducing the features of both types of human tumours.

Methods

Patients and tumour material

Human liver samples: A series of 354 liver tumour samples, including 10 early HCCs, 337 HCCs and seven malignant transformations of HCA (hepatocellular adenoma) into HCC (HCC on HCA), were collected from 325 patients treated surgically in Europe. The study was approved by the appropriate institutional review boards (CCPRB Paris Saint-Louis, 1997, 2004 and 2010, approval number 01-037; Bordeaux, 2010-A00498-31) and all patients gave informed consent in accordance with French law. All samples were immediately frozen in liquid nitrogen and stored at –80 °C. Clinical data, biological and histological parameters are described in the supplemental material.

Quantitative RT-PCR and RNA-sequencing

We assessed mRNA levels by quantitative RT-PCR on 354 HCC samples. We reverse transcribed 500 ng of total RNA with the High-Capacity Transcription Kit (Life Technologies) and assessed gene expression in predesigned TaqMan assays (*MICA*: Hs00741286_m1; *MICB*: Hs00792952_m1; *ATM*: Hs01112326_m1; *ATR*: Hs00354807_m1; Life Technologies) on Fluidigm 96 dynamic arrays with the BioMark Real-Time PCR system. Expression levels (Ct values) were assessed with Fluidigm Real-Time PCR Analysis software (4.1.3) and gene expression data were expressed by the $2^{-\Delta\Delta Ct}$ method, relative to ribosomal 18S (R18S) and the expression level of the corresponding gene in five normal liver samples. Published RNA-seq data for 200 tumours were used to evaluate *ULBP1* and *ULBP2* expression in HCC patients (see **Table 2**) (40,41). The sequencing data reported in this paper have been deposited in the EGA (European Genome-phenome Archive) database under accession numbers EGAS00001002879, EGAS00001001284 and EGAS00001003837.

Mice and HCC induction

All animal studies were approved by the *Ministère de l'Enseignement Supérieur, de la Recherche et de l'Innovation* (MESRI) and the *Direction Départementale des Services Vétérinaires de Paris* (agreement no. 75-956) and by the Mouse Facility Core laboratories (CRC UMRS1138). All mice (strain C57Bl6J) were kept in well-controlled animal housing facilities and had free access to tap water and food pellets. The various HCC mouse models used here to model HCC tumorigenesis (strain C57Bl6J) have been described elsewhere (42). *Lpk-myc+* transgenic mice, which express the *c-myc* oncogene in the liver under the control of rat L-PK regulatory

regions, developed HCC with *Ctnnb1* mutations; we established homogeneous groups of these mice consisting exclusively of littermates (43). In our study, the *Lpk-myc+* model is referred to here as the β -catenin mutated HCC model. In this model, unlike the intestine, in which the *c-myc* oncogene is a crucial target of the Wnt signalling pathway driving tumorigenesis, studies with a combination of several molecular approaches (ChIP-seq, RNA-Seq) showed that *c-myc* was not a target of Wnt signalling in the liver. In this model, *c-myc* and β -catenin signalling co-operate to drive liver carcinogenesis, and all *Lpk-c-myc* tumours harbour *Ctnnb1*-activating mutations (43).

Cohorts of male age-matched wild-type (WT) mice received a single intraperitoneal injection of diethylnitrosamine (DEN) (Sigma) (25 mg kg⁻¹ body weight) to induce HCC, or PBS, at 14 days of age (44). HCC development was studied at 12 to 14 months in *Lpk-myc+* mice and mice with DEN-induced HCC.

We also analysed Wnt/ β -catenin signalling through inducible hepatocyte-specific inactivation of the *Apc* gene, in *Apc* ^{Δ Hep} (*Apc*^{*flox/flox*}, *TTR-Cre Tam*) mice; the control mice in this case were *Apc*^{*flox/flox*}, Cre-negative (42,45–47). This model reproduces the initial step of β -catenin oncogenesis and is based on tamoxifen-inducible hepatospecific inactivation of the tumor suppressor gene adenomatous polyposis coli (*Apc*^{-/-}). All hepatocytes of the liver lobule display aberrant β -catenin activation after tamoxifen injection. The *Apc* gene was inactivated by a single i.p. injection of 1.5 mg tamoxifen (MP Biomedicals, France) in 9- to 12-week-old mice (42,45–47).

Nonparenchymal cell isolation and flow cytometry

Livers were harvested and the nonparenchymal cell (NPC) fraction was extracted on a Percoll gradient, as previously described (42,47).

Cells were incubated with anti-CD16/CD32 antibodies (Fcγ III/II receptor, clone 2.4G2, eBiosciences) before staining with labelled antibodies, according to the manufacturer's recommendations. Cells were subjected to fluorescence-activated cell sorting (FACS) analysis with the following antibodies: anti-CD45/Ly5 (30-F11), anti-TCR chain (H57-597), anti-NK1.1 (PK136), anti-CD4 (RM4-5), anti-CD8α (53-6.7) and anti-NKG2D (CX5) antibodies, all purchased from BD Pharmingen/Horizon or from eBioscience. CD1d-α-galactosylceramide-loaded (CD1d-α-GalCer-loaded) tetramers (APC-labelled) for the identification of invariant NKT cells were provided by the NIH Tetramer Core Facility, Bethesda Maryland USA. Data were acquired with a Fortessa FACS flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc.).

Preparation of total RNA and quantitative PCR analysis

Total RNA was extracted from mouse liver tissue or cell lines (AML12 and Hepa1.6) with TRIzol (Invitrogen), and 2 µg was used as a template for cDNA synthesis (MMLV, Life Technologies). Purified RNA was then reverse-transcribed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed with the Fast Start Quantitative PCR (q-PCR) kit with SYBR Luminaris Color HiGreen qPCR master mix (Thermo Fisher Scientific) and specific primers. The reactions were performed in 96-well plates in a LightCycler 480 instrument (Roche) with 40 cycles. We determined the relative amounts of the mRNAs studied by the second-derivative maximum method, with LightCycler 480 analysis software and normalization against 18s rRNA, used as an invariant control for all studies. Data are expressed as means ± standard deviation of the mean. The statistical significance of differences was assessed in Students *t* test in Prism 5.0

software (GraphPad Software, San Diego, CA). *P* values <0.05 were considered statistically significant.

Immunohistochemistry

Tissues were fixed by incubation in 4% formalin overnight at 4 °C, and were then embedded in paraffin wax. For immunohistochemistry, liver sections (5 µm) were deparaffinised. Antigen retrieval was performed by incubating liver sections in Tris-based buffer (Vector Laboratories) in a microwave at 850 Watts for 20 min after boiling. Tissue sections were blocked by incubation with the Avidin/Biotin blocking kit (Vector Laboratories) for 15 min each at room temperature and with normal horse serum (2.5%) for 20 min at room temperature. Tissue sections were then incubated overnight at 4 °C with the anti-Rae1 pan-specific antibody (R&D AF1136) at a concentration of 10 µg/mL or with antibodies directed against glutamine synthase (BD Transduction Laboratories, clone 6, 1/400). Endogenous peroxidase was blocked by treating the sections with 3% hydrogen peroxide for 20 min at room temperature. Tissue sections were incubated with biotinylated anti-goat IgG antibody (1:250 dilution, Vector Laboratories, CA, USA) for 30 minutes. Specific binding was detected with the ABC reagent (Vector Laboratories) and DAB (Vector Laboratories) and the slides were counterstained with haematoxylin.

TCF4 ChIP-sequencing

The ChIP protocol was adapted from a previous study (48), as previously described (49). The ChIP-seq data were deposited under accession number GSE35213 in the Gene Expression Omnibus database. β -catenin and TCF-4 binding to the Rae

promoter, relative to the immunoglobulin isotype control, was assessed on the basis of SYBR green fluorescence, with the following oligonucleotides: FW-GACTTTCCACAAACGCACAA, Rev-GAAACTGCCCGTTACCAC designed according to the sequence of the TCF-4 peak identified upstream from the Rae-1 gene.

Editing of Ctnnb1 Exon 3 with CRISPR-Cas 9 technology

We purchased the murine hepatocyte cell line AML12 cell line from ATCC-CRL-2254™. AML12 cells were cultured in DMEM/F-12 (1:1) + GlutaMAX, supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics. The exon 3 region of CTNNB1 was deleted with CRISPR-Cas9 technology, leading to an activation of beta-catenin signalling in the cells. Clones with deletions of exon 3 were selected on puromycin and were passaged more than 10 times. Cells were harvested and collected for RNA extraction, as described above.

Silencing of Ctnnb1 by a siRNA strategy in Ctnnb1-mutated Hepa1.6 cells

Hepa 1.6 cells (ATCC, CRL-1830) harbouring Ctnnb1-activating mutations were cultured in DMEM supplemented with 5% heat-inactivated FCS. They were plated at a density of 300,000 cells per well and transfected one day later with 20 nM small-interfering RNA (siRNA) against β -catenin (QiagenSI00942039) or a control siRNA (Dharmacon D-001210-01-05) in the presence of Lipofectamine 2000 (Thermo Fisher Scientific). Molecular analyses were performed 72 h post transfection. Total RNA was extracted with TRIzol® reagent (Thermo Fisher Scientific), as described above.

Statistical analysis

Data were visualized and statistical analyses were performed with PRISM software (GraphPad Software, Inc., La Jolla, CA) and R software version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org>) with the Bioconductor package. Comparisons of continuous variables between two or more groups were performed with Mann-Whitney tests. Correlations between continuous variables were assessed by Pearson's correlation tests if both variables were normally distributed and the assumptions of linearity and homoscedasticity were respected, or by Spearman's rank correlation tests. All tests were two-tailed and *P*-values < 0.05 were considered significant.

