Well-differentiated liver cancers reveal the potential link between ACE2 dysfunction and metabolic breakdown

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Angiotensin-converting enzyme 2 (ACE2) is the receptor of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) causing Coronavirus disease 2019 (COVID-19). Transmembrane serine protease 2 (TMPRSS2) is a coreceptor. Abnormal hepatic function in COVID-19 suggests specific or bystander liver disease. Because liver cancer cells express the ACE2 viral receptor, they are widely used as models of SARS-CoV-2 infection in vitro. Therefore, the purpose of this study was to analyze ACE2 and TMPRSS2 expression and localization in human liver cancers and in non-tumor livers. We studied ACE2 and TMPRSS2 in transcriptomic datasets totaling 1503 liver cancers, followed by high-resolution confocal multiplex immunohistochemistry and quantitative image analysis of a 41-HCC tissue microarray. In cancers, we detected ACE2 and TMPRSS2 at the biliary pole of tumor hepatocytes. In whole mount sections of five normal liver samples, we identified ACE2 in hepatocyte’s bile canaliculi, biliary epithelium, sinusoidal and capillary endothelial cells. Tumors carrying mutated β-catenin showed ACE2 DNA hypomethylation and higher mRNA and protein expression, consistently with predicted β-catenin response sites in the ACE2 promoter. Finally, ACE2 and TMPRSS2 co-expression networks highlighted hepatocyte-specific functions, oxidative stress and inflammation, suggesting a link between inflammation, ACE2 dysfunction and metabolic breakdown.

Abbreviations

ACE2: Angiotensin-converting enzyme 2
Ang: Angiotensin
CLEC4M: C-Type Lectin Domain Family 4 Member M
DPP4: Dipeptidyl Peptidase 4
COVID-19: Coronavirus disease 2019
CTNNB1: β-Catenin
HCC: Hepatocellular carcinoma
LSECs, MERS-CoV: Middle East Respiratory Syndrome Coronavirus
SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2
TCGA: The Cancer Genome Atlas
TMPRSS2: Transmembrane serine protease 2

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) causes the Coronavirus disease 2019 (COVID-19)¹–³. SARS-CoV-2 is an enveloped RNA betacoronavirus phylogenetically similar to SARS-CoV and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV). Although ~ 80% of SARS-CoV-2-infected individuals develop asymptomatic self-limited upper airway forms of COVID-19, approximately 15% need hospitalization and 5% develop severe disease with acute respiratory distress syndrome, immune dysregulation and a “cytokine storm” with disseminated intravascular coagulation⁴–⁵. Approximately 50% of hospitalized patients present comorbidities: hypertension, type-2 diabetes and coronary heart disease²–⁴,⁶.

The baseline prevalence of chronic liver disease in COVID-19 was about three percent in two meta-analyses assembling 3301 patients²,⁶, but the mean prevalence of COVID-19 related liver dysfunction was about 20%.

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Liver dysfunction correlates with the extent of pulmonary lesions\(^\text{10}\). Patients with hepatocyte-type dysfunction at admission are at higher risk of progressing to severe COVID-19\(^\text{11}\). After admission, antiviral drugs such as lopinavir and ritonavir are risk factors for liver damage\(^\text{11}\). Autopsies suggested that SARS-CoV-2 infects and replicates within hepatocytes in severe COVID-19 cases\(^\text{12-14}\) and SARS-CoV-2 RNA has been detected in the respiratory epithelium, kidney, liver, heart, brain, blood and stools in severe COVID-19 cases\(^\text{5,16}\). In addition, crown-like viral particles with complete envelope and typical spikes have been observed in lung, kidney\(^\text{16,17}\) and in hepatocytes\(^\text{14}\) suggesting multiorgan targeting of COVID-19.

Cell entry of SARS-CoV and SARS-CoV-2 depends on the binding of the viral spike (S) protein to the Angiotensin-converting enzyme 2 (ACE2) and on the serine protease TMPRSS2 for S protein fusion\(^\text{18,19}\). The rate of SARS-CoV-2 infection correlates with cell surface ACE2 expression\(^\text{18,19}\). Importantly, HuH-7\(^\text{18,20}\) human hepatocellular carcinoma (HCC) and HepG2 hepatoblastoma\(^\text{20}\) cells are widely used models of SARS-CoV-2 infection because they express cell surface ACE2 and can be infected at high titers.

