Received: 4 October 2021 | Accepted: 29 November 2021

DOI: 10.1002/ijc.33898

TUMOR MARKERS AND SIGNATURES

IJC INTERNATIONAL JOURNAL of CANCER

Comprehensive clinicopathologic study of alpha fetoprotein-expression in a large cohort of patients with hepatocellular carcinoma

Dirk Andreas Ridder[1] | Arndt Weinmann[2] | Mario Schindeldecker[1,3] | Lana Louisa Urbansky[1] | Kristina Berndt[1] | Tiemo Sven Gerber[1] | Hauke Lang[4] | Johannes Lotz[5] | Karl J. Lackner[5] | Wilfried Roth[1] | Beate Katharina Straub[1]

[1]Institute of Pathology, University Medical Center of the Johannes Gutenberg University, Mainz, Germany

[2]Department of Internal Medicine, University Medical Center of the Johannes Gutenberg University, Mainz, Germany

[3]Tissue Biobank, University Medical Center of the Johannes Gutenberg University, Mainz, Germany

[4]Department of General, Visceral and Transplant Surgery, University Medical Center of the Johannes Gutenberg University, Mainz, Germany

[5]Institute for Laboratory Medicine and Clinical Chemistry, University Medical Center of the Johannes Gutenberg University, Mainz, Germany

Correspondence
Dirk Andreas Ridder and Beate Katharina Straub, Institute of Pathology, University Medicine Mainz, Langenbeckstr. 1, 55131 Mainz, Germany.
Email: dirk.ridder@unimedizin-mainz.de (D. A. R.) and
Email: beate.straub@unimedizin-mainz.de (B. K. S.)

Funding information
Deutsche Forschungsgemeinschaft, Grant/Award Number: 1160/1-2

Abstract

Alpha fetoprotein (AFP) is the most widely used diagnostic and prognostic serum biomarker for hepatocellular carcinoma (HCC). Despite its wide clinical use, a systematic clinicopathologic study comparing AFP expression in HCC in situ with serum AFP concentrations has not yet been conducted. To analyze AFP expression in a large cohort of patients by immunohistochemistry, we employed a comprehensive tissue microarray with 871 different HCCs of overall 561 patients. AFP immunoreactivity was detected in only about 20% of HCC core biopsies, whereas 48.9% of the patients displayed increased serum values (>12 ng/mL). Immunostaining of whole tumor slides revealed that lack of detectable immunoreactivity in core biopsies in a subgroup of patients with elevated AFP serum concentrations is due to heterogeneous intratumoral AFP expression. Serum AFP concentrations and AFP expression in situ were moderately correlated (Spearman's rank correlation coefficient .53, $P = 1.2e-13$). High AFP expression detected in serum (>227.3 ng/mL) or in situ predicted unfavorable prognosis and was associated with vascular invasion, higher tumor grade and macrotrabecular-massive tumor subtype. Multivariate and ROC curve analysis demonstrated that high AFP concentrations in serum is an independent prognostic parameter and represents the more robust prognostic predictor in comparison to AFP immunostaining of core biopsies. The previously published vessels encapsulating tumor clusters (VETC) pattern turned out as an additional, statistically independent prognostic parameter. AFP-positivity was associated with increased tumor cell apoptosis, but not with increased vascular densities. Additionally, AFP-positive tumors displayed increased proliferation rates, urea cycle dysregulation and signs of genomic instability, which may constitute the basis for their increased aggressiveness.

Abbreviations: AFP, alpha fetoprotein; ASH, alcoholic steatohepatitis; BCLC, Barcelona Clinic Liver Cancer; CAD, carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase and dihydroorotase; CPS1, carbamoyl-phosphate synthetase 1; GS, glutamine synthetase; HBX, hbv x protein; HCC, hepatocellular carcinoma; HCV, hepatitis c virus; HR, hazard ratio; IRS, immunoreactive score; ROC, receiver operating characteristic; TMA, tissue microarray; VETC, vessels encapsulating tumor cell clusters.

[Correction added on December 27, 2021 after first online publication: Projekt DEAL funding statement has been added.]

KEYWORDS
alpha fetoprotein, biomarker, hepatocellular carcinoma, prognosis, VETC

What's new?

While multiple biomarkers have shown promise in hepatocellular carcinoma (HCC) diagnosis and surveillance, alpha fetoprotein (AFP) remains the most clinically important and most widely used, despite poor sensitivity and specificity. Here, to better understand the clinicopathological relevance of AFP in HCC, comprehensive immunohistochemical analyses on AFP expression were performed, revealing moderate correlations between serum AFP concentrations and AFP expression in situ. AFP immunoreactivity was not detected in many patients with elevated serum AFP concentrations, due primarily to intratumoral heterogeneity. AFP expression was correlated with additional clinical and morphological parameters, including vascular invasion, higher tumor grade and macrotrabecular-massive tumor subtype.

1 | INTRODUCTION

Hepatocellular carcinoma (HCC) is the most commonly diagnosed malignant neoplasia of the liver and the fourth leading cause of cancer-related mortality worldwide.[1] Despite poor sensitivity and specificity, alpha fetoprotein (AFP) is still the most widely used serum marker for the diagnosis and detection of HCC. However, especially small tumors often present with normal serum concentrations and elevated AFP concentrations may also stem from nonhepatocellular tumors or benign diseases.[2,3] Therefore, its use has not been generally recommended in current guidelines.[2,4] Besides its diagnostic use, increased AFP serum concentrations also represent a robust prognostic biomarker in HCC that has yet to be matched.[5] Therefore, serum AFP concentrations have also been proposed to be included in a prognostic model to select patients suitable for liver transplantation (LTX) otherwise excluded by the Milan criteria,[2,6] although this has not been widely translated into clinical practice. However, in the most recent German guidelines for the treatment of HCC it has been included that patients with AFP serum concentrations above 1000 ng/mL should receive neoadjuvant therapy before LTX. Furthermore, a rise in serum AFP above 1000 ng/mL during a bridging therapy is considered a contraindication for LTX.[7]

AFP is a 70 kDa glycoprotein that is produced by the yolk sac and the fetal liver during embryonic development representing the fetal analog of human albumin.[3] Under normal physiologic conditions, AFP serum concentrations rapidly decline after birth and remain low during the entire life span.[3] The AFP gene is a member of the albumin gene family and its expression is thought to be primarily regulated at the transcriptional level.[5] Under physiological conditions, AFP expression is under inhibitory control of the transcription factor p53.[8] Mutations of the *TP53* gene, which are found in a considerable number of HCCs,[9] or mutations of the p53 binding element within the AFP repressor may result in reactivation of AFP expression in HCC cells.[3] Additionally, the HBV x protein (HBx) has been shown to induce AFP by blocking the suppressing effect of p53 on the AFP promoter. Demethylation of the AFP gene promoter may also result in reactivation of AFP transcription.[8] In addition to these regulatory processes at the level of transcription, also posttranslational modifications with impact on AFP protein concentrations have been reported. Acetylation of certain lysine residues within the AFP protein, for example, has recently been shown to increase its stability by reducing ubiquitination.[10]

The analysis of mice with a germline deletion of the AFP gene has revealed that AFP is dispensable for embryonic development, although female homozygous AFP-KO mice are not fertile.[11] AFP is thought to ensure the transport of various molecules, such as certain heavy metals (copper and nickel), bilirubin, fatty acids and certain drugs.[3] Furthermore, AFP has been demonstrated to inhibit apoptosis by interacting with caspase-3, preventing its activation by cleavage.[10,12] Moreover, AFP has been shown to promote cell proliferation, migration and cancer cell invasion by activating PI3K/AKT/mTOR signaling.[13] Furthermore AFP has also been implied in angiogenesis, as antiangiogenic therapy was demonstrated to be more effective in HCC patients with elevated AFP serum concentrations and the finding that AFP-positive HCCs show increased activation of VEGF signaling.[5,14,15] However, whether these effects are direct or indirect remains to be determined.

Despite its widespread clinical use, a systematic clinicopathologic study of AFP immunohistochemistry in HCC has not yet been conducted. Therefore, we created a comprehensive tissue microarray comprising more than 850 tumors from a clinicopathologically well characterized cohort of 561 patients including primary and recurrent tumors, metastases and tumor thrombi as well as normal/nonneoplastic livers of the same patients, and correlated immunohistochemically detected AFP expression with comprehensive clinicopathological and additional immunohistochemical parameters.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

Tissue samples from 561 HCC patients that underwent tumor resection at the University Medical Center Mainz from 1997 to 2017 were provided by and in accordance with the regulations of the Tissue

Biobank of the University Medical Center Mainz. Clinical data of HCC patients, including survival, were retrieved from a prospectively populated clinical database.[16] Patient records and informations were anonymized and de-identified prior to analysis. The mean duration of follow-up was 55.2 months.

