**Supplementary Materials & Methods, Tables and Figures**

**Cancer testis antigen expression in hepatocellular carcinoma and paired tumor-free liver tissue**

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**Materials & Methods**

Liver and healthy tissue samples

*Cirrhotic and healthy (liver) tissues*

Freshly frozen healthy liver tissues (n=21) were obtained during liver transplantation from donor liver grafts at the end of cold ischemic storage. Archived freshly frozen tissue samples of non-cancerous cirrhotic livers (n=35) were retrieved from the tissue bank of the Department of Pathology, Erasmus Medical Center Rotterdam. The non-cancerous cirrhotic liver tissues had been retrieved from patients who underwent liver transplantation for liver cirrhosis in our center between May 2007 and June 2017. The etiology of the cirrhosis was determined by information from medical records, laboratory tests and pathological examination of the explanted livers. Cirrhotic livers with malignancies, diagnosed by pathological examination, were excluded.

RNA isolated from fresh frozen healthy adrenal gland (R1234004-50), artery (HR-810), brain (R1234035-50), colon (R1234090-50), heart (R1234122-50), lung (R1234152-50), muscle (R1234171-50), ovary (HR-406), pancreas (R1234188-50), skin (R1234218-50), small intestine (R1234226-50), stomach (HR-302), testis (R1234260-50), throat (R1234263-10), thymus (HR-702), thyroid (R1234265-50), trachea (R1234160-50), urinary bladder (R1234010-50) and uterus (R1234274-50) tissues were purchased from AMS Biotechnology Ltd, Abingdon, UK. Bone marrow derived from a healthy donor (Department of Hematology, Erasmus MC), healthy kidney tissue obtained from a donor kidney (Department of Internal Medicine, Erasmus MC) and RNA of healthy testis tissues (Department of Pathology, Erasmus MC) were kindly provided. Lymph node and spleen tissues were collected from samples retrieved during liver transplantation in our center in September 2019.

Quantitative real-time PCR

RNA was isolated using the NucleoSpin® RNA isolation kit of Macherey-Nagel (Dueren, Germany) according to manufacturer’s instructions. RNA (4 ug) was reverse-transcribed into cDNA using PrimeScript™ RT master Mix (Perfect Real Time, Takara, cat# RR036A), according to the manufacturer’s instructions. RT-qPCR was performed using SYBR™ Green PCR Master Mix (ThermoFisher) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems), using 12.5 ng cDNA per reaction, with the following conditions: 50°C for 2 minutes, 95°C for 2 minutes, then 38 cycles of 95°C for 15 seconds, 58-62°C for 15 seconds (according to the Tm of the primers), 72°C for 1 minute, and then finally for the Melt Curve stage 95°C for 15 seconds, 60°C for 1 minute and a 0.7°C step-wise increase until 95°C was reached. All Ct-values over 35 were considered negative. The level of target gene expression relative to the geometric mean of three control genes (HPRT1, GUSB, PMM1)2 was calculated by 2-ΔΔT method, after which a cut-off of 0.001 was used to define expression. All amplifications were performed in at least two technical repeats. Means of technical replicates were used for analysis. Primers were designed with Primer Blast (NCBI), efficiency was determined by dilution of cDNA and product length was determined by gel electrophoresis.

Immunohistochemistry

The FFPE blocks of the HCC and TFL tissues were examined by a pathologist (MD) to mark tumor and tumor-free liver tissues. A TMA Grand Master (2.5; 3D Histech) was used to create tissue microarrays (TMA). Three tissue cores of 1 mm were taken of each tissue and placed in a recipient formalin block. Immunohistochemistry (IHC) was performed using an automated, validated and accredited staining system (Ventana Benchmark ULTRA, Ventana Medical Systems, Tucsen, AZ, USA) using the optiview universal DAB detection Kit (cat.760-700, Ventana Medical Systems). In brief, following deparaffinization and heat-induced antigen retrieval tissue sections were incubated with each of the primary antibodies according to their optimized incubation time and concentration **(Supplementary Table S2)**. The antibodies were titrated using testis as a positive control tissue and placenta and spleen as negative control tissues. Incubation was followed by hematoxylin II counter stain for 12 minutes and then a blue colouring reagent for 8 minutes according to the manufacturer’s instructions (Ventana Medical Systems, Tucsen, AZ, USA). The stained TMAs were then scanned using a Nanozoomer (Hamamatsu), and analyzed using NDP.view2 software (Hamamatsu).

**Search query:**

(("cancer testis antigen"[All Fields] OR ((("cancer"[All Fields] OR "neoplas\*"[All Fields]) AND ("testis"[All Fields] OR "testes"[All Fields]) AND ("Antigens, Neoplasm"[Majr] OR "antigen\*"[All Fields] OR "Ags"[All Fields] OR "ag"[All Fields] OR "gene"[All Fields] OR "genes"[All Fields] OR "antigen\*"[All Fields])))) AND (("Carcinoma, Hepatocellular"[Majr]) OR "Fibrolamellar hepatocellular carcinoma" [Supplementary Concept] OR "liver cell carcinoma"[All Fields] OR "liver cancer"[All Fields] OR "hepatocellular carcinoma cell line"[All Fields] OR (("liver"[All Fields] OR "hepat\*"[All Fields]) AND ("carcinoma\*"[All Fields] OR "ca"[All Fields] OR "cas"[All Fields] OR "cancer\*"[All Fields])) OR "hepatocarcinom\*"[All Fields])) AND "Humans"[Mesh]

Pubmed search 04-10-2018

**Supplementary Table S1. Primer sequences and annealing temperatures (Tm) used for RT-qPCR.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Primer | Tm | Forward Primer | Reverse Primer | Product Length |
| CAGE1 | 60 | TCATCCGAAGTCCATGACCA | GACTCTTCCTGGAGTGGTTG | 118 |
| CBLL2 | 62 | TTCCACCAGAACAGCACACC | AACGGTTTCCCACTGGATGG | 146 |
| CCDC83 | 60 | AGGAGGGCAGGCCTTTTTAATC | TCCATTGTGCTGGTTAGCTATGA | 148 |
| CPXCR1 | 60 | CAGCCAGTCATACTATCCTC | CTACAGTCATTAGGAGGCTC | 118 |
| CSAG2/3 | 58 | GGAGTGGGCCAACACTATCC | GGCTGTCCGAAGAGAGACTG | 123 |
| CT45 | 62 | ATGCACATCACTCCCAGGTG | TTGTTTCCTTGCTGGAGGAGA | 147 |
| CT47A1 | 60 | ACCTAGACGCAGCAGAGGT | AACTTGAACACTGTCACATACATCC | 141 |
| CTAG1A/B | 60 | GGCTTCAGGGCTGAATGGA | TGTTGCCGGACACAGTGAAC | 191 |
| Cxorf48 | 60 | CTGGCAACGTGCCTCTAAAAG | AAGATGGCGAGGCACAACAT | 110 |
| DDX53 | 60 | GTTGGTGTGGTCATTGGTTAC | CGCTTTGGCCTTTGCTTTCAT | 144 |
| DPPA2 | 62 | CAATCTCCTTCCATCCCAGGGT | ACCAGTGTCAAAATCACACTTTCC | 118 |
| DUSP21 | 62 | TTGTCAATGCCTCGGTGGAA | CGAGTCACGAGCATCGGTAA | 86 |
| FAM46D | 60 | AGCCTTAACGGATGAAGGAAAA | AAACTCCAGCTAGTGAAACTCC | 92 |
| FATE1 | 62 | ATGGAGCTTGGATCTCGGTC | CTCAGCATTCTGGGCTTTGG | 155 |
| FBXO39 | 60 | TGATAGATCTCCTGCCCACCT | CTCGTCGAGTGACTCATGGTT | 83 |
| FMR1NB | 60 | TCCTGCTGTTCGTGTGCTAC | TCAGCAAAGCTTCCAATGCG | 147 |
| FTHL17 | 60 | ATCAACAGCCACATCACGCT | CATTTTGTCGTCCGACAGGC | 132 |
| GAGE1 | 60 | ACCTGAGTCATCTTAAAACATGTGA | AGTAAACATGAAGCAGAGTGCC | 80 |
| GPC3 | 60 | AACCATGTCTATGCCCAAAGGT | CCAGAGCCTCCAATGCACTC | 108 |
| GUSB | 58 | CAGGTGATGGAAGAAGTGG | GTTGCTCACAAGGTCACAG | 171 |
| HORMAD1 | 60 | CAACGAATCTAGCATGTTGTC | CACAATCACCATCCTTAAAACC | 188 |
| HPRT1 | 58 | GCTATAAATTCTTTGCTGACCTGCTG | AATTACTTTTATGTCCCCTGTTGACTGG | 140 |
| LUZP4 | 60 | CTTCGTTTCGGAAGCTAACGC | CTCCGATGGCGATGTCTATGA | 217 |
| MAGEA1 | 60 | AGAAGCGAGGTTTCCATTCTGA | GGAATCCTGTCCTCTGGGTTG | 116 |
| MAGEA2 | 62 | CTCCAGCTTCTCGACTACCATC | GACTCCAGGTCGGGAAACATTC | 148 |
| MAGEA3 | 62 | ATCTTCAGCAAAGCTTCCAGT | GGTGGCAAAGATGTACAAGTGG | 93 |
| MAGEA4 | 58 | GAGCTTCTGCGTCTGACTCG | TGTCTGCTCAGAACCTTGTCTC | 85 |
| MAGEA8 | 60 | GGTCGGCTTGAGATCGGCT | CCTCAGCTTGACTGCTACTACTG | 150 |
| MAGEA9B | 60 | GCTTGATACCGGTGGAGGAG | GGTTAGCCTGTCCCGAGAAC | 124 |
| MAGEA10 | 62 | GAGATCGGCTGAAGAGAGCG | ACTCTTGTCAGATCCTGCGAC | 140 |
| MAGEB1 | 60 | TGAAGTAGTGAGCAGCCAAGA | GCTGGCAGCACCAATAAATGT | 172 |
| MAGEB2 | 58 | TCCTGACTTCCGCTTTGGAGGC | GCACGGAGCTTACTCTTCTGACC | 135 |
| MAGEB3 | 60 | CTACCCAAACCTCTTCTCAGCC | AGACCCTGGATCCTCCCTCTA | 144 |
| MAGEB6 | 62 | ACCCTTGTCAGCAAGCTAGG | GATCACAACCAGGAGCGACA | 99 |
| MAGEC1 | 62 | GGCCATCTTGGGAGTCTGAA | TGGAGCACCTTGAAGACTGG | 106 |
| MAGEC2 | 62 | GGAGTCAAGGCCTGTTGGAT | GGGAGGCATGACGACTTCTT | 84 |
| PAGE1 | 62 | GGCTGAAGTTGTGAAATATGGGT | CTGCAGATGCTCCCTCATCC | 177 |
| PAGE5 | 62 | TGATGTCAGGGAGGGGACTC | TGGTTTCAGTCTTCATTTGTCTTGG | 105 |
| PASD1 | 62 | TGCAGAGGTTGAGCAGTATGG | GGATTCACCTCAGGCTCACC | 153 |
| PLAC1 | 60 | ACACAGCAAGTTCCTTCTTCC | GAGGATTTCTTCTTCTGGCAGC | 118 |
| PMM1 | 58 | CGAGTTCTCCGAACTGGAC | CTGTTTTCAGGGCTTCCAC | 86 |
| RNF17 | 60 | GGACAATGCAGTGGTCCAAAG | AGGAGCACCAAGAGAATCGAA | 137 |
| SAGE1 | 58 | CCTTAGCTGACTCTGGTGCTC | GACTCGTTTGAAGTGGAGAAGC | 150 |
| SLCO6A1 | 62 | TGGCCTTGGGTGTAAGCTATG | ATCCAACAACGTCCTGTGTG | 136 |
| SPANXA | 62 | ATGATGCCGGAGACCCCAAC | GTGGTCATTCAGCAGTTCCTCT | 144 |
| SPANXC | 60 | CGCTACAGGAGGAACGTGAA | ATTCCTCCTCCTCCATTTGG | 100 |
| SPANXN3 | 62 | ACCAGAATCATGGAACAGCCAA | TGTTTGGTACCTCTTGCATCTC | 106 |
| SYCP1 | 62 | CTATCTGTGGACATCTGCCAA | TTGGTTTTGTTGGTGTCTTCAC | 80 |
| TEKT5 | 62 | GGTCCATGACAACGTGGAGA | TGCTGAGCATCCCGGTTATC | 126 |
| TFDP3 | 60 | TTGGAGGTGTGTTCACGACG | CTGAGATCCACCGGAGCTTG | 113 |
| TPPP2 | 60 | GCAAAGTCAAGGCCAAGAACG | CTGGACTCTTCCCTTTGAAGC | 99 |
| TSPY | 62 | ACAAGATTGCTGAGTCCCCTG | TCAACAACTGGGAGTCCCCT | 149 |
| ZCCHC13 | 62 | TGCTACAACTGTGGGAGAAGC | TGACGATCACAGTCACGAGC | 122 |

**Supplementary Table S2. Antibodies used for immunohistochemistry.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Antibody** | **Host Species** | **Dilution** | **Company** | **Clone** | **Lot number** | **Procedure** | **Ab incu-bation at 37˚C** |
| PAGE1 | Rabbit | 1:1000 | Sigma-Aldrich | Polyclonal | R04065 | Optiview CC1 32' | 32 minutes |
| TSPY | Rabbit | 1:200 | Sigma-Aldrich | Polyclonal | R59337 | Optiview CC1 32' | 32 minutes |
| MAGEA9 | Mouse | 1:50 | Prof. Y. Fradet, Québec, Canada3 | 14A11 | N/A | Optiview CC1 32' | 32 minutes |
| MAGEC2 | Rabbit | 1:500 | Sigma-Aldrich | Polyclonal | A115364 | Optiview CC1 32' | 32 minutes |
| CT47A1 | Rabbit | 1:8000 | Sigma-Aldrich | Polyclonal | R39285 | Optiview CC1 32' | 32 minutes |
| MAGEA1 | Mouse | 1:50 | Santa Cruz | MA454 | B0507 | Optiview CC1 32' | 32 minutes |
| MAGEB2 | Rabbit | 1:500 | Sigma-Aldrich | Polyclonal | R109336 | Optiview CC1 32' | 32 minutes |
| SLCO6A1 | Rabbit | 1:200 | Sigma-Aldrich | Polyclonal | R72094 | Optiview CC1 32' | 32 minutes |
| MAGEC1 | Mouse | 1:3200 | Santa Cruz | CT7-33 | A1807 | Optiview CC1 32' | 32 minutes |