Results

The expression of the NKG2D ligands, *MICA* and *MICB*, in human HCC is associated with tumour aggressiveness and poor patient outcome

We investigated the possible association of *MICA* and *MICB* expression with specific clinical features of HCC, by RT-qPCR analyses assessing the expression of these genes in 354 patient samples (37). We first determined the expression levels of these genes by diagnosis, as follows: early HCC ($N=10$), HCC emerging from HCA (HCC on HCA; $N=7$) and HCC ($N=337$), with normal liver (NL) samples ($N=5$) as a control (**Fig. 1A**). We found that both *MICA* and *MICB* were significantly more strongly expressed in early HCC and HCC/HCA than in NL ($N=5$). Moreover, both *MICA* and *MICB* were more strongly expressed in HCC than in early HCC and HCC on HCA (**Fig. 1A**). We also checked that the levels of expression of the *MICA* and *MICB* were positively correlated ($R=0.677$, $p<0.0001$) suggesting that these two genes located in the HLA class I region of chromosome 6 are co-induced during liver tumorigenesis (**Fig. S1A**). We then evaluated the expression of *MICA* and *MICB* in HCC as a function of Edmondson-Steiner grade criteria, distinguishing four specific liver cancer stages and depicting tumour cell differentiation. We found that the expression of both *MICA* and *MICB* was positively associated with tumour grade and low levels of differentiation (**Fig. 1B**, **Fig. S1B**). These ligands are known to be regulated principally at the transcriptional level (23).

Finally, we used a five-gene molecular score to predict the outcome of patients with HCC (50), identifying two groups of HCC: P1, corresponding to HCC with a good prognosis and P2, corresponding to HCC associated with bad prognosis and early tumour recurrence. Interestingly, HCC patients with poor outcomes displayed the highest levels of expression of both *MICA* and *MICB* (**Fig. 1C**).

Collectively, our data show that the expression of the NKG2D ligands *MICA* and *MICB* in human HCC is associated with tumour aggressiveness and poor patient outcome.

The NKG2D ligands *MICA* and *MICB* are less strongly expressed in HCCs displaying chromosomal stability

We investigated whether the expression of *MICA/B* was associated with specific genetic alterations, by evaluating *MICA* and *MICB* expression as a function of the molecular classification of human HCCs (51). This classification distinguishes three groups, G1 to G3, characterised by aggressive HCC tumours with high levels of chromosomal instability and *TP53* mutations (51,52), and three groups, G4 to G6, associated with a better prognosis, chromosomal stability and a well-differentiated tumour phenotype. *CTNNB1*-mutated HCCs belong to groups G5-G6 and have no inflammatory infiltrates. We found that the expression levels of both *MICA* and *MICB* were significantly lower in HCC samples from groups G4 to G6 than in samples from groups G1 to G3 (**Fig. 2A**).

We also studied *MICA* and *MICB* expression levels as a function of the presence or absence of prevalent mutations (*TERT*, *TP53*, *CTNNB1*) within the landscape of genetic alterations of HCC (5,53). We found that *TP53*-mutated HCCs had significantly higher levels of *MICA/B* expression than non-mutated HCCs (**Fig. 2C**). The expression of these genes did not differ significantly between HCCs with and without *CTNNB1* or *TERT* mutations (**Fig. 2C, Fig. S1A** respectively).

Finally, as tumours with *TP53* mutations are known to display chromosomal instability leading to high levels of DNA damage, we investigated the relationship between the expression of *MICA/B* and expression of the *ATM/ATR* genes, two master regulators

of the DNA damage response known to regulate the expression of NKG2D ligands (23).

We found a positive correlation between the levels of expression of the *MICA/B* and *ATM/ATR* genes (**Fig. S2B**). Our data show that *MICA/B* are more strongly expressed in HCCs with *TP53* mutations and chromosomal instability, and that their expression is correlated with that of DDR-related genes.

The expression of *ULBP1/2* ligands in human HCCs is weaker in tumours with stable chromosomes than in those displaying chromosomal instability and is associated with *CTNNB1* mutation status

We extended our analysis to the expression of *ULBP* ligands, which have been little studied in HCC. We investigated whether the expression of *ULBPs* had characteristics in common with the expression of *MICA/B*, by comparing the expression of *ULBP1* and *2* according to our previous RNAseq dataset with the molecular classification of human HCCs (40). As for *MICA/B*, we found that the expression levels of the *ULBP1* and *ULBP2* genes were correlated ($R=0.49$, $p<0.0001$) (**Fig. S3A**). Moreover, although not significant, the expression levels of both *ULBP1* and *2* tended to be higher in HCC tumours with lower levels of differentiation and higher grades (**Fig. S3B, C**).

Using the five-gene molecular score predicting the outcome of patients with HCC (50), we found that HCC patients with poor outcomes displayed the highest levels of expression for both *ULBP1* and *ULBP2* (**Fig. 3A**). Moreover, the level of expression of these genes was significantly lower in groups G4 to G6 than in HCCs displaying chromosomal instability (groups G1 to G3) and was lower than that in normal livers (**Fig. S4A**). An analysis of the expression of *ULBP1/2* in HCC samples as a function

of mutational status showed that *ULBP1/2* expression levels were significantly higher in HCCs with *TP53* mutations than in HCCs without such mutations (**Fig. 3B**), but that there was no significant difference between HCCs with and without *TERT* mutations, as for *MICA/B* (**Fig. S4B**). Interestingly, *ULBP1/2* expression levels were significantly lower in HCCs with *CTNNB1* mutations, suggesting that β -catenin signalling may downregulate the expression of *ULBP1/2* ligands (**Fig. 3B**). Consistent with these observations, we found that *ULBP1* expression was inversely correlated with the expression levels of three positive β -catenin target genes: *GLUL*, *LGR5* and *TBX3* (**Fig. 3C**).

Collectively, these findings indicate that the level of expression of *ULBP1/2* ligands in human HCCs is higher in tumours with *TP53* mutations and lower in those with *CTNNB1* mutations, suggesting that β -catenin signalling downregulates the expression of these genes.

***CTNNB1*-mutated HCCs in mice have low levels of NKG2D ligand expression**

We investigated the molecular mechanisms regulating the expression of NKG2D ligands in two mouse models of liver tumorigenesis reproducing the features of the two major groups of human HCCs. The diethylnitrosamine (DEN)-induced HCC model mimics aggressive unstable tumours displaying preferential Ras mutation and without *Ctnnb1*-activating mutations (44), whereas the Lpk-myc⁺ model mimics HCC tumours with *Ctnnb1* mutations and stable chromosomes (42,43,47). We evaluated the level of expression of NKG2D ligands in these models. Consistent with human data, we found that mRNA levels for the mouse NKG2D ligands (Rae-1 δ , Rae-1 ϵ , human *ULBP* orthologs) were strongly upregulated in aggressive HCCs relative to control livers without tumours (**Fig. 4A**). However, as in human HCC, the expression

of these ligands was strongly downregulated in HCCs with *Ctnnb1* mutations, suggesting a role for beta-catenin signalling in this downregulation (**Fig. 4A**). Furthermore, the NKG2D (Klrk1) receptor was significantly upregulated on lymphocytes (in terms of the percentage of cells expressing this receptor and the expression level per cell) in HCCs with *Ctnnb1* mutations relative to DEN-induced HCCs (**Fig. S5**).

We performed additional investigations with the *Apc*-KO mouse model (*Apc*^{ΔHep}), in which the beta-catenin pathway is activated in all hepatocytes (without *Ctnnb1* mutation) (42,45,46). We found that mRNA levels for NKG2D ligands (Rae-1δ, Rae-1ε) were significantly downregulated in *Apc*^{ΔHep} livers relative to livers in which beta-catenin signalling was not activated (**Fig. 4B**). We validated our observations at the protein level, by immunohistochemical staining of Rae-1 on paraffin-embedded liver sections. We found that Rae-1 ligands were not expressed in specific areas in which beta-catenin signalling was turned on (stained for glutamine synthase, a target of beta-catenin) in control livers (**Fig. 4C**). Rae-1 ligands were expressed in hepatocytes, except for those surrounding the pericentral vein (constitutive activation of beta-catenin signalling), absent from hepatocytes in which *Apc* was knocked out and in tumour hepatocytes with *Ctnnb1* mutations, but expressed in HCCs without mutations affecting beta-catenin (DEN-induced HCC).

Collectively, these data strongly suggest that beta-catenin signalling downregulates the expression of Rae-1 NKG2D ligands in hepatocytes.

Beta-catenin inhibits Rae-1 NKG2D ligands by binding to TCF4 on the Rae1-promoter

We investigated the molecular mechanisms of beta-catenin-mediated Rae1 downregulation, by performing ChIP-Seq experiments on purified primary hepatocytes displaying beta-catenin activation (Apc-KO, Apc^{ΔHep}) and control hepatocytes (GSE35213) (**Fig. 5A**). Beta-catenin has no DNA-binding domain. The ChIP-seq experiment was therefore performed with an antibody against the preferred partner of beta-catenin in hepatocytes, TCF4 (49). The Chip-seq data showed a significant induction of TCF4 binding to the Rae-1 promoter region in hepatocytes displaying beta-catenin activation (Apc-KO mouse model, Apc^{ΔHep}) than in control hepatocytes (**Fig. 5A**). We confirmed this enrichment in TCF-4 binding upstream from the Rae-1 promoter by ChIP-qPCR on another panel of Apc^{ΔHep} hepatocytes (**Fig. 5A**). These molecular data demonstrate that beta-catenin directly inhibits the expression of Rae-1 NKG2D ligands through binding to TCF4 on the Rae-1 promoter in hepatocytes.

We used two different cellular systems to confirm that Rae-1 ligand expression was a negative target of β-catenin signalling: a non-mutated hepatocyte cell line (αML) and a hepatoma cell line (Hepa1.6) harbouring activating *Ctnnb1* mutations. We first edited exon 3 of the β-catenin gene with CRISPR-Cas9 technology in αML cells, to activate β-catenin signalling. A significant downregulation of Rae-1 ligands (Rae-1δ, Rae-1ε) was observed in β-catenin-activated αML cells relative to control αML cells, supporting the conclusions drawn from the ChIP-seq analysis (**Fig. 5B**). We then performed a siRNA experiment on the Hepa1.6 cell line, in which we showed that Rae-1δ expression was upregulated when β-catenin was silenced.

Overall, we provide strong evidence in both humans (*ULBP1/2*) and mice (Rae-1) that the expression of NKG2D ligands is significantly weaker in HCCs with *CTNNB1*

mutations, revealing a downregulation of these NKG2D ligands by beta-catenin activation.