As liver cancer cells are in vitro models for the study of SARS-CoV-2 entry and cytopathic effects, we extensively studied mRNA, protein expression and localization of the viral receptors ACE2 and TMPRSS2 in human HCCs. This characterization will be useful to design in vitro studies on virus entry, replication and metabolic alterations in liver cells.

**Results**

**Higher expression of ACE2 and TMPRSS2 mRNAs in HCCs with mutated β-catenin.** We first searched for relationships of ACE2 and TMPRSS2 mRNA expression levels with HCC aggressiveness. To this end, overall and disease-free survival analyses were carried out in the TCGA dataset (n = 370). After applying the exclusion criteria described in Supplementary Fig. 1a, the survival dataset consisted of 256 patients. In consistency with previous reports\(^\text{21-23}\), higher ACE2 and TMPRSS2 mRNA expression were associated with better overall and disease-free survival (Supplementary Fig. 1b and 2a). ACE2 and TMPRSS2 mRNAs were also detected in 47 non-tumor livers in the TCGA dataset. Whereas ACE2 mRNA expression was 1.7-fold higher in non-tumors livers than in HCCs, TMPRSS2 expression did not differ between both groups (Supplementary Fig. 2b).

In HCCs, the expression of genes associated with relatively good prognosis is frequently preserved in well-differentiated tumors because they maintain the phenotype of adult hepatocytes\(^\text{24,25}\). To verify if this applied to ACE2 and TMPRSS2, we measured their expression levels in HCCs classified according to their likeness to normal hepatocytes. Thus classified, HCCs are divided into two major classes, namely “low-proliferation” and “high-proliferation”\(^\text{25}\). Four HCC subclasses result from the interaction of, on one hand, the metabolic phenotype and, on the other hand, the proliferation/differentiation ratios of tumor cells\(^\text{24,26}\). Hence, in the “low-proliferation” class of well-to-moderately differentiated HCCs, the portal-type (PP) and perivenous-type (PV) subclasses refer to the preservation of the metabolic zonation functions of normal hepatocytes\(^\text{24,26}\). At the opposite end of the spectrum, in the “high-proliferation” class of moderately-to-poorly differentiated HCCs, the extracellular-matrix-type (ECM) and STEM-type subclasses, refer to tumors developing an important stromal and vascular support and expressing cancer stem cell markers\(^\text{24,26}\). In this context, we found higher ACE2 mRNA expression in perivenous-type HCCs than in the other subclasses (Fig. 1a; Supplementary Fig. 2c) in both the Desert’s cDNA microarray meta-dataset (1133 HCCs)\(^\text{36}\) (Fig. 1a) and in the TCGA dataset (370 HCCs) (Supplementary Fig. 2c). Of note, ACE2 mRNA expression was higher in HCCs expressing previously validated β-catenin pathway activation signatures\(^\text{26,27}\) (Fig. 1b) and in HCCs carrying sequenced β-catenin (CTNNB1) activating mutations (Supplementary Fig. 2c). In turn, TMPRSS2 was expressed at higher levels in perivenous-type HCCs (Supplementary Fig. 2c, d), which we previously demonstrated to carry wild-type CTNNB1 and to belong to the “low-proliferation” class of well-to-moderately differentiated HCCs that preserve the perivenous metabolic functions of normal hepatocytes\(^\text{26}\). The MERS-CoV receptor, DPP4, was also associated with β-catenin pathway activation (Supplementary Fig. 2e–g). ACE2 mRNA was hypomethylated in tumors carrying CTNNB1 mutations (Fig. 1c), which is consistent with transcriptional activation of the ACE2 gene. By contrast, TMPRSS2 was hyper-methylated in HCCs carrying CTNNB1 mutations (Supplementary Fig. 3a; Supplementary Table 1), which is consistent with transcriptional repression. Neither ACE2 nor DPP4 mRNA expression were related TERT and TP53 gene mutations, which are the two most frequent mutations in HCCs (Supplementary Fig. 3c, d). In turn, TMPRSS2 mRNA levels were higher in HCCs carrying wild-type TP53 (Supplementary Fig. 3d), which is consistent with the above-described higher expression of TMPRSS2 in the well-differentiated, non-proliferative class of HCCs, where TP53 gene mutations are rare\(^\text{26,28}\). Indeed, TP53 mutations are most frequent in poorly-differentiated HCCs (S2\(^\text{25}\), G3\(^\text{38}\)), showing an aggressive, cancer stem cell phenotype\(^\text{26,31}\).