**Supplementary Table S3. Results of the literature search and overview of included articles.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Study | Gene(s) | Population | Detection method | Outcome |
| Wei Y, et al. Int J Oncol. 20184 | MAGEA9 | HCC patients (n=90; China) | IHC | IHC: 40/90 (44%) MAGEA9+ |
| Jiao Y, et al. PLoS One. 20175 | TFDP3 | HCC cell line (HepG2) and normal human hepatocyte cell line (L-02) and HCC patients (China) | RT-qPCR and IHC | mRNA and IHC: HepG2 and L02 are both TFDP3+, expression is higher in HepG2. Also protein expression in HCC patients. |
| Liu, et al. Cancer Lett. 20176 | CTCFL | HCC cell lines (HepG2, SMMC-7721, Huh7, HCCLM3, PLC/PRF/5), normal human hepatocyte cell lines (L-02 and WRL68) and HCC patients (n=25; China) | RT-qPCR and IHC | RT-qPCR: all cell lines positive, expression higher in HCC cell lines than normal human hepatocyte cell lines  IHC: 18/25 (72%) CTFCLhigh and 7/25 (28%) CTCFLlow |
| Xie, et al. Drug target. 20177 | TTK | Review | n.a. | Liu, Oncotarget 2015: 118/152 (77.63%) of HCC patients mRNA TTK+ – China |
| Charoentong, et al. Cell Rep. 20171 | BRDT, CAGE1, CCDC83, CPXCR1, CSAG2, CT45A1, DDX53, DPPA2, FMR1NB, FTHL17, GAGE1, LUZP4, MAGEA1, MAGEA2, MAGEA3, MAGEA4, MAGEA5, MAGEA9, FAM46D, MAGEB1, MAGEB2, MAGEB3, MAGEC1, PAGE1, PASD1, POTEA, POTEB, POTED, SLCO6A1, SPANXC, SPANXN3, SSX3, SSX5, SSX7, TSPY2, TSPY3, TSSK6, XAGE2, ZNF645, | The Cancer Genome Atlas (TCGA); including 363 HCC patients | RNA sequencing | Aforementioned genes are all correlated with CD4 and/or CD8 T cells in HCC |
| Kido, et al. J Genet Genomics. 20168 | TSPY | TCGA and refers to Kido, et al. 2014 IHC: male HCC patients (n=287; TMA purchased from US Biomax) and RT-qPCR: male HCC patients (n=32; China) | TCGA: RNA seq  Kido, et al. 2014: RT-qPCR and IHC | This paper researches the TSPY co-expression network (TCN), which is activated in 30% of HCCs (TCGA).  Kido, et al. 2014: RT-qPCR: 15/32 (46.9%) TSPY+  IHC: 55/287 (19.2%) TSPY+ |
| Fu, et al. Int J Clin Exp Pathol. 20159 | ACRBP | HCC cell lines (Bel-74041, HepG22, QGY-77033, QGY-77014, BEL-74025, SMMC-77216) | RT-PCR1-6, IHC1,2 and WB1,2 | RT-PCR: 6/6 cell lines  IHC: 2/2 cell lines  WB: 2/2 cell lines |
| Wang, et al. Int J Clin Exp Pathol. 201510 | MAGEA3, MAGEA4, MAGEC2, NY-ESO-1 | HCC cell lines (LO2, HepG2, Hep3B, Huh7, SMMC-7721) and HCC patients (China; n=142) | RT-PCR and IHC | Cell lines: 4/5 cell lines for 4 TAAs.  HCC patients: 112/142 (78.9%) MA3+, 48/142 (33.8%) MA4+, 106/142 (74.6%) MC2+, 20/142 (14.1%) NY-ESO-1+. No expression in TFL.  IHC: 108/142 (76.1%) MA3+, 44/142 (31.0%) MA4+, 99/142 (69.7%) MC2+, 19/142 (13.4%) NY-ESO-1+ |
| Sideras, et al. Br J Cancer. 201511 | MAGEA1, MAGEA3/4,  MAGEA10, MAGEC1, MAGEC2, NY-ESO-1, SSX2, SP17 | HCC patients (Netherlands; n=133) | IHC | 9.8% MAGEA1+, 3.0% MAGEA3/4+, 7.5% MAGEA10+, 17.3% MAGEC1+, 19.5% MAGEC2+, 3.8% NY-ESO-1+, 0% SSX2+, 87% SP17+. No expression in TFL, except SP17 (88.0%) |
| Melis, et al. J Transl Med. 201412 | NUF2, TTK, MAGEA3, CEP55 | HBV+ HCC patients (n=10; Italy) | RT-PCR | Expression of al 4 TAAs in 10 patients, both in HCC and TFL, but higher in HCC. |
| Li, et al. J Transl Med. 201413 | TSPY | HCC cell lines (HepG2, SMMC7721, Huh7, MHCC97L, MHCC97H, HCCLM3) and HCC patients (n= 52;China) | RT-PCR | 6/6 cell lines and expression of TSPY in male HCC tissues, but not female HCC tissues |
| Deng, et al. Hepatology. 201414 | DUSP21, CT45, ZCCHC13, MAGEA9, MAGEB6, PIHD3, PNMA5, MPC1L, IL13RA1 | HCC patients (n=24; China?) | RT-PCR | 8/24 (33.3%) DUSP21+, 7/24 (29.2%) CT45+, 4/24 (16.7%) ZCCHC13+, 3/24 (12.5%) MAGEA9+, 3/24 (12.5%) MAGEB6+, 4/24 (16.7%) PIHD3+, 6/24 (25%) PNMA5+, 6/24 (25%) MPC1L+, 1/24 (4.2%) IL13RA1+ |
| Xia, et al. Int J Clin Exp Pathol. 201315 | SP17, MAGEC1, NY-ESO-1 | HCC patients (n=45; China) | IHC | 16/45 (35.6%) MAGEC1+, 7/45 (15.6%) NY-ESO-1+, 36/45 (80%) SP17+ |
| Zhou, et al. Oncol Rep. 201316 | FAM9C | HCC cell lines (SSMC-7721, QGY-7703, BEL-7404, BEL-7405, YY-8103, Huh7) and HCC patients (n=46; China) | RT-qPCR and IHC | RT-qPCR: 25/46 HCC patients have upregulation of FAM9C in T compared to TFL  Cell lines: 2/6 FAM9C+  IHC showed nuclear staining (T>TFL) |
| Chen, et al. Genet Test Mol Biomarkers. 201317 | CTCFL | HCC cel lines (SMMC-7721, BEL-7402, Huh7, HepG2) and HCC patients (n=105; China) | RT-PCR, IHC and WB | Cell lines: 3/4 CTCFL+ (RT-PCR and WB)  HCC patients: 58/105 (55.2%) CTCFL+ (IHC) |
| Song, et al. Oncol Rep. 201218 | AKAP3, CTp11 | HCC cell lines (SNU-354, SNU-398, SNU-423, SNU-449, HepG2) and HCC patients (n= 10; Korea) | RT-PCR | 5/10 (50%) AKAP3+, 1/9 (11.1%) CTp11+ |
| Li, et al. Bull Cancer. 201219 – no full text | CABYR-c | HCC patients (n=20; China) | RT-PCR and WB | Both mRNA and protein expression are significantly higher in HCC compared to TFL |
| Yoon, et al. Tohoku J Exp Med. 201120 | RNF17 | HCC patients (n=28; Korea), CCA patients (n=5) and combined HCC-CCA (n=8) – Korea | RT-qPCR | 4/28 (14.3%) HCC RNF17+, 1/5 (20%) CCA RNF17+, 2/8 (25%) combined HCC/CCA RNF17+. No expression in TFL. |
| Tseng, et al. Oncol Rep. 201121 | CABYR-a/b, CABYR-c/d, CABYR-e | HCC cell lines (HepG2, Huh7) and HCC patients (n=16; Taiwan) | RT-PCR and WB | Cell lines: 2/2 expressed CABYR-a/b and CABYR-c/d  HCC patients: 7/16 (43.8%) CABYR-a/b+, 14/16 (87.5%) CABYR-c/d+, 0/16 (0%) CABYR-e+ |
| Wang, et al. Oncol Rep. 200921 | NY-ESO-1, CTAG2, SSX1 | HCC patients (n=64; China) | RT-PCR | 19/64 (29.7%) NY-ESO-1+, 29/64 (45.3%) CTAG2+, 24/64 (37.5%) SSX1+ |
| Riener, et al. Int J Cancer. 200922 | MAGEA4, MAGEC1, MAGEC2, GAGE, NY-ESO-1 | HCC patients (n=146; Switzerland), CCA (n=50), GBC (n=32) | IHC | HCC: 0/146 (0%) MAGEA4+, 17/146 (12%) MAGEC1+, 50/146 (34%) MAGEC2+, 16/146 (11%) GAGE+, 3/146 (2%) NY-ESO-1+.  No expression in CCA.  GBC: 4/32 (13%) MAGEC2+, 1/32 (3%) GAGE+, 1 (3%) NY-ESO-1+, 0/32 MAGEC1/MAGEA4+ |
| Lu, et al. Chin Med J. 200723 | NY-ESO-1, SSX1 | HCC patients (n=36; China) | RT-PCR | 4/36 (11.1%) NY-ESO-1+, 22/36 (61.1%) SSX1+ |
| Wu, et al. Life Sci. 200624 | SSX2, SSX5 | HCC patients (n=36; China) | RT-PCR | 13/36 (36.1%) SSX2, 17/36 (47.2%) SSX5 |
| Watanabe, et al. Cancer Sci. 200525 | IGSF11 | HCC cell line (Alexander, Huh7, HepG2, SNU475) | RT-PCR | HCC cell lines: 3/4 IGSF11+ |
| Yin, et al. Br J Cancer. 200526 | TSPY | HCC cell lines (hep-hcc-1, hep-hcc-2, hep-hcc-HLE, Hep3B, COS7) and HCC patients (n= 57;China) | RT-PCR | 20/57 (35%) TSPY+ |
| Shi, et al. Br J Cancer. 200527 | DDX53 | HCC patients (n=33; China) | RT-PCR | 13/33 (39.4%) DDX53+ |
| Peng, et al. Cancer Lett. 200528 | MAGEA1, MAGEA3, MAGEA4, MAGEA10, SSX1, SSX2, SSX4, SSX5, NY-ESO-1, MAGEB1, MAGEB2, MAGEC1, MAGEC2, SYCP1 | HCC patients (n=73; China) | RT-PCR | 51/73 (69.9%) MAGEA1+, 35/73 (47.9%) MAGEA3+, 6/30 (20%) MAGEA4+, 11/30 (36.7%) MAGEA10+, 29/43 (67.4%) SSX1+, 26/73 (35.6%) SSX2+, 21/43 (48.8%) SSX4+, 13/43 (30.2%) SSX5+, 31/73 (42.5%) NY-ESO-1+, 13/25 (52%) MAGEB1+, 15/25 (60%) MAGEB2+, 12/25 (48%) MAGEC1+, 17/25 (68%) MAGEC2+, 10/30 (33.3%) SYCP1+ |
| Sato, et al. Int J Oncol. 200529 – no full text | NY-ESO-1, CTAG2 | HCC patients – Japan | RT-PCR and IHC | IHC: 3/10 (30%) NY-ESO-1+ - all 10 samples expressed NY-ESO-1 mRNA  1/6 (16.7%) CTAG2+ - all 6 samples expressed CTAG2 mRNA |
| Yang, et al. Lab Invest. 200530 | FATE | HCC patients (n=35; China) | RT-PCR and IHC | RT-PCR: 10/15 (66%) FATE+  IHC: 7/35 (20%) FATE+ |
| Dong, et al. Biochem Cell Biol. 200431 – no full text | FATE | HCC patients (China) | RT-PCR | 25% of HCC samples FATE+ |
| Dong, et al. Br J Cancer. 200432 | ZNF165 | HCC patients (n=42; China) | RT-PCR | 22/42 (52%) ZNF165+ |
| Zhao, et al. World J Gastroenterol. 200433 | MAGEA1, MAGEC2, SSX1, SPANXC | HCC patients (n=105; China) | RT-PCR | 79/105 (75.2%) MAGEA1+, 59/105 (56.2%) MAGEC2+, 76/105 (72.4%) SSX1+, 66/105 (62.9%) SPANXC+ |
| Li, et al. Lab Invest. 200334 – no full text | MAGEC2 | HCC patients (n=70; China) | IHC | 26/70 (37.1%) MAGEC2+ |
| Dong, et al. Br J Cancer. 200335 | FATE, TPTE | HCC patients (n=62; China) | RT-PCR | 41/62 (66%) FATE1+, 24/62 (39%) TPTE+ |
| Luo, et al. Cancer Immun. 200236 | MAGEA1, MAGEA3, MAGEA4, GAGE, NY-ESO-1, SSX1, SSX2, SSX4, SYCP1, LUZP4 | HCC patients (n=21; China) | RT-PCR | 4/21 (19%) MAGEA1+, 5/21 (24%) MAGEA3+, 1/21 (4.8%) MAGEA4+, 8/21 (38%) GAGE+, 0/21 (0%) NY-ESO-1+, 8/21 (38%) SSX1+, 2/21 (9.5%) SSX2+, 2/21 (9.5%) SSX4+, 6/21 (29%) SYCP1+, 4/21 (19%) LUZP4+ |
| Wang, et al. J Immunol. 200237 | MAGEC2, TFDP3 | HCC patients (n=20; China) | RT-PCR | 14/20 (70%) MAGEC2+, 5/17 (29.4%) TFDP3+ |
| de Wit, et al. Int J Cancer. 200238 | DSCR8 | HCC cell lines (Hep3B, HepG2, PLC/RPF/5, Huh7) | RT-PCR | 1/4 cell lines DSCR8+ |
| Ono, et al. Proc Natl Acad Sci U S A. 200139 | ACRBP | HCC patients (n=5; Japan) | RT-PCR | 2/5 (40%) ACRBP+ |
| Chen, et al. Cancer Lett. 200140 | SSX1, SSX2, SSX4, SSX5, SYCP1, NY-ESO-1 | HCC patients (n=30; Taiwan) | RT-PCR | 24/30 (80%) SSX1+, 14/30 (46.7%) SSX2+, 22/30 (73.3%) SSX4+, 10/30 (33.3%) SSX5+, 2/30 (6.7%) SYCP1+, 11/30 (36.7%) NY-ESO-1+ |

**Supplementary Table S4. Frequency table of healthy liver tissues (n=21) expressing mRNA of the CTAs.** Colors correlate to the percentage of positive healthy liver tissues.

|  |  |  |  |
| --- | --- | --- | --- |
|  | **mRNA+ healthy liver (%)** |  | **mRNA+ healthy liver (%)** |
| **CAGE1** | 0.0 | **MAGEA10** | 0.0 |
| **CBLL2** | 42.9 | **MAGEB1** | 19.0 |
| **CCDC83** | 0.0 | **MAGEB2** | 0.0 |
| **CPXCR1** | 0.0 | **MAGEB3** | 28.6 |
| **CSAG2/3** | 85.7 | **MAGEB6** | 28.6 |
| **CT45** | 14.3 | **MAGEC1** | 4.8 |
| **CT47A1** | 0.0 | **MAGEC2** | 0.0 |
| **Cxorf48** | 0.0 | **NYESO1** | 0.0 |
| **DDX53** | 47.6 | **PAGE1** | 0.0 |
| **DPPA2** | 0.0 | **PAGE5** | 100.0 |
| **DUSP21** | 23.8 | **PASD1** | 0.0 |
| **FAM46D** | 0.0 | **PLAC1** | 0.0 |
| **FATE1** | 33.3 | **RNF17** | 4.8 |
| **FBXO39** | 100.0 | **SAGE1** | 0.0 |
| **FMR1NB** | 0.0 | **SLCO6A1** | 0.0 |
| **FTHL17** | 42.9 | **SPANXA** | 66.7 |
| **GAGE1** | 23.8 | **SPANXC** | 38.1 |
| **HORMAD1** | 100.0 | **SPANXN3** | 0.0 |
| **LUZP4** | 0.0 | **SYCP1** | 47.6 |
| **MAGEA1** | 0.0 | **TEKT5** | 100.0 |
| **MAGEA2** | 23.8 | **TFDP3** | 33.3 |
| **MAGEA3** | 14.3 | **TPPP2** | 100.0 |
| **MAGEA4** | 0.0 | **TSPY** | 0.0 |
| **MAGEA8** | 0.0 | **ZCCHC13** | 38.1 |
| **MAGEA9** | 0.0 |  |  |

**Supplementary Table S5. Expression of excluded CTAs in HCC patients and in cirrhotic liver tissues without malignancy.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **mRNA-positive HCC (%)1** | **mean in mRNA-+ HCC**  **(range)2** | **Relative expression HCC**  **(compared to testis)3** | **mRNA-positive TFL (%)4** | **mean in mRNA-+ TFL (range)5** | **Relative expression TFL**  **(compared to testis)6** | **mRNA-positive cirrhotic tissue7** |
| **CCDC83** | 0.00 | #VALUE! |  | 0.00 | #VALUE! |  | 0 |
| **CPXCR1** | 2.02 | 0.001 (0.001-0.001) | 0.00378 | 0.00 | #VALUE! |  | 0 |
| **Cxorf48** | 8.25 | 0.157 (0.002-0.991) | 1.839 | 2.04 | 0.012 (0.001-0.023) | 0.139 | 2.9 |
| **DPPA2** | 1.03 | 0.135 (0.135-0.135) | 1.204 | 0.00 | #VALUE! |  | 0 |
| **FAM46D** | 5.15 | 0.003 (0.002-0.004) | 0.058 | 0.00 | #VALUE! |  | 2.9 |
| **FMR1NB** | 6.19 | 0.031 (0.002-0.124) | 0.022 | 1.02 | 0.088 (0.088-0.088) | 0.062 | 0 |
| **LUZP4** | 6.19 | 0.106 (0.001-0.49) | 0.242 | 1.02 | 0.279 (0.279-0.279) | 0.636 | 0 |
| **MAGEA4** | 6.19 | 0.803 (0.001-2.559) | 28.036 | 0.00 | #VALUE! |  | 0 |
| **MAGEA8** | 3.09 | 0.014 (0.004-0.022) | 5.115 | 0.00 | #VALUE! |  | 0 |
| **PASD1** | 2.02 | 0.017 (0.007-0.026) | 0.007 | 2.00 | 0.02 (0.019-0.021) | 0.009 | 0 |
| **PLAC1** | 4.12 | 0.014 (0.001-0.041) | 0.146 | 0.00 | #VALUE! |  | 0 |
| **RNF17** | 21.65 | 0.053 (0.001-0.507) | 0.04570 | 13.27 | 0.023 (0.002-0.134) | 0.01964 | 5.7 |
| **SAGE1** | 4.12 | 0.086 (0.008-0.15) | 0.505 | 3.06 | 0.19 (0.006-0.543) | 1.117 | 0 |
| **SPANXN3** | 1.01 | 0.004 (0.004-0.004) | 52.644 | 3.00 | 0.002 (0.001-0.003) | 27.174 | 0.0 |

1Percentage of hepatocellular carcinomas (HCC) expressing mRNA of the excluded CTAs – meaning a Ct-value <35 and relative expression > 0.001 (n=100); 2Mean relative expression (relative to the geometric mean of the 3 household genes- GUSB, HPRT1, PMM1) level in HCCs expressing the CTA and range; 3Mean relative expression of the CTA in HCC expressing the CTA, relative to the relative mean expression in 3 testis tissues; 4Percentage of paired tumor-free liver tissues (TFL) expressing mRNA of the excluded CTAs (n=100); 5Mean relative expression level in TFLs expressing the CTA and range; 6Mean relative expression of the CTA in TFL expressing the CTA, relative to the relative mean expression in 3 testis tissues; 7Percentage of non-cancerous cirrhotic liver tissues expressing the CTA (n=35)

**Supplementary Table S6. Patient characteristics of HCC-patients included in protein expression analysis.**

|  |  |
| --- | --- |
| Characteristic | HCC patients (n=76) |
| **Age at surgery (years)** | |
| Mean ± SD | 60.4 ± 14.4 |
| Median (range) | 64 (16-82) |
| **Sex – no. (%)** | |
| Male | 47 (61.8) |
| Female | 29 (38.2) |
| **Race – no. (%)** | |
| White | 64 (84.2) |
| African | 6 (7.9) |
| Asian | 5 (6.6) |
| Not reported | 1 (1.3) |
| **Etiology – no. (%)** | |
| No known liver disease | 21 (27.6) |
| Alcohol | 17 (22.4) |
| Hepatitis B | 9 (11.8) |
| NASH | 8 (10.5) |
| Hepatitis C + Alcohol | 6 (7.9) |
| Hepatitis B + Alc/HepC/HepD/NASH | 6 (7.9) |
| Hepatitis C | 5 (6.6) |
| Fibrolamellar HCC | 3 (4.0) |
| Hemochromatosis + NASH | 1 (1.3) |
| Autoimmune hepatitis | - |
| Primary sclerosing cholangitis | - |
| **Hepatitis status – no. (%)** | |
| Hepatitis B or C positive | 26 (34.2) |
| Chronic Hepatitis B | 15 (19.7) |
| Chronic Hepatitis C | 12 (15.8) |
| **Cirrhosis – no. (%)** | |
| Yes | 23 (30.3) |
| No | 53 (69.7) |
| **Tumor differentiation – no. (%)** | |
| Good | 8 (10.5) |
| Moderate | 41 (54.0) |
| Poor | 14 (18.4) |
| Unknown | 13 (17.1) |
| **Vascular invasion – no. (%)** | |
| Yes | 40 (52.6) |
| No | 29 (38.2) |
| Unknown | 7 (9.2) |
| **Number of lesions – no. (%)** | |
| 1 | 40 (52.6) |
| >1 | 36 (47.4) |
| Median (range) | 1 (1-11) |
| **Size of largest lesion (cm)** | |
| Mean ± SD | 7.4 ± 5.2 |
| Median (range) | 6.1 (1-24) |
| **AFP level before resection (ug/l)** | |
| Mean ± SD | 64965 ± 401956 |
| Median (range) | 9.5 (2-3118700) |

**Supplementary Table S7. Cox regression analysis of HCC recurrence and HCC-specific survival based on CTA mRNA expression in HCC tumors**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | HCC recurrence | | HCC survival | |
|  | Univariate analysis | | Univariate analysis | |
| Variable | HR (95% CI) | p-value | HR (95% CI) | p-value |
| ≥1 CTA in tumor | 1.8 (0.86-3.6) | 0.12 | 1 (0.41-2.6) | 0.94 |
| ≥2 CTAs in tumor | 1.1 (0.65-1.9) | 0.67 | 0.86 (0.39-1.9) | 0.7 |
| ≥3 CTAs in tumor | 1.1 (0.62-1.8) | 0.85 | 0.74 (0.34-1.6) | 0.47 |
| Number of CTAs in tumor (numeric) | 1 (0.96-1.1) | 0.29 | 1 (0.87-1.1) | 1 |
| >1 tumor | 1.2 (0.68-2) | 0.56 | 1.1 (0.49-2.4) | 0.83 |
| >2 tumors | 2.6 (1.3-4.9) | **0.0042** | 1.8 (0.69-4.9) | 0.22 |
| Cirrhosis | 1.6 (0.89-2.8) | 0.12 | 1.5 (0.66-3.4) | 0.33 |
| Chronic viral hepatitis | 2.3 (1.3-4) | **0.0031** | 3.3 (1.5-7.2) | **0.0032** |
| Vascular invasion | 1.3 (0.72-2.3) | 0.41 | 2.2 (0.96-4.9) | 0.063 |
| Tumor > 5 cm | 1.3 (0.74-2.3) | 0.37 | 2.3 (0.9-5.7) | 0.081 |
| AFP > 200 ug/l | 1.9 (1-3.4) | **0.034** | 2.7 (1.2-6) | **0.013** |
| AFP > 400 ug/l | 2.4 (1.3-4.5) | **0.0051** | 3.3 (1.5-7.3) | **0.0038** |

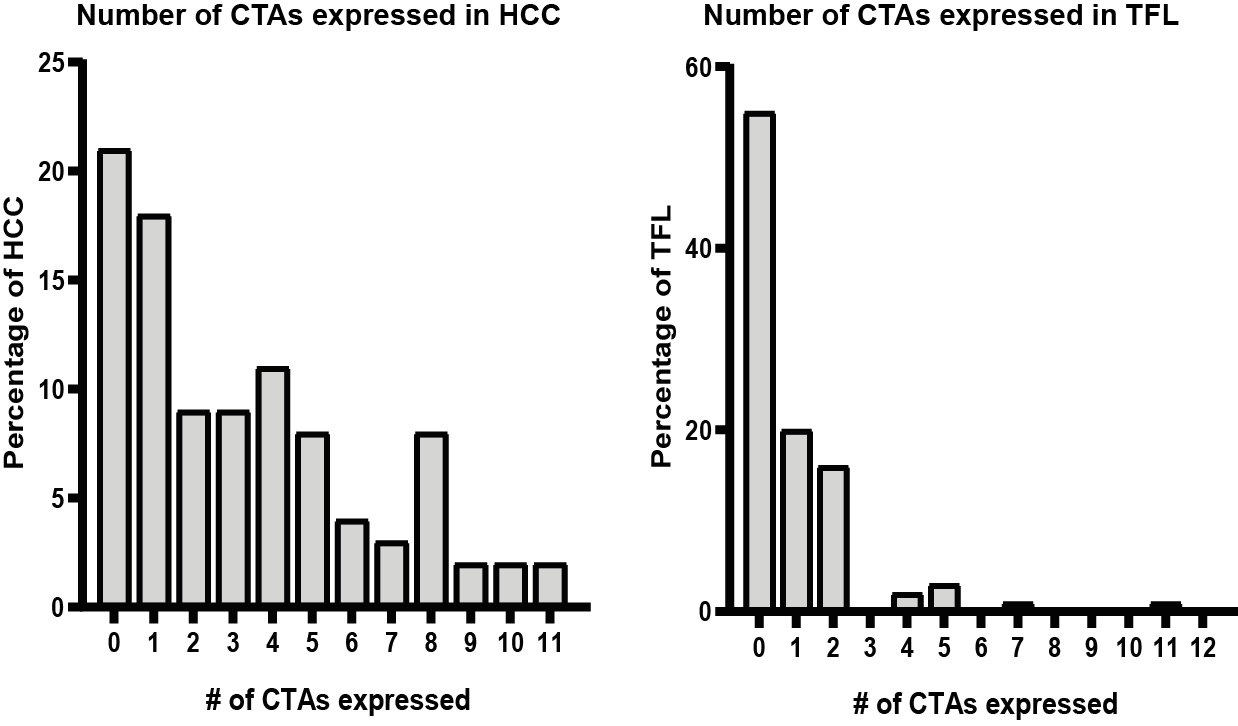
Abbreviations: HR, hazard ratio; CI, confidence interval; CTA, cancer-testis antigen; AFP , alphafetoprotein

**Supplementary Table S8. Cox regression analysis of HCC recurrence and HCC-specific survival based on CTA protein expression in TFL**

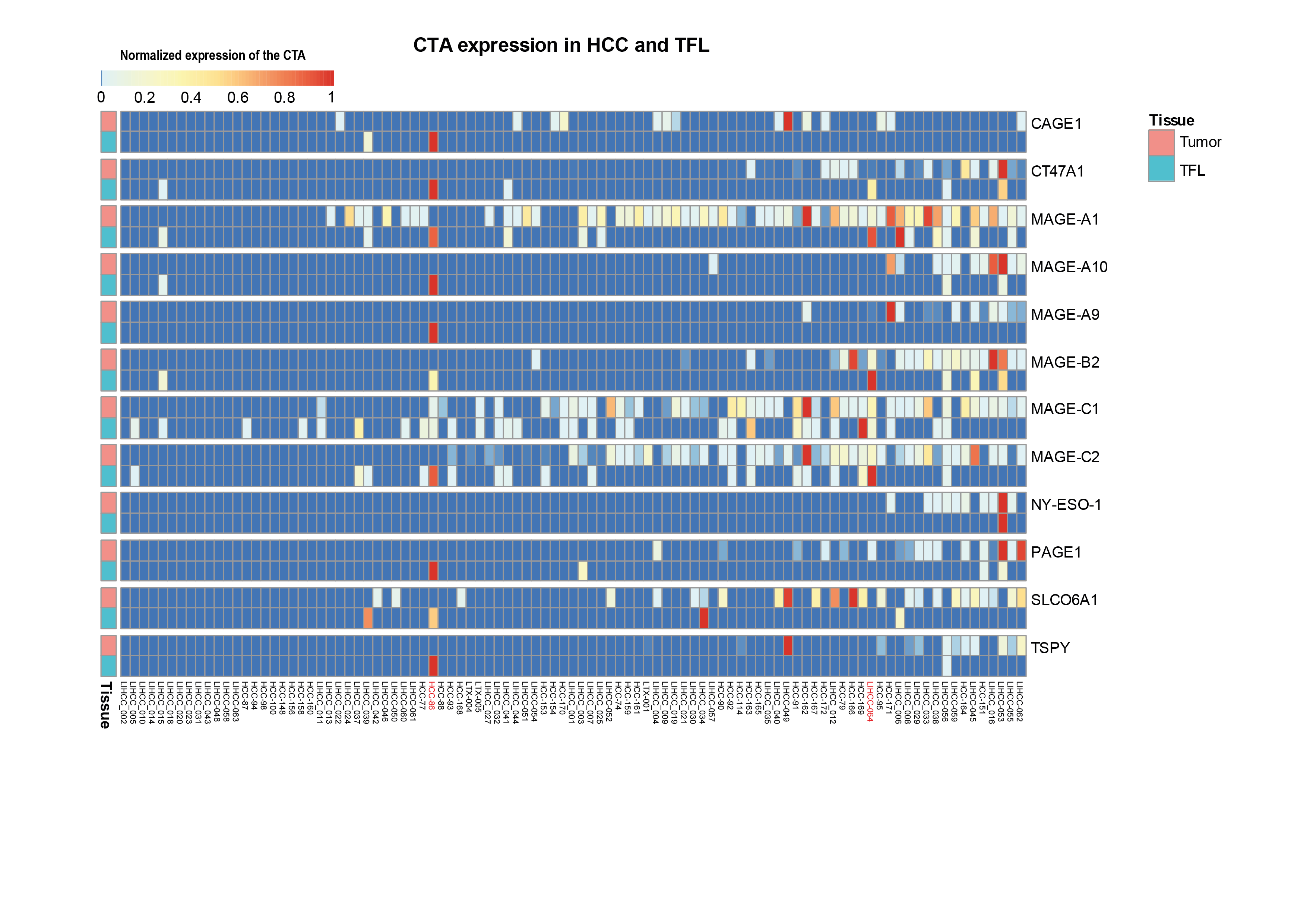
|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Recurrence | | | | HCC-specific survival | | | |
|  | Univariate analysis | | Multivariate analysis | | Univariate analysis | | Multivariate analysis | |
| Variable | HR (95% CI) | p-value | HR (95% CI) | p-value | HR (95% CI) | p-value | HR (95% CI) | p-value |
| ≥1 CTA in TFL | 1.9 (0.98-3.8) | 0.056 | 2.5 (1.2-5.0) | **0.01** | 3.5 (1.4-8.6) | **0.0071** | 5.0 (1.9-13.1) | **0.001** |
| Number of CTAs in TFL (numeric) | 1.9 (1-3.6) | **0.035** |  |  | 3.5 (1.6-7.9) | **0.0024** |  |  |
| >2 tumors | 3.3 (1.5-7.2) | 0.0028 | 2.5 (1.1-5.7) | **0.04** | 2.1 (0.69-6.4) | 0.19 |  |  |
| Chronic viral hepatitis | 2.9 (1.5-5.6) | 0.0017 | 2.6 (1.3-5.4) | **0.009** | 3.4 (1.4-8.6) | **0.0081** | 4.8 (1.9-12.6) | **0.001** |
| Vascular invasion | 1 (0.5-2.1) | 0.93 |  |  | 1.1 (0.43-2.6) | 0.89 |  |  |
| Tumor > 5 cm | 1.1 (0.57-2.2) | 0.73 |  |  | 1.7 (0.65-4.5) | 0.28 |  |  |
| AFP > 400 ug/l | 1.9 (0.92-4) | 0.083 |  |  | 2.4 (0.93-6.1) | 0.07 |  |  |