Expression of the NKG2D receptor and ULBP1 is associated with the presence of inflammatory cells in HCC

Given that NKG2D ligands were upregulated in aggressive inflamed HCCs and that ULBP1/2 was downregulated in *CTNNB1*-mutated HCC with low levels of inflammation, we investigated whether there was a relationship between the NKG2D system and inflammatory status in humans, as recently reported in mice (54). We monitored the expression of KLRK1 (NKG2D receptor) in our cohort of HCC patients. We found that KLRK1 was more strongly expressed in HCC samples displaying lymphocytic inflammation (**Fig. 6A**). Moreover, using MCP counter analysis to determine the nature of inflammatory infiltrates within the tumour microenvironment, we found a strong association between KLRK1 expression and endothelial cell and immune population signatures (B cells, T cells, cytotoxic cells, myeloid dendritic cells, neutrophils and NK cells). We also found a weak association between ULBP1 expression and these immune populations, except for NK cells.

Thus, the expression of both the ligands and receptor of NKG2D is associated with immune cell signatures in liver tumours, consistent with the notion that the NKG2D system is intrinsically coupled to inflammation in HCC (54).

Discussion

The worldwide incidence of HCC is similar to patient mortality, highlighting the poor prognosis of this cancer. Therapeutic options for HCC remain limited. Immunotherapy has yielded spectacular results for other solid tumours (breast, melanoma, prostate, lung), that were previously incurable (55–57). Immunotherapy strategies for HCC are currently being investigated, but the results have been poor (58). Original ways of counteracting HCC progression are, therefore, urgently needed.

In this study, we investigated the expression of NKG2D ligands (human *MICA/B*, *ULBP1/2* and murine *Rae-1*) in both human and mice HCCs. These ligands are known to be involved in tumour immunosurveillance, and this work took the genomic features of liver tumours into account for the first time. Our work provides compelling evidence to suggest that the expression of NKG2D ligands is associated with aggressive liver tumorigenesis and that the level of expression of these ligands is lower in HCCs displaying chromosomal stability (G4/G5/G6) than in those displaying chromosomal instability (G1/G2/G3). We also found that *ULBP1/2* expression was specifically downregulated in HCCs with *CTNNB1* mutations relative to normal liver, and that the level of *ULBP1/2* expression was inversely correlated with the level of expression of β -catenin target genes. We provide clear evidence, at the molecular level, in mouse models, for the downregulation of *Rae-1* ligands, the orthologs of *ULBPs*, by beta-catenin. We found that expression of the NKG2D receptor and the *ULPB1* ligand was associated with the presence of immune cell signatures, suggesting an intricate link with the inflammation status of the HCC tumor.

We show here, for the first time, that the level of expression of *MICA/B* and *ULPB1/2* is higher in HCCs associated with a poor prognosis and early tumour recurrence (within two years of resection) (50,59). NKG2D ligands can be shed into the

bloodstream, and the presence of their soluble form in patient serum samples has been associated with poor prognosis for other cancers (60). The levels of these ligands in the blood could therefore be used as a marker of disease progression for HCC patients.

We show here that NKG2D ligands are more strongly expressed in group G1/G2/G3 HCCs. These HCCs are aggressive tumours with high levels of inflammation (hot tumours) (6,52). Chronic inflammation in the liver creates a permissive environment favouring HCC development. Indeed, it underlies aggressiveness by enhancing cellular proliferation, angiogenesis and tumour invasiveness (61). Inflammatory signals in the peritumoural tissue of HCC patients have been shown to be associated with a poorer prognosis and tumour recurrence (62–65).

Using the DEN-induced HCC mouse model, Sheppard et al. (13,54), provided genetic evidence of a deleterious role of the NKG2D system, which has sustained tumour-promoting effects (54). Indeed, they showed that the engagement of the NKG2D receptor by its cognate ligands increased the recruitment of inflammatory cells in the liver, increasing tumour aggressiveness (54). Our data show that the expression of the NKG2D receptor and *ULBP1* is associated with the presence of inflammatory cells in human HCC tumours and with specific immune cell signatures corresponding to various populations, from lymphocytic to myeloid immune cells.

In our two mouse models of HCC, the proportion of NKG2D-expressing immune cells and the level of NKG2D expression were higher in HCCs with *Cttnb1* mutations than in DEN-induced HCCs. Based on these data, and given the almost total extinction of NKG2D ligand expression in HCCs with *Cttnb1* mutations, we propose that immune cells express higher levels of NKG2D receptors at their surface to facilitate the sensing of very small amounts of ligands.

We suggest that the activation of the NKG2D system contributes to a strong inflammatory response, exacerbating liver tissue damage and underlying HCC aggressiveness and poor patient outcome.

The immune response to NKG2D ligand expression should therefore be investigated further, to identify innovative targets for HCC treatment.

HCCs with *CTNNB1* mutations are characterized by an absence of inflammatory infiltrates (cold tumours) (10,11,66). Indeed, recent data for analyses of the immunological landscape of HCCs have highlighted the existence of a specific group of HCCs expressing markers of immune responses referred to as the “immune class” and excluding HCCs with *CTNNB1* mutations (10). However, the molecular basis of this lack of inflammation in tumours with *CTNNB1* mutations remains a matter of debate. Nevertheless, it has recently been shown that oncogenic beta-catenin activation in melanoma tumours inhibits T-cell infiltration (67). We suggest here that the low levels of NKG2D ligands in tumours with *CTNNB1* mutations and the demonstration that beta-catenin signalling partly inhibits NKG2D ligands may account for the less inflamed and less aggressive phenotype of these tumours.

Further studies are required to determine whether re-expressing Rae-1 ligands in *Cttnb1*-mutated mouse HCCs could convert these cold tumours into potentially aggressive hot HCCs.

Abbreviations: HCC, hepatocellular carcinoma, TME, tumour microenvironment, NKG2D ligands, natural killer group 2 D ligands.

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Figure legends and tables

Table 1: Details of the cohort of HCC patients (354) studied in Fluidigm experiments (e.g. MICA/MICB).

Table 2: Details of the cohort of HCC patients (200) for whom RNA-sequencing data were available for the analysis of ULBP1 and ULBP2 expression.

Fig. 1. *MICA* and *MICB* expression is upregulated during human HCC development and strongly associated with tumour grade.

(A) The levels of mRNA for NKG2D ligands (*MICA* and *MICB*) were assessed in normal liver (NL) ($N=5$), early HCC (hepatocellular carcinoma) ($N=10$), HCC on HCA (hepatocellular adenoma) ($N=7$), and HCC ($N=337$). The fold-change (\log_2) in gene expression is presented relative to the level of expression in normal liver. The black lines correspond to the mean values in normal liver. The significance of differences in expression was assessed in Mann-Whitney tests: non-significant, ns; $*P<0.05$; $***P<0.001$.

(B) The levels of mRNA for NKG2D ligands (*MICA* and *MICB*) were determined in HCCs classified according to Edmondson-Steiner grade. Grade I = very well differentiated; Grade II = moderately differentiated, Grade III = poorly differentiated; Grade IV = undifferentiated HCC. The fold-change (\log_2) in gene expression is presented relative to the level of expression in normal liver. The black lines correspond to the mean values. The significance of differences in expression was assessed in Mann-Whitney tests: non-significant, ns; $*P<0.05$; $**P<0.01$; $***P<0.001$.

(C) The levels of mRNA for NKG2D ligands (*MICA* and *MICB*) were determined in normal liver (NL) ($N=5$) and in HCC, with a five-gene molecular score used to predict the outcome of patients. P1 ($N=226$) and P2 ($N=127$). The fold-change (\log_2) in gene expression is presented relative to the level of expression in normal liver. The black lines correspond to the mean values. The significance of differences in expression was assessed in Mann-Whitney tests: non-significant, ns; $*P<0.05$; $***P<0.001$.

Fig. 2. The expression of *MICA* and *MICB* is strongly upregulated in group G1/G2/G3 HCCs and is weaker in group G4/G5/G6 HCCs displaying chromosomal stability

(A) The levels of mRNA for NKG2D ligands (*MICA* and *MICB*) were assessed in normal liver (NL) ($N=5$), G1/G2/G3 ($N=112$), and G4/G5/G6 ($N=232$) HCCs. The fold-change (\log_2) in gene expression is presented relative to the level of expression in normal liver. The black lines correspond to the mean values. The significance of differences in expression was assessed in Mann-Whitney tests: non-significant, ns; ** $P<0.01$; *** $P<0.001$.

(B) The levels of mRNA for NKG2D ligands (*MICA* and *MICB*) were assessed in HCCs with ($N=61$) and without ($N=277$) *TP53* mutations, and in HCCs with ($N=166$) and without ($N=260$) *CTNNB1* mutations. The fold-change (\log_2) in gene expression is presented relative to the level of expression in normal liver. The black lines correspond to the mean values. The significance of differences in expression was assessed in Mann-Whitney tests: non-significant, ns; *** $P<0.001$.

Fig. 3. The expression of *ULBP1* and *ULBP2* is specifically downregulated in groups of HCCs with stable chromosomes.

(A) The levels of mRNA for NKG2D ligands (*ULBP1* and *ULBP2*) were assessed in normal liver (NL) ($N=3$) and in HCC, with a five-gene molecular score used to predict patient outcome. P1 ($N=87$) and P2 ($N=94$). The fold-change (\log_2) in gene expression is presented relative to the level of expression in normal liver. The black lines correspond to the mean values. The significance of differences in expression was assessed in Mann-Whitney tests: non-significant, ns; * $P<0.05$; *** $P<0.001$.

(B) The levels of mRNA for NKG2D ligands (*ULBP1* and *ULBP2*) were assessed in HCCs with ($N=128$) and without ($N=72$) *TP53* mutations, and in HCCs with ($N=60$) and without ($N=140$) *CTNNB1* mutations. The fold-change (\log_2) in gene expression is presented relative to the level of expression in normal liver. The black lines correspond to the mean values. The significance of differences in expression was assessed in Mann-Whitney tests: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

(C) Correlation between the levels of mRNA for *ULBP1* and *ULBP2* and those for *GLUL*, *LGR5* or *TBX3* in human HCC samples ($N=354$). Single values were plotted and Pearson's product-moment correlation coefficient was determined. Statistical significance was assessed in Student's *t*-test.