In line with the higher ACE2 and DPP4 mRNA expression in HCCs showing increased β-catenin pathway activation and CTNNB1 activating mutations, in silico analysis of 5,000 base pairs upstream of the transcription start sites of ACE2 and DPP4 DNAs revealed putative T-cell factor 4/LEF-1 consensus transcription factor binding sites, respectively (Fig. 1d; Supplementary Fig. 4a, b).

**ACE2 is predominantly immunodetected in perivenous-type HCCs carrying CTNNB1 mutations and preserving hepatocyte polarization.** In consistency with the above findings, scoring of immunohistochemical signal for ACE2 and for the β-catenin target gene GLUL (glutamine synthetase) in a tissue microarray of 41 HCCs (Fig. 1e, f; Supplementary Table 5) revealed that the expression of ACE2 and GLUL were correlated (Spearman’s R = 0.62; p < 0.001; n = 38). Sanger sequencing of the mutational hotspot in the 3rd exon of CTNNB1 revealed that HCCs carrying CTNNB1 activating mutations expressed 5.1- and 3.3-fold higher levels of GLUL and ACE2 proteins than those carrying wild-type CTNNB1 (Fig. 1e–h; Supplementary Table 5). To confirm whether tumors expressing the highest levels of ACE2 protein corresponded to perivenous-type HCCs, we applied our previously described β-catenin pathway activation score\(^\text{26}\). It results from an equation based on...
Figure 1. High ACE2 levels in well-differentiated, non-proliferative HCCs, with mutated β-catenin (CTNNB1) and hypomethylated DNA. (a) and (b) ACE2 mRNA expression in the Désert’s meta-dataset of 1133 HCCs. (a) HCC subclasses: PP, periportal-type; PV, perivenous-type; ECM, extracellular-matrix-type and STEM, stem-cell-type HCCs. (b) ACE2 mRNA expression according to β-catenin activation levels, assessed as described. (c) ACE2 DNA is hypomethylated in HCCs carrying mutated CTNNB1 (TCGA dataset). (d) TCF/LEF-1 binding sites, responding to β-catenin transcriptional activation in the proximal ACE2 DNA sequence upstream the transcription start site, according to the PROMO program (TRANSFAC database). (e) and (f) Immunohistochemical detection (brown signal) of ACE2 (e) and GLUL (glutamine synthetase, (f) in an HCC tissue microarray (TMA). Slides were slightly counterstained with hematoxylin (blue). Three 1-mm in diameter spots were punched from each formalin-fixed paraffin-embedded routine liver tissue block (n = 41 HCCs; 2 normal liver controls). Digital slides were acquired with a 20X objective. (g) ACE2 and GLUL immunohistochemical signal scoring in HCCs, according to the tumor’s CTNNB1 mutational status, (MUT) versus wild-type (WT); and β-catenin activation scores, (High) versus (Low), using the median value as a cut-off (High, β-catenin activation score > 4). TMA scores are shown as mean (red bar) ± 95% confidence intervals (blue bars). Each dot corresponds to the average out of triplicate tissue cores from each HCC. Statistical differences between means calculated with Mann–Whitney U test. Tumor numbers for each group are indicated. Only were scored those HCCs for which at least two spots were exploitable. TMA33 and β-catenin activation26 scoring were performed as we previously described. Average GLUL and ACE2 scores, CTNNB1 mutational status and β-catenin activation scores for each HCC are provided in Supplementary Table 5. (h) Higher magnification from the indicated spots in (e) and (f).
mRNA expression data of five genes as follows: β-catenin activation score = [(GLUL × LGR5 × ODOM) × (VNN1 × HAL)]\(^{32,33}\). Thus, we measured mRNA expression for these five genes by real-time PCR on mirror frozen samples from the same HCCs as those included in the TMA, applying our previously described method\(^{26,28,32}\). GLUL and ACE2 protein expression were correlated with β-catenin activation scores (GLUL, \(R = 0.69, p < 0.001, n = 41\); ACE2, \(R = 0.51, p = 0.001, n = 38\)). β-catenin activation scores ranged from –2 to 20, median = 4 (Supplementary Table 5). As previously described\(^{26}\), high scores (above the median), define perivenous-type HCCs. On average, GLUL and ACE2 proteins were respectively 4.0- and 2.6-fold higher in HCCs with high β-catenin activation scores (Fig. 1g). These results indicate that HCCs preserving a perivenous-type metabolic phenotype express higher level of ACE2 protein\(^{26}\).

HCCs with mutated CTNNB1 belong to the class of well-to-moderately differentiated tumors that preserve adult hepatocyte features\(^{25}\). Thus, they are frequently cholestatic and contain pseudo-glandular structures with bile plugs, where hepatocytes are polarized and preserve the basal and apical poles\(^{31}\). Co-immunolabeling for ACE2 and ABC2 (a.k.a. MRP2, a marker of the biliary pole in hepatocytes) in HCCs, detected ACE2 at the biliary pole and within the lumen of pseudo-glandular tumor structures and in slit-like bile canaliculi (Fig. 2a,b). By contrast, ACE2 was not detected at the basal hepatocyte pole, which was highlighted by the capillary endothelial cell marker CD34 (Fig. 2c). To quantify ACE2 protein expression at the biliary pole of HCC cells, we measured the fluorescence area for separate ACE2 and ABC2 signals and for dual (ACE2 + ABC2) staining in 96 TMA spots corresponding to 32 HCCs. To correct for differences in cell densities among HCCs, we plotted specific signal areas as the fraction of the DAPI area (i.e., nuclear staining) (Fig. 2d,e). Mean ± SD ABC2/DAPI or ACE2/DAPI ratios were: ABC2/DAPI, 33 ± 27%; ACE2/DAPI, 16 ± 34%; (ACE2 + ABC2)/DAPI, 3 ± 6%. Of note, 19 ± 25% of all ACE2 expression (i.e., (ACE2 + ABC2)/ACE2) was located at the biliary poles of HCC cells (Fig. 2d,e).

In tumors without CTNNB1 mutations, only 6 ± 9% of all ACE2 was localized at the biliary poles. By contrast, in tumors with germline mutations CTNNB1, 31 ± 27% of all ACE2 was detected at the biliary poles (Fig. 2d,e). The mean dual (ACE2 + ABC2)/DAPI signal was 35 folds higher in HCCs carrying mutated than in tumors carrying wild-type CTNNB1 (mean ± SD: wild-type, 0.0018 ± 0.004; mutated, 0.063 ± 0.07; \(p = 0.002\)), thus confirming that HCCs carrying mutated CTNNB1 are enriched in ACE2 expression and indicating that ACE2 is particularly associated with the biliary pole in tumor cells preserving polarized hepatocyte-like features. Adding robustness to these data, dual (ACE2 + ABC2) expression was correlated with β-catenin activation scores (Spearman’s \(R = 0.58, n = 31; p = 0.0005\)).

TMPRSS2 was also detected at the apical pole of some of the pseudo-glandular formations or within slit-like trabecular structures and in cell–cell borders (Supplementary Fig. 5). In contrast with ACE2, the signal for TMPRSS2 was mild in HCCs, suggesting low protein expression levels, which were unrelated to CTNNB1 mutations. Neither ACE2 nor TMPRSS2 (not shown) were detected in myofibroblasts as suggested by dual ACE2 and ACTA2 staining (Supplementary Fig. 5).