2.2 | Immunohistochemistry

A tissue microarray (TMA) was created, comprising at least two cores of primary tumor and surrounding nonneoplastic liver tissue of each patient, as well as of relapse tumors, lymph node and distant metastases and larger tumor thrombi if available.[17] Antigen retrieval was performed using Tris/EDTA buffer, pH 9 (Dako, Santa Clara, California, #8024) or citrate buffer, pH = 6.1 (Dako, #GV805) or cell conditioning solution 1 (Roche, Mannheim, Germany, #950-124). Following antigen retrieval, tissue microarray slides were incubated with the respective antibodies (see Table S1). Staining was performed with an automated staining system (DAKO Autostainer plus, Agilent Technologies, Santa Clara, California) and the Dako EnVision FLEX staining system (Agilent Technologies) in accordance with the manufacturers' instructions. TMA slides were digitalized using the NanoZoomer-Series Digital slide scanner (Hamamatsu Photonics, Hamamatsu, Japan) prior to image analysis. Immunoreactivity was either rated semiquantitatively according to Remmele et al[18] or in case of Ki67 and CD34, digital image analysis was performed using the HALO platform (Indica Labs, Corrales, New Mexico) including the TMA module and the CytoNuclear v1.6 module. Missing or erroneous cores, for example, with extensive necrosis, were excluded from the analysis. In case of Ki67, positive cell nuclei were counted; in case of CD34, the stained area was quantified.

2.3 | Serum chemistry

Between 1997 and 2007 AFP serum concentration was determined by an immunoassay based on Time Resolved Amplification of Cryptate Emission (CIS-Bio Kryptor, Brahms, Henningsdorf, Germany). Since 2007 AFP serum concentration was determined by a luminescence immunoassay on Abbott Architect i2000 analyzers (Abbott Diagnostics, Wiesbaden, Germany). Comparison of the two assays showed an excellent correlation. However, results obtained by the Abbott assay were systematically higher. The following regression equation was obtained from 1330 routine patient samples in the concentration range <1000 ng/mL analyzed by both methods in parallel: $AFP_{Abbott} = 11\,746 \times AFP_{Brahms} - 0.5708$ with $r = .9981$.

2.4 | Statistical analysis

All statistical analyses were performed using the R environment for statistical computing (version 4.0.3).[19] The nonparametric Mann-Whitney *U* test was used to compare differences between two independent groups when dependent variables were either continuous or ordinal. The Wilcoxon signed-rank test was employed to determine whether two dependent samples were selected from populations having the same distribution. The Kruskal-Wallis test was applied to compare two independent groups, which consist of one dependent scale variable and one explanatory nominal variable with three or more levels. Benjamini-Hochberg corrections were applied to counterbalance the effects of multiple testing and control for the false discovery rate. Categorical variables were compared with the χ^2 test or Fisher's exact test. Spearman's correlation was used to examine linear correlations between two continuous variables showing a non-normal distribution. *P*-values ≤.05 were considered statistically significant. AFP protein expression and AFP serum concentrations were dichotomized by either receiver operating characteristic (ROC) curve analysis or by utilizing the Charité cut-off finder functions to provide a significant distinction between the high and low protein expression levels based on survival outcome.[20] Overall survival was calculated as the interval between initial diagnosis and death regardless of etiology or the last follow-up and analyzed with the Kaplan-Meyer method; differences were evaluated by the log-rank test. Univariate and multivariate COX regression analysis was conducted using the functions coxph from the R package survival (version 3.2.7). Variable selection was performed by employing the stepwise backward model selection by the Akaike information criterion (AIC) method from the R package MASS (version 7.3.53)[21] to create a significant multivariate Cox model. ROC curves were used to determine the biomarker potential of AFP detected on core biopsies and by serum analysis relative to 5 year survival in HCC.

3 | RESULTS

3.1 | AFP expression in HCCs in situ correlates with AFP serum concentrations despite intratumoral heterogeneity

To analyze AFP protein expression in a large number of HCCs by immunohistochemistry, we created a tissue microarray (TMA) with 871 HCCs of 561 patients with comprehensive clinical and pathological data.[17] By immunohistochemistry, we detected AFP in 19.3% of the primary HCCs. Using the immunoreactive score[18] (IRS) for semiquantitative assessment of AFP expression, 5.2% of the primary HCCs showed strong (IRS = 9-12), 5.4% intermediate (IRS = 5-8) and 8.7% at least weak AFP expression (IRS = 1-4) (Figure 1A). In 448 of the 561 patients (79.9%), preoperative AFP serum concentrations were available. In 48.9% of the patients, AFP concentrations were elevated (>12 ng/mL). AFP serum concentrations correlated moderately with IRS of AFP detected by immunohistochemistry (Figure 1B). Also in the independent Cancer Genome Atlas (TCGA) cohort[9] analyzed for comparison, AFP mRNA levels correlated moderately with AFP serum concentrations (Figure S1A). In our cohort, in patients with serum-positive HCCs (AFP serum concentration > 12 ng/mL), serum concentrations moderately correlated with tumor size (Figure 1C).

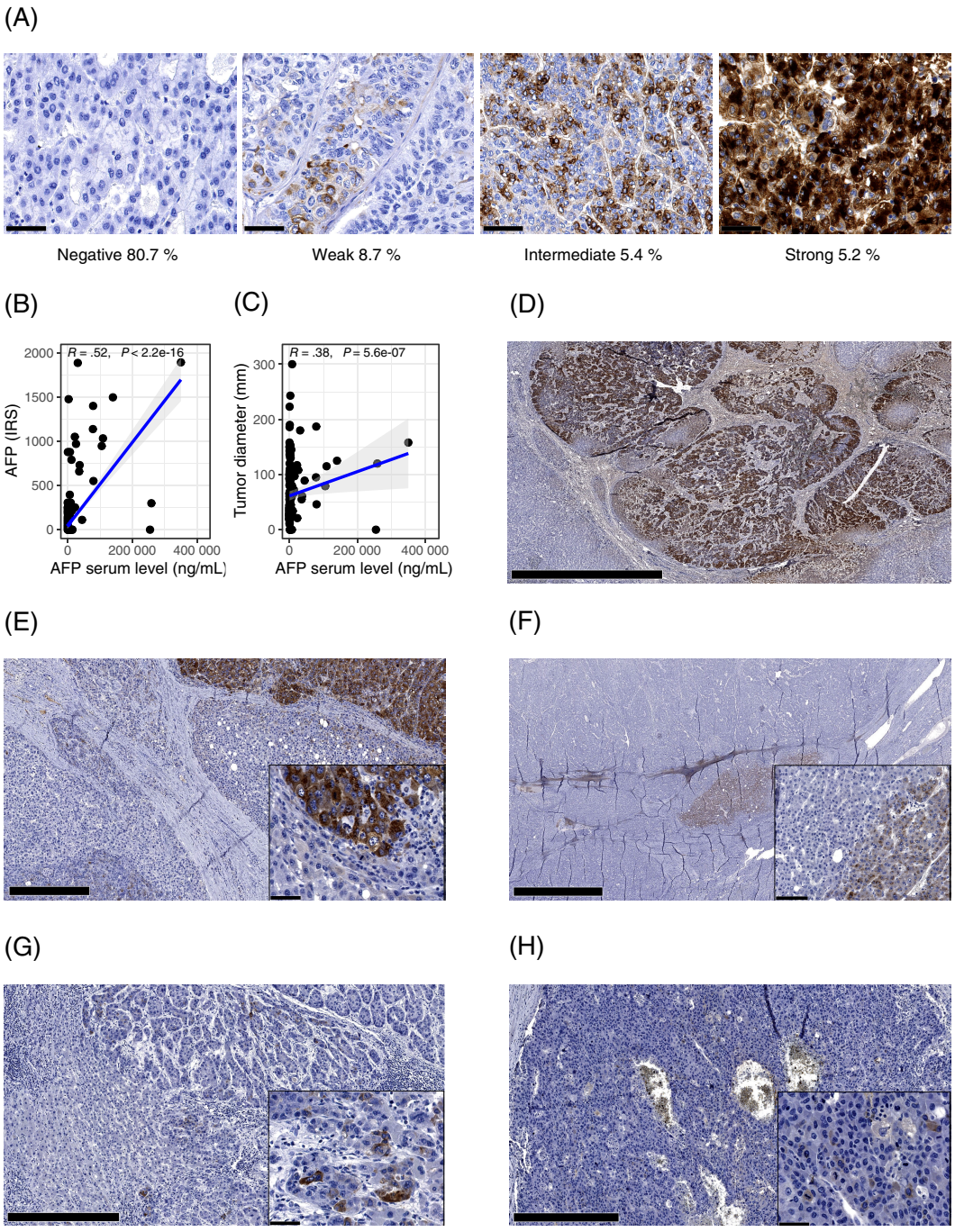

FIGURE 1 AFP expression levels in HCC determined by immunostaining of core biopsies correlate with AFP serum concentrations and tumor size despite considerable intratumoral heterogeneity. (A) Representative images of immunohistochemical stainings of core biopsies of primary HCCs with different expression levels of AFP. The percentage of tumors with the respective expression levels is indicated. Scale bar: 50 μm. (B) Scatter plot of the immunoreactive score for AFP (*y* axis) and AFP serum concentrations (*x* axis). (C) Scatter plot of tumor diameter (*y* axis) and AFP serum concentrations (*x* axis), restricted to patients with an AFP serum concentration >20 ng/mL. (D) Representative immunohistochemical staining of a primary HCCs with strong homogeneous AFP expression. Scale bar: 2.5 mm. (E-H) Immunohistochemical stainings of primary HCCs with heterogeneously distributed AFP expression. In (G) and (H), only singular cells show AFP expression. Scale bar: 500 μm (E,G,H) or 2.5 mm (F)