Fig. 4. Expression of the *Rae-1* δ and *Rae-1* ϵ NKG2D ligands is strongly downregulated in HCCs displaying β -catenin activation

(A) The levels of mRNA for NKG2D ligands (*Rae-1* δ and ϵ) were assessed by real-time RT-qPCR on DEN-induced HCC ($N=9$), HCCs with β -catenin mutations ($N=8$) and control liver ($N=7$). Gene expression was normalized against 18s rRNA for each sample. Data are expressed as the mean \pm SEM, with significance determined in Mann-Whitney tests: * $P<0.05$; ** $P<0.01$ *** $P<0.001$.

(B) The levels of mRNA for NKG2D ligands (*Rae-1* δ and ϵ) were assessed by real-time RT-qPCR in *Apc* ^{Δ Hep} livers ($N=14$) and control liver ($N=11$). Gene expression was normalized against 18s rRNA for each sample. Data are expressed as the mean \pm SEM, with significance determined in Mann-Whitney test: non significant, ns; ** $P<0.01$.

(C) Paraffin-embedded liver sections obtained from *Apc* ^{Δ Hep}, DEN-induced HCCs, HCCs with β -catenin mutations (Lpk-myc+) and control livers were stained for glutamine synthase (GS) and Pan-*Rae-1*. Scale bars: 100 μ m.

Fig. 5. The expression of the *Rae-1* δ and *Rae-1* ϵ NKG2D ligand genes is directly inhibited by β -catenin signalling

(A) Genomic environment of the *Rae-1* gene (UCSC Genome Browser) and ChIP-seq peaks at the *Rae-1* promoter. The crude reads for the ChIP-seq analysis of *Apc* ^{Δ Hep} and control livers against TCF4 (data deposited under accession number GSE32513 in the Gene Expression Omnibus database). qPCR was performed to amplify the *Rae-1* promoter. The data are expressed as the mean \pm SEM, with significance assessed in Mann-Whitney tests: ** $P<0.01$.

(B) The levels of mRNA for NKG2D ligands (*Rae-1* δ and ϵ) were assessed by real-time RT-qPCR in CRISPR-Cas9 exon 3 β -catenin-activated α ML cells and CRISPR-Cas9 *rosa* control α ML cells. Gene expression was normalized against 18s rRNA for each sample. The data are expressed as the mean \pm SEM, with significance assessed in Mann-Whitney tests: * $P<0.05$; ** $P<0.01$.

Fig. 6. Expression of the NKG2D receptor and ULBP1 is associated with the presence of inflammatory cells in HCC

(A) The levels of mRNA for KLRK1 were assessed in HCC tumors with (Yes; $N=28$) and without (No; $N=91$) lymphocytic infiltrates. The fold-change (\log_2) in gene expression is presented relative to the level of expression in normal liver. The black lines correspond to the mean values. The significance of differences in expression was assessed with Mann-Whitney tests: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

(B) Corrplot showing Spearman's rank correlations between the expression levels of *KLRK1*, *ULBP1*, *ULPB2* and those of the endothelial, B-cell, T-cell, cytotoxic cell, myeloid dendritic cell, neutrophil and NK cell signatures found in RT-qPCR data for HCC samples. Red circles indicate significant positive correlations. Blue circles indicate negative correlations. The intensity of the color indicates the magnitude of the rho correlation coefficient. P values are shown at the bottom of each cell.