In five non-tumor samples from patients undergoing resection of colon cancer metastases and with minimal inflammatory changes in the liver, ACE2 was co-detected with the sinusoidal endothelial cell marker CLEC4M (a.k.a. DC-SIGNR) (Fig. 3a,c,e) and the bile canaliculi marker ABC2 (a.k.a. MRP2) (Fig. 3b,d,f). The presence of ACE2 within bile canaliculi is consistent with the facts that the extracellular part of ACE2 can be cleaved off the cell surface by ADAM17, thus shedding functional ACE2 fragments\(^{34}\); and that ACE2 is an abundant component of the normal human bile proteome\(^{25}\). Also, ACE2 was co-detected with CD34 in capillary vessels of the periporal vascular plexus (Fig. 3g). By contrast, ACE2 was not detected in CD68-positive sinusoidal cells (Kupffer cells, Fig. 3h) or ACTA2-positive vascular smooth muscle cells or myofibroblasts (Fig. 3i,j). As expected, the antibodies used detected high levels of ACE2 in the apical intestinal epithelium, Bowman’s capsule and convoluted tubules in the kidney; in turn, TMPRSS2 was detected in the apical compartment of the epithelial lining of prostatic glands (Supplementary Fig. 5).

In summary, dual labeling of HCC and of non-neoplastic liver with cell-type specific antibodies showed ACE2 in the epithelial lining of bile ducts and in bile canaliculi, i.e., the biliary pole of normal and differentiated tumor hepatocytes. In non-neoplastic liver, CLEC4M-positive sinusoidal endothelial cells and CD34-positive capillary endothelial cells of the periporal vascular plexus also contain ACE2.

**ACE2 and TMPRSS2 co-expression networks highlight metabolic functions typical of normal hepatocytes.** By Weighted Gene Correlation Network Analysis\(^{36}\) comparing periportal-type (\(n = 342\)) and perivenous-type (\(n = 225\)) HCCs within the Désert’s dataset (\(n = 1133\)), we found that ACE2 and TMPRSS2 were related through interacting metabolic functions (Fig. 4a), including detoxification, aminoacid catabolism, lipid metabolism, fatty acid oxidation and catabolism of organic compounds, which is probably related to the carboxypeptidase functions of ACE2, involved in the degradation of inflammatory polypeptides\(^{25,28}\) (Fig. 4b). These findings are in line with the above-described expression of ACE2 and TMPRSS2 in well-to-moderately differentiated HCCs preserving the metabolic programs of adult hepatocytes. Next, we specifically investigated the ACE2 co-expression network in HCCs and analyzed associated gene functions (Supplementary Tables 2, 3, 4). Not surprisingly, ACE2 was associated with hepatocyte-specific functions such as aminoacid metabolism, xenobiotic detoxification, fatty acid uptake and oxidation. High ACE2 expression was associated with low expression levels of oxidative stress markers and cytokine-mediated inflammatory signals (Supplementary Fig. 7a, b). These findings raise the hypothesis of a link between inflammation, ACE2 dysfunction and metabolic breakdown that may be relevant to the pathogenesis of COVID-19.
Figure 2. ACE2 is detected in the biliary pole of moderately-to-well-differentiated HCCs with trabecular or pseudo-glandular patterns. Combined immunoperoxidase and immunofluorescence analysis of a tissue microarray containing 41 HCCs and two normal liver controls, spotted in triplicates. Representative images are shown. Immunoperoxidase signal (brown) is counterstained with hematoxylin (blue). ACE2 appears in red by immunofluorescence, other markers in green and nuclei in blue (DAPI staining). (a), (b) ACE2 colocalizes with the apical hepatocyte marker ABCC2 (a.k.a. MRP2) (arrows). (c) Tumor capillary vessels are marked with CD34. Confocal digital images were acquired with a 40X objective. Images are Z-stacks of four 500 nm focusing steps. (d) Quantification of ACE2, ABCC2, dual (ABCC2 + ACE2) staining and the ratio of (ACE2 + ABCC2) × ACE2⁻¹, representing the fraction of ACE2 protein expression detected as dual (ACE2 + ABCC2) staining, i.e., the fraction of overlap of ACE2 with ABCC2. Values for individual TMA spots from 32 HCCs are expressed as the fraction of DAPI staining of nuclei, to correct for natural variations in cell density in each spot. Means ± 95% confidence intervals are shown for each condition. The statistical significance between groups was calculated with Kruskal–Wallis test (p < 0.0001), followed by the post-hoc Dunn’s test as indicated. (e) Sample scan from a TMA spot showing examples for the different signals quantified in (d). (f) The fraction of overlapping (ACE2 + ABCC2) over total ACE2 signal (i.e., HCC cells expressing ACE2 at the biliary pole), is higher in HCCs carrying CTNNB1 mutations. Each data point represents the mean of two to three cores for each tumor. Statistical significance calculated by Mann–Whitney U test.
Figure 3. ACE2 is detected in sinusoidal endothelial cells, bile ducts and capillaries of the periportal plexus in non-tumor livers. Co-immunostaining for ACE2 and the indicated markers in three non-tumor livers from patients undergoing resection of colon cancer metastasis. Representative images are shown. ACE2 appears in red by immunofluorescence, other markers in green and nuclei in blue (DAPI staining). (a), (b) Low power views (X5 magnification) show ACE2 in a bile duct (BD) and in a zone with mild inflammatory infiltration (arrow) and dystrophic bile ducts (BDs). The sinusoidal endothelial cell marker CLEC4M (a.k.a. DC-SIGNR) and the bile canaliculi marker ABCC2 (a.k.a. MRP2) show lobular staining at this magnification. Pt, portal tract. (c), (d) At X30 magnification, ACE is detected in sinusoidal endothelial cells, which can be identified by comparing CLEC4M (c) and ABCC2 (d) staining. Digital images were acquired with a microscope scanner and a X40 objective and exported at the indicated magnifications (Nanozoomer, Hamamatsu Photonics). (e), (f) Confocal scans of whole tissue sections as Z-stacks of 4 X 500 nm focusing steps at X40 (e) and X143 (f). ACE2 is detected in CLEC4M-positive sinusoidal endothelial cells (e) and within ABCC2-positive bile canaliculi (f). ACE2-positive bile ducts (BDs) are seen in (e). (g) At 28X magnification, ACE2 is sparsely detected in CD34-positive endothelial cells of the periportal vascular plexus (arrows). (h) At 24X magnification, no evidence of ACE2 detection in CD68-positive Kupffer cells. (i), (j) No evidence of ACE2 detection in vascular smooth muscle (i, 10X magnification) or in ACTA2 (a.k.a alpha smooth muscle actin)-positive cells within portal tract stroma (j, 20X magnification). ACE2-positive cells within the portal tract are ACTA2 negative (arrows).
Discussion

In this work, we show that ACE2 and TMPRSS2 mRNAs and proteins are detected at higher levels in well-to-moderately differentiated HCCs than in poorly differentiated tumors. In particular, ACE2 is detected at the biliary pole in tumors preserving hepatocyte polarization. By contrast, in non-tumor livers, ACE2 is detected not only in the epithelial lining of bile ducts and within hepatocyte's bile canaliculi, but also in the CLEC4M-positive sinusoidal endothelium and the CD34-positive capillary vessels of the periportal plexus. TMPRSS2, in turn, is not detected in normal liver, but is detected at the biliary pole in well-to-moderately differentiated HCCs, which is consistent with the functional association of ACE2 and TMPRSS2 through periportal and perivenous metabolic

Figure 4. (a) Connections between the ACE2 and the TMPRSS2 expression networks in the Désert's meta-dataset, in non-proliferative-moderately-to-well-differentiated, periportal-type and perivenous-type HCCs. The co-expression network was identified by Weighted Gene Correlation Network Analysis and the WGCNA R package, followed by visualization with Cytoscape. Genes co-expressed with ACE2, light-blue nodes with black borders and black links; genes co-expressed with TMPRSS2, white nodes with red borders and black links; genes co-expressed with both ACE2 and TMPRSS2, light-blue nodes with red borders and blue links. Link thickness is proportional to the correlation coefficient between a given node and ACE2 or TMPRSS2. (b) Functional analysis and gene ontology enrichment of the gene co-expression network shown in b are visualized with Express Analysis from Metascape. Gene ontology (GO) terms, families and P values are indicated.
biotic detoxification (CTNNB1) mutations, ACE2 DNA is hypomethylated, and ACE2 mRNA expression upregulated. Conversely, TMPRSS2 mRNA is lower in HCCs carrying mutated CTNNB1 and its DNA hypermethylated. This finding agrees with the fact that only ACE2 (and DPP4, but not TMPRSS2) contain TCF/LEF-1 binding sites that are predicted to respond to β-catenin transcriptional activation. Neither ACE2, nor DPP4 or TMPRSS2 are associated to TERT or TP53 gene mutations. On the contrary, HCCs containing mutated TP53 show relatively lower levels of TMPRSS2 (Supplementary Fig. 3D). This association does not necessarily imply a direct regulation of TMPRSS2 by TP53, but may be coincidental. In fact, well-differentiated, perportal-type HCCs express the highest levels of TMPRSS2 and, as we previously showed, have the lowest prevalence of TP53 mutations24. Nonetheless, the relationships of ACE2 and TMPRSS2 with histological, transcriptomic and genomic HCC subclasses converge to demonstrate that tumors preserving hepatocyte-like features, such as hepatocyte trabeculae with bile canaliculi and/or pseudo-glandular structures with an apical biliary pole, express higher levels of the SARS-CoV-2 receptor and co-receptor couple.