However, 57.5% of the patients with increased AFP serum concentrations (>12 ng/mL, partially also clearly elevated values) lacked significant immunohistochemically detected AFP expression in respective core biopsies (Figure S1B). We hypothesized that serum AFP concentrations may be more sensitive compared to AFP immunohistochemistry, or that AFP expression within a single HCC may be heterogeneous, and TMA cores although done twice on the same HCC, may not be representative for the whole HCC tissue. Therefore, we performed AFP staining of 16 whole tissue slides of surgical specimens of patients with elevated serum AFP, without detectable AFP expression in the

respective core biopsies. Only 1 out of 16 whole tissue slides showed quite homogenous strong expression (Figure 1D), in two slides a patchy staining was observed (Figure 1E,F). In six slides only focal intermingled single cells displayed AFP expression (Figure 1G,H). In 7 out 16 investigated whole slides no AFP immunoreactivity was detected (not shown), pointing to AFP expression outside of the investigated tissue. There was no specific immunoreactivity for AFP in the surrounding tissue. Taken together, intratumoral AFP expression is rather heterogeneously distributed and is moderately correlated with serum AFP concentrations.

3.2 | AFP expression as determined by immunohistochemistry in core biopsies predicts unfavorable clinical outcome, but is less predictive than measurement of AFP serum concentrations

As increased AFP concentrations in serum have been demonstrated to be a powerful prognostic tool,[5] we wanted to compare the prognostic effect of AFP immunostaining of core biopsies to AFP detection in serum. We performed a receiver operating characteristic (ROC) curve analysis on the immunoreactive AFP scores to identify the ideal cut-off score, which turned out to be >0. Immunohistochemical AFP expression in core biopsies of primary HCCs, even if minimal, predicted shorter overall survival (HR 1.63 [1.26, 2.11], $P < .001$) (Figure 2A). In the study cohort, the ideal cut-off for AFP in serum was >10.17 ng/mL according to ROC curve analysis, which was also associated with shorter overall survival (HR 1.54 [1.21, 1.97], $P < .001$) (Figure 2B). As a second statistical method to dichotomize the patient cohort, we utilized the Charité cut-off finder function,[20] which resulted in a cut-off score of 6.25 for the IRS (HR 2.62 [1.77, 3.87], $P < .0001$) and 227.3 ng/mL for AFP serum concentration (HR 2.04 [1.56, 2.66], $P < .0001$). The respective Kaplan-Meier plots are shown in Figure 2C,D. We gained similar results in the independent TCGA cohort analyzed for comparison (Figure S1F,G).

It has been recommended to test rather higher AFP concentrations (>200 or >400 ng/mL) in investigations regarding clinical outcome.[2] Therefore, we included the cut-off scores determined by the Charité cut-off finder function (227.3 ng/mL for AFP serum concentrations and 6.25 for IRS) in the multivariate analysis. Increased AFP concentrations in serum turned out as a statistically independent prognostic factor, whereas increased AFP expression as determined by immunohistochemistry on core biopsies was not significant in multivariate analysis (Table 1).

Furthermore, in order to compare prognostic prediction of AFP serum concentrations to immunostaining of core biopsies, we analyzed the area under the ROC curve for 5 year survival, which was considerably larger for detection of AFP concentrations in serum (Figure 2F) compared to AFP immunohistochemistry of core biopsies (Figure 2E). In our opinion, this is mainly caused by sampling error due to heterogeneous expression of AFP, as mentioned above, and may be improved when whole slides of surgical specimens are evaluated.

3.3 | Correlation of AFP expression with clinicopathological parameters

We then correlated immunohistochemically detected AFP expression in situ and AFP concentrations in serum with clinicopathologic parameters, employing the different cut-off values mentioned above (Table S2 and S3). AFP-positive HCCs significantly more often presented with vascular invasion detected by imaging and also histology-proven microvascular and macrovascular invasion, in accordance with various studies.[2,5] As well, HCCs with vascular invasion demonstrated increased AFP expression (Figure 3A). In 29 patients, intravascular HCC thrombi were analyzed, but no significant difference in AFP expression was detected when compared to the respective primary HCCs (Figure 3B), arguing against a locally increased risk for vascular invasion in tumor areas with AFP expression and for AFP expression in more aggressive tumors. Additionally, we did not find significant differences in AFP expression in recurrent HCCs, HCC lymph node and distant metastases, when compared to the respective primary HCCs (Figures 3C and S1C,D). AFP positivity was associated with higher tumor grade (Figure 3D). Specifically, AFP expression was additionally positively correlated with the size of cell nuclei ($R = .34$, $P = 2.94e-15$) and was negatively correlated with roundness of cell nuclei ($R = -.35$, $P = 3.64e-17$).

Recently, different histological subtypes of HCCs with characteristic morphologies and distinct prognoses have been proposed.[22] Macrotrabecular-massive (MTM) growth pattern, which is a known independent prognostic factor for poor clinical outcome,[23] was associated with higher AFP expression (Figure 3E,F). Vice versa, HCCs of MTM subtype were significantly overrepresented among HCCs with AFP expression (Tables S2 and S3). AFP-positive tumors less often showed glutamine synthetase (GS) overexpression (IRS for GS > 9), which is regarded as a surrogate marker for *ctnnb1* mutations or activated WNT signaling respectively[24] (Table S2), although the difference was less pronounced when dichotomizing according to AFP serum concentrations (Table S3). These findings are in accordance to previous studies.[25,26] We also analyzed the prevalence of the vessels encapsulating tumor clusters (VETC) pattern (as determined by CD34 immunohistochemistry; Figure 3G), another known independent prognostic morphological parameter[27] with regard to AFP expression. We detected a trend toward overrepresentation of the VETC pattern among AFP-positive HCCs detected by immunohistochemistry (Table S2) and a significantly larger proportion of VETC-positive tumors among HCCs with elevated AFP serum concentrations (Table S3).

Interestingly, HCCs occurring in livers with alcoholic steatohepatitis (ASH) displayed significantly lower AFP expression levels detected both by immunohistochemistry (Figure 3H) and in serum (Figure 3I). In line with this finding, AFP-positive HCCs were underrepresented among HCCs in ASH patients (Tables S2 and S3). Furthermore, HCCs with clearly elevated AFP serum concentrations (>227.3 ng/mL, see above) were overrepresented in noncirrhotic livers (Table S3). Patients suffering from HCV infection significantly more often presented with slightly increased AFP serum concentrations, but showed clearly increased AFP serum concentrations to a