Figure 1

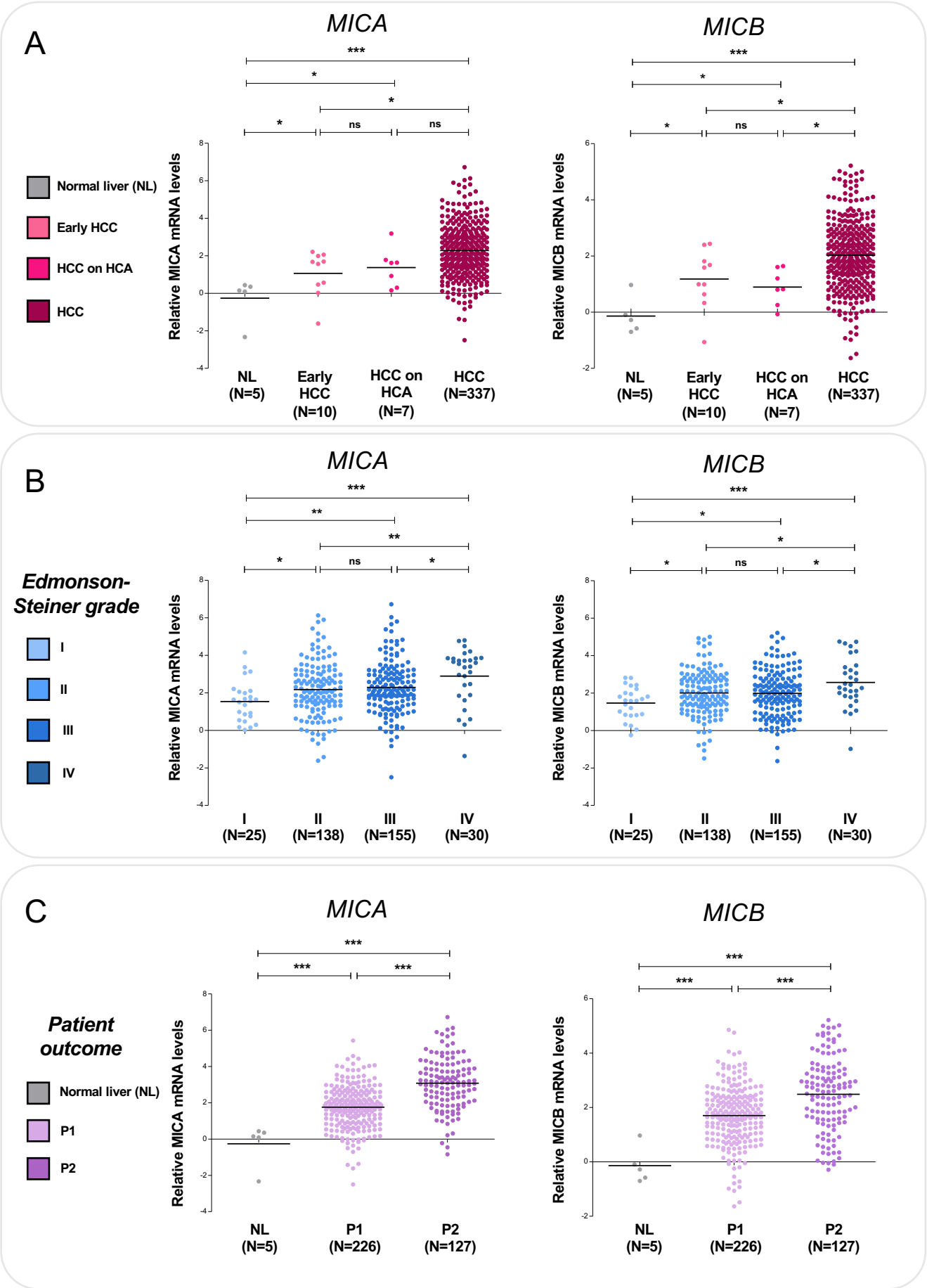


Figure 2

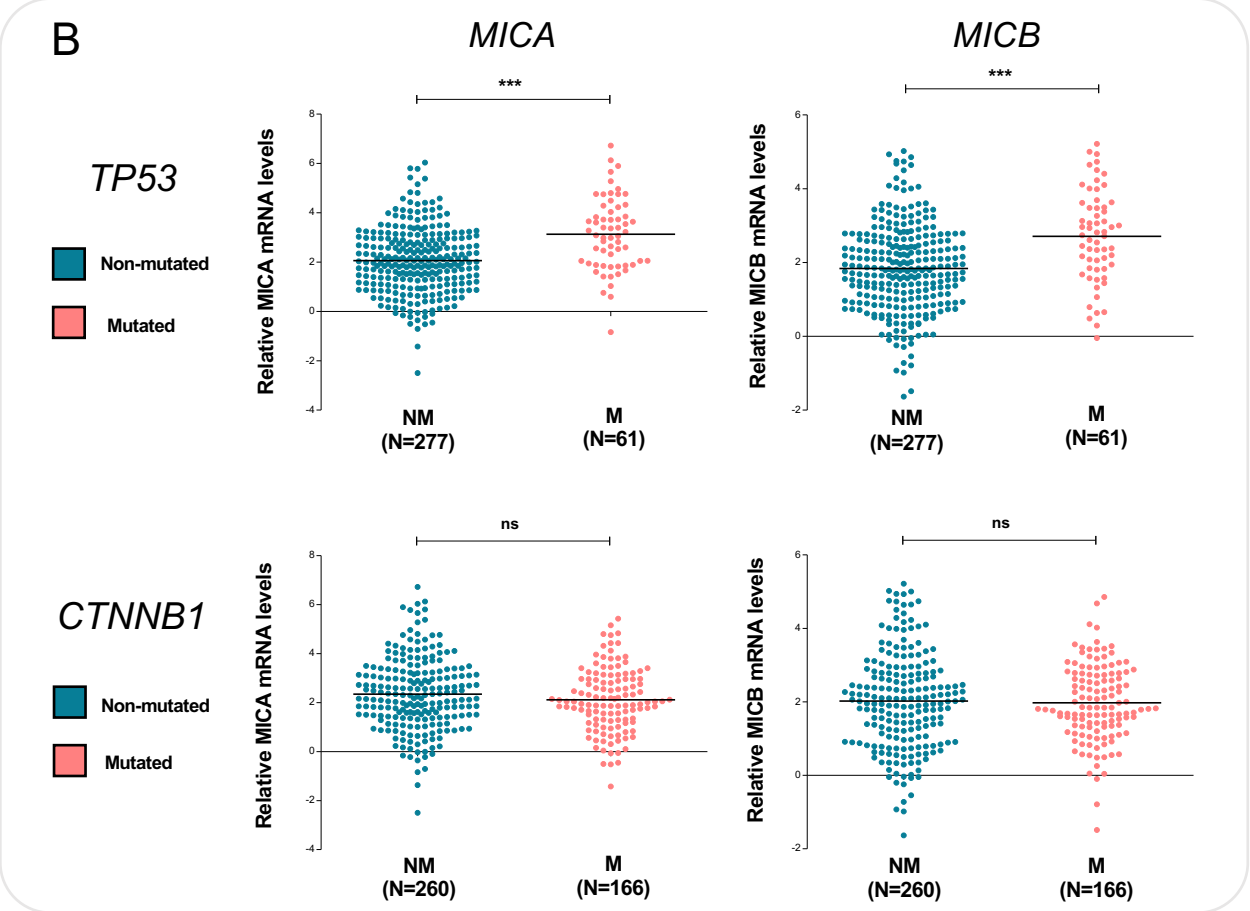
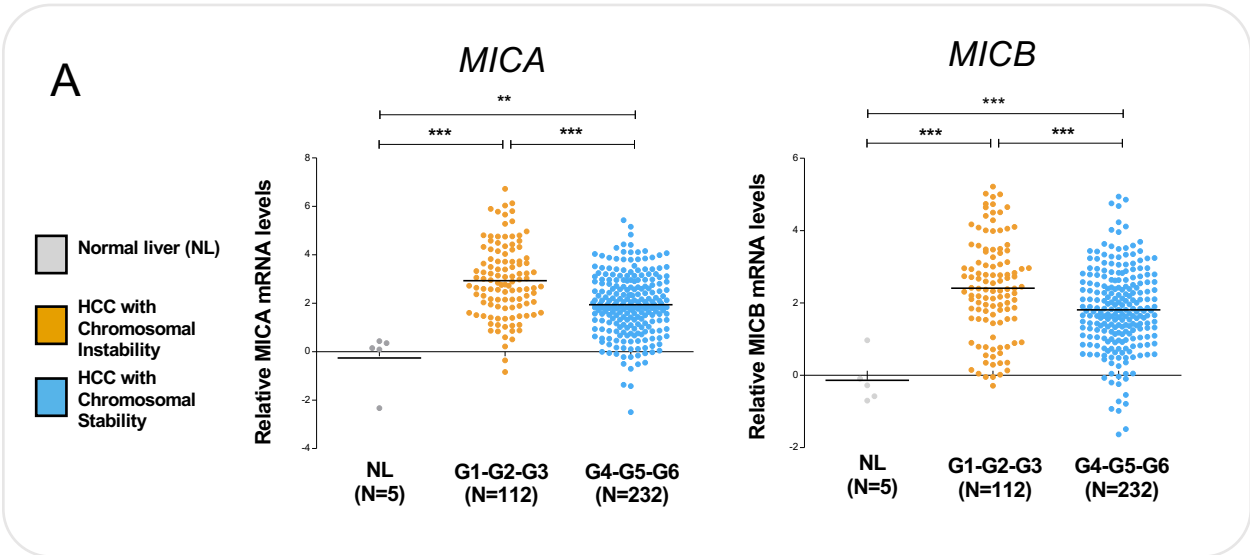