The above findings agree with further observations in this work: the ACE2 co-expression network included liver-enriched genes involved in hepatocyte-specific functions, such as aminoacid metabolism (GNMT), xeno-biotic detoxification (CYP3A4), fatty acid uptake, bile acid re-conjugation (SLC2A5), enterohepatic circulation of bile acids and cholesterol homeostasis (SLC10A1). ACE2 was also co-expressed with SIRT1 and CD36 and the fatty acid oxidation enzymes ACADL, EHHADH, ACADM and EC12, and alternative glucose metabolism pathways (CRY1), as well as enzymes involved in gluconeogenesis (PCK2). This network is in line with the role of ACE2 as a regulator of fatty acid uptake in hepatocytes47, -Mas1-sirtuin1-CD36 axis39. Indeed, ACE2 knock-out mice show low levels of peripheral lipid uptake, but high hepatocyte uptake of triglycerides, resulting in steatosis, lipid peroxidation, oxidative stress and inflammation68. In turn, the induction of oxidative stress by ACE2 deficiency may be related to its effects on glucose and lipid metabolism because the ACE2/Ang, -/Mas axis increases glucose uptake, decreasing insulin resistance. Thus, ACE2 knock-out mice exhibit high oxidative stress, whereas exposure to Ang1-7 reduces oxidative stress in liver cells through insulin signaling66. Taken together, this body of evidence matches the metabolic landscape of HCCs carrying activating CTNNB1 mutations. These tumors oxidize fatty acids as a source of energy to feed the oxidative phosphorylation pathway43, but resist to oxidative stress67 and suppress inflammation68.

ACE2 expression was related to gene functions that smoother infection. Indeed, ACE2 was related to low levels of the inflammation-related transcripts BIRC3, MSN, MMP9, LTb, TNFRSF4, IL4R, CCL20 and IL32, among others (Supplementary Table 2). ACE2 exerts anti-inflammatory effects through carboxyterminal cleavage of vasoactive polypeptides, such as Apelin-13, which induces vasodilation, and des-arg9-bradykinin, a neutrophil chemotacticant44. In turn, deletion of ACE2 leads to tissue injury and oxidative stress, through upregulation of the AT1 receptor and inflammatory cytokines like IL1b, IL6, IL17, RANTES, ICAM1, TNFa, TNFRSF1A and downregulation of the anti-inflammatory cytokine IL1045. This picture is surprisingly similar to that seen in the spectrum going from immune dysregulation to macrophage activation syndrome in moderate-to-severe COVID-1966.

The anti-inflammatory role of ACE2 goes well with its up-regulation in response to liver injury, such as bile duct ligation66 and with the evidence that ACE2 produced by hepatocytes limits fibrogenesis through degradation of AngII to Ang1-7. In human liver cirrhosis, ACE2 protein is increased by > 90 folds in hepatocytes. In vitro, hypoxia increases ACE2 activity in hepatocytes, enhancing acetycholine-induced vasodilatation66. As hypoxia activates Wnt/β-catenin signaling, it would be interesting to investigate whether ACE2 expression in perivenous hepatocytes depends on β-catenin34,46.

This body of matching data suggests that ACE2 may regulate energy metabolism, protecting against oxidative stress and inflammation. As both TMPRSS2 and ACE2 are functionally connected through metabolic pathways in the liver, it may be reasonable to hypothesize that ACE2 depletion resulting from SARS-CoV-2 infection18 may contribute to metabolic dysfunction and inflammation. Our results point to the relevance of further studies on the signaling pathways and pathological contexts leading to cell surface ACE2 availability and to the possible impact of viral infection on the disruption of the ACE2 metabolic network.

