Expression of Cancer testis Antigens in Tumor-adjacent Normal Liver Predicts Post-resection Recurrence of Hepatocellular Carcinoma

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Abstract

Background: High recurrence rates after resection of hepatocellular cancer (HCC) with curative intent and lack of effective therapy for advanced disease impair clinical outcomes of HCC. Cancer/testis antigens (CTAs) are suitable targets for cancer immunotherapy if selectively expressed in tumor cells. The aims of this study were to establish a panel of CTAs that are frequently and selectively expressed in tumors of HCC-patients, and to investigate whether CTAs might be expressed in tumor-free liver tissues of HCC-patients.

Methods: Surgically-resected tumor and paired tumor-free (TFL) tissues of HCC patients (n=100), healthy livers (n=21), and other healthy tissues (n=22 different tissues) were assessed for mRNA expression of 49 carefully selected CTAs by RT-qPCR. Protein expression of 5 CTAs was determined by immunohistochemistry (n=78).

Results: Twelve CTAs were expressed at mRNA level in ≥10% of HCC-tumor tissues and not in healthy tissues except testis. In tumors, mRNA and protein of ≥ 1 CTA was expressed in 78% and 71% of HCC-patients, respectively. In TFL, CTA mRNA and protein expression was found in 45% and 30% of HCC-patients, respectively. Interestingly, CTA expression in TFL was an independent negative prognostic factor for HCC-recurrence and survival after tumor resection.

Conclusions: We established a novel panel of 12 testis-restricted CTAs expressed in tumors of most HCC-patients, that can be safely used for immunotherapeutic targeting of HCC. The increased risk of HCC recurrence in patients with CTA expression in TFL suggests that CTA-expressing (pre-)malignant cells may be a source of HCC recurrence. Therefore, immunotherapeutic targeting of these antigens should be considered as adjuvant therapy to prevent HCC-recurrence after tumor resection.

Lay summary:

Expression of multiple defined cancer testis antigens in non-cancerous liver tissue is associated with significantly increased cancer-recurrence and worse patient survival after tumor resection. We propose that immunotherapeutic targeting of these antigens may prevent HCC recurrence after tumor resection.

Background

Liver cancer is the fourth leading cause of cancer related death, with hepatocellular carcinoma (HCC) being the most common subtype.(1) HCC is often diagnosed at advanced stage and these patients can only be offered palliative therapies.(2, 3) Recently immune checkpoint inhibitors (CPIs) that block PD-1 or PD-L1, either as single treatment or in combination with a monoclonal antibody against vascular endothelial growth factor, have been shown to induce objective responses in advanced HCC patients, but unfortunately the majority of patients does not respond.(4–6)

CPI therapy generally shows higher efficacy in patients with high tumor mutational load, extensive tumor CD8 T-cell infiltration, and pre-existing systemic anti-tumor T-cell immunity.(7, 8) Immunomodulatory forms of induction chemotherapy or radiotherapy to transform immunologically “cold” tumors, that lack T cell infiltration, into “hot” tumors are being studied to sensitize patients to CPI therapy.(9, 10) Also combinations of CPIs with therapeutic vaccination to enhance systemic anti-tumor T-cell immunity are being studied.(11–13) Therapeutic vaccination has shown potential to elicit systemic anti-tumor T cell responses in advanced HCC,(14, 15) and may therefore be an effective method to sensitize HCC patients for CPI therapy. However, such trials have not been conducted in HCC so far.

A prerequisite of a safe and effective vaccine is that the targets are immunogenic and exclusively expressed in tumor cells, to prevent auto-immune side effects. Cancer testis antigens (CTA) are a family of proteins that are highly expressed in immune-privileged germ cells, whereas their expression in other healthy tissues is partially or completely silenced. In addition, CTAs can become aberrantly expressed in cancer cells of various histological subtypes.(16) As CTAs have also shown to be immunogenic, these antigens are considered as suitable shared tumor antigens for cancer immunotherapy, including therapeutic vaccination.(16, 17) Based on their expression profile in adult healthy tissues, they are classified into testis-restricted, testis/brain-restricted and testis-selective CTAs, the latter category showing expression in somatic tissues, although often at lower levels.(18) As testis-restricted CTAs are solely expressed in immune-privileged germ cells, they can therefore be safely applied for cancer immunotherapy. (18, 19)

Although early stage HCC-patients are treated by surgical resection or radiofrequency ablation with curative intent, recurrence rates are high, and currently no therapies to prevent recurrence are available. Early recurrence likely originates from occult metastases in non-cancerous liver tissue at the time of resection, whereas late recurrences are more likely to represent *de novo* tumors.(20–22) To identify patients at risk of HCC recurrence, it remains of great importance to identify such occult (pre-)malignant lesions or cells in the non-cancerous liver tissue at the time of resection. We hypothesized that CTAs might not only be expressed in tumors, but also in occult (pre-)malignant lesions or cells present in non-cancerous liver tissues of HCC patients, and that these may be (at least partially) responsible for HCC recurrence after tumor resection. We further reasoned that if this hypothesis is correct, adjuvant therapies to eradicate CTA-expressing (pre-)malignant cells from the remaining liver tissue may be a valuable tool in prevention of HCC recurrence after tumor resection.

The aims of this study were: 1) To establish a panel of CTAs that are frequently expressed in tumors of HCC patients but not in any healthy tissue except testis, for immunotherapeutic purposes such as therapeutic vaccination; 2) To determine whether CTAs are also expressed in non-cancerous liver tissues of HCC-patients, and whether such expression is associated with HCC recurrence after tumor resection.

Patients, Materials & Methods

HCC patients and tissues

A total of 100 archived surgically-resected fresh frozen tumor tissue samples and paired tumor-free liver (TFL) tissue samples obtained at a distance of > 2 cm from the tumors, as well as 76 formalin-fixed paraffin-embedded (FFPE) paired tumor and TFL tissues, of HCC patients were collected after surgery or retrieved from the archives of the Department of Pathology, Erasmus Medical Center Rotterdam and the Dutch nationwide pathology archives (PALGA), respectively. The included HCC patients underwent hepatic resection (n = 97 and n = 73 for fresh frozen and FFPE samples, respectively) or liver transplantation (n = 3 for both fresh frozen and FFPE samples) for HCC in our center between February 1995 and September 2017, and diagnosis of HCC was confirmed by pathological examination. Medical records were reviewed for clinicopathological variables and date of first recurrence, HCC-specific death and last follow-up. This study was approved by the local ethics committees and adhered to the 1975 Declaration of Helsinki.

Further details of these and other included tissues can be found in the supplementary materials and methods.

Selection of CTAs

A literature search to identify CTAs reported to be expressed in HCC was conducted in PubMed on October 4th, 2018. A summary of this search is provided in Fig. 1A and the query in the Supplementary data. Papers written in English that described CTA expression in HCC patients and/or HCC cell lines were included. In addition, the CTA database (http://www.cta.lncc.br/) was consulted to find additional CTAs expressed in HCC and one relevant paper was added.(23)

Quantitative real-time PCR

RNA was isolated from the frozen tissues and RT-qPCR was performed. The sequences, Tm-values and product lengths of the used primers are provided in **Supplementary Table S1**, and detailed methods can be found in the supplementary data file.

Immunohistochemistry

Protein expression was determined by immunohistochemistry (IHC) on tissue microarrays (TMA), that contained three 1 mm cores of each tumor and TFL tissue, as described in the supplementary data file as is the immunohistochemical staining method. The stained TMAs were scored blindly by two researchers, based on intensity of the staining (0 [none], 1 [low], 2 [intermediate], 3 [strong]) and the percentage of positive tumor cells or hepatocytes (A [< 10%], B [10–50%], C [50–90%], D [> 90%]). If less than 5 positive cells per core were observed, the core was scored as 0, and cores smaller than 50% of the original surface were excluded. The score per core was the product of the intensity and the percentage of positive cells (A = 0.1, B = 0.3, C = 0.7 and D = 1). The final score was the average score of the three cores.

Statistical analysis

All statistical analyses were performed using Graphpad (Version 8.2.1 for Windows, San Diego, CA) and R Statistical software (Version 3.6.1 for Windows, Foundation for Statistical Computing, Vienna, Austria).

The correlation analysis was performed in RStudio with the ‘corplot’ package, using Pearson’s correlation coefficient. For creating heatmaps, RStudio was used with the ‘gplots’ and ‘pheatmap’ packages. Survival analysis was performed by the Kaplan-Meier method and the Cox proportional hazards model. Time to event was calculated from the day of surgery. Used statistical tests are indicated in the figures. P-values < 0.05 were considered significant.

Results

Patient characteristics

The clinicopathological characteristics of the 100 HCC patients, 35 cirrhotic patients and 21 healthy controls analyzed for mRNA expression are listed in Table 1. The majority of HCC-patients are Caucasian (82%), 34% of patients had cirrhosis, and 33% of patients had no underlying liver disease.

Table 1

Patient characteristics. Patient characteristics of patients included in mRNA analysis. [1]data not available of 3 patients; [2]Patient with AFP of 48 ug/l was diagnosed with metastatic serous ovarian carcinoma 21 months after LTx

Characteristic	HCC patients (n = 100)	Cirrhotic patients (n = 35)	Healthy controls (n = 21)
Age at surgery (years)			
Mean ± SD	59.9 ± 14.5	46 ± 12.9	51 ± 16.2
Median (range)	63.5 (11–82)	47 (21–68)	52 (13–88)
Sex – no. (%)			
Male	63 (63)	24 (68.6)	12 (57.1)
Female	37 (37)	11 (31.4)	9 (42.9)
Race – no. (%)			
White	82 (82)	31 (88.6)	-
African	9 (9)	3 (8.6)	-
Asian	8 (8)	1 (2.9)	-
Not reported	1 (1)	-	21 (100)
Etiology – no. (%)			
No known liver disease	33 (33)	5 (14.3)	NA
Alcohol	21 (21)	5 (14.3)	NA
Hepatitis B	12 (12)	5 (14.3)	NA
NASH	8 (8)	5 (14.3)	NA
Hepatitis C + Alcohol	8 (8)	-	NA
Hepatitis B + Alc/HepC/HepD/NASH	6 (6)	-	NA
Hepatitis C	6 (6)	5 (14.3)	NA
Fibrolamellar HCC	4 (4)	-	NA
Hemochromatosis + NASH/Alcohol	2 (2)	-	NA
Autoimmune hepatitis	-	5 (14.3)	NA
Primary sclerosing cholangitis	-	5 (14.3)	NA
Hepatitis status – no. (%)			

Characteristic	HCC patients (n = 100)	Cirrhotic patients (n = 35)	Healthy controls (n = 21)
Hepatitis B or C positive	32 (32)	10 (28.6)	0 (0)
Chronic Hepatitis B	18 (18)	5 (14.3)	0 (0)
Chronic Hepatitis C	15 (15)	5 (14.3)	0 (0)
Cirrhosis – no. (%)			
Yes	34 (34)	35 (100)	0 (0)
No	66 (66)	0 (0)	21 (100)
Tumor differentiation – no. (%)			
Good	12 (12)	NA	NA
Moderate	52 (52)	NA	NA
Poor	18 (18)	NA	NA
Unknown	18 (18)	NA	NA
Vascular invasion – no. (%)			
Yes	49 (49)	NA	NA
No	42 (42)	NA	NA
Unknown	9 (9)	NA	NA
Number of lesions – no. (%)			
1	56 (56)	NA	NA
> 1	44 (44)	NA	NA
Median (range)	1 (1–11)		
Size of largest lesion (cm)			
Mean ± SD	7 ± 5.8	NA	NA
Median (range)	5.5 (1–34)	NA	NA
AFP level before resection (ug/l)			
Mean ± SD	51403 ± 351136	7.3[1] ± 9.7	NA
Median (range)	9 (1-3118700)	3.5[1] (1-48[2])	NA

Selection of 26 CTAs after literature study and exclusion of those expressed in healthy liver

Using a query to identify publications on CTAs expressed in HCC tissue, 281 publication records were obtained through the PubMed search and one relevant paper(23) was added. After removal of non-English publications, 270 publications were screened on title and abstract, of which 231 papers were excluded. Full texts were screened of the remaining 39 studies, which all met the inclusion criteria **(**Fig. 1A**)**. In these 39 studies, expression of 73 different CTAs in HCC was reported; mRNA expression of 51, protein expression of 1 and both mRNA and protein expression of 21 CTAs **(Supplementary Table S3)**. In addition, the CTA database (http://www.cta.lncc.br/) was consulted, which resulted in identification of 34 other CTAs expressed in HCC; 27 by mRNA, 4 by protein and 3 by protein and mRNA expression. Furthermore, 38 CTAs identified by the CTA database had already been identified in the literature search **(**Fig. 1B**)**. Consecutively, to exclude expression of these 107 CTAs in healthy tissues, studies using next-generation sequencing to quantify mRNA expression levels in samples obtained from a large array of healthy tissues and organs, provided by the FANTOM consortium,(24) Human Protein Atlas (HPA) consortium(25) and genome-based tissue expression (GTEx) consortium, summarized on www.proteinatlas.org, and the genome-wide analysis of CTA mRNA expression by Hofmann, *et al.*(18) were consulted, which led to the exclusion of 47 CTAs due to expression in non-germline tissues **(**Fig. 1B**)**.

To verify the absence of expression in healthy adult non-germline tissues, the expression of the remaining 60 CTAs was first determined in 21 healthy liver tissues by RT-qPCR. For 11 CTAs it was not feasible to design specific primers, due to high sequence homology with other genes. Of the 49 CTAs tested, 23 were expressed in healthy livers, with prevalence rates varying from 14–100%, and therefore excluded from further analysis. Twenty-four CTAs showed undetectable mRNA expression levels in healthy livers. Two CTAs (MAGEC1 and RING finger protein 17 [RNF17]) were each found to be expressed in 1 out of 21 tested healthy livers (with very low relative expression levels of 0.005 and 0.002 respectively), and therefore not excluded **(**Fig. 1C **and Supplementary Table S4).** These 26 CTAs were selected for further study.

A panel of 12 CTAs is expressed in more than 10% of HCC tumors and not in healthy tissues

The mRNA expression of these 26 CTAs was determined in 100 paired HCC tumors and TFL and in 35 non-malignant cirrhotic liver tissues. Thirteen CTAs were expressed in tumors of > 10% of HCC patients at variable expression levels (Table 2, Fig. 2A **and Supplementary Table S5)** and selected for further study. To verify the absence of these 13 CTAs in healthy adult non-germline tissues, mRNA expression was determined in 23 types of healthy adult tissues other than liver **(**Fig. 2B**)**. Most tissues did not express any CTA, except for ovary which expressed five CTAs. Four CTAs were expressed at very low relative expression levels in ovary (MAGEB2 0.002, cancer/testis antigen family 47 member A1 [CT47A1] 0.002, MAGEC1 0.003 and MAGEC2 0.002). However, RNF17 had a higher relative expression level (0.097) and was also expressed in other tissues (thyroid, adrenal gland, bladder, brain, throat, trachea, ovary and thymus), and was therefore excluded from further analysis.

Table 2

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

	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 tissue[7]
CAGE1	14.4	0.082 (0.003–0.711)	0.188	2.0	0.009 (0.003–0.015)	0.020	0
CT47A1	26.8	1.311 (0.001–20.565)	0.632	6.1	0.255 (0.01–0.769)	0.123	0
MAGEA1	58.6	0.403 (0.003–1.926)	4.170	13.0	0.055 (0.005–0.188)	0.567	0
MAGEA9	14.1	0.41 (0.001–4.953)	2.848	1.0	0.035 (0.035–0.035)	0.243	0
MAGEA10	12.4	0.123 (0.002–0.518)	1.080	4.1	0.028 (0.004–0.088)	0.249	0
MAGEB2	24.2	0.395 (0.002-2.4)	0.761	6.0	0.053 (0.018–0.127)	0.102	0
MAGEC1	47.5	0.109 (0.001–0.841)	0.407	32.0	0.047 (0.002–0.466)	0.174	28.6
MAGEC2	55.6	0.692 (0.001–9.305)	1.542	19.0	0.041 (0.003–0.28)	0.091	25.7
NYESO1	10.1	0.13 (0.007–1.04)	0.525	1.0	0.018 (0.018–0.018)	0.071	0

	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 tissue[7]
PAGE1	18.2	0.37 (0.002–2.225)	1.001	5.0	0.059 (0.009–0.179)	0.159	2.9
SLCO6A1	25.8	0.095 (0.002–0.411)	0.053	4.1	0.011 (0.004–0.017)	0.006	2.9
TSPY*	21.0	0.827 (0.004–7.401)	34.135	4.8	0.218 (0.001–0.641)	9.012	4.2

Among the 12 remaining CTAs (Table 2) were 6 members of the MAGE gene family (MAGEA1, MAGEA9, MAGEA10, MAGEB2, MAGEC1 and MAGEC2). MAGEA1, MAGEC1 and MAGEC2 were most frequently expressed, with expression rates between 48% and 59% of the tumors. Other CTAs that were expressed in more than 10% of tumors are cancer antigen 1 (CAGE1; 14%), CT47A1 (27%), New York esophageal squamous cell carcinoma 1 (NYESO1;10%), PAGE family member 1 (PAGE1; 18%), solute carrier organic anion transporter family member 6A1 (SLCO6A1; 26%) and testis-specific Y-encoded protein 1 (TSPY; in 21% of male HCC patients and 0% of female HCC patients, as expected from a gene located on the Y-chromosome).(26) Co-expression of CTAs in HCC tumors was visualized by a correlation plot in Fig. 2D. Three co-expression clusters could be identified; 1) MAGEB2, MAGEA10, PAGE1, NYESO1 and CT47A1; 2) SLCO6A1, TSPY and CAGE1; and 3) MAGEA1, MAGEC2 and MAGEC1. MAGEA9 did not show co-expression with other CTAs.

Thus, based on mRNA expression data, we identified a panel of 12 CTAs prevalently expressed in tumors of HCC-patients, but not in healthy adult tissues except testis. Seventy-eight percent of tumors expressed at least one of these 12 CTAs, 59% expressed at least 2 CTAs, 50% expressed at least 3 CTAs, and 40% 4 or more CTAs (Fig. 2C **and Supplementary Figure S1)**.

CTAs are expressed in tumor-free liver tissues of HCC patients

Despite the TFL being located at least 2 cm away from the tumor and being classified as tumor-free by a pathologist, all 12 CTAs were expressed in these tumor-free liver tissues of HCC patients, although at significantly lower levels (Table 2 **and** Fig. 2A**)**. Forty-five percent of patients expressed at least one CTA in TFL (**Supplementary Figure S1**). The CTAs most frequently expressed in TFL were MAGEA1 (13% of patients), MAGEC1 (32%) and MAGEC2 (19%). The latter two were also found to be expressed in approximately 25% of cirrhotic liver tissues of HCC-patients without liver cancer, suggesting that their expression may be activated during early (pre-)malignant transformations in the liver. Interestingly, when

a particular CTA was detected in TFL, it was often also present in the tumor (**Supplementary Figure S2**); 85% of patients that expressed any CTA in TFL, also had CTA expression in tumor. For example, LIHCC-064 expressed 7 CTAs in tumor, of which 5 were also expressed in TFL, suggesting that CTA-expressing cells in TFL were derived from the primary tumor.

CTA mRNA expression in tumor correlates with vascular invasion and PAGE1 is associated with HBV

To assess whether CTA expression in tumors was associated with certain etiologies or clinical factors, a clustering analysis of the patients based on the relative expression of the CTAs was performed **(Supplementary Figure S3B)**. Patients with vascular invasion clustered, and had higher relative CTA expression levels and a higher number of CTAs expressed in their tumors compared to patients without vascular invasion in general. The majority of tumors that did not express any CTA, did not display vascular invasion (17/22 patients, 77%).

To study this in more detail, we also analyzed the CTA expression per etiology or known clinicopathological risk factors. Hepatitis C infection, serum alpha-fetoprotein (AFP)-level (≤ 400 ng/ml vs > 400 ng/ml) and differentiation grade were not associated with CTA expression in tumors **(Supplementary Figure S4)**. However, tumors in cirrhotic livers had significantly less frequent expression of MAGEC1, whereas HBV-related tumors expressed PAGE1 more frequently. In line with the clustering analysis, tumors with vascular invasion significantly expressed MAGEA9, MAGEC1 and SLCO6A1 more frequently, and the number of CTAs expressed per tumor was also significantly higher **(**Fig. 2E**)**.

CTAs are expressed on protein level in HCC tumors and TFL

Consecutively, we examined protein expression of these CTAs in tumor and TFL tissues of 78 HCC-patients of which FFPE blocks were available (patient characteristics are shown in **Supplementary Table S6**). Protein expression of MAGEA1, MAGEA10, MAGEC1, MAGEC2 and NYESO1 in HCC tumors has previously been reported by our group.(27) For CAGE1 no suitable IHC antibodies are available. The MAGEB2 IHC Ab showed reliable staining in testis tissue, however, we could not detect any positive cells in HCC and TFL tissues. TSPY and SCLO6A1 Abs demonstrated an unspecific staining pattern and a punctate staining that did not allow for quantification of positive cells, respectively, and were therefore discarded **(Supplementary Figure S5)**.(26)

CT47A1, PAGE1, MAGEA9, MAGEC2 and MAGEA1 were detected at protein level in tumor tissues (CT47A1 in 14%, PAGE1 in 23%, MAGEA9 in 11%, MAGEC2 in 59% and MAGEA1 in 34% of tumors; Fig. 3). Seventy-one percent of HCC tumor tissues expressed at least one of these CTA on protein level **(**Fig. 3C**)**. MAGEA9 was not expressed in any TFL tissue, while we observed expression of CT47A1, PAGE1, MAGEC2 and MAGEA1 in hepatocytes in 1%, 3%, 17% and 9% of TFL tissues, respectively, but at significantly lower expression levels than in tumors **(**Fig. 3B**)**. Thirty percent of patients expressed at least one protein in their TFL tissue. Most protein expression was focal, as illustrated by the observation that in most patients only part of the tumor cores included in the TMA showed protein expression (**Supplementary Figure S6)**.

CTA protein expression in tumors showed similar associations with etiological and clinicopathological factors as CTA mRNA expression in tumors, but significance was not reached (**Supplementary Figure S7**). HCC tumors with vascular invasion tended to have more CTA protein expressed than those without and PAGE1 protein tended to be more prevalently expressed in HBV-related tumors.

In conclusion, the CTAs that were studied for protein expression, also showed protein expression in tumors and, except MAGEA9, also in TFL.

CTA expression in TFL is correlated with HCC recurrence and HCC-specific survival after surgical resection

Finally, we analyzed whether CTA expression in tumors and/or TFL was associated with HCC recurrence and HCC-specific survival. Both survival analysis and cox-regression analysis did not show any association of CTA mRNA expression in tumor tissues and postsurgical prognosis in this cohort (**Supplementary Tables S7 and Supplementary Figure S8**), and neither did CTA protein expression (data not shown). However, expression of CTA mRNA in TFL **(**Fig. 4A**)** was negatively associated with both HCC recurrence and HCC-specific patient survival after surgical resection (Fig. 4B; **Supplementary Figure S8**). Early recurrence, defined as HCC recurrence within 2 years, was observed in 64% of patients with CTA expression in TFL versus 40% in those without. Two-year HCC-specific survival rates were 71% and 89% in patients with and without CTA expression in TFL, respectively. CTA protein expression (Fig. 4C) was associated with poor postsurgical outcome as well (Fig. 4D). In multivariate analysis both CTA mRNA and protein expression in TFL were independent prognostic factors for HCC recurrence (hazard ratio [HR] 2.48 and 2.47 for mRNA and protein expression, respectively) and HCC-specific survival (HR 2.32 and 4.99, respectively; Table 3 **and Supplementary Table S8**).

Table 3

CTA mRNA-expression in TFL is an independent prognostic factor of HCC recurrence and HCC-specific survival. Univariate and multivariate analyses of factors associated with recurrence and survival according to the cox proportional hazard model. Abbreviations: AFP, alphafoetoprotein; 95% CI, 95% confidence interval; CTA, cancer testis antigen; HR, hazard ratio; TFL, tumor-free liver.

	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	2.3 (1.3-4.0)	**0.0034**	2.5 (1.47–4.5)	**0.003**	2.4 (1.1–5.4)	**0.03**	2.3 (1.0-5.3)	**0.044**
≥ 2 CTAs in TFL	2.1 (1.2–3.7)	**0.013**			1.7 (0.7–3.9)	0.22		
≥ 3 CTAs in TFL	4.2 (1.9–9.4)	**0.00053**			5.1 (1.9–14)	**0.0015**		
Number of CTAs in TFL (numeric)	1.3 (1.2–1.5)	**2.0E-05**			1.3 (1.1–1.5)	**0.0011**		
> 1 tumor	1.2 (0.7-2.0)	0.56			1.1 (0.5–2.4)	0.83		
> 2 tumors	2.6 (1.3–4.9)	**0.0042**	2.4 (1.2–4.7)	**0.02**	1.8 (0.7–4.9)	0.22		
Cirrhosis	1.6 (0.9–2.8)	0.12			1.5 (0.7–3.4)	0.33		
Chronic viral hepatitis	2.3 (1.3-4.0)	**0.0031**	2.7 (1.5-5.0)	**0.001**	3.3 (1.5–7.2)	**0.0032**	4.63 (2.0-10.8)	**0.0004**
Vascular invasion	1.3 (0.7–2.3)	0.41			2.2 (0.96–4.9)	0.063		
Tumor > 5 cm	1.3 (0.7–2.3)	0.37			2.3 (0.9–5.7)	0.081		
AFP > 200 ug/l	1.9 (1.0-3.4)	**0.034**			2.7 (1.2-6)	**0.013**		

	Recurrence				HCC-specific survival			
AFP > 400 ug/l	2.4 (1.3–4.5)	**0.0051**	3.0 (1.5–5.8)	**0.001**	3.3 (1.5–7.3)	**0.0038**	4.0 (1.7–9.4)	**0.002**

Two examples of protein expression in TFL are shown in Fig. 4E. In the first patient (left panel) MAGEC2 expressing cells were scattered across the TFL, whereas in the second patient (right panel) small foci of PAGE1 expressing cells could be observed. All TFL tissues with CTA expression were reassessed by a medical pathologist to verify the absence of histologically detectable HCC metastasis. Except for extensive vascular invasion in the tumor of the second patient (Fig. 4E) no histological indications for the presence of malignant cells in TFL were present.

In conclusion, we found that CTA expression in TFL is an independent negative prognostic factor of both HCC recurrence and HCC-specific survival. This may indicate that occult CTA-expressing (pre-)malignant cells are present in the remaining liver tissue after tumor resection and that these cells are at least partially responsible for HCC recurrence after surgery.

Discussion

We established a novel panel of 12 CTAs, that are each expressed in at least 10% of HCC tumors, while none of them are expressed in healthy tissues except immune-privileged testis. Based on mRNA analysis, approximately 80% of our HCC-patients expressed one or more of these antigens in their tumor tissues, whereas protein expression of five of these CTAs was detected in tumors of approximately 70% of these patients. This CTA-panel can therefore be safely applied for immunotherapeutic purposes in the majority of Western HCC-patients. In addition, we found that 45% of HCC-patients expressed one or more of the 12 CTAs of our panel in their histologically tumor-free liver tissue, and that expression in TFL was associated with more HCC recurrence and worse patient survival. These data suggest that occult CTA-expressing (pre-)malignant cells may remain present in non-cancerous liver tissue after tumor resection, and that these cells might be at least partially responsible for HCC recurrence after surgery.

CTA expression in tumors of HCC-patients has been studied before, however, as demonstrated by the results of our literature study (**Supplementary Table S3**), most studies investigated only a few CTAs, determined either RNA or protein expression but not both, and most importantly, did not exclude CTAs expressed in healthy tissues (Fig. 1B **and C, Supplementary Tables S4**). As far as we are aware, the present study is the most comprehensive investigation of CTA-expression in tumor and paired TFL tissues of HCC-patients performed. Moreover, we determined both RNA and protein expression and excluded any CTA that showed up to 4-log lower RNA expression compared to the mean of 3 reference genes in any healthy tissue, in order to prevent therapy-induced auto-immunity in future clinical applications. As 59% of HCC-patients expressed at least 2 CTAs, 50% expressed at least 3 CTAs, and 40% expressed 4 or more CTAs in their tumors, our CTA-panel enables therapeutic targeting of multiple CTAs in

most HCC-patients, which is important to prevent escape of tumor cells that do not express a particular CTA from therapy-induced immunity.

Currently, somatic mutation-derived neo-antigens are considered to be the most promising candidate antigens for therapeutic vaccination in cancer patients.(28–30) However, Dong, et al. recently showed that in multifocal HCC neo-antigens are unique to every tumor lesion, whereas CTA expression was conserved between lesions. (31). These data indicate that effective therapeutic vaccination with neo-antigens in these patients requires analysis of mutations in every lesion and design of a vaccine consisting of neo-antigens expressed in different lesions. In addition, Dong et al found that, compared to neo-antigens, CTAs are expressed at higher levels in tumors of HCC-patients, and that CTA peptides eluted from tumor-expressed MHC class I molecules could evoke expansion of patient T cells *in vitro*. Therefore, CTAs may represent more suitable antigens for vaccination in multifocal HCC compared to neo-antigens. Moreover, since expression of the same CTAs is shared by different HCC patients, the use of CTAs for therapeutic vaccination allows use of off-the-shelve vaccine antigens, which can applied faster, which is important for patients with fast growing tumors, and may be more cost-effective than vaccines consisting of neo-antigens which require design and production of a personalized vaccine for every individual patient.

Importantly, several CTAs of our panel, such as the MAGE-family members, TSPY and CAGE1, are functionally involved in tumorigeneses and cancer progression by modulating gene expression, regulating mitosis and tumorigenic signaling.(16, 17) Involvement of these CTAs in cancer progression may prevent antigen loss upon therapeutic targeting.(32) Their role in cancer progression is further supported by data showing that CTA expression is more prevalent in advanced tumors.(33, 34) In HCC, MAGEA9 expression was related to the presence of distant metastasis and was an independent negative prognostic factor for disease-free survival and overall survival.(35) We found increased CTA expression, including MAGE-A9, in tumors with vascular invasion, which is associated with worse prognosis in HCC. (36) However, in this cohort we did not find any association between CTA expression in tumor tissues and survival, like Liang et al. and previous work by our group reported.(27, 37) Both studies found that patients with more tumor antigens expressed in their HCC tumor, had a better post-surgical prognosis of HCC-patients. This difference may be related to the different panels of CTA that were investigated in the referred studies and in our present study. Moreover, in contrast to our Western HCC-patient cohort, the patient cohort in the study by Liang et al. consisted mainly of chronic HBV patients.

Expression of CTAs in tumor-free liver tissues of HCC patients has been sparsely investigated before (**Supplementary Table S3**). Our study is the first to analyze CTA expression in tumor-free liver tissues of HCC-patients both at RNA and protein level. Moreover, this study is the first to analyze in HCC, or in any other type of cancer, whether CTA expression in non-cancerous tumor-surrounding tissues is associated with post-operative HCC recurrence and patient survival. To our surprise, we observed RNA expression of one or more of the 12 CTAs of our panel in histologically tumor-free liver tissues of 45% of our HCC-patients, while protein expression of one or more of 4 of these CTAs was detected in non-cancerous liver tissues of 40% of patients. Most notably, we found that expression in TFL was associated with faster and

more HCC recurrence as well as worse patient survival after tumor resection. Aufhauser et al.(38) hypothesized that early HCC recurrence (< 2 years) after tumor resection in HCC patients is the consequence of occult multi-focality present at the time of tumor resection, but failed to find markers to identify such occult metastases. The 2-year recurrence rate in our cohort was significantly higher in patients with CTA-expression in TFL compared to patients without CTA-expression in TFL. Moreover, CTA mRNA expression profiles in TFL were similar to those in the corresponding tumors, and our preliminary immunohistochemical data show that CTA-expressing cells in TFL were either single cells or small foci. Based on these observations, we hypothesize that CTA-expressing cells in TFL of patients with early HCC recurrence represent intra-hepatic metastases. This hypothesis is supported by a study performed in colorectal cancer patients with liver metastasis. In TFL, they detected low frequencies of somatic mutations that were also observed in matched tumor samples, despite appearing normal histologically. Since these mutations were not found in the matched blood samples, it was hypothesized that either tumor DNA or tumor cells diffused or migrated into the surrounding normal tissue.(39) However, the authors did not correlate this to either HCC recurrence or survival. Conversely, CTA-expressing cells in TFL of HCC-patients with late HCC recurrence may represent *de novo* tumor-initiating cells. In this respect, it is interesting that we also observed CTA-expression in cirrhotic livers of non-HCC patients. A future study is needed to investigate whether such patients have an increased risk of developing HCC. Similarly, a previous study detected MAGE-antigen expression in lung tissues of former smokers at risk for NSCLC development.(40) Clearly, future research is required to further characterize CTA-expressing cells in tumor-free tissues.

Most therapeutic cancer vaccination studies have been performed in advanced cancer patients with high tumor load in which an immunosuppressive tumor microenvironment has been established, and showed modest clinical results. Based on our data showing the presence of scattered single CTA-expressing cells and small foci of CTA-expressing cells in TFL of almost half of resected HCC-patients, therapeutic vaccination with CTA after tumor resection might be a promising approach to prevent HCC recurrence in such patients. Compared to vaccination in advanced cancer, we expect that the low tumor load remaining after resection of detectable tumors may enhance the probability of effective immunological eradication of CTA-expressing (pre-)malignant cells. Analysis of expression of our panel of 12 CTAs in resected non-malignant liver tissue may allow identification of HCC-patients at risk of HCC recurrence.

A prerequisite for therapeutically targeting antigens by vaccination, is that they are immunogenic. Most of the CTAs included in our panel have previously been proven immunogenic in cancer patients.(41) More specifically in HCC patients, we and other research groups have demonstrated the presence of MAGEA1-, MAGEA10-, MAGEC2- and NY-ESO-1-specific T-cells, both in blood and in tumors. (42–46). In addition, NY-ESO-1 and TSPY-specific IgG have been detected in HCC-patients,(47, 48) while CT47A1-, PAGE1- and SLCO6A1-specific antibodies were recently detected in NSCLC patients.(49)

We acknowledge several limitations of this study. First, since the etiologies of HCC differ geographically, this CTA-panel might not be applicable to non-Western HCC-populations. Secondly, protein expression of CAGE1, MAGEB2 and TSPY needs to be confirmed. Thirdly, the reported association between CTA

expression in TFL and cancer recurrence has to validated in another cohort. Finally, future research is required to investigate whether CTA-expressing cells in TFL are really (pre-)malignant cells that can give rise to cancer recurrence.

Conclusions

We established a panel of 12 testis-restricted CTAs that are expressed in almost 80% of HCC patients. In addition, we demonstrated expression of these CTAs in tumor-free liver tissues of 45% of HCC-patients. The negative association between expression of these CTAs in TFL and HCC-recurrence and survival, combined with immunohistochemical data, suggest that CTA-expressing cells remain present in the liver after tumor resection and are at least partially responsible for HCC recurrence. Therefore, immunotherapeutic targeting of CTA-expressing cells by vaccination or other immunotherapeutic strategies, such as tumor-immune cell engaging bispecific antibodies, TCR-engineered T cells or CAR T cells, should be considered as adjuvant therapy to prevent HCC recurrence after tumor resection in HCC patients with CTA expression in TFL. In addition, this panel of CTAs can probably also be used as therapeutic targets to treat advanced HCC patients.

List Of Abbreviations

AFP, alpha-foetoprotein; CAGE1, cancer antigen 1; CPI, checkpoint inhibition; CT47A1, cancer/testis antigen family 47 member A1; CTA, cancer testis antigen; FFPE, formalin-fixed paraffin-embedded; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; IHC, immunohistochemistry; MAGE, melanoma antigen; NAFLD, non-alcoholic fatty liver disease; NSCLC, non-small cell lung cancer; NYESO1, New York esophageal squamous cell carcinoma 1; PAGE1, PAGE family member 1; RNF17, RING finger protein 17; SLCO6A1, solute carrier organic anion transporter family member 6A1; TFL, tumor-free liver; TMA, tissue microarray; TSPY, testis-specific Y-encoded protein 1.

Declarations

Ethics approval and consent to participate

This study was approved by the local medical ethics committee of Erasmus MC, Rotterdam (MEC-2009-012).

Consent for publication

NA

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare no potential conflicts of interest.

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Author contributions

LN and JK conceived the idea and designed the study. LN, ZG, HO and SM performed most of the experiments. PB, LCC, GZ and TB provided assistance with experiments. LN analyzed the data and wrote the manuscript. MD conducted sample analysis and provided clinicopathological data. JIJ provided patient samples and obtained consent from patients. JK, DS and MB supervised the study. QP critically reviewed the manuscript. LN, ZG and JK revised the manuscript with input from all authors.

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Figures

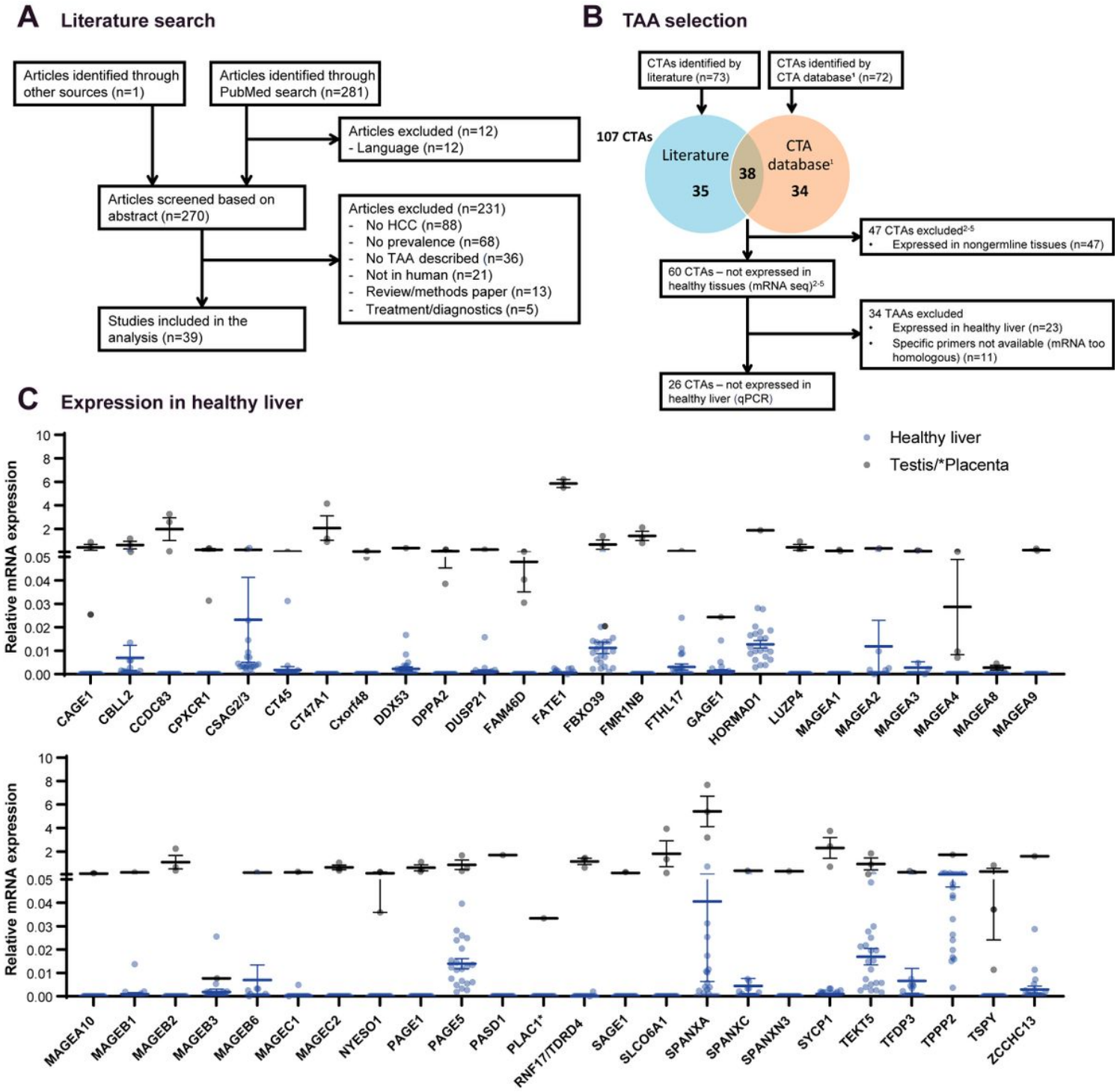

Figure 1

Selection of CTAs. A/B. Study Flow Diagram. C. Relative mRNA expression of selected CTAs in healthy donor livers (n=21) in blue and in the respective positive control tissues in black. Control tissues were: placenta (for PLAC1; n=1) or testis (all other CTAs; n=1-3). 1http://www.cta.lncc.br/, 2Hofmann, et al. 3FANTOM consortium, 4HPA consortium, 5GTEx consortium.(18, 25, 50, 51)

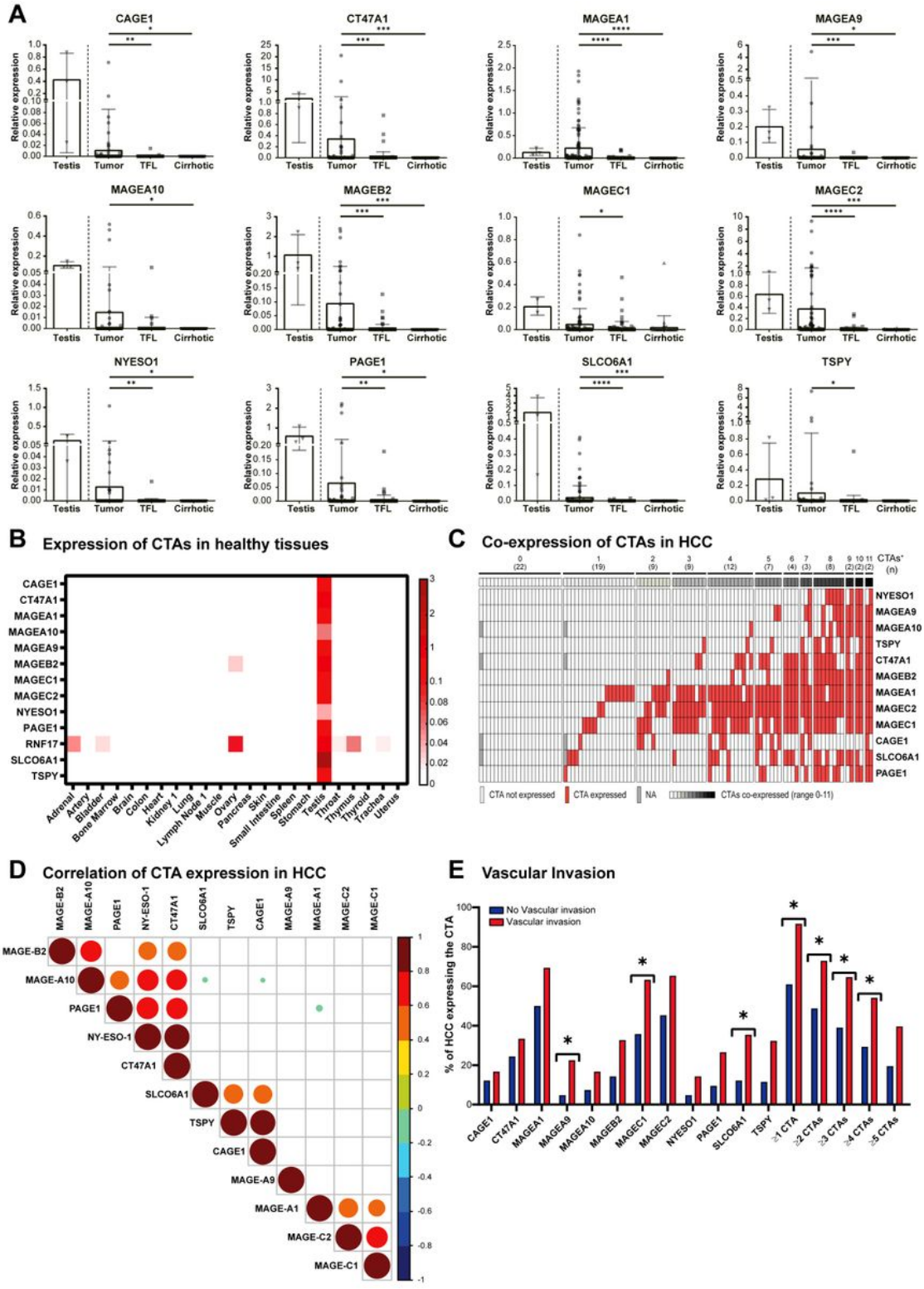

Figure 2

Panel of 12 CTAs expressed in >10% of HCC tumors, but not in healthy tissues. mRNA expression of 12 CTAs in 100 paired HCC and TFL tissues, 35 cirrhotic tissues and 22 different adult healthy tissues, as determined by RT-qPCR. A. mRNA expression of the 12 CTAs that are expressed in more than 10% of HCCs and not in healthy tissues. Dots show individual patient tissues, bars show the mean relative expression level, and error bars show the standard deviation. Wilcoxon signed-rank test, * p<0.05,

**p<0.01, ***p<0.001. B. Heatmap indicating relative mRNA expression levels of all CTAs that are expressed in >10% of HCCs, in healthy adult tissues. C. Heatmap indicating co-expression of CTA mRNA in tumor tissue D. Correlation matrix plot showing significant correlations (p<0.05) between expression of CTAs. Both colors, as indicated by the color bar, and size of the circles indicate the Pearson's correlation coefficient. Data is ordered by hierarchical clustering. E. Expression of CTAs in tumors with and without vascular invasion. Chi-squared test, *p<0.05, **p<0.01.

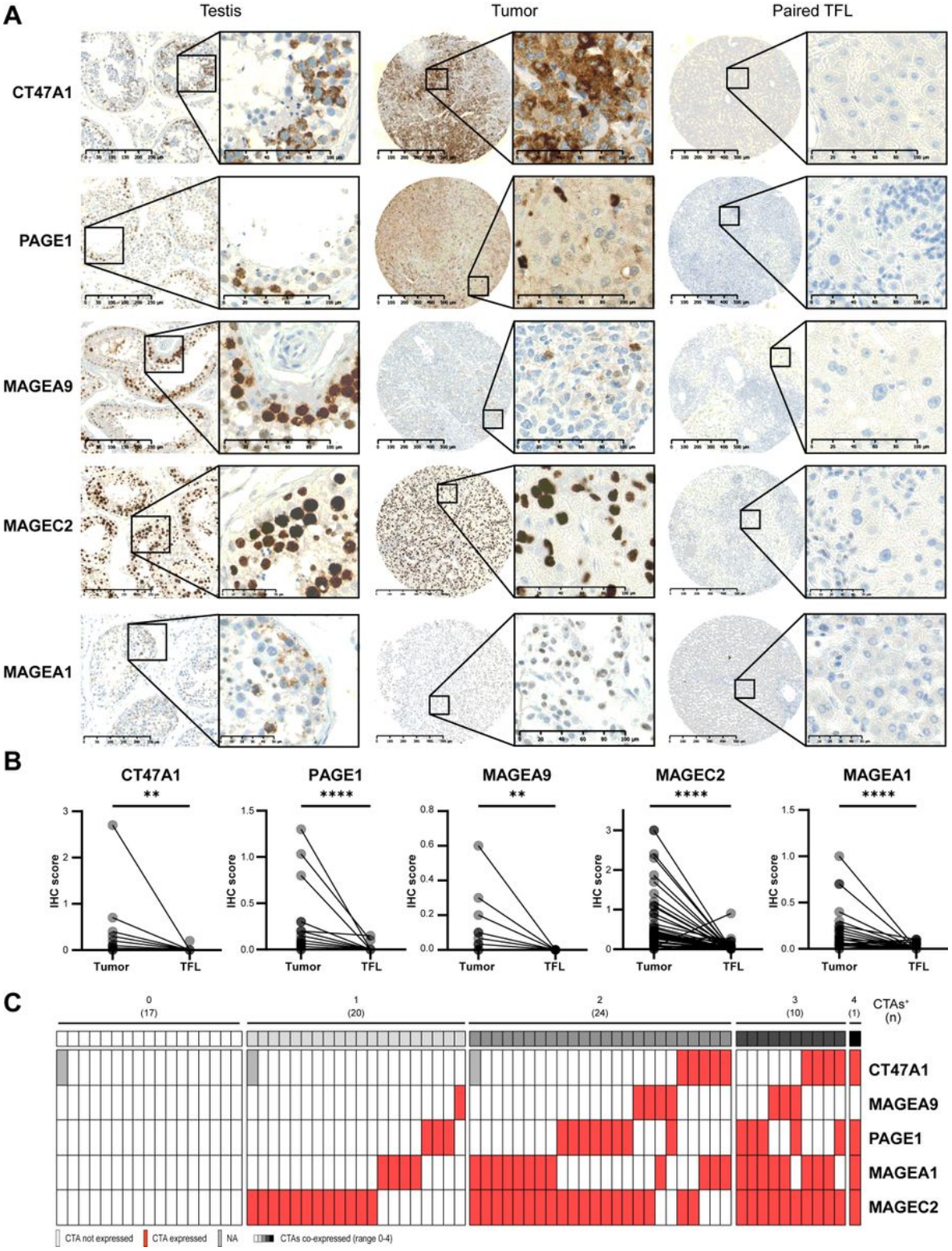

Figure 3

Proteins CT47A1, PAGE1, MAGEA9, MAGEC2 and MAGEA1 are expressed in HCC tumors and TFL. TMAs of tumor and TFL tissues were immunohistochemically stained to study the protein expression of aforementioned CTAs. A. Representative examples of immunohistochemical stains in testis, a positive HCC tumor tissue and the paired TFL tissue. B. Protein expression scores of CT47A1, PAGE1, MAGEA9, MAGEC2 and MAGEA1 in tumors and paired TFL (n=78). Wilcoxon signed-rank test, **p<0.01, ****p<0.0001. C. Heatmap indicating co-expression of CTA proteins in tumor tissue. TMA slides were scanned by a Nanozoomer (Hamamatsu), and analyzed by NDP.view2 software (Hamamatsu).

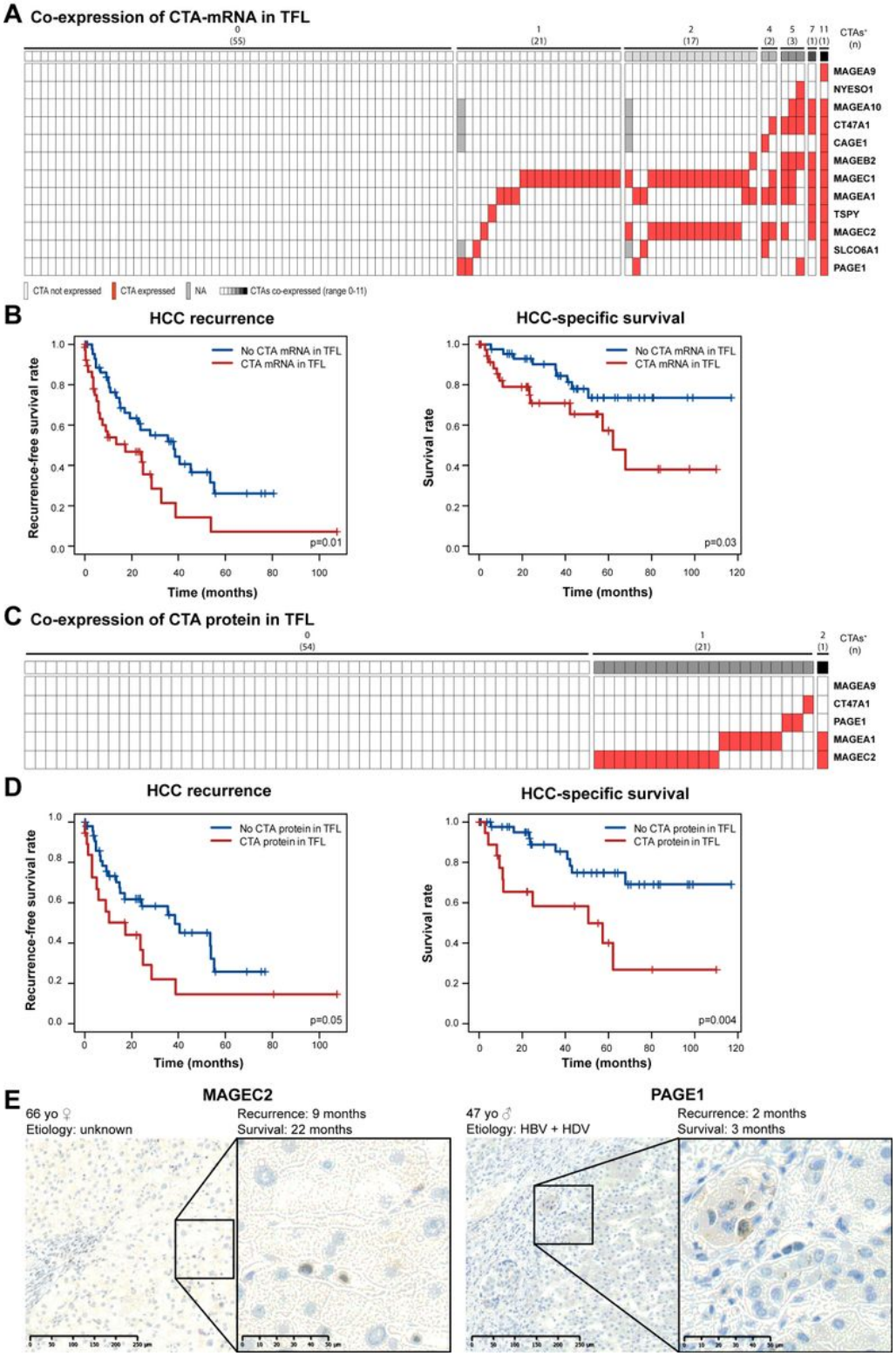

Figure 4

Both mRNA and protein expression of CTAs in TFL are associated with HCC recurrence and HCC-specific survival. A. Heatmap indicating co-expression of CTA mRNA in tumor-free liver tissue. B. HCC recurrence and HCC-specific survival in HCC patients by CTA mRNA expression in TFL. Plus-signs indicate censored data. Cox-Mantel log-rank test. C. Heatmap indicating co-expression of CTA protein in tumor-free liver tissue. D. HCC recurrence and HCC-specific survival in HCC patients by CTA protein expression in TFL. Plus-signs indicate censored data. Cox-Mantel log-rank test. E. Representative example of IHC staining of MAGEC2 and PAGE1 protein expression in TFL, and accompanying patient data. TMA slides were scanned by a Nanozoomer (Hamamatsu), and analyzed by NDP.view2 software (Hamamatsu).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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