Figure 3

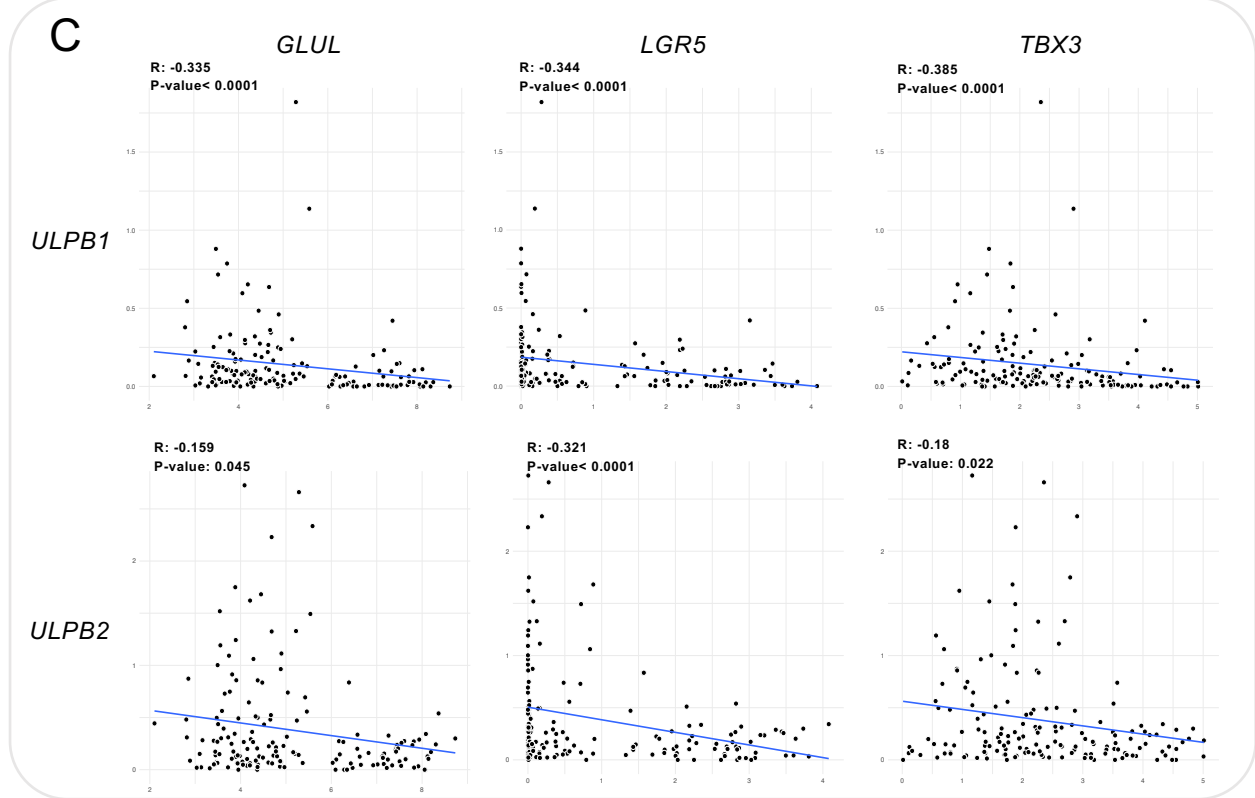
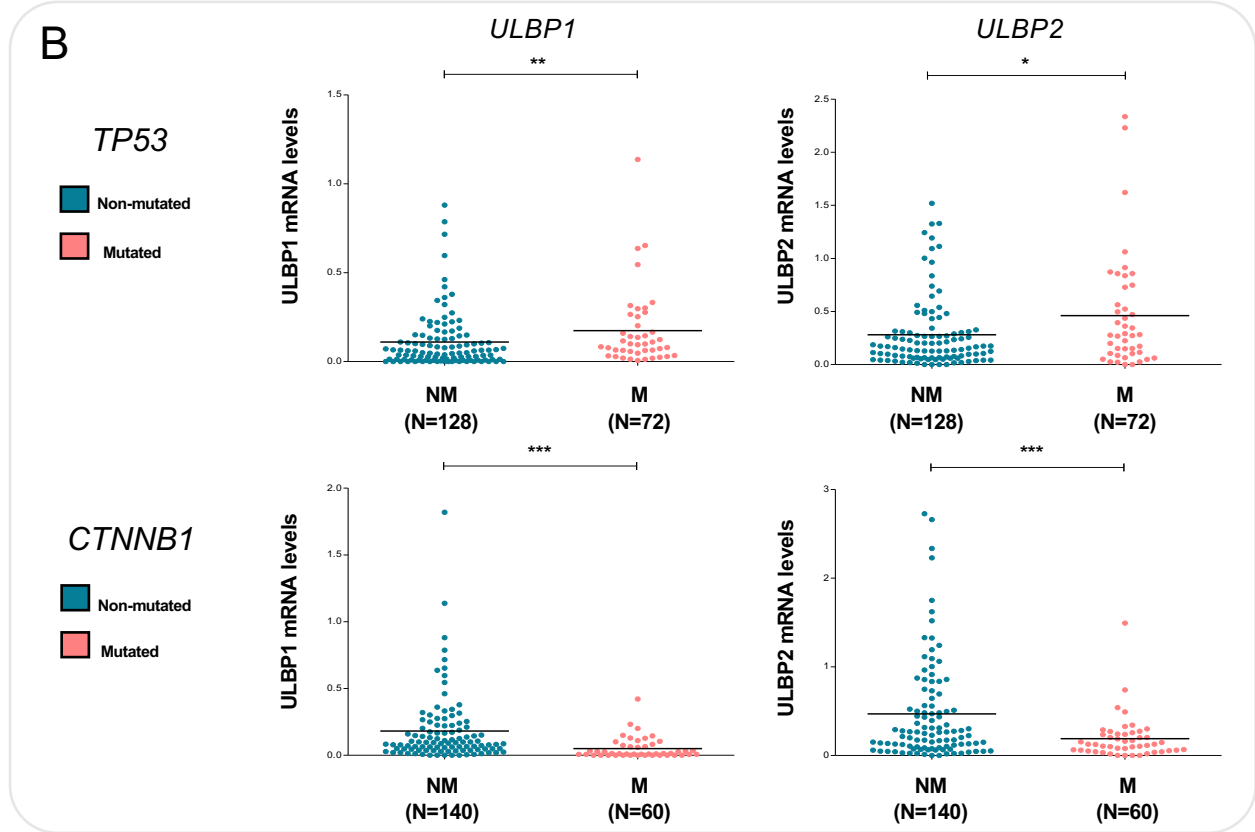
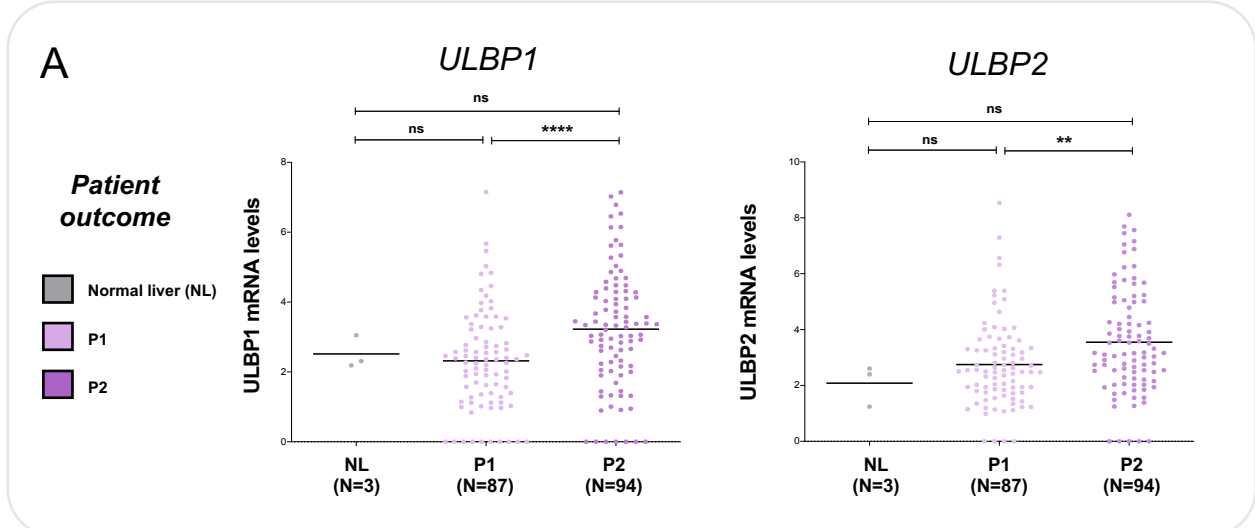
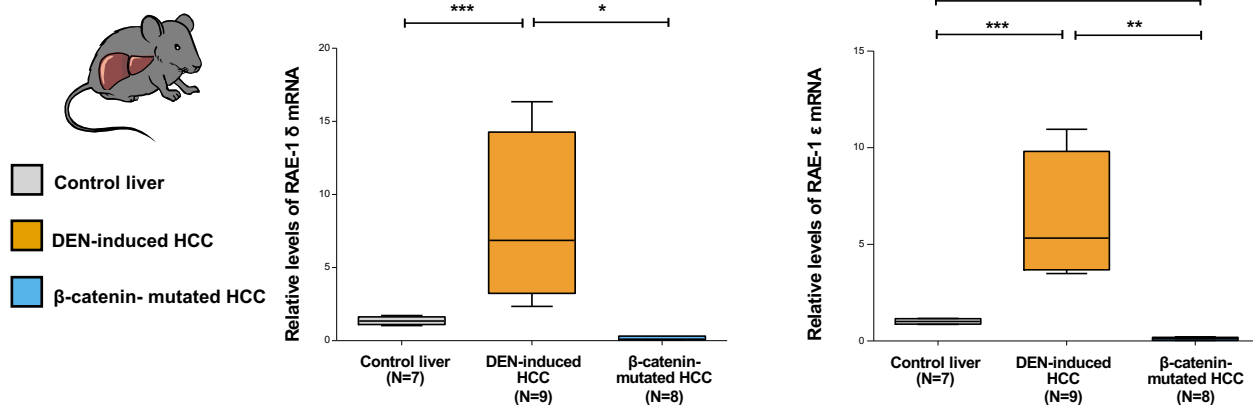
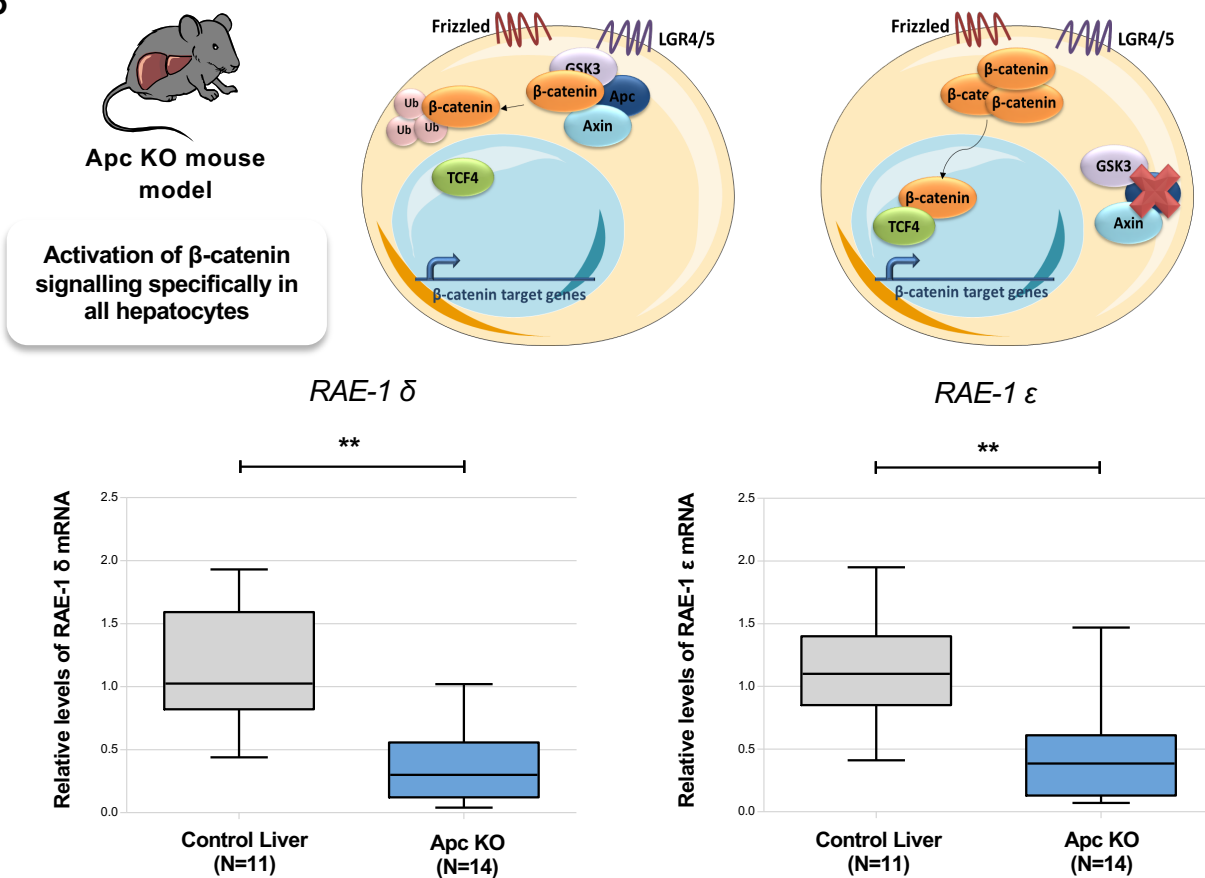


Figure 4

A



B



C

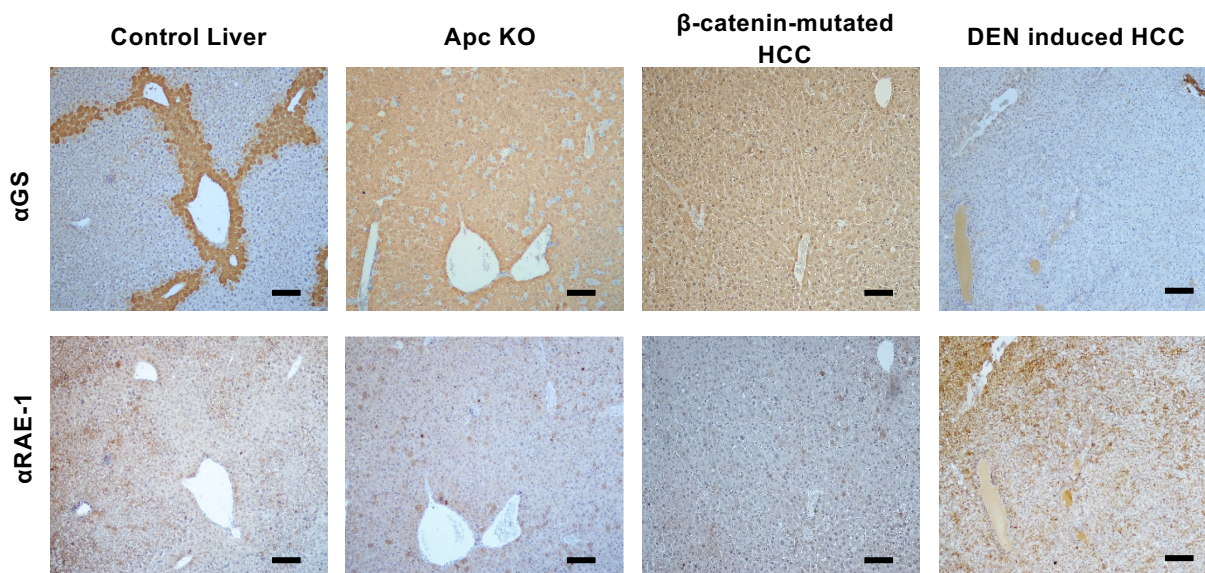


Figure 5

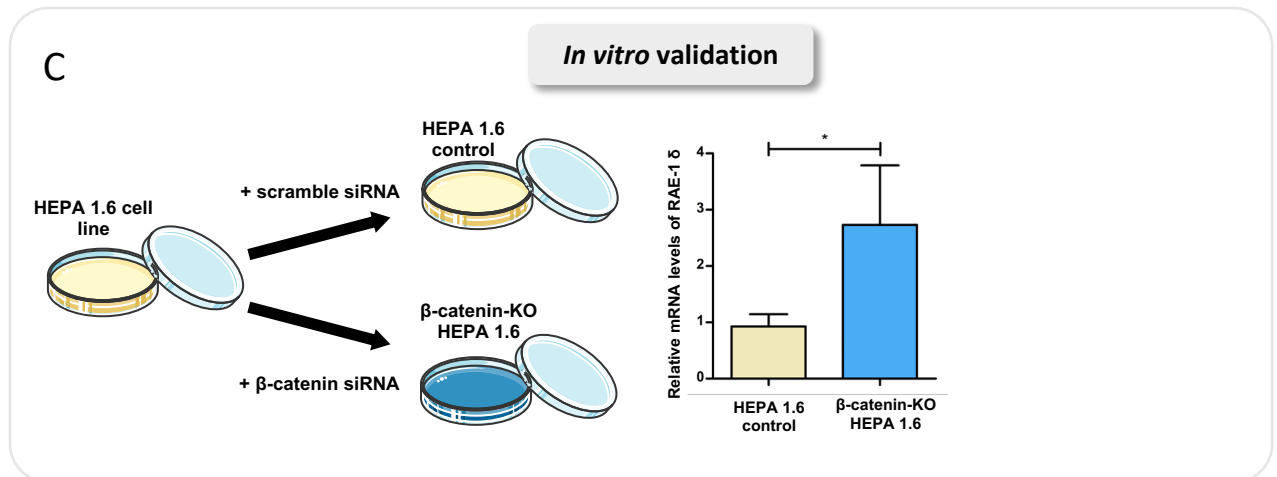
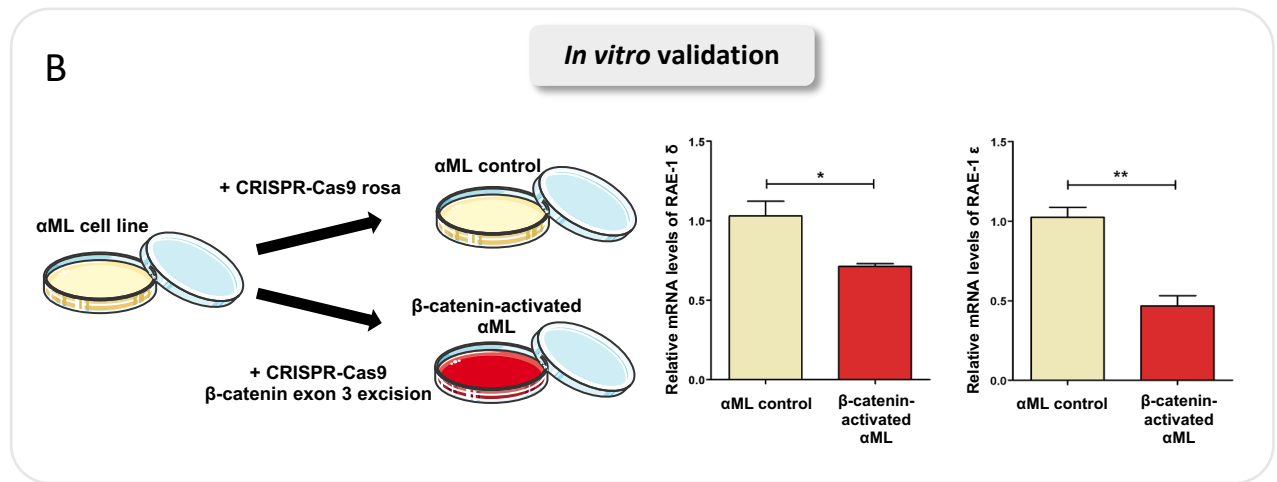
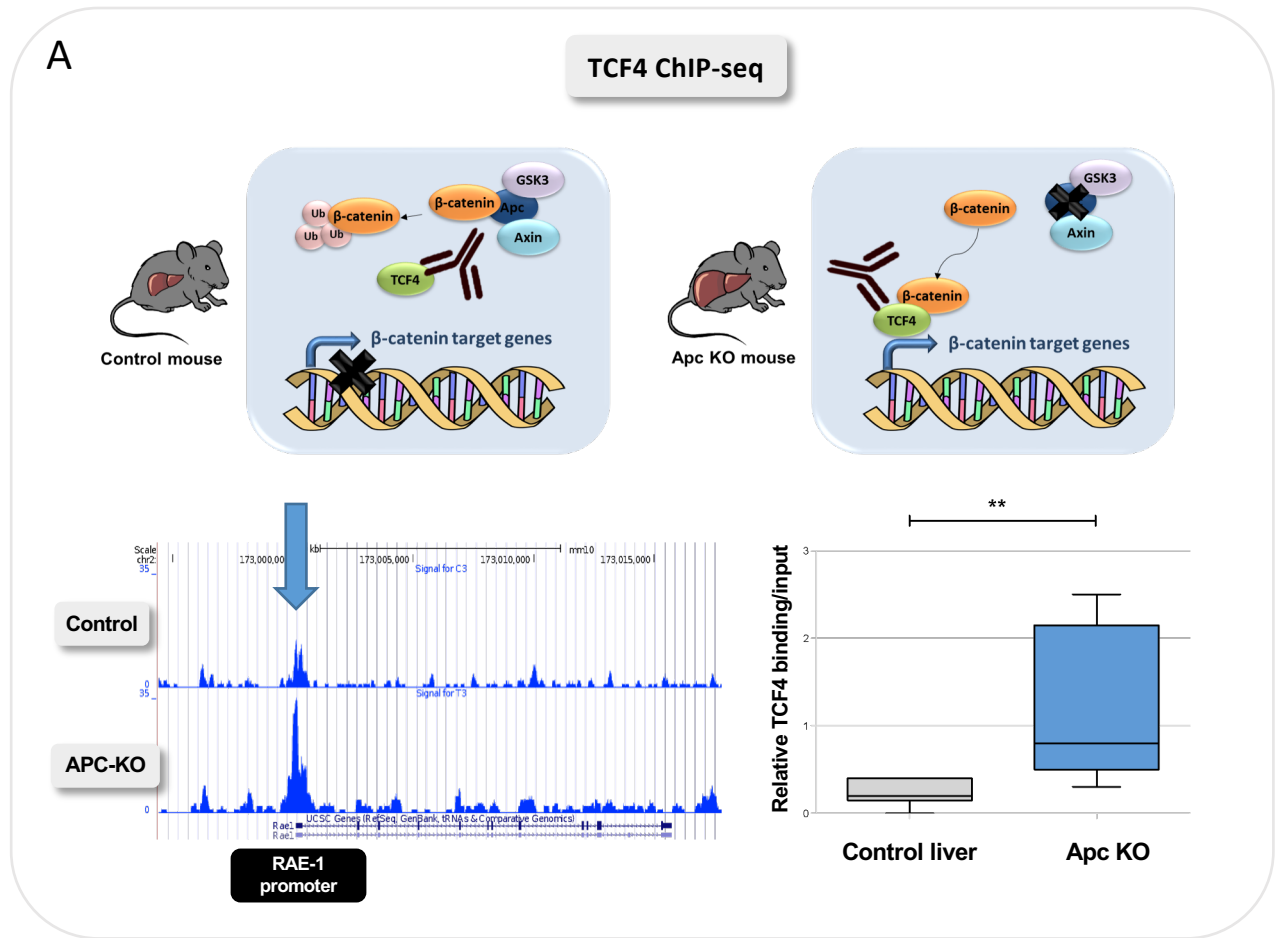
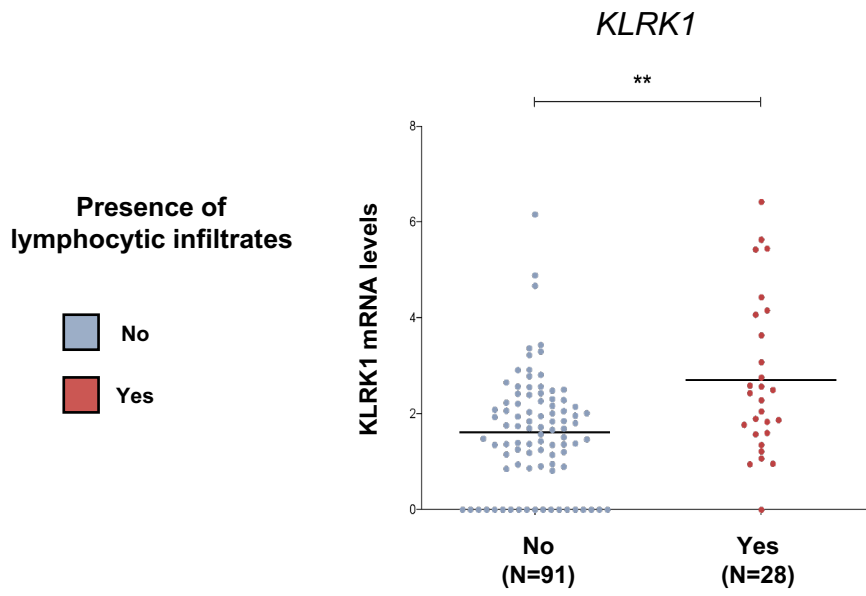
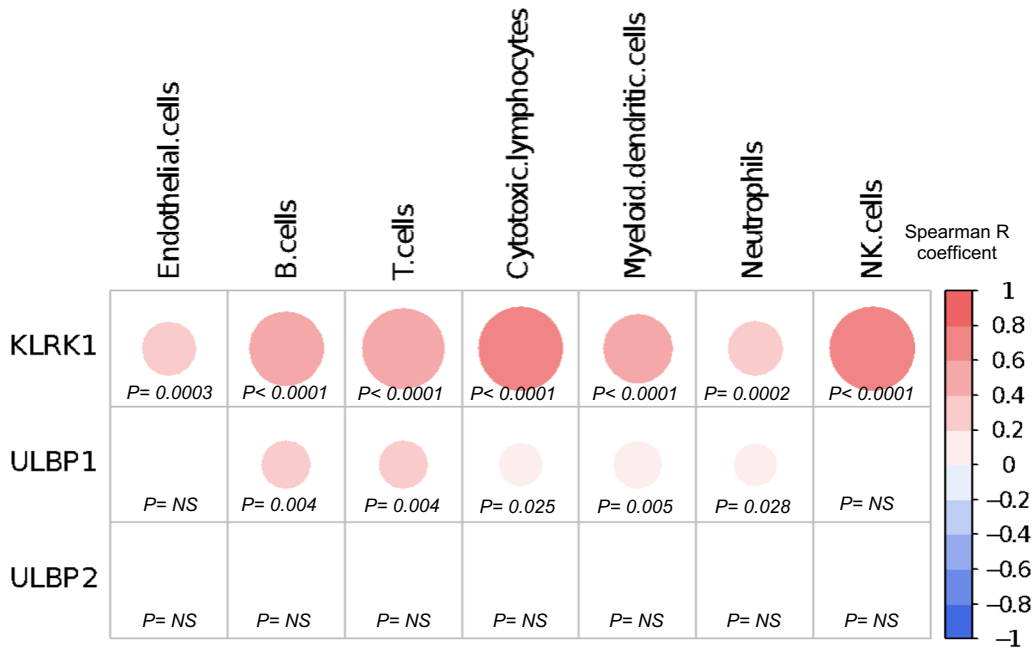


Figure 6

A

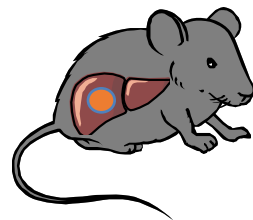
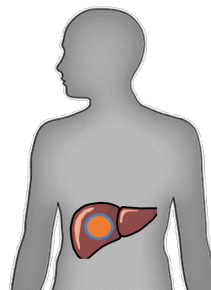


B

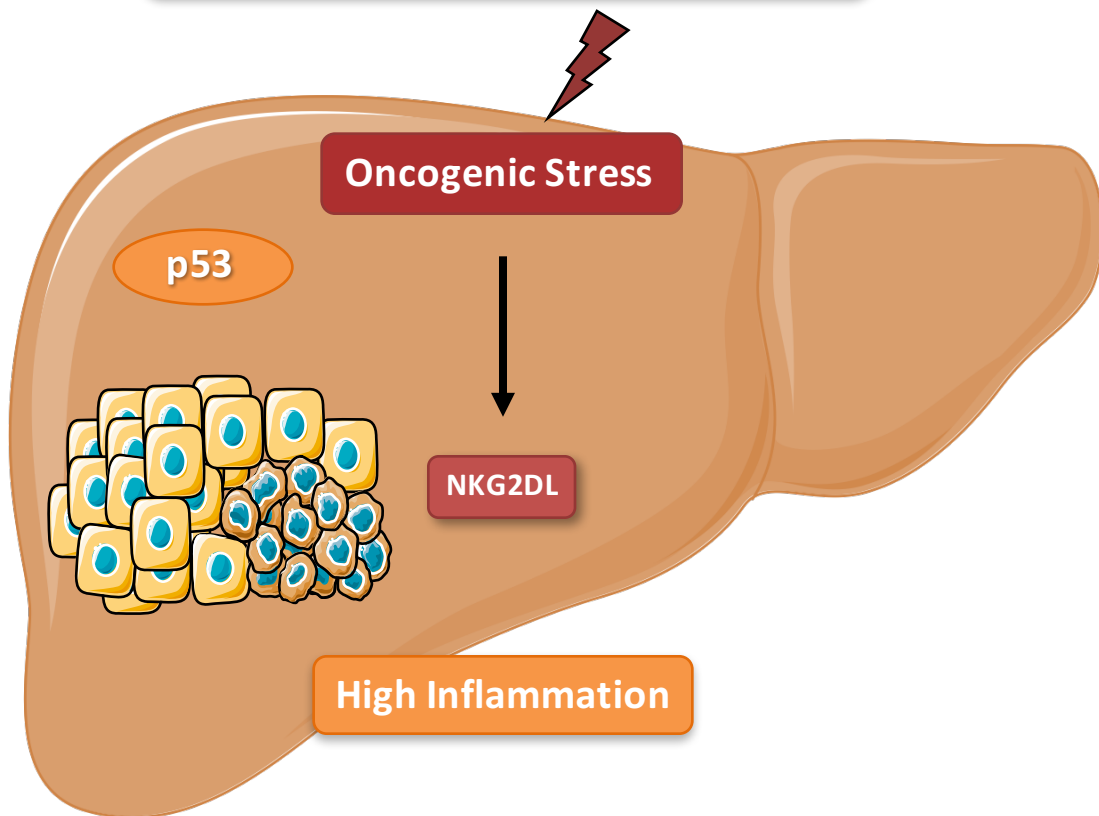


Variables		Data available	Numbers (%), Median (Min-Max)
Analysis per patient (n=325)			
Age (years)		324	64 (18-90)
Gender (Male)		324	267 (82,4%)
Hepatitis B		323	63 (19,5%)
Hepatitis C		324	68 (21%)
High alcohol intake		324	147 (45,4%)
Metabolic syndrome		321	50 (15,6%)
Without etiology		324	43 (13,3%)
AFP<20 ng/mL		306	129 (42,2%)
Analysis per tumor (n=354)			
Histological Diagnosis	Early HCC	354	10 (2,8%)
	HCC on HCA		7 (2%)
	HCC		337 (95,2%)
Metavir score (fibrosis of non-tumor liver)	F0-F1	354	114 (32,2%)
	F2-F3		86 (24,3%)
	F4		154 (43,5%)
G1-G6	G1	353	26 (7,4%)
	G2		39 (11,1%)
	G3		47 (13,3%)
	G4		125 (35,4%)
	G5		70 (19,8%)
	G6		46 (13%)
Edmondson III-IV		348	185 (53,2%)
WHO	Good	348	106 (30,5%)
	Medium		194 (55,7%)
	Bad		48 (13,8%)
5-gene score	Good	353	226 (64%)
	Bad		127 (36%)
<i>TERT</i> promoter mutations		338	214 (63,3%)
<i>CTNNB1</i> mutations		339	125 (36,9%)
<i>TP53</i> mutations		347	61 (17,6%)

		LICA-FR (n=200)	
Variable		n	Total (%)
Age (years)	median [min-max]	200	64 (18-94)
Gender	Male	200	157 (79%)
Etiology	Alcohol	198	72 (36%)
	Hepatitis B	200	52 (26%)
	Hepatitis C	198	31 (16%)
	Metabolic Syndrome	196	39 (20%)
	Without known etiology	199	25 (13%)
Fibrosis stage	F1-F2		92 (46%)
	F2-F3	200	45 (22.5%)
	F4		63 (31.5%)
Edmonson grading	I-II	200	59 (30%)
	III-IV		139 (70%)
G1G6 (Boyault S. et al., Hepatology 2007)	G1		24 (12%)
	G2		28 (14%)
	G3	197	50 (25%)
	G4		40 (20%)
	G5		31 (16%)
	G6		24 (12%)
WHO (differentiation)	good		32 (18%)
	medium	177	106 (60%)
	weak		39 (22%)
5 gene score (Nault JC et al., Gastroenterology 2015)	P1	181	87 (48%)
	P2		94 (52%)
TERT alterations	Mutated	198	101 (51%)
CTNNB1 alterations	Mutated	200	60 (30%)
TP53 gene alterations	Mutated	200	72 (36%)

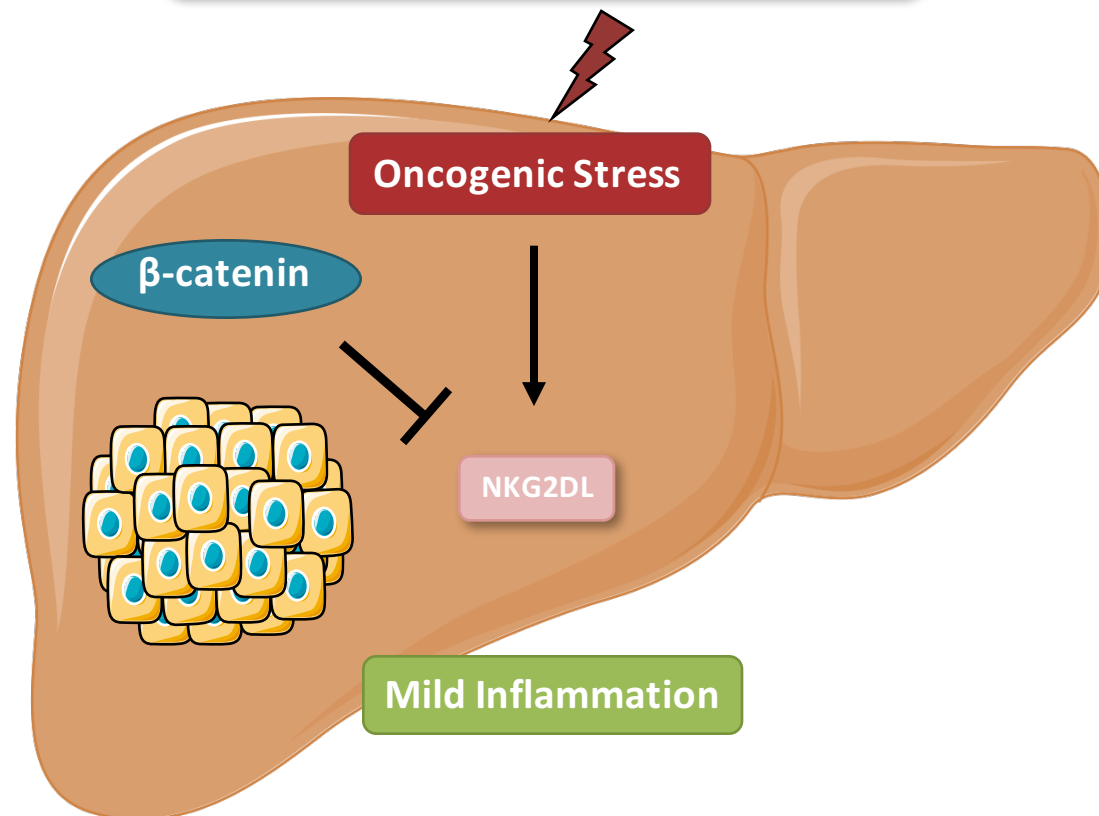


Chromosomal Instability



HCC
High aggressiveness

Chromosomal Stability



HCC
Low aggressiveness