Materials and methods

Patients, samples and datasets. Human hepatocellular carcinoma (HCC): immunohistochemistry was performed on a tissue microarray (TMA) built from formalin-fixed-paraffin-embedded (FFPE) routine tissue blocks of 41 HCC cases and two histologically normal liver controls, for which the exon 3 of β-catenin (CTNNB1) had been Sanger-sequenced, as we previously described36. In addition, three formalin-fixed, paraffin-embedded non-tumor liver tissue blocks from patients undergoing resection of liver metastases from extra-hepatic cancers were added to the immunohistochemistry analysis. To calculate β-catenin activation scores based on the mRNA expression of GLUL, LGR5, ODAM, VNN1 and HAL, we retrieved real-time PCR mRNA expression data from our previously published work36 corresponding to frozen fragments of the 41 tumors arrayed in the TMA. Quality control matching of frozen and FFPE fragments has been previously described in an 80 HCC dataset including the 41-tumor subset32,33. Tissue samples and patient data were collected and anonymized after obtaining written informed consent from the patients. De-identification was performed in accordance with the Health Insurance Portability and Accountability Act (HIPAA) Privacy Rule. The study was approved by INSERM’s Institutional Advisory Board (approval number 19-630). Research involving human research participants has been performed in accordance with the Declaration of Helsinki. All experiments were performed in accordance with...
relevant guidelines and regulations. Two different immunohistochemical methods were used to detect primary antibodies: peroxidase-labeled secondary antibodies, revealed by a chromogenic substrate or multiplex immunofluorescence followed by analysis with a confocal slide scanner. The Desert's microarray meta-dataset composed of 1133 human HCCs was previously described\(^\text{26}\). In this dataset, HCCs expressing a β-catenin-activated transcriptomic program were identified by a 5-gen signature composed of GLUL, LGRS, ODAM, VNN1 and HAL, which predicts activating CTNNB1 mutations with high sensitivity (0.86–0.91) and specificity (0.83–1.0)\(^\text{36}\). The publicly available TCGA dataset was composed of 370 HCCs and 47 matching non-tumor samples. RNA sequencing, mutation and methylation data were extracted as described\(^\text{26}\), using the TCGAAbioblinks R package. Raw mRNA expression was median-normalized (DESeq R package). DNA methylation was quantile-normalized (preprocessCore R package); probes with B-value over 0.8 or under 0.2 were respectively considered as hypermethylated or hypomethylated. The R-package Limma was used to identify differential methylation.

See Supplementary Information for a detailed description of immunohistochemical methods and statistical analyses and Supplementary Table 6 for primary, secondary antibodies, fluorochromes used, epitope unmasking and incubation conditions.

Data availability

Datasets analyzed in this study are available at The Cancer Genome Atlas or at the Gene Expression Omnibus databases. Accession numbers and standard, publicly available R packages used are provided in the main text and supplementary files. The procedures applied for merging nine publicly available transcriptomic datasets into a meta-dataset totaling 1133 hepatocellular carcinomas have been previously published\(^\text{26}\). A filtered, normalized and batch-effect-corrected user-friendly version of this meta-dataset is available from the corresponding author upon reasonable request.

Received: 9 August 2021; Accepted: 8 December 2021

Published online: 03 February 2022

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Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-03710-0.

Acknowledgements

We thank Caroline Aninat, Latifa Bousarghin, Pierre-Jean Ferron and Olivier Loréal for insightful discussions; Michèle Le Guennec, Patricia Jouas, Adina Pascu and Thomas Poussou for logistics and administrative support.

We thank Caroline Aninat, Latifa Bousarghin, Pierre-Jean Ferron and Olivier Loréal for insightful discussions; Michèle Le Guennec, Patricia Jouas, Adina Pascu and Thomas Poussou for logistics and administrative support.

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Funding

Inserm; Univ Rennes 1; Ministère de l’Enseignement Supérieur; Institut National du Cancer, grant # INCA_12688; Ligue Nationale Contre le Cancer 2018, Comités d’Ille-et-Vilaine et Vendée.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-03710-0.

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