Abbreviations: HR, hazard ratio; CI, confidence interval; CTA, cancer-testis antigen; TFL, tumor-free liver; AFP , alphafetoprotein

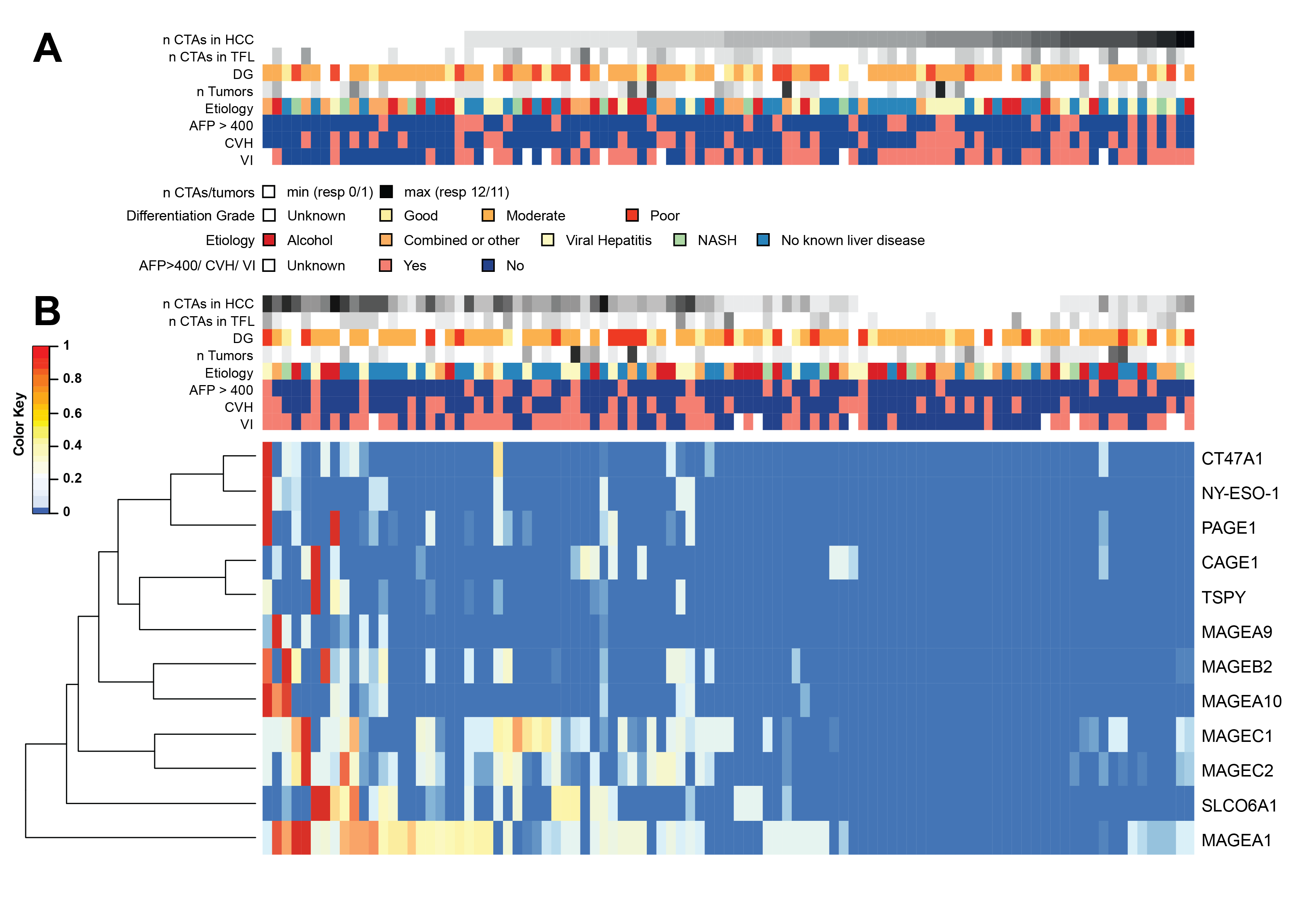
**Supplementary Figure S1. Number of CTAs co-expressed in HCC tumors and TFL, based on mRNA expression.**



**Supplementary Figure S2**



**Supplementary Figure S2. Heatmap of CTA expression in HCC and TFL.** Heatmap of mRNA expression of the 12 CTAs expressed in ≥ 10% of HCCs (rows), in HCC and TFL for every patient (columns). Patients were ordered by number of CTAs expressed in each individual tumor. The –ΔCt values were used and for normalization this data was scaled between 0 and 1 [((x-(min(x))/(max(x)-min(x)))]. Colors correspond to the value between 0 and 1 and patients LIHCC-064 and HCC-86 are highlighted in red. Heatmap was made in R, using the pheatmap package.

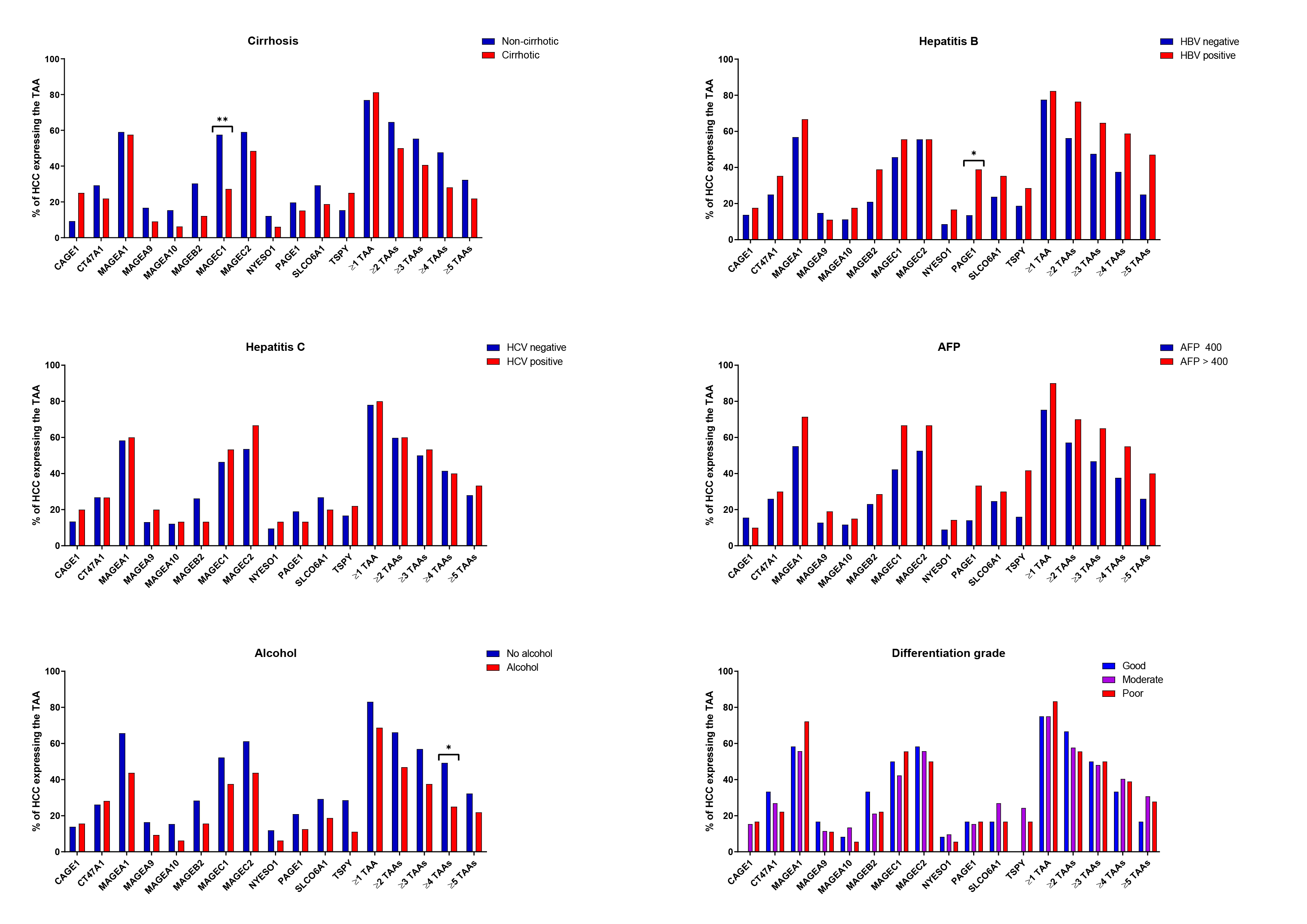
**Supplementary Figure S3**

**Supplementary Figure S3. Hierarchical clustering of mRNA expression of the CTAs in tumors shows clustering by vascular invasion of the HCC** **A.** Figure depicting clinical parameters in the rows and patient in the columns. Data is ordered by number of CTAs expressed in each individual tumor **B.** mRNA expression was normalized between 0 and 1 for every CTA and then both samples and genes were hierarchically clustered. Heatmap was made in R, using the gplots package.

Abbreviations: n CTAs in HCC , Number of CTAs expressed in HCC; n CTAs in TFL , Number of CTAs expressed in TFL; DG , Differentiation Grade; n Tumors , number of tumors; CVH , Chronic viral hepatitis; VI , Vascular Invasion.

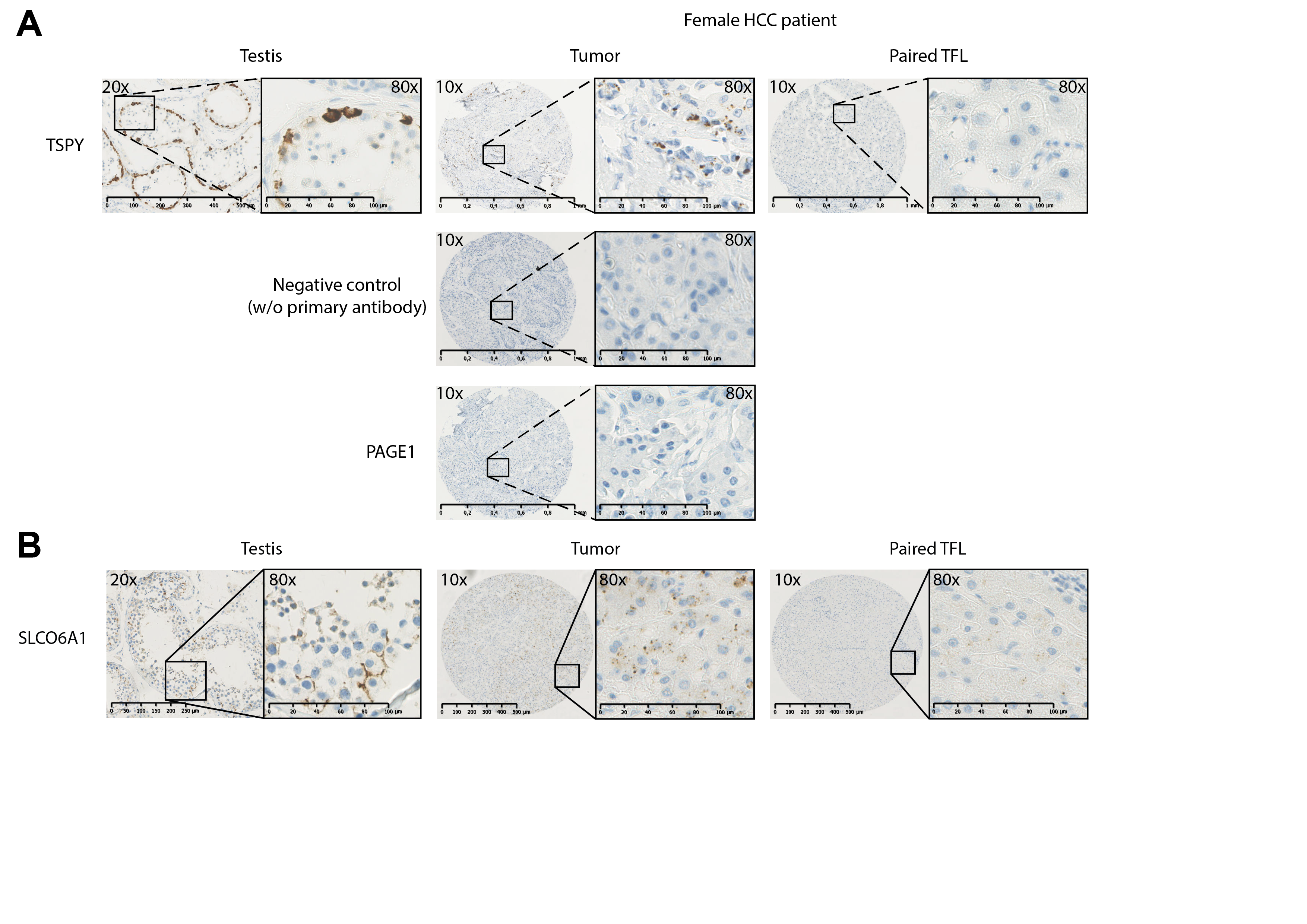
**Supplementary Figure S4**

**Supplementary Figure S4. CTA mRNA expression in HCC tumors based on clinicopathological parameters.** Chi-square test \*p<0.05, \*\*p<0.01



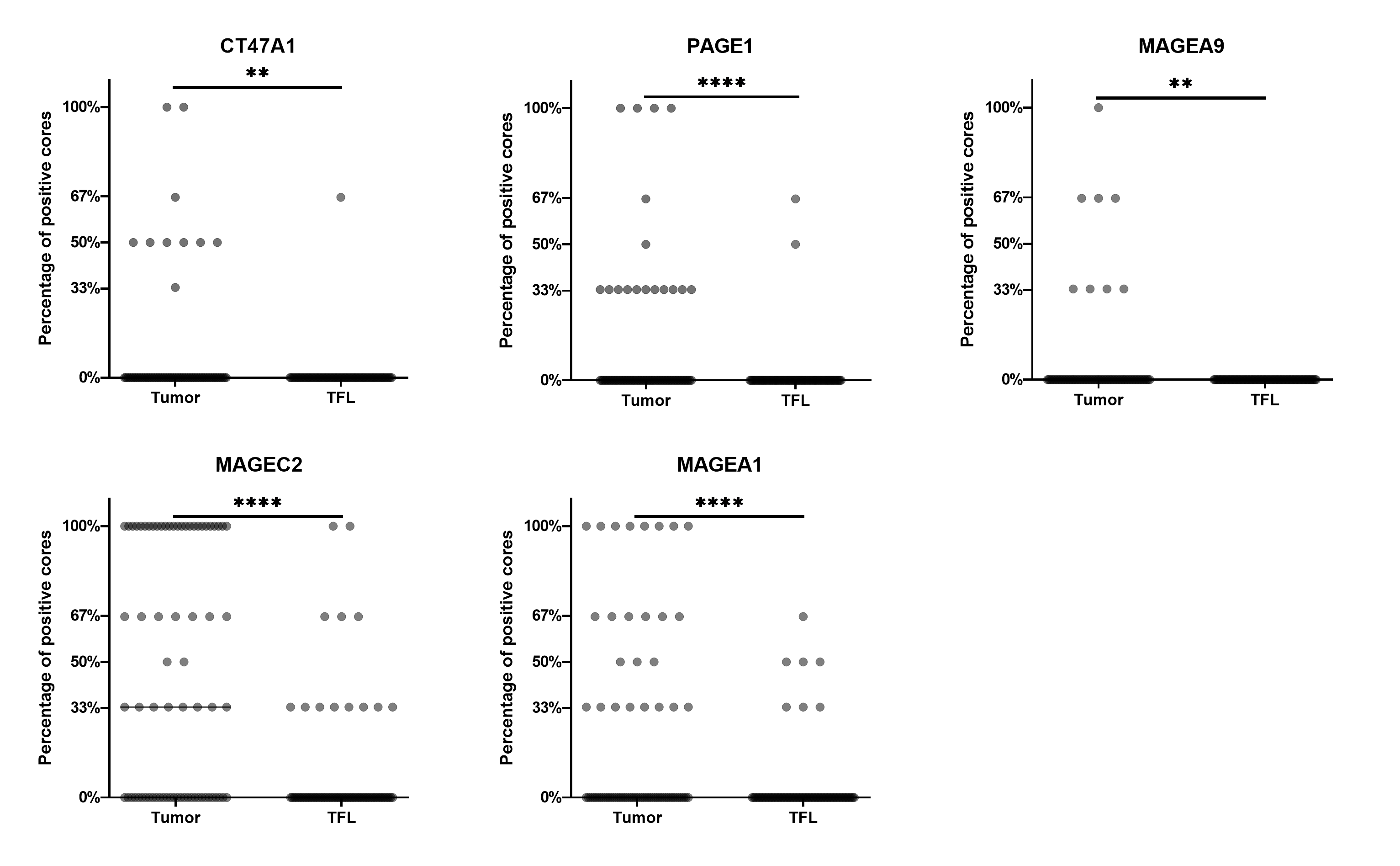
Abbreviations: AFP, alpha-fetoprotein

**Supplementary Figure S5**



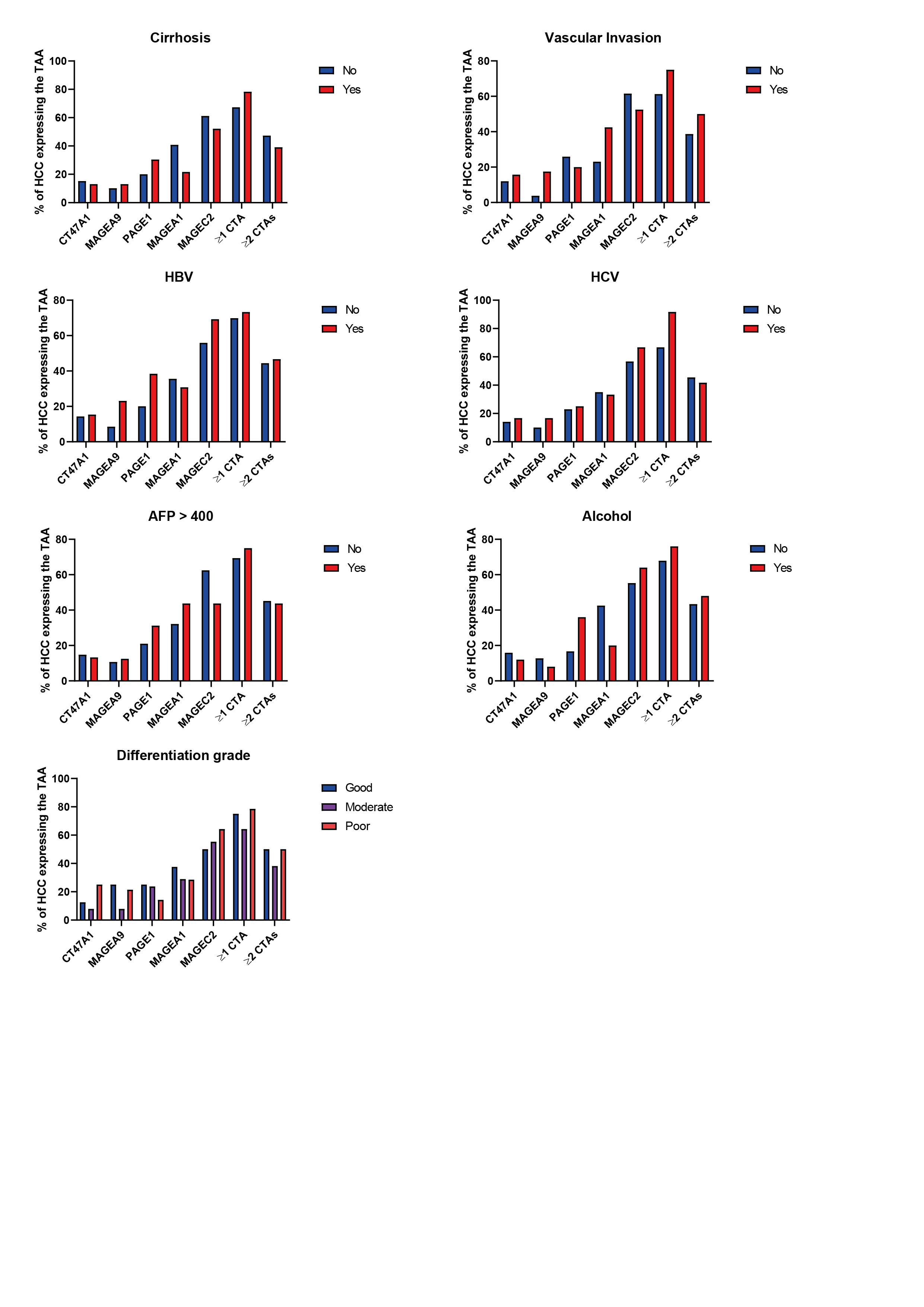
**Supplementary Figure S5. TSPY expression in female HCC tumors and SLCO6A1 expression. A.** TSPY protein expression was determined by IHC. TSPY is expressed in spermatogonia of normal testis, as expected.41 However, TSPY protein expression was also found in two female HCC patients, of which one example is shown above. The staining is absent in the negative control and in the PAGE1 stained core. TSPY is encoded by the y-chromosome, expression in women is thus biologically impossible. **B.** Representative example of immunohistochemical stains of SLCO6A1 in testis, a positive HCC tumor tissue and the paired TFL tissue.

**Supplementary Figure S6**



**Supplementary Figure S6. Proteins are focally expressed in most tumors.** Protein expression was determined on TMAs, which had 3 cores of each tumor and TFL. The graphs display the percentage of cores containing protein-expressing cells (a score ≥1A). Most tumors and TFL focally express the proteins, illustrated by not all cores being positive. Wilcoxon signed-rank test. \*\* P<0.01, \*\*\*\* P<0.0001

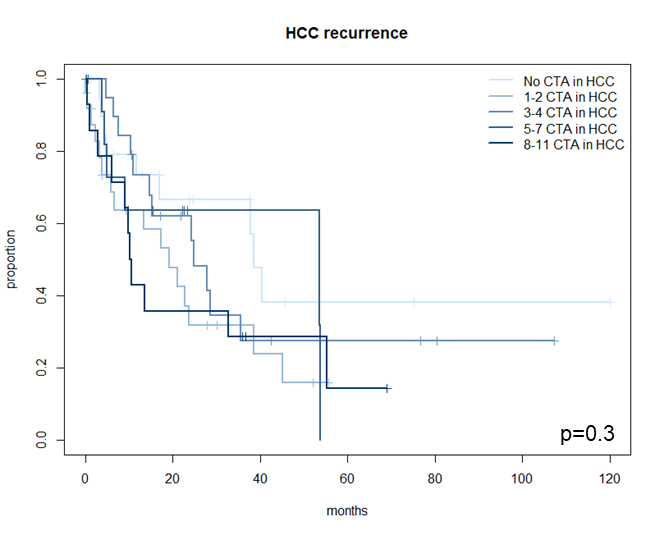
**Supplementary Figure S7**

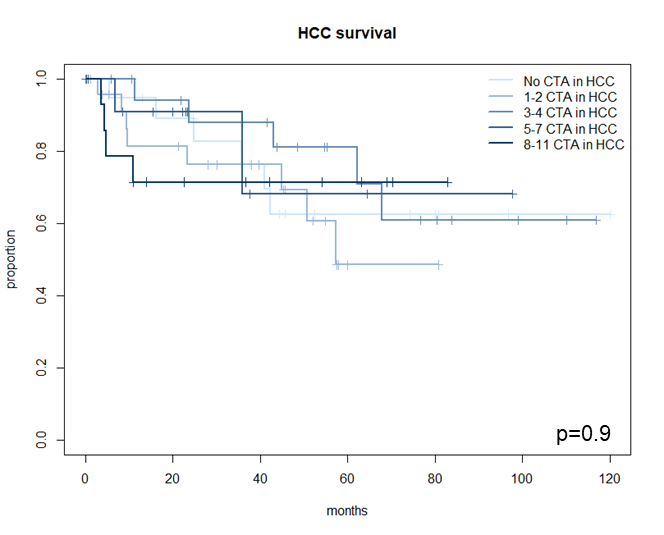


**Supplementary Figure S7. CTA protein expression in HCC tumors based on clinical parameters.** Protein expression in tumor tissue per clinical parameter. Chi-square test .

Abbreviations: HBV , Hepatitis B virus; HCV , Hepatitis C Virus; AFP >400 , Alphafetoprotein > 400 ug/L

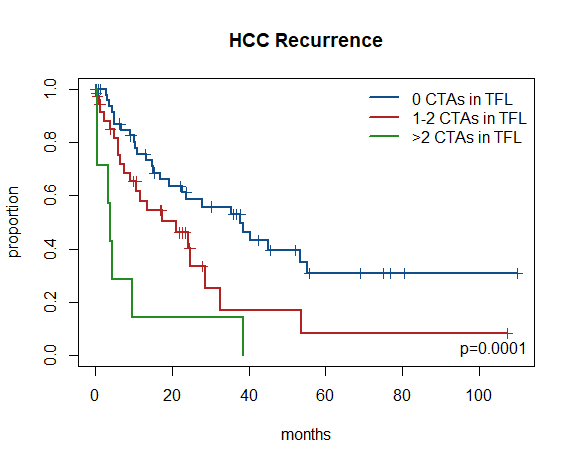
**Supplementary Figure S8.**



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**Supplementary Figure S8. HCC recurrence and HCC-specific survival by CTA mRNA-expression in tumor tissue.** Log-rank test.

**Supplementary Figure S9.**



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**Supplementary Figure S9. HCC recurrence and HCC-specific survival by number of CTAs expressed (based on mRNA expression) in TFL.** Log-rank test.

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