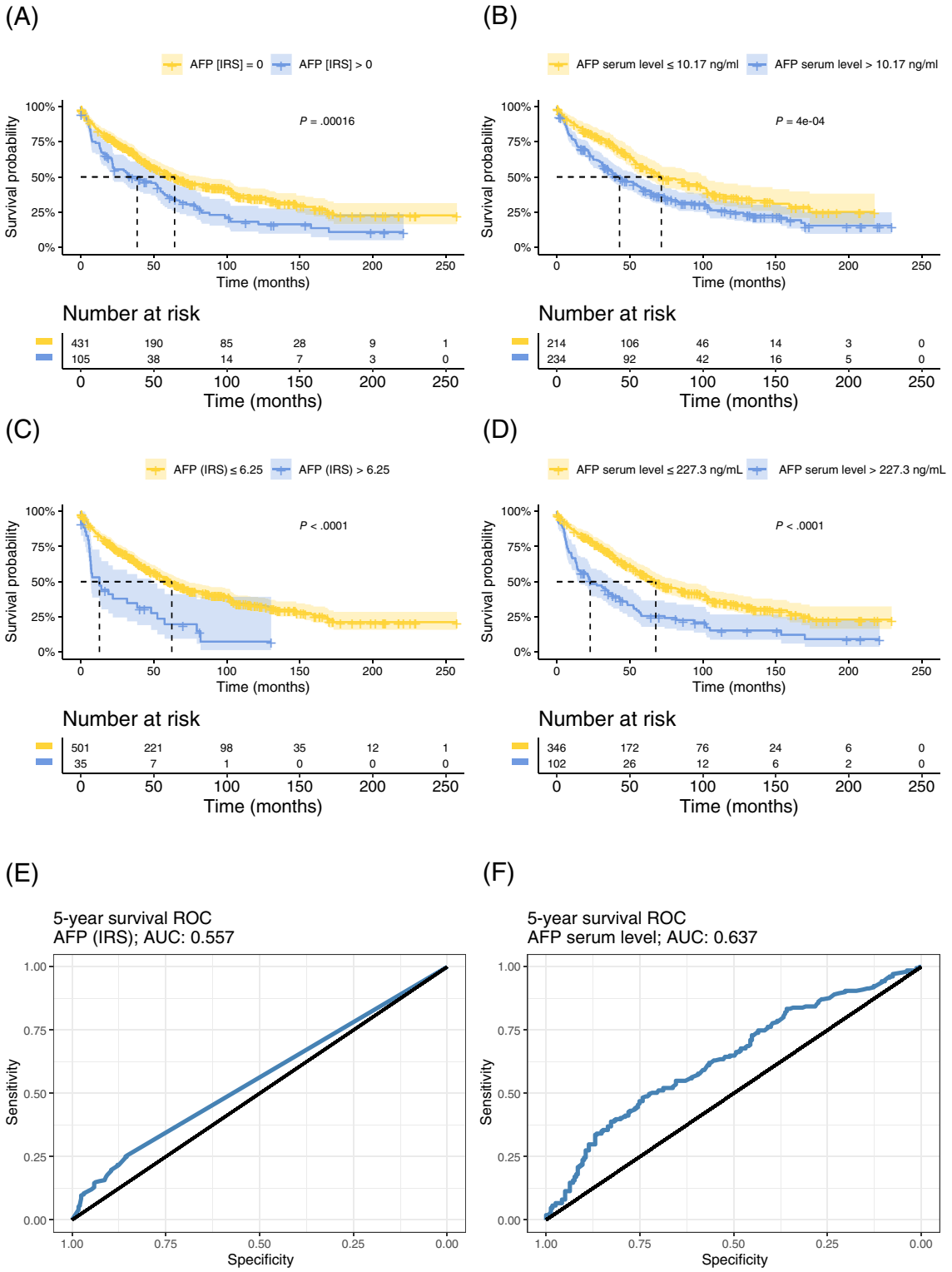

FIGURE 2 Immunohistochemically detected AFP expression in core biopsies predicts unfavorable clinical outcome and is less predictive than measurement of AFP serum concentrations. (A) Kaplan-Meyer plot of overall survival in HCC patients according to immunohistochemically detected AFP (IRS > 0 vs IRS = 0). (B) Kaplan-Meyer plot of overall survival in HCC patients according to AFP serum concentration (>10.17 vs ≤10.17 ng/mL). (C) Kaplan-Meyer plot of overall survival in HCC patients according to immunohistochemically detected AFP (IRS > 6.25 vs IRS ≤ 6.25). (D) Kaplan-Meyer plot of overall survival in HCC patients according to AFP serum concentration (>227.3 vs ≤227.3 ng/mL). (E) ROC curves for 5-year survival according to immunohistochemically detected AFP expression (n = 536). (F) ROC curves for 5-year survival according to AFP serum concentrations (n = 448)

TABLE 1 Univariate and multivariate Cox regression analyses of prognostic factors (AIC-based selection)

	Univariate				Multivariate			
	HR	Conf.low	Conf.high	*P* value	HR	Conf.low	Conf.high	*P* value
Clinical features								
Age (≥60 vs <60)	1.42	1.13	1.79	<.01				
Sex (male vs female)	1.06	0.81	1.38	.66				
C2 (true vs false)	0.88	0.69	1.11	.26				
NASH (true vs false)	1.03	0.67	1.59	.90				
Hemochromatosis (tr. vs f.)	0.70	0.41	1.19	.19				
HBV (pos. vs neg.)	0.89	0.68	1.18	.41				
HCV (pos. vs neg.)	1.08	0.83	1.40	.59				
Child-Pugh (B/C vs A)	0.98	0.75	1.27	.86				
ECOG (PST1-4 vs PST0)	1.41	1.12	1.78	<.01				
BCLC new (B-D vs A)	1.83	1.34	2.49	<.001	1.80	1.22	2.66	.003
Albumin (high vs low)	0.55	0.42	0.73	<.0001				
TNM M (M1 vs M0)	2.67	1.42	5.02	<.01				
AFP serum (> vs ≤227.3 ng/mL)	2.04	1.56	2.66	<.001	1.69	1.25	2.27	<.001
Pathologic features								
LCI (yes vs no)	0.86	0.69	1.08	.20	0.91	0.76	1.28	.910
Grading (G3 vs G1/G2)	1.51	1.16	1.96	<.01				
TNM N (N1 vs N0)	1.69	0.80	3.59	.17				
VI (micro vs none)	1.45	1.10	1.90	<.01				
VI (macro vs none)	3.19	2.39	4.26	<.0001				
Macrotrabecular subtype	1.97	1.35	2.87	<.001	1.46	0.91	2.36	.120
VETC (pos. vs neg.)	1.51	1.14	2.00	<.01	1.50	1.11	2.05	.009
GS (≥9 vs <9)	0.77	0.58	1.03	.08				
AFP (IRS >6.25 vs ≤6.25)	2.62	1.77	3.87	<.001				

similar extent than nonHCV patients (Table S3). However, the mean value of AFP serum concentrations was significantly lower in HCC patients with HCV infection (Figure 3J).

We did not observe significant differences in AFP expression with respect to other etiologies of underlying liver disease such as hemochromatosis, hepatitis B and D, primary biliary cholangitis or other clinical parameters such as nicotine abuse and gender, and found no correlation to liver enzymes, albumin, CA19-9 and urea serum concentrations (data not shown).

3.4 | Correlation of AFP expression with molecular parameters

In order to further investigate the underlying mechanism of increased malignant potential of AFP-positive tumors, we performed additional immunohistochemical stainings and correlated expression of different biomarkers with AFP expression. As one possible characteristic of increased malignancy of AFP-positive HCCs, increased angiogenic activity has been postulated.[5,14] We quantified the CD34-positive area as a measure for microvessel density and did not observe a significant difference with respect to AFP positivity in the HCCs (Figure 4A). Another mechanism potentially explaining the increased aggressiveness of AFP-positive HCCs that has been discussed in the literature is AFP-mediated inhibition of activation of caspase-3.[10,12] Acetylation of AFP has been demonstrated to result in reduced caspase-3 cleavage resulting in inhibition of apoptosis. In contrast to this hypothesis, we found even higher levels of cleaved caspase-3 in AFP-positive HCCs in our collective indicating increased apoptosis in AFP-positive tumors (Figure 4B). This also held true, when we performed subgroup analyses matched for histological tumor grade (Figure S1E), in order to exclude a selection bias, as G3 HCCs were overrepresented among AFP-positive tumors (Tables S2 and S3). Therefore, according to our large HCC collective, increased angiogenesis and decreased apoptosis seem rather unlikely to constitute the molecular basis for increased malignant behavior of AFP-positive HCCs.

As AFP has also been shown to promote tumor cell proliferation in vitro,[13] we also analyzed the number of Ki67-positive cells and detected a significantly increased proliferation rate in AFP-positive tumors (Figure 4C). Increased proliferation may be due to specific molecular alterations in AFP-positive tumors. Recently, we and others

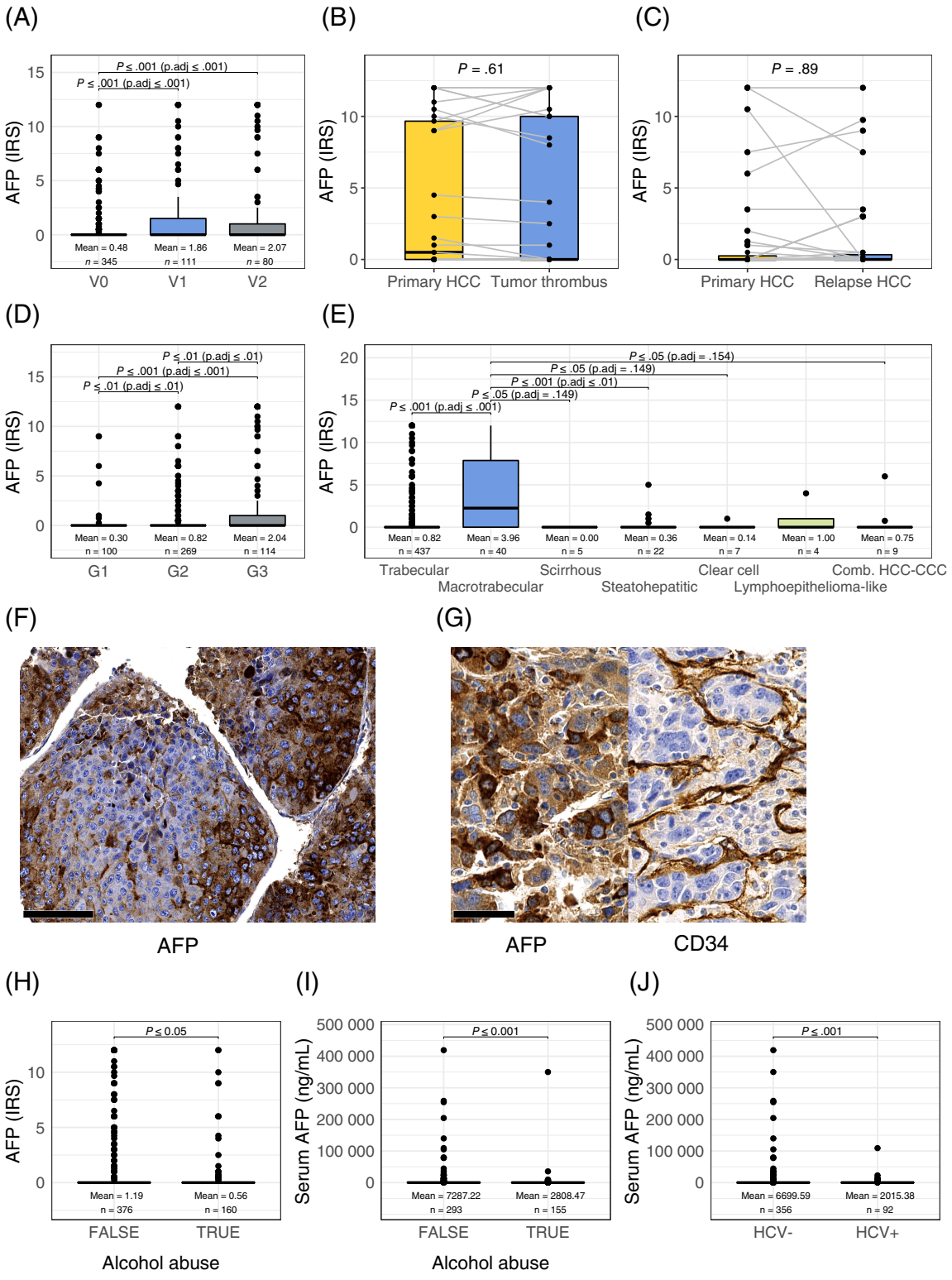

FIGURE 3 Association of AFP expression with clinico-pathologic parameters. (A) Quantification of AFP immunostaining with respect to vascular invasion. (B) Quantification of AFP immunostaining in primary HCCs and tumor thrombi of the same patients (n = 29, paired analysis). (C) AFP expression detected by immunostaining in primary HCCs and the respective relapse tumors of the same patients (n = 39, paired analysis). (D) Quantification of AFP immunostaining with respect to tumor grade. (E) Quantification of CAD expression according to morphological tumor subtype. (F) Representative immunostaining for AFP in a tumor with macrotrabecular-massive tumor subtype with trabeculae being more than 10 cell layers thick. Scale bar: 100 μm. (G) Representative images of an AFP-positive HCC with VETC-pattern (left panel: AFP, right panel: CD34). Scale bar: 50 μm. (H) Quantification of AFP immunostaining with respect to alcohol abuse. (I) AFP serum concentrations with respect to alcohol abuse. (J) AFP serum concentrations of patients with HCCs that had developed upon HCV infection vs other etiologies

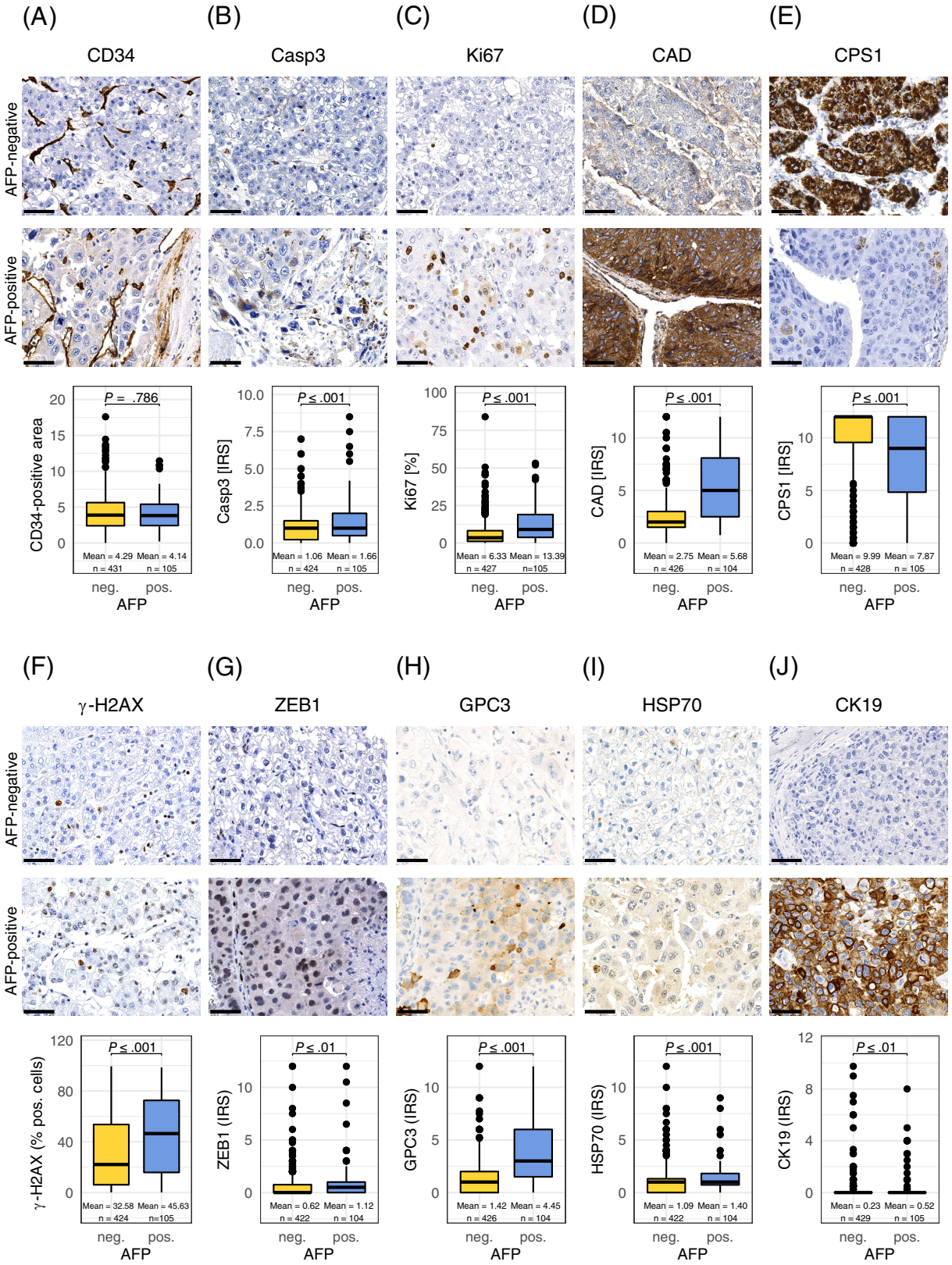

FIGURE 4 Association of AFP expression with additional molecular parameters. (A) to (J) Representative images of immunostainings for the indicated proteins (A: CD34, B: Caspase 3, C: Ki67, D: CAD, E: CPS1, F: γ-H2AX, G: ZEB1, H: GPC3, I: HSP70 and J: CK19) in AFP-negative (upper panel) and AFP-positive (middle panel) HCCs as determined by AFP immunostaining. The lower panels display the respective quantifications of the stainings for the indicated proteins according to the presence or absence of AFP immunoreactivity

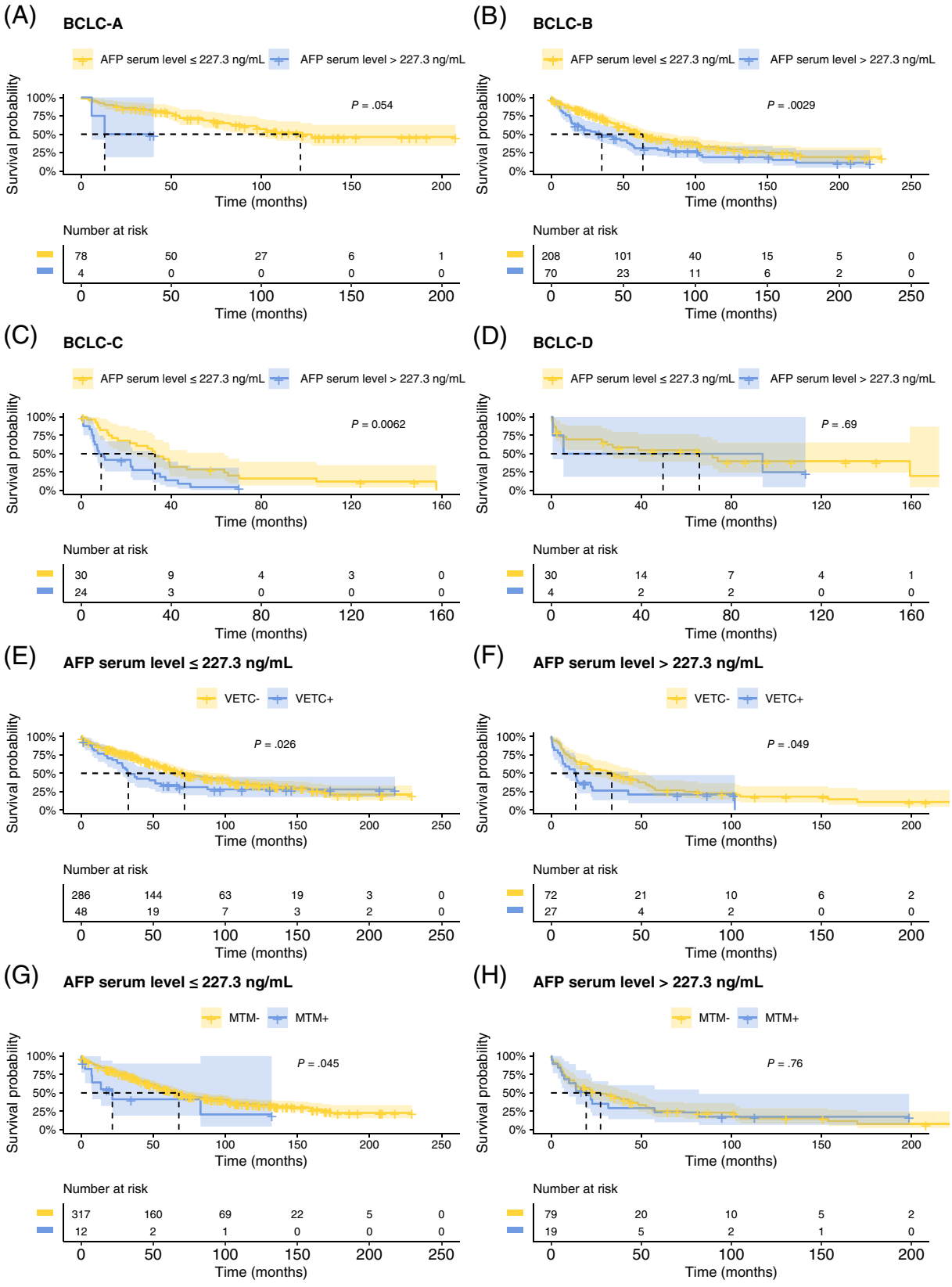

FIGURE 5 Survival analysis according to AFP serum concentrations in clinically relevant subgroups. (A-D) Kaplan-Meyer plot of overall survival according to AFP serum concentration (>227.3 vs ≤227.3 ng/mL) and BCLC stage. (E,F) Kaplan-Meyer plot of overall survival according to AFP serum concentration (>227.3 vs ≤227.3 ng/mL) and presence or absence of the VETC pattern. (G,H) Kaplan-Meyer plot of overall survival according to AFP serum concentration (>227.3 vs ≤227.3 ng/mL) and presence or absence of MTM subtype

have demonstrated that urea cycle dysregulation is a common phenomenon in multiple types of cancer, including HCC, that is associated with poor prognosis.[17,28] High expression of the multifunctional enzyme carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase and dihydroorotase (CAD) and reduced expression of carbamoyl-phosphate synthetase 1 (CPS1) as a measure for urea cycle dysregulation have been demonstrated to predict unfavorable prognosis.[17] AFP-positive tumors as detected by immunohistochemistry showed significantly higher CAD expression and significantly reduced CPS1 levels compared to AFP-negative tumors (Figure 4D,E), indicating an increased level of urea cycle perturbation in AFP-positive tumors. Increased proliferation and apoptosis have also been shown to induce replicative stress resulting in increased DNA damage.[29] Therefore, we also analyzed the levels of histone H2A.X phosphorylated at Ser139 (also known as γ-H2AX), a marker for DNA double strand breaks, according to AFP expression.[29,30] AFP-positive HCCs showed significantly increased γ-H2AX staining (Figure 4F), which is in line with previously published results that AFP-positive tumors show increased chromosomal instability.[31]

AFP-positive tumors also displayed significantly increased expression of the transcription factor ZEB1 and the proteoglycan glypican 3 (GPC3), that both have been demonstrated to play a role in cell motility and metastasis[32,33] (Figure 4G,H). Furthermore, two other established prognostic markers associated with poor clinical outcome such as heat-shock protein 70 (HSP 70) and CK19, a marker for hepatic progenitor cells,[33] were significantly overexpressed in AFP-positive HCCs (Figure 4I,J). When we analyzed all additional immunohistochemical parameters mentioned in Figure 4 according to AFP serum concentrations, we gained similar results (Figure S2).

3.5 | Survival analysis according to AFP serum concentrations in clinically relevant subgroups

As we found measurement of AFP in serum to be superior to assessment of core biopsies by immunohistochemistry, we performed Kaplan-Meyer analyses according to AFP serum concentrations to analyze the effect of AFP positivity in clinically relevant subgroups. We chose the same cut-off of 227.3 ng/mL that had been determined by the cut-off finder function[20] and that we used in multivariate analysis for all further analyses, as rather high AFP concentrations (>200 or >400 ng/mL) have been recommended for analyses regarding prognosis assessment.[2] The Barcelona Clinic Liver Cancer (BCLC) staging system is the most widely used algorithm for prognosis assessment and treatment allocation of HCC patients, at least in Europe and the United States.[2] Therefore, we performed subgroup analyses according to BCLC stage and AFP serum concentrations (Figure 5A-D). As expected, in the BCLC stages B and C, we found a significant prognostic effect of high amounts of AFP detected in serum (Figure 5B,C). In the BCLC stages A and D, only four patients had increased AFP concentrations in serum in each subgroup, which precluded a meaningful statistical analysis (Figure 5A,D).

Recently, the VETC pattern has been identified as an independent predictor of unfavorable outcome associated with increased vascular invasion.[27] When we analyzed overall survival according to high (Figure 5E) and low (Figure 5F) AFP serum concentrations, we found significantly reduced survival in patients with tumors displaying the VETC pattern, no matter whether they showed high AFP concentrations or not. This is also reflected by the fact that presence of the VETC pattern proved to be a statistically independent prognostic factor in the multivariate analysis of our dataset (Table 1). Furthermore, we analyzed survival according to presence or absence of macrotrabecular-massive (MTM) subtype, a morphological pattern that has been previously associated with poor prognosis,[23] and elevated AFP serum concentrations. The MTM subtype was only predictive in HCCs with low AFP serum concentrations (Figure 5G,H) and, in line with this finding, did not prove to be a statistically independent prognostic factor in multivariate analysis (Table S3). The majority of HCCs of MTM subtype showed high AFP serum concentrations (19/31). Therefore, knowledge of the MTM subtype in liver biopsies may be useful in patients with low AFP serum concentrations, whereas the determination of the VETC pattern by CD34 immunohistochemistry may give additional prognostic information regardless of AFP expression status.

4 | DISCUSSION

We here describe a comprehensive clinicopathologic study on AFP expression in situ and in serum in 871 HCCs in 561 patients enrolled at the University Medicine Mainz from years 1997 to 2017. In the investigated patient cohort, AFP serum concentrations moderately correlated with AFP expression determined by immunohistochemistry and also with tumor size. We can therefore show that AFP concentrations in fact stem from the tumor itself and not from the surrounding liver parenchyme reacting to the tumor. High AFP concentrations in serum or in situ predicted short overall survival. However, AFP detection in serum proved to be the more robust prognostic parameter and was a statistically independent prognostic parameter in multivariate analysis, whereas immunohistochemically detected AFP expression was not. This is most likely due to the heterogeneous intratumoral AFP expression and the resulting lack of representativity of liver core biopsies. Intratumoral heterogeneity is indeed well known to occur in a considerable proportion of HCCs and may result from genomic heterogeneity as well as nongenomic heterogeneity, including differences in DNA methylation patterns, noncoding RNAs and transcriptional regulation.[34-36] The molecular basis for intratumoral differences in AFP expression are currently not well understood,[5] but recently it has been demonstrated that methylation of the AFP promoter correlates well with AFP mRNA and serum concentrations,[25,37] which may represent the key regulatory mechanism. Interestingly, AFP has recently also been proposed as a target for immunotherapy.[38] In this respect, heterogeneous AFP expression in HCCs may present a major limitation of this strategy.

In our large HCC collective, we also reproduced published data, showing that AFP expression was significantly associated with

microvascular and macrovascular invasion, higher tumor grading, as well as with HCC of MTM subtype, which has previously been reported as an independent prognostic factor.[5,31] Additionally, we detected an association of AFP expression with the VETC pattern. Both MTM[23,31] and VETC pattern[27] have been published as independent factors associated with vascular invasion and short overall survival. Interestingly, according to our data, presence of the MTM subtype predicted unfavorable prognosis only in AFP-negative HCCs and thereby did not act as an independent prognostic parameter in multivariate analysis. In contrast, the VETC pattern proved to be a statistically independent parameter and was significantly associated with short overall survival. Indeed, determination of the VETC pattern in liver core biopsies may therefore add valuable information on prognosis and clinical course in patients with both AFP-positive and AFP-negative HCCs. In immunohistochemical investigation of tumor thrombi, we did not find a significant difference in AFP expression when compared to the respective primary HCCs, indicating that AFP-positive cells of a tumor with heterogeneously distributed AFP-expression not preferentially invade the vasculature. Rather, heterogenous AFP expression may be a consequence of other factors conferring invasive potential.

Furthermore, we investigated additional parameters that have been postulated to mediate the increased aggressiveness of AFP-expressing tumor cells. According to mechanistic studies in cell lines, AFP has been demonstrated to inhibit apoptosis by reducing caspase-3-cleavage.[10] Yet, our in situ data in a large HCC collective actually even point to the contrary. AFP-positive HCCs presented significantly higher levels of caspase-3-activation than AFP-negative tumors. Inhibition of apoptosis by AFP therefore does not seem to mediate the increased aggressiveness of AFP-positive HCCs. However, regulation of apoptosis is a complex process with numerous proteins involved and is not solely regulated at the level of caspase-3, although it represents a major proapoptotic effector.[39] Furthermore, antiapoptotic effects in early stages of hepatocarcinogenesis cannot be excluded, although dysplastic nodules and early HCCs rather do not show AFP expression.[40,41]

Following another possible hypothesis, increased angiogenesis may mediate the increased malignant potential of AFP-positive HCCs, which is based on the finding that antiangiogenic therapy was demonstrated to be more effective in HCC patients with elevated AFP serum concentrations and that AFP-positive HCCs showed increased activation of VEGF signaling.[5,14,15] AFP positivity in our large patient collective was not accompanied by an increased vascular density as determined by CD34 staining. Although this finding only represents a snapshot, and angiogenesis and tumor growth are dynamic processes, it points to the idea that the detrimental effects of VEGF signaling may not necessarily be solely mediated by effects on the vasculature, but possibly also by acting on tumor or immune cells as has been suggested previously.[42,43]

Differences in tumor cell metabolism in AFP-positive HCCs resulting in increased tumor cell proliferation may also play an important role.[37] In our study, we unraveled that AFP-positive tumors showed increased proliferation and significantly lower CPS1 and significantly higher CAD levels, indicating increased urea cycle dysregulation, a phenomenon that has been associated with unfavorable prognosis and has recently been demonstrated in a variety of malignant tumors, including HCC.[17,28] Yet how these processes are regulated, especially with respect to the role of AFP is currently not known. Differential methylation may play a role here, too.[17,37,44] Additionally, we found an increased expression of γ-H2AX, a marker for DNA double strand breaks, in AFP-positive tumors, indicating increased chromosomal instability, possibly due to replicative stress.[29] Chromosomal instability has recently been shown to drive metastasis and may explain the increased metastatic capacity of AFP-positive tumor cells.[45]

Furthermore, we confirmed previously published results that AFP-positive HCCs showed increased expression of the hepatic progenitor cell marker CK19 and of the established prognostic markers GPC3, HSP70 and ZEB1, that all have been associated with increased invasive capacity, vascular invasion and metastasis.[32,33,46] Especially CK19,[46] but also ZEB1[47] and GPC3[48] have been implied in cancer stemness of HCC cells. AFP expression may identify HCCs with stem cell behavior as is also the case in mixed hepatocellular and cholangiocarcinoma, as AFP is a protein physiologically expressed in the fetus as well as neoplastically in hepatoblastoma and germ cell tumors of the ovary and testis.

In summary, we here provide a comprehensive immunohistochemical study of AFP expression in a large collective of HCC patients having analyzed its relation to several clinical, morphological and immunohistochemical parameters. Furthermore, we have undertaken mechanistic considerations on AFP-positive tumors based on in vitro experiments. Given the tremendous differences in prognosis and clinical course of AFP-positive compared to AFP-negative HCCs, and evidence from preclinical studies, that AFP promotes tumor progression and metastasis,[3,49,50] further studies are urgently needed to gain more insight in the mechanisms underlying the regulation of AFP expression and to investigate whether AFP only represents a bystander or a true effector mediating tumor aggressiveness.

ACKNOWLEDGMENTS

We thank Sabine Jakubowski, Bonny Adami and Silke Mitschke for excellent technical assistance in immunohistochemistry of TMAs. TMA slides were provided by the tissue biobank of the University Medical Center Mainz. The study was funded in part by a grant of the DFG to STR 1160/1-2. DAR is supported by the Clinician Scientist Fellowship "Else Kröner Research College: 2018_Kolleg.05." Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

ETHICS STATEMENT

The study was approved by the local ethics committee (Ethik-Kommission der Landesärztekammer Rheinland-Pfalz, 837.146.17 [10980], as well as addendum 2018-13857_1 to DAR and BKS). Informed consent was obtained from all subjects involved in the study.

ORCID

Beate Katharina Straub https://orcid.org/0000-0002-4857-1561

REFERENCES

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394-424.
2. European Association for the Study of the Liver. EASL clinical practice guidelines: management of hepatocellular carcinoma. *J Hepatol*. 2018; 69:182-236.
3. Sauzay C, Petit A, Bourgeois A-M, et al. Alpha-foetoprotein (AFP): a multi-purpose marker in hepatocellular carcinoma. *Clin Chim Acta*. 2016;463:39-44.
4. Heimbach JK, Kulik LM, Finn RS, et al. AASLD guidelines for the treatment of hepatocellular carcinoma. *Hepatology*. 2018;67:358-380.
5. Galle PR, Foerster F, Kudo M, et al. Biology and significance of alpha-fetoprotein in hepatocellular carcinoma. *Liver Int*. 2019;39:2214-2229.
6. Notarpaolo A, Layese R, Magistri P, et al. Validation of the AFP model as a predictor of HCC recurrence in patients with viral hepatitis-related cirrhosis who had received a liver transplant for HCC. *J Hepatol*. 2017;66:552-559.
7. Leitlinienprogramm Onkologie (Deutsche Krebsgesellschaft, Deutsche Krebshilfe, AWMF). Diagnostik und Therapie des Hepatozellulären Karzinoms und biliärer Karziome, Langversion 2.0, 2021, AWMF Registernummer: 032/053OL. https://www.leitlinienprogramm-onkologie.de/leitlinien/hcc-und-billiäre-karzinome
8. Zheng Y, Zhu M, Li M. Effects of alpha-fetoprotein on the occurrence and progression of hepatocellular carcinoma. *J Cancer Res Clin Oncol*. 2020;146:2439-2446.
9. Cancer Genome Atlas Research Network. Comprehensive and integrative genomic characterization of hepatocellular carcinoma. *Cell*. 2017;169:1327-1341.
10. Xue J, Cao Z, Cheng Y, et al. Acetylation of alpha-fetoprotein promotes hepatocellular carcinoma progression. *Cancer Lett*. 2020;471:12-26.
11. de Mees C, Laes J-F, Bakker J, et al. Alpha-fetoprotein controls female fertility and prenatal development of the gonadotropin-releasing hormone pathway through an antiestrogenic action. *Mol Cell Biol*. 2006;26:2012-2018.
12. Li M, Li H, Li C, et al. Alpha fetoprotein is a novel protein-binding partner for caspase-3 and blocks the apoptotic signaling pathway in human hepatoma cells. *Int J Cancer*. 2009;124:2845-2854.
13. Wang S, Zhu M, Wang Q, et al. Alpha-fetoprotein inhibits autophagy to promote malignant behaviour in hepatocellular carcinoma cells by activating PI3K/AKT/mTOR signalling. *Cell Death Dis*. 2018;9:1027.
14. Mitsuhashi N, Kobayashi S, Doki T, et al. Clinical significance of alpha-fetoprotein: involvement in proliferation, angiogenesis, and apoptosis of hepatocellular carcinoma. *J Gastroenterol Hepatol*. 2008; 23:e189-e197.
15. Zhu AX, Kang Y-K, Yen C-J, et al. Ramucirumab after sorafenib in patients with advanced hepatocellular carcinoma and increased α-fetoprotein concentrations (REACH-2): a randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet Oncol*. 2019;20:282-296.
16. Weinmann A, Koch S, Niederle IM, et al. Trends in epidemiology, treatment, and survival of hepatocellular carcinoma patients between 1998 and 2009: an analysis of 1066 cases of a German HCC Registry. *J Clin Gastroenterol*. 2014;48:279-289.
17. Ridder DA, Schindeldecker M, Weinmann A, et al. Key enzymes in pyrimidine synthesis, CAD and CPS1, predict prognosis in hepatocellular carcinoma. *Cancers*. 2021;13:744.
18. Remmele W, Stegner HE. Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. *Pathologe*. 1987; 8:138-140.
19. R Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing; 2020.
20. Budczies J, Klauschen F, Sinn BV, et al. Cutoff finder: a comprehensive and straightforward web application enabling rapid biomarker cutoff optimization. *PLoS One*. 2012;7:e51862.
21. Venables WN, Ripley BD. Modern applied statistics with S [internet]. In: Venables WN, Ripley BD, eds. *Modern Applied Statistics with S*. New York, NY: Springer; 2002.
22. Lokuhetty D. *WHO Classification of Tumours*; WHO: Geneva, Switzerland, 2019; ISBN 978-92-832-4499-8.
23. Ziol M, Poté N, Amaddeo G, et al. Macrotrabecular-massive hepatocellular carcinoma: a distinctive histological subtype with clinical relevance. *Hepatology*. 2018;68:103-112.
24. Zucman-Rossi J, Benhamouche S, Godard C, et al. Differential effects of inactivated Axin1 and activated beta-catenin mutations in human hepatocellular carcinomas. *Oncogene*. 2007;26:774-780.
25. Montal R, Andreu-Oller C, Bassaganyas L, et al. Molecular portrait of high alpha-fetoprotein in hepatocellular carcinoma: implications for biomarker-driven clinical trials. *Br J Cancer*. 2019;121:340-343.
26. Yim SY, Lee J-S. The genomic landscape and its clinical implications in hepatocellular carcinoma. *J Liver Cancer*. 2019;19:97-107.
27. Renne SL, Woo HY, Allegra S, et al. Vessels encapsulating tumor clusters (VETC) is a powerful predictor of aggressive hepatocellular carcinoma. *Hepatology*. 2020;71:183-195.
28. Lee JS, Adler L, Karathia H, et al. Urea cycle dysregulation generates clinically relevant genomic and biochemical signatures. *Cell*. 2018; 174:1559-1570.e22.
29. Boege Y, Malehmir M, Healy ME, et al. A dual role of caspase-8 in triggering and sensing proliferation-associated DNA damage, a key determinant of liver cancer development. *Cancer Cell*. 2017;32:342-359.e10.
30. Kuo LJ, Yang L-X. Gamma-H2AX: a novel biomarker for DNA double-strand breaks. *In Vivo*. 2008;22:305-309.
31. Calderaro J, Ziol M, Paradis V, Zucman-Rossi J. Molecular and histological correlations in liver cancer. *J Hepatol*. 2019;71:616-630.
32. Zhou Y-M, Cao L, Li B, et al. Clinicopathological significance of ZEB1 protein in patients with hepatocellular carcinoma. *Ann Surg Oncol*. 2012;19:1700-1706.
33. Zacharakis G, Aleid A, Aldossari KK. New and old biomarkers of hepatocellular carcinoma. *Hepatoma Res*. 2018;4:65.
34. Lu L-C, Hsu C-H, Hsu C, Cheng A-L. Tumor heterogeneity in hepatocellular carcinoma: facing the challenges. *Liver Cancer*. 2016;5: 128-138.
35. Hlady R, Sathyanarayan A, Thompson J, et al. Integrating the Epigenome to identify novel drivers of hepatocellular carcinoma. *Hepatology*. 2019;69:639-652.
36. Zhang Q, Lou Y, Bai X-L, Liang T-B. Intratumoral heterogeneity of hepatocellular carcinoma: from single-cell to population-based studies. *World J Gastroenterol*. 2020;26:3720-3736.
37. Chen W, Peng J, Ye J, Dai W, Li G, He Y. Aberrant AFP expression characterizes a subset of hepatocellular carcinoma with distinct gene expression patterns and inferior prognosis. *J Cancer*. 2020;11:403-413.
38. Nakagawa H, Mizukoshi E, Kobayashi E, et al. Association between high-avidity T-cell receptors, induced by α-fetoprotein-derived peptides, and anti-tumor effects in patients with hepatocellular carcinoma. *Gastroenterology*. 2017;152:1395-1406.
39. van Opdenbosch N, Lamkanfi M. Caspases in cell death, inflammation, and disease. *Immunity*. 2019;50:1352-1364.
40. Fujioka M, Nakashima Y, Nakashima O, Kojiro M. Immunohistologic study on the expressions of [alpha]-fetoprotein and protein induced by vitamin K absence or antagonist II in surgically resected small hepatocellular carcinoma. *Hepatology*. 2001;34:1128-1134.
41. Wee A. Diagnostic utility of immunohistochemistry in hepatocellular carcinoma, its variants and their mimics. *Appl Immunohistochem Mol Morphol*. 2006;14:266-272.

42. Ceci C, Atzori MG, Lacal PM, Graziani G. Role of VEGFs/VEGFR-1 signaling and its inhibition in modulating tumor invasion: experimental evidence in different metastatic cancer models. *Int J Mol Sci*. 2020;21:1388. doi: 10.3390/ijms21041388
43. Yang J, Yan J, Liu B. Targeting VEGF/VEGFR to modulate antitumor immunity. *Front Immunol*. 2018;9:978. doi: 10.3389/fimmu.2018.00978
44. Liu H, Dong H, Robertson K, Liu C. DNA methylation suppresses expression of the urea cycle enzyme carbamoyl phosphate synthetase 1 (CPS1) in human hepatocellular carcinoma. *Am J Pathol*. 2011;178:652-661.
45. Bakhoum SF, Ngo B, Laughney AM, et al. Chromosomal instability drives metastasis through a cytosolic DNA response. *Nature*. 2018;553:467-472.
46. Zhuo J-Y, Lu D, Tan W-Y, Zheng S-S, Shen Y-Q, Xu X. CK19-positive hepatocellular carcinoma is a characteristic subtype. *J Cancer*. 2020;11:5069-5077.
47. Tsai S-C, Lin C-C, Shih T-C, et al. The miR-200b-ZEB1 circuit regulates diverse stemness of human hepatocellular carcinoma. *Mol Carcinog*. 2017;56:2035-2047.
48. Ho DWY, Yang ZF, Yi K, et al. Gene expression profiling of liver cancer stem cells by RNA-sequencing. *PLoS One*. 2012;7:e37159.
49. Chen T, Dai X, Dai J, et al. AFP promotes HCC progression by suppressing the HuR-mediated Fas/FADD apoptotic pathway. *Cell Death Dis*. 2020;11:822.
50. Lu Y, Zhu M, Li W, et al. Alpha fetoprotein plays a critical role in promoting metastasis of hepatocellular carcinoma cells. *J Cell Mol Med*. 2016;20:549-558.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Ridder DA, Weinmann A, Schindeldecker M, et al. Comprehensive clinicopathologic study of alpha fetoprotein-expression in a large cohort of patients with hepatocellular carcinoma. *Int. J. