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Somatic mutations activating STAT3 in human inflammatory hepatocellular adenomas

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Abbreviations:

CRP	C-Reactive Protein
CSF1R	colony stimulating factor 1 receptor
CTNNB1	catenin (cadherin-associated protein), beta 1
EGFR	epidermal growth factor receptor
EP	empty plasmid
gp130	glycoprotein 130
HCA	hepatocellular adenoma
HCC	hepatocellular carcinoma
HNF1 α	hepatocyte nuclear factors 1 α
IHCA	inflammatory hepatocellular adenoma
IL6ST	interleukin 6 signal transducer
Luc	luciferase
ORF	open reading frame
PDGFR	platelet-derived growth factor receptors
SAA	serum amyloid A
SH2	Src Homology 2
siRNA	small interfering RNA
SOCS3	suppressor of cytokine signaling 3
Src	sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
TAD	transactivation domain

Abstract

Inflammatory hepatocellular adenomas (IHCA) are benign liver tumors and 60% of these have *IL6ST* (gp130) mutations that activate interleukin 6 (IL-6) signaling. Here we report that 12% of IHCA subsets lacking *IL6ST* mutations harbor somatic *STAT3* mutations (6/49). Most of these mutations are amino acid substitutions in the SH2 domain that directs *STAT3* dimerization. In contrast to wild-type *STAT3*, IHCA *STAT3* mutants constitutively activated the IL-6 signaling pathway independent of ligand in hepatocellular cells. Indeed, the IHCA *STAT3* Y640 mutant homodimerized independent of IL-6 and was hypersensitive to IL-6 stimulation. This was associated with phosphorylation of tyrosine 705, a residue required for IL-6-induced *STAT3* activation. Silencing or inhibiting the tyrosine kinases JAK1 or Src, which phosphorylate *STAT3*, impaired constitutive activity of IHCA *STAT3* mutants in hepatocellular cells. Thus, we identified for the first time somatic *STAT3* mutations in human tumors, revealing a new mechanism of recurrent *STAT3* activation and underscoring the role of the IL-6-*STAT3* pathway in benign hepatocellular tumorigenesis.

Introduction

Inflammatory hepatocellular adenomas (IHCA) are benign liver tumors that predominantly develop in women and that are frequently associated with obesity and alcohol abuse (Bioulac-Sage et al., 2007; Zucman-Rossi et al., 2006). These tumors are characterized by overexpression of the acute-phase inflammatory response in tumor hepatocytes and highly polymorphous inflammatory infiltrates. We recently showed that 60% of IHCA activate STAT3 (Signal Transducers and Activators of Transcription type 3) via somatic heterozygous activating mutations in *IL6ST* that encodes the gp130 co-receptor and signal transducer of IL-6 family cytokines (Rebouissou et al., 2009).

Inflammatory adenoma is a homogenous disease, where gp130 mutated and non-mutated IHCA are similar in their expression profiles, in particular regarding the expression of acute phase inflammatory response target genes (Rebouissou et al., 2009). To identify genetic alterations causing the inflammatory phenotype, defined as an acute-phase inflammatory response with elevated levels of Serum Amyloid A (SAA) and C-Reactive protein (CRP) observed in non-mutated gp130 IHCA, we used a candidate gene strategy. We focused on *STAT3* as it encodes a key nuclear transcription factor that induces the acute-phase inflammatory response in hepatocytes. Moreover, *STAT3* has oncogenic roles in epithelial cell tumorigenesis (Grivennikov et al., 2009; Grivennikov et al., 2010; Yu et al., 2009). *STAT* proteins are latent transcription factors activated by phosphorylation, typically following the binding of extracellular ligands to cytokine receptors that activate associated JAK kinases, via growth factor receptors having intrinsic tyrosine kinase activity (e.g., EGFR, PDGFR and CSF1R), as well as by non-receptor tyrosine kinases such as Src family kinases (Levy and Darnell, 2002).

STAT3, one of seven STAT family members, is activated by gp130 in response to interleukin-6 (IL-6) (Inoue et al., 1997; Zhong et al., 1994). STAT3 is persistently phosphorylated in many human cancer cell lines and in epithelial cells and primary tumors, including hepatocellular carcinoma, breast, prostate, and head and neck cancers, and also in several hematological malignancies. *STAT3* mutations have never been described in human tumors, yet we reasoned that alterations activating its function would explain the inflammatory phenotype of IHCA lacking mutations in *gp130*.

Results and Discussion

Identification of *STAT3* mutations in IHCA

Among 114 screened hepatocellular adenomas, we identified seven *STAT3* mutations in 6 tumors (Table 1 and Supplementary Fig. S1a-b). All of these adenomas were inflammatory (6 out of 75 IHCA) and no additional somatic mutations were identified in *HNF1A*, *CTNNB1* or *IL6ST*, genes recurrently altered in IHCA and HCA (Bluteau et al., 2002; Rebouissou et al., 2009; Zucman-Rossi et al., 2006). Thus, *STAT3* IHCA mutations are exclusive from all other known genetic alterations in hepatocellular tumors. *STAT3* mutated IHCA were associated with obesity ($n = 4$) and/or alcohol abuse ($n = 3$) and multiple (≥ 4) nodules were present in half of these cases. All *STAT3* mutations were somatic, as they were not observed in adjacent non-tumor liver tissues that were steatotic in 5/6 cases. All mutations were also monoallelic and led to amino acid substitutions in 5 cases or to in-frame insertions of 1 to 4 amino acids in the remaining two cases. In one case (#379) we identified three nucleotide mutations that led to amino acid substitutions at codons 502 and 658. Sequencing cloned *STAT3*

cDNA from this tumor showed that the three different nucleotide mutations were carried by the same allele. We also showed in three different cases (#379, #966 and #1351) that the mutations were harbored by all of the isoforms of STAT3, including splicing of nucleotides 2099-2101 and 2145-2194 (STAT3 β) that lead to p.Ser701del and p.Thr716PhefsX8, respectively (Supplementary Fig. S1c) (Schaefer et al., 1995). Sequencing *STAT3* RT-PCR products in mutated tumors showed that all cases expressed the normal and mutated *STAT3* alleles at comparable levels (Supplementary Fig. S1a-b). Thus, the expression of one mutated *STAT3* allele is a rare but recurrent genetic event in IHCA that is selected for during benign hepatocellular tumorigenesis.

Four of the seven identified mutations were located in the domain of STAT3 (residues 585-688) that shares homology with Src-homology-2 (SH2) domains (Fig. 1a). The STAT3 SH2 domain mediates STAT3 dimerization via binding of phosphotyrosine residue Y705 (Shuai et al., 1994; Wen et al., 1995). Moreover, three of these mutations clustered in a hotspot in the SH2 domain at codons 657 and 658. Interestingly, introduction of cysteine at residues 662 and 664 promotes STAT3 dimerization and creates a constitutively active transcription factor (Stat3-C) (Bromberg et al., 1999). The fourth mutation, Y640F, was in the PYTK motif conserved in STAT1, STAT2 and STAT3. Interestingly, substitution of the corresponding Y631 to phenylalanine in STAT2 promotes type I IFN signaling (Constantinescu et al., 2008; Scarzello et al., 2007). The three other mutations found in IHCA were distributed throughout the protein; the altered leucine-78 that contributes to dimerization (Chen et al., 2003), glutamate-166 that is part of helix α 1 involved in the interaction with gp130 (Zhang et al., 2000a) and aspartate-502 that is located in the α -helical “connector” domain. Remarkably, none of the

mutations identified in IHCA were similar to the 150 germline inactivating *STAT3* heterozygous mutations described in patients with Job's syndrome that causes the hyper-IgE syndrome (Holland et al., 2007; Minegishi et al., 2007).

IHCA mutations activate STAT3 in absence of IL-6

To investigate possible functional consequences of these *STAT3* IHCA mutations, we introduced the seven different IHCA mutations into a *STAT3 α* (full length) cDNA and analyzed their effects in Hep3B cells, a hepatocellular carcinoma line that activates the acute inflammatory phase following treatment with IL-6 (Coulouarn et al., 2005). The results were also validated in two additional hepatocellular cell lines (HepG2 and Huh7). As described (Bromberg et al., 1999), in the absence of IL-6 ligand and serum, overexpression of wild-type *STAT3* (WT) alone was not sufficient to activate a *STAT3* reporter (Fig. 1b) nor the downstream acute-phase inflammatory genes (Fig. 1c). In contrast, the six IHCA mutants and the mouse *Stat3-C* mutant (Bromberg et al., 1999) activated the *STAT3* reporter in the absence of IL-6 and induced targets typical of the acute inflammatory response, including *CRP* and *SOCS3* (Fig. 1b and 1c). Moreover, activation of *STAT3* was proportional to the amount of the transfected mutant *STAT3* (Fig. 1d). The last IHCA mutant D502Y/K658Y, identified in tumor #379, has two mutations on the same allele. The D502Y mutation did not impart constitutive activity, whereas K658Y activated the inflammatory response. Further, the dual mutant (D502Y + K658Y) produced a hyperactive *STAT3* mutant compared to K658Y alone (Fig. 1e). Thus, mutation at D502 alone is not sufficient to activate *STAT3* but cooperates with the mutation at K658 present in the SH2 domain.

IHCA STAT3 mutants are constitutively phosphorylated on Tyr705 and translocate to the nucleus

STAT3 activation requires phosphorylation of tyrosine 705, dimerization and translocation to the nucleus. Accordingly, in *STAT3* mutated IHCA, immunohistochemical analyses demonstrated marked overexpression of phospho-Tyr705-STAT3 in the nucleus of these tumor hepatocytes (Fig. 2a). Similarly, in Hep3B transfected cells, we showed that *STAT3* mutants L78R, E166Q, Y640F and G656_Y657insF were constitutively phosphorylated on Tyr705 and translocated to the nucleus, whereas overexpressed wild-type *STAT3* was unphosphorylated and cytoplasmic in the absence of cytokine (Fig. 2b-d). *STAT3* IHCA mutants did not exhibit increased phosphorylation of serine 727, a second residue also involved in *STAT3* activation (Wen et al., 1995), when compared to IL-6 activated wild-type *STAT3* (Supplementary Fig. S2a). Moreover, in *STAT3* Y640 and G656 mutants substitution of tyrosine 705 with phenylalanine totally abrogated activation of the inflammatory response. Thus, tyrosine phosphorylation is essential for activation of IHCA mutant *STAT3* (Fig. 2e).

IHCA STAT3 mutants dimerize in absence of cytokine

A critical step in STAT3 activation is the formation of homodimers through reciprocal phospho-Tyr705-SH2 domain interactions. To assess dimerization by IHCA STAT3 mutants, we performed co-immunoprecipitation assays with epitope-tagged STAT3 proteins. The Y640F mutant homodimerized or heterodimerized with wild-type STAT3 independent of IL-6 (Fig. 3a). Moreover, mutant homodimers were highly phosphorylated at tyrosine 705. In contrast, homodimers of wild-type STAT3 were only faintly detected and were not phosphorylated on tyrosine 705. As expected, IL-6 treatment does not enhance dimer formation, but slightly increases tyrosine 705 phosphorylation (Haan et al., 2000). In addition, overexpression of wild-type STAT3 impaired the activity of the STAT3 mutant Y640F in a dose dependent manner (Fig. 3b). Thus, wild-type STAT3 can compete with the mutant STAT3 and impair the formation of active IHCA mutant homodimers. Interestingly, analysis of the STAT3 homodimer structure (Becker et al., 1998) showed that 4 out of 6 STAT3 mutants targeted amino acids (Y640, K658, Y657 and Y657-M660) that reside near the transcription activation domain (TAD, residues 701-722) (Fig. 3c and Supplementary Fig. S2b). For example, Tyr-640 engages in hydrophobic interactions with two-fold related Phe-710 and residues 656-659 pack against TAD residues 711-716 (Fig. 3c and Supplementary Fig. S2b). These modifications could thus enhance mutant STAT3 function, and they must also somehow facilitate phosphorylation of tyrosine 705 by kinases in hepatocytes, which would then drive dimerization and nuclear localization of mutant STAT3. Following cytokine exposure, phosphorylated STAT3 homodimers translocate to the nucleus where they bind to specific IL-6 responsive elements to activate the transcription of

target genes (Hattori et al., 1990). In Hep3B cells, nuclear Y640 and G656 STAT3 mutants bind to recognition elements found in endogenous STAT3 target genes in the absence of IL-6 whereas, as expected, wild-type STAT3 was defective in binding in the absence of IL-6 (Fig. 3d).

IHCA STAT3 mutants are hyper-responsive to IL-6

To investigate the role of the IL-6-JAK pathway in the activation status of IHCA STAT3 mutants, we tested the effects of increasing doses of IL-6 on their activity. Indeed, even low doses of IL-6 (1 ng/ml) dramatically augmented the constitutive activity of the Y640 STAT3 mutant when compared to the wild-type STAT3 (Fig. 4a). This hypersensitive response to IL-6 signaling was confirmed for L78, E166, G656 and Y657 STAT3 mutants (Fig. 4b and Supplementary Fig. S2c). Together with gp130, JAK1 is known as the major kinase that phosphorylates and activates STAT3 in IL-6 signaling. Accordingly, silencing JAK1 by siRNA abolished the hyper-stimulation of the STAT3 mutants by IL-6 (Fig. 4b).

Activity of IHCA STAT3 mutants requires Src

In the absence of IL-6 signaling, silencing of gp130 or JAK2 did not affect the constitutive activity of IHCA STAT3 mutants (Fig. 4c-d). In contrast, JAK1 knockdown by siRNA partially impaired constitutive IHCA STAT3 mutant activity (Fig. 4e). Similarly, AG490, an inhibitor that disables the kinase activity of both JAK1 and JAK2, Ruxolitinib, a selective inhibitor of Jak1, and curcumin, a STAT3 inhibitor, partially decreased the

activity of IHCA STAT3 mutants (Supplementary Fig. S3a-b). In contrast, treatment with SrcI-1 kinase inhibitor dramatically impaired both the constitutive and IL-6 induced activation of IHCA STAT3 mutants (Fig. 4f and Supplementary Fig. S3c). Two other Src kinase inhibitors also impaired the activity of IHCA STAT3 mutants (Supplementary Fig. S3d-e). Thus, phosphorylation and activation of mutant STAT3 does not require gp130 or JAK2 but requires JAK1 and Src signaling. Depending on cell context, STAT3 can be activated directly by Src family kinases or via Jak1 (in NIH 3T3 cells) as an intermediate kinase between Src and STAT3 (Zhang *et al.*, 2000). Our findings suggest that in hepatocellular cell lines, the requirement of Jak1 is necessary for maximal activation of IHCA STAT3 mutants, whereas Src is essential for their constitutive activation.

STAT3-mutated IHCA are similar to gp130-mutated IHCA

In vivo, expression analyses of 16 IHCA revealed no significant differences in 28 target genes of IL-6 and interferon signalling pathways in STAT3 mutated adenomas ($n=5$) versus gp130 mutated IHCA ($n = 11$; Fig. 4g). Thus, the functional effects of mutating STAT3 and gp130 in human hepatocellular adenomas appear redundant.

In the liver, activation of the IL-6-STAT3 signaling is necessary for the acute-phase response during inflammation (Lutticken *et al.*, 1994) and liver regeneration, particularly following transplantation (Cressman *et al.*, 1996). The connection between inflammation and tumorigenesis has been extensively investigated over the past decade (see ref. (Grivennikov and Karin, 2010) for review). Depending of the model, inflammation can trigger tumor initiation or function to enhance tumor progression and/or metastasis. During these processes and in numerous tumor types, STAT3 activation has been

recognized as a major oncogenic event. However, in our cohort, STAT3 activating mutations are found in 8% of the inflammatory hepatocellular adenoma, but were not present in a series of 218 malignant liver tumors, including hepatocellular carcinomas and cholangiocarcinomas ($n = 11$). Thus, STAT3 activating mutations appear to be largely or quasi-exclusively associated with benign proliferation of hepatocytes in human.

Collectively our findings show that most of the genetic alterations of HCA subtypes, (HNF1A inactivation, β -catenin activation or activation of STAT3 via mutation of STAT3 or gp130), are associated with benign hepatocyte tumorigenesis, where there remains a largely controlled rate of tumor cell proliferation. At present, only β -catenin activating mutations are associated with malignant transformation to hepatocellular carcinoma (Zucman-Rossi et al., 2006), and here there are likely additional cooperating mutations that lead to frank malignancy. Regardless, to our knowledge this is the first identification of somatic mutation of *STAT3* in human tumors, and these findings underscore the important role of STAT3 activation in hepatocellular benign tumorigenesis and reveal a new mechanism of recurrent STAT3 activation.

Materials and Methods

Tumors and Patients

A series of 364 hepatocellular tumors including 114 hepatocellular adenoma (HCA), 218 common hepatocellular carcinoma (HCC), 11 cholangiocarcinomas and 20 hepatocholangiocarcinomas were previously collected (according to French ethical guidelines) and characterized (Bioulac-Sage et al., 2007; Zucman-Rossi et al., 2006). Briefly, patients with HCA were predominantly female (80%) with a mean age of 39 years; among them 13% presented mutations of *CTNNB1* gene, 19% of *HNF1 α* , 26% displayed IHCA-associated *IL6ST* mutations, 39.5% showed IHCA without *IL6ST* mutations and 9.6% were non classified adenomas. Patients with HCC were predominantly male (79%), with a mean age of 64 years, and 34.3% of these presented with cirrhosis. The underlying risk factors of patients with HCC were chronic hepatitis B (19%), chronic hepatitis C (25%) and heavy alcohol intake (38%). Patients with cholangiocarcinoma were predominantly male (72%) and were slightly younger (mean age: 61 years) than patients with common HCC. Patients with hepatocholangiocarcinoma were also predominantly male (89%) and had a median age of 61 years. All patients have given consent according to French law, and the ethical committee of Saint Louis Hospital approved the study.

Tumor and non-tumor liver samples were frozen immediately after surgery or biopsy and stored at -80°C . Tissues samples were also included in 10% formalin, paraffin-embedded and stained with haematin-eosin and masson trichrome. The

diagnosis of HCA, HCC, cholangiocarcinoma and hepatocholangiocarcinoma was performed on established histological criteria (1995).

Quantitative RT-PCR

Quantitative RT-PCR was performed as described (Rebouissou et al., 2009) using pre-designed primers and probe sets from Applied Biosystems (listed in Supplementary Table S1). Ribosomal 18S RNA (*R18S*) was used to normalize expression data and the $2^{-\Delta\Delta CT}$ method was applied.

DNA sequencing

DNA sequencing was performed as described (Rebouissou et al., 2009) using primers described in Supplementary Table S2. All HCA samples were previously sequenced for *CTNNB1* (exons 2-4), *HNF1A* (exons 1-10), and *IL6ST* (exon 6). All HCC samples were previously sequenced for *CTNNB1* (exons 2-4) and *P53* (exons 2-10) as described (Rebouissou et al., 2009; Zucman-Rossi et al., 2006). All mutations were validated by sequencing a second independent PCR product on both strands. In all cases the somatic origin of the mutation found in tumor was verified by sequencing the corresponding adjacent, normal liver sample.

Generation of STAT3 mutants

A full-length STAT3 open reading frame cloned in pCMV6-XL4 vector was purchased from OriGene (SC124165, NM_139276). Mutagenesis reactions were performed using the QuickChange XL site-directed mutagenesis kit (Stratagene) using primers described

in Supplementary Table S3. All constructs were verified by sequencing. The pCMV6-XL4 expression vector plasmid was used as a control. Stat3-C plasmid was purchased from Addgene, Inc. (plasmid 8722).

Cell culture

Hep3B, Huh7 and HepG2 cells (ATCC) were grown in DMEM containing 10% FCS supplemented with penicillin (100 units/mL) and streptomycin (0.1 mg/mL). For transfections, cells were plated 16 hr prior to transfection to produce monolayers that were 60% confluent, and these were transfected by using either Lipofectamine™ LTX (plasmid alone) or Lipofectamine™ 2000 (plasmid and siRNA) according to the manufacturer's instructions (Invitrogen). Transfection efficiency was monitored by measuring the level of either wild-type and mutated *STAT3* mRNA using quantitative RT-PCR or western blot analysis. The same *STAT3* expression level was observed in wild-type *STAT3* and IHCA mutant *STAT3* transfected cells. For luciferase assays, Hep3B cells were co-transfected with a *STAT3* luciferase reporter expressing a firefly luciferase reporter gene that contains three copies of a *STAT3* consensus binding site linked to a minimal thymidine kinase promoter (kindly provided by Dr. H. Gascan, Institut National de la Santé et de la Recherche Médicale UMR564, Angers, France) (pSIEM-Luc 1µg, (Coqueret and Gascan, 2000)) and the expression plasmid for wild-type or IHCA mutant *STAT3* (1µg). One day after transfection, cells were starved in a free-serum medium for 3 hr, then left untreated or treated with IL-6 (100 ng/ml) for 3 hr, and they were then lysed and the luciferase activity determined according to the manufacturer's recommendations (Promega). The activities were normalized to the protein amounts in each cell lysate. All analyses were performed in triplicate.

Knockdown with siRNA

The efficiency of knockdown using siRNAs was monitored by measuring the level of either *JAK1*, *JAK2* or *IL6ST* mRNA using quantitative RT-PCR (Supplementary Fig. S3f) or by western blot analysis. Knockdown efficiency was greater than 80%. JAK1 siRNA (S7646), JAK2 siRNA (S7651) and IL6ST (gp130) siRNA (S229006) were purchased from Applied Biosystems; siRNA control (Block-IT Alexa Fluor Red) was purchased from Invitrogen.

Pharmacological treatments

Cells were exposed, in serum-free medium, for 6 hr to Tyrphostin AG490 (Sigma France), 9 hr to Src Inhibitor-1 (Sigma France), Src Inhibitor-5 (JS Res Chemical Trading, Germany) or PP2 (Sigma), 18 hr to Ruxolitinib (INCB-018424, JS Res Chemical Trading, Germany) and 24 hr to Curcumin (Sigma). In the last 3 hr of treatment, cells were stimulated with IL-6 (100ng/ml), or not, as indicated.

Western blot analysis

Western blot analyses were performed as described (Rebouissou et al., 2009) using antibodies specific for STAT3 (Cell Signaling Technology, diluted 1:500) and phospho-STAT3 Tyr705, (Cell Signaling Technology, diluted 1:200). For dimerization assays, cell lysates were incubated with Immobilized Protein G agarose (Pierce) and anti-flag (Cell signaling technology, 1:50) or anti-myc (Cell signaling technology, 1:200) antibody, at 4°C overnight, prior to western blot analysis.

Immunohistochemistry

Immunohistochemistry was performed using a Dako autostainer, on paraffin sections of 10% fixed tumor tissue using a monoclonal anti-phospho-STAT3 Tyr705 (Cell Signaling Technology, 1:50). For each immunohistochemical procedure, antigen retrieval was performed in citrate buffer, detection was amplified by the Dako Envision system.

STAT3 DNA-binding assays

One day after transfection, cells were starved in a serum-free medium overnight, and the TransFactor Extraction Kit (Clontech Inc.) was used to prepare nuclear extracts following the manufacturer's protocol. Nuclear extracts were analyzed for STAT3 DNA binding activity using the TransFactor Universal STAT3-specific kits (Clontech Inc.), which is an ELISA-based method.

Immunofluorescence

24 hr after transfection cells were fixed in 4% formaldehyde for 15 min, washed with phosphate-buffered saline (PBS), then permeabilized with 0.1% triton for 15 min. Cells were saturated with 2% BSA for 30 min, washed with PBS, then incubated with primary antibody overnight at 4°C. After three washes with PBS, cells were incubated with secondary antibodies for 1 hr. The slides were washed, then mounted with VECTASHIELD® Mounting Medium with DAPI (Vector labs). Immunofluorescence images were obtained using a Carl Zeiss HBO 100 optic microscope. All data from one experiment were collected at the same intensity and background level. The following monoclonal antibodies were used: rabbit anti-phospho-STAT3 Tyr705 (Cell Signaling

Technology, 1:200), mouse anti-total STAT3 (Cell Signaling Technology, 1:100) and the secondary antibodies were anti-mouse (Invitrogen, 1:200) and anti-rabbit (Invitrogen, 1:200).

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Figure 1. Gain-of-function mutations in STAT3 in IHCA. **a**, Distribution of STAT3 mutations identified in IHCA are represented according to the different protein domains (SH2: Src-homology-2 domain; TAD: Transactivation domain). * indicates the two mutations that are found on the same allele in tumor #379. Stat3-C location is indicated in grey. **b**, STAT3 mutants L78R (L⁷⁸), E166Q (E¹⁶⁶), Y640F (Y⁶⁴⁰), D502Y K658Y (D⁵⁰²/K⁶⁵⁸), G656_Y657insF (G⁶⁵⁶), Y657_M660dup (Y⁶⁵⁷) and Stat3-C (S^C) or control STAT3 wild-type (WT) and empty plasmid (EP) were transfected in Hep3B, HepG2 and Huh7 cells expressing a STAT3-driven luciferase (Luc) reporter construct. STAT3 activation was measured after 6 hr of serum starvation and when indicated were treated for 3 hr with IL-6 (100 ng/ml). Shown is the Luc activity (mean) determined from triplicate co-transfections (\pm s.d.) relative to pSIEM-luc alone (EP) without IL-6. **c**, Graphs are qRT-PCR results showing the induction of endogenous *CRP*, *STAT3* and *SOCS3* mRNA after overexpression of mutant STAT3 relative to unstimulated empty-plasmid-transfected Hep3B cells (EP) (mean \pm s.d.). **d**, Endogenous *CRP* mRNA expression in Hep3B cells transfected with increasing amounts of expression plasmids encoding WT or E¹⁶⁶ STAT3 mutant (dotted line: mock-transfected cells). **e**, Activity of the STAT3 mutants, including D502Y (D⁵⁰²), K658Y (K⁶⁵⁸) and the double mutant D502Y K658Y (D⁵⁰²/K⁶⁵⁸) were evaluated using STAT3-driven Luc in Hep3B cell line. Data are from triplicate transfections (\pm s.d.) relative to pSIEM-luc alone (EP). ns, not significant. *, ** and *** indicate differences between groups at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (two-tailed *t*-test).

Figure 2. Subcellular localization and role of tyrosine 705 phosphorylation in STAT3 IHCA mutants. **a**, Immunohistochemistry of phospho-STAT3-Y705 in STAT3-mutated IHCA and adjacent non-tumor liver (NTL). **b-d**, Vectors driving the expression of STAT3 mutants, including L78R (L⁷⁸), E166Q (E¹⁶⁶), Y640F (Y⁶⁴⁰), G656_Y657insF (G⁶⁵⁶) or wild-type (WT) STAT3 were transfected into Hep3B cells. **b**, Expression of total and phosphorylated (*pY705*) STAT3 proteins were assessed by western blot and bands were quantified with *ChemiDoc* (Bio-Rad) using the Quantity One software; bars show the expression of *pY705* STAT3 relative to total STAT3. **c, d**, Subcellular localization of total and phosphorylated (*pY705*) STAT3 proteins were analyzed by western blot (**c**) and by immunofluorescence (**d**). Results are representative of three independent experiments. Scale bars = 20 μ m. **e**, *CRP* mRNA expression after transfection of wild-type (WT), Y705F (Y⁷⁰⁵), Y640F (Y⁶⁴⁰), Y640F/Y705F (Y⁶⁴⁰/Y⁷⁰⁵), G656_Y657insF (G⁶⁵⁶) or G656_Y657insF/Y705F (G⁶⁵⁶/Y⁷⁰⁵) STAT3 in unstimulated Hep3B cells. Data are from triplicate transfections (\pm s.d.) relative to empty plasmid (EP). ns, not significant. *, ** and *** indicate differences between groups at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (two-tailed *t*-test).

Figure 3. Dimerization of STAT3 IHCA mutants. **a**, Flag- and Myc-tagged constructs encoding either WT or mutant Y640 STAT3 were co-transfected (1:1) into Hep3B cells treated, or not, with IL-6 (100 ng/ml). STAT3 dimer formation was detected after immunoprecipitation using the anti-Flag antibody, followed by immunoblot (IB) analysis with Myc, Flag and *pY705*-STAT3 specific antibodies. Results are representative of three independent experiments. **b**, Effects of increasing WT STAT3 expression (wedge) on the constitutive activity of Y⁶⁴⁰ mutant STAT3 without IL-6. The graph plots the mean

level of expression of *SOCS3* and *CRP* relative to cells transfected with EP (\pm s.d.). Units are plasmid μ g. **c**, Several STAT3 IHCA mutations cluster near the transcription activation domain (TAD) of STAT3 (crystal structure PDB: 1BG1)(Becker et al., 1998). Residues 640, 656-660 (shown in green) that are mutated in IHCA tumors, the phosphorylated tyrosine (Tyr-705, black), as well as TAD residues 711-716 (blue) are shown in stick representation. One polypeptide chain and both TADs are shown as a cartoon. The two-fold related molecule, except the TAD, is shown as a surface representation. For residues of the TAD, Tyr-710 of the two-fold related molecule is labeled while residues 711-716 are not for clarity. **d**, DNA-binding activity of 10 μ g of nuclear extracts of Hep3B cells engineered to express wild-type (WT), Y⁶⁴⁰ or G⁶⁵⁶ STAT3 mutants. Bars indicate the DNA-binding activity to consensus STAT3 oligonucleotides relative to activity measured in cells transfected with empty plasmid (EP). ns, not significant. *, ** and *** indicate differences between groups at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (two-tailed t-test). Data are from triplicate transfections (\pm s.d.).

Figure 4. Role of IL-6-JAK pathway and Src kinases on STAT3 IHCA mutant activation. STAT3 mutants Y⁶⁴⁰ and E¹⁶⁶ were over-expressed in Hep3B cells. Activation of STAT3 is shown by luciferase (Luc) activity (mean) determined from triplicate transfections (\pm s.d.) relative to pSIEM-luc alone (EP) with serum starvation. **a**, Hep3B cells were exposed to increasing concentrations of IL-6. **b**, STAT3 mutants (E¹⁶⁶ and Y⁶⁴⁰) or empty vector (EP) were co-transfected with JAK1 siRNA (+) or control siRNA (-) into Hep3B cells exposed to IL-6 (100 ng/ml). **c**, STAT3 mutants (E¹⁶⁶ and Y⁶⁴⁰) or empty vector (EP) were co-transfected with gp130 siRNA (+) or control siRNA (-) into Hep3B cells. **d**, STAT3 mutants (E¹⁶⁶ and Y⁶⁴⁰) or empty vector (EP) were co-transfected with JAK2 siRNA (+) or control siRNA (-) into Hep3B cells. **e**, STAT3 mutants (E¹⁶⁶ and Y⁶⁴⁰) or empty vector (EP) were co-transfected with JAK1 siRNA (+) or control siRNA (-) into Hep3B cells. **f**, STAT3 mutants (E¹⁶⁶ and Y⁶⁴⁰) or empty vector (EP) were transfected into Hep3B cells exposed to Src inhibitor 1 (SrcI-1) (10 μ M, 9h). ns, not significant. *, ** and *** indicate differences between groups at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (two-tailed *t*-test). Data are from triplicate transfections (\pm s.d.). **g, Expression profiles of STAT3 mutated IHCA and gp130 mutated IHCA.** Comparison was carried out for the 28 genes validated by quantitative RT-PCR that were significantly differentially expressed between IHCA and non-tumorous livers. 5 STAT3 mutated IHCA (in grey) were compared to 11 gp130 mutated IHCA (in white). Results are expressed as mean \pm s.d.; differences between groups are not significant (two-tailed Mann-Whitney test).

Supplementary Figure S1. Somatic DNA alterations identified on STAT3 gene and distribution of mutations according to the different isoforms. **a-b**, Selection of forwards and reverse sequences in three mutated IHCA (T) and their matching non-tumor DNA (N) showing the somatic origin of all mutations identified (arrows). Amino acids and nucleotides are numbered according to *STAT3* cDNA ORF. Sequences of cDNA show the expression of both normal and mutated *STAT3* alleles in tumors. **c**, Schematic representation of *STAT3* isoforms obtained by sequencing full-length cloned *STAT3* cDNA from tumor #379 and according to the different protein domains (SH2:

Src-homology-2 domain; TAD: Transactivation domain). Mutations were harbored by all the different STAT3 isoforms, including or not splicing of nucleotides 2099-2101 leading to in-frame Ser701 deletion (S701del); and splicing of nucleotides 2145-2194 leading to a frame shift and a premature termination stop, eight codons downstream (p.Thr716PhefsX8) (STAT3 β) (Schaefer et al., 1995). The two mutations (D502Y/K658Y) found are carried by the same allele.

Supplementary Figure S2. a, Serine 727 phosphorylation of STAT3 mutants.

Vectors driving the expression of STAT3 mutants, including L78R (L⁷⁸), E166Q (E¹⁶⁶), Y640F (Y⁶⁴⁰), or wild-type (WT) STAT3 were transfected into Hep3B cells. Expression of total and phosphorylated (pS727) STAT3 proteins were analyzed by western blot and the bands were quantified with *ChemiDoc* (Bio-Rad) using the Quantity One software. Bars show the expression of pS727 STAT3 relative to total STAT3. Results are representative of three independent experiments. **b, Several STAT3 mutations in IHCA tumors reside near the TAD and Tyr-705.** The crystal structure (PDB entry 1bg1) reveals that several of residues mutated or duplicated in IHCA tumors (Tyr-640 and 656-660) reside near the TAD. The DNA, one entire polypeptide chain, and both TADs are shown as a cartoon (*bottom*) and are colored as shown in the primary structure (*top*): the *N*-terminal domain that mediates cooperative DNA binding, grey, residues 1-130 and not part of the crystal structure; the coiled-coil domain, red, residues 130-320; the DNA binding domain, orange, residues 321-465; a linker region, yellow, residues 466-585; the SH2 domain, green, residues 586-689; and the transcriptional activation domain (TAD), blue, residues 689-722. The crystallographically related residues 136-688 are shown as a surface representation; carbon atoms are in white, nitrogen in blue, oxygen in red, and sulfur in yellow. Residues mutated or duplicated in inflammatory tumors (166, 502, 640, 656-660) are shown as spheres (carbon in white, nitrogen in blue, and oxygen in red) and some are labeled. The phosphorylated tyrosine (Tyr-705) is also shown as a sphere in both two-fold related molecules with the carbon drawn in black. For clarity, residues labeled on the molecule shown in surface representation are identified by their residue number. **c, Effect of IL-6 on STAT3 mutant activity.** Hep3B transfected with EP, WT or STAT3 mutants (L⁷⁸, G⁶⁵⁶ or Y⁶⁵⁷) with or without IL-6 exposure (100 ng/ml). Graph plots the luciferase activity (Luc) relative to cells transfected with pSIEM-Luc alone (EP) \pm s.d. (transfections in triplicate). ns, not significant. *, ** and *** indicate differences between groups at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (two-tailed t-test).

Supplementary Figure S3. Effect of inhibitors on STAT3 IHCA mutants activation.

a, Hep3B cells transfected with STAT3 mutants (E¹⁶⁶ and Y⁶⁴⁰) or empty vector (EP) treated with 100 ng/ml of IL-6 were exposed 3 hr to increasing concentrations of AG490 or 18 hr to increasing concentrations of Ruxolitinib. The graph plots the luciferase (Luc) activity relative to the transfected cells not treated with the inhibitor. **b,** Y⁶⁴⁰ STAT3 mutant was over-expressed in Hep3B and exposed to increasing concentration of STAT3 inhibitor Curcumin during 24 hr. The graph plots the luciferase (Luc) activity relative to the transfected cells not treated with the inhibitor. **c,** STAT3 mutants E¹⁶⁶ or Y⁶⁴⁰ were over-expressed in Hep3B and exposed to Src inhibitor 1 (Srci-1) (10 μ M, 9 hr) and IL-6 (100 ng/ml, during the last 3 hr of treatment). The graph plots the luciferase (Luc) activity relative to the transfected cells not treated with the inhibitor. **d,** STAT3

mutants E¹⁶⁶ or Y⁶⁴⁰ were over-expressed in Hep3B and exposed to Src inhibitor 5 (SrcI-5) or to PP2, 10 μ M during 9 hr. The graph plots the luciferase (Luc) activity relative to the transfected cells not treated with the inhibitor. All results are from triplicate transfections (\pm s.d.) relative to pSIEM-luc alone (EP) with serum starvation. ns, not significant. *, ** and *** indicate differences between groups at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively (two-tailed t-test). **e**, Vectors driving the expression of Y640F (Y⁶⁴⁰) STAT3 mutant or empty vector (EP) were transfected into Hep3B cells and exposed to Src inhibitor 1 (SrcI-1) (10 μ M, 9 hr) and IL-6 (100 ng/ml, during the last 3 hr of treatment). Expression of total and phosphorylated (pY705) STAT3 proteins were analyzed by western blot. **f, Knockdown efficiency by JAK1 siRNA.** Hep3B cells were co-transfected with Y⁶⁴⁰ STAT3 mutant or empty vector (EP) and JAK1 siRNA (+) or control siRNA (-). Shown is the endogenous *JAK1* mRNA expression determined from triplicate transfections (\pm s.d.) relative to EP with serum starvation. Remaining *JAK1* expression in JAK1 siRNA transfected cells was less than 20% of control.

Table 1. STAT3 somatic mutations identified in inflammatory hepatocellular tumours

Tumours	Nucleotide change	Amino acid change	Mutant ID
CHC341T	T233>G	L78>R	L ⁷⁸
CHC379T	G1504>T	D502>Y	D ⁵⁰²
	A1972>T; G1974>T	K658>Y	K ⁶⁵⁸
CHC574T	G496>C	E166>Q	E ¹⁶⁶
CHC966T	A1919>T	Y640>F	Y ⁶⁴⁰
CHC1021T	T1969_G1980dup	Y657_M660dup	Y ⁶⁵⁷
CHC1351T	C1968>T; 1969_1970insTTT	G656_Y657insF	G ⁶⁵⁶

Codons and mutated nucleotides are numbered according to *STAT3* cDNA ORF

Supplementary Table S1. TaqMan® pre-designed gene expression assays used for quantitative RT-PCR analyses

Assay ID	Gene Symbol	Gene Name
Hs99999901_s1	18S	Eukaryotic 18S rRNA
Hs00187845_m1	BCL2A1	BCL2-related protein A1
Hs00154109_m1	BIRC3	baculoviral IAP repeat-containing 3
Hs00355476_m1	CCL20	chemokine (C-C motif) ligand 20
Hs00265044_m1	CRP	C-reactive protein, pentraxin-related
Hs00170586_m1	FGB	fibrinogen beta chain
Hs00221783_m1	HAMP	Hepcidin
Hs00153153_m1	HIF1A	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)
Hs00219575_m1	HLA-DRA	major histocompatibility complex, class II, DR alpha
Hs00428366_m1	HLA-E	major histocompatibility complex, class I, E
Hs00166223_m1	IFNGR1	interferon gamma receptor 1
Hs00194264_m1	IFNGR2	interferon gamma receptor 2
Hs00985639_m1	IL6	interleukin 6 (interferon, beta 2)
Hs01075667_m1	IL6R	interleukin 6 receptor
Hs00185375_m1	IRF7	interferon regulatory factor 7
Hs00196051_m1	ISGF3G	interferon-stimulated transcription factor 3, gamma
Hs00234567_m1	JAK2	Janus kinase 2
Hs00544758_m1	PMSB8	proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)
Hs00544762_m1	PSMB9	proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2)
Hs01667582_m1	SAA2	serum amyloid A2
Hs00705164_s1	SOCS1	suppressor of cytokine signaling 1
Hs00269575_s1	SOCS3	suppressor of cytokine signaling 3
Hs00162154_m1	SPINK1	serine peptidase inhibitor, Kazal type 1
Hs00234829_m1	STAT1	signal transducer and activator of transcription 1, 91kDa
Hs00237139_m1	STAT2	signal transducer and activator of transcription 2, 113kDa
Hs00374280_m1	STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)
Hs00388682_m1	TAP1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)
Hs00900055_m1	VEGFA	vascular endothelial growth factor A

Supplementary Table S2. List of primers used in PCR and sequencing

F: forward primer; R: reverse primers

localisation	Sequence	Primer name
STAT3 cDNA amplification		
22 bp upstream of exon 1	TGGGACCCCTGATTTTAGCA	1F-STAT3
92 bp downstream of stop	TTCAGATGATCTGGGGTTTGG	4R-STAT3
STAT3 cDNA sequencing		
22 bp upstream of exon 1	TGGGACCCCTGATTTTAGCA	1F-STAT3
exon 6	TCACTCACGATGCTTCTCCG	1R-STAT3
exon 5	CCTCAAGAGTCAAGGAGACAT	2F-STAT3
exon 12	CCTCAGGGTCAAGTGTGTTGAA	2R-STAT3
exon 11	TCTCAGAGGATCCCGAAATT	3F-STAT3
exon 19	CCTCCTTCTTTGCTGCTTTCA	3R-STAT3
exon 18	AAAGTACATCCTGGCCCTTTG	4F-STAT3
92 bp downstream of stop	TTCAGATGATCTGGGGTTTGG	4R-STAT3
STAT3 gDNA amplification and sequencing		
intron 1	GAATGGGTTATAGCATCAGG	STAT3-ADNg-ex2-F
intron 2	CTCAGGTAATGATGCTCACG	STAT3-ADNg-ex2-R
intron 4	TTTTCTGTTCCCAAGGAAAT	STAT3-ADNg-ex5-F
intron 5	CCCGCCTTAAGATCTAAACAGA	STAT3-ADNg-ex5-R
intron 14	CCCCACTCCCTCCGCAGACC	STAT3-ADNg-ex16-F
intron 16	GCGGGAGGGAGAAGGGGTGAA	STAT3-ADNg-ex16-R
intron 19	TGGGCAGTTTTCTCTGAGATGACCT	STAT3-ADNg-ex20-F
intron 20	CCCCCAGGTTATTCAGGCATTTG	STAT3-ADNg-ex20-R
intron 20	TGGCTTCCTCCAGCTCTGCTTACTG	STAT3-ADNg-ex21-22-F
intron 22	GAGCATCACACAAAGGGGACCAACT	STAT3-ADNg-ex21-22-R
intron 22	GCCTGGGCCCTGTCTCTGAA	STAT3-ADNg-ex23-F
intron 23	TGGGGTTTGGCTGTGTGAGGG	STAT3-ADNg-ex23-R

Supplementary Table S3. List of primers used in site-directed mutagenesis

STAT3 c.233T>G p.L78R	
STAT3 c.233T>G p.L78R -F	5'-tgcaagagtcgaatgttcgctatcagcacaatctacg-3'
STAT3 c.233T>G p.L78R -R	5'-cgtagattgtgctgatagcgaacattcgactcttgca-3'
STAT3 c.496G>C p.E166Q	
STAT3 c.496G>C p.E166Q -F	5'-gatctagaacagaaaatgaaagtgttacagaatctccaggatg-3'
STAT3 c.496G>C p.E166Q -R	5'-catcctggagattctgtaccacttcattttctgttctagatc-3'
STAT3 c.1504G>T p.D502Y	
STAT3 c.1504G>T p.D502Y -F	5'-ccaattggaacctggatcaagtggccgagg-3'
STAT3 c.1504G>T p.D502Y -R	5'-cctcggccacttgataccagggtccaattgg-3'
STAT3 c.1972_1974delinsTAT p.K658Y	
STAT3 c.1972_1974delinsTAT p.K658Y -F	5'-ttgctgaaatcatcatgggctattatcatggatgctaccaatattcc-3'
STAT3 c.1972_1974delinsTAT p.K658Y -R	5'-ggatattggtagcatccatgatataatagcccatgatgattcagcaa-3'
STAT3 c.1919A>T p.Y640F	
STAT3 c.1919A>T p.Y640F -F	5'-cagtcctggaaccattcacaagcagcagct-3'
STAT3 c.1919A>T p.Y640F -R	5'-agctgctgctttgtgaatggtccacggactg-3'
STAT3 c.1969_1980dup p.Y657_M660dup	
STAT3 c.1969_1980dup p.Y657_M660dup -F	5'-tttgctgaaatcatcatgggctataagatcatgtataagatcatggatgctacc-3'
STAT3 c.1969_1980dup p.Y657_M660dup -R	5'-ggtagcatccatgatcttatacatgatcttatagcccatgatgattcagcaaa-3'
STAT3 c.1968delinsTTTT p.G656_Y657insF	
STAT3 c.1968delinsTTTT p.G656_Y657insF -F	5'-ttgctgaaatcatcatgggttttataagatcatggatgctacc-3'
STAT3 c.1968delinsTTTT p.G656_Y657insF -R	5'-gtagcatccatgatcttataaaaaccatgatgattcagcaa-3'
STAT3 p.Y705F	
STAT3 p.Y705F -F	5'-gtagcgtgccccattcctgaagaccaag-3'
STAT3 p.Y705F -R	5'-cttggtcttcaggaatggggcagcgtac-3'
STAT3-Flag	
STAT3-Flag -F	5'-gctacctccccatggattataaagatgatgatgataaatgagggccctatggagctgagaacgg-3'
STAT3-Flag -R	5'-gttctcagctccatagggccctcatttatcatcatcatcttataatccatggggaggtagcgc-3'
STAT3-Myc	
STAT3-Myc -F	5'-gctacctccccatggaacaaaaacttatttctgaagaagatcttgagggccctatggagctgagaacgg-3'
STAT3-Myc -R	5'-gttctcagctccatagggccctcaaagatcttctcagaaataagttttgttccatggggaggtagcgc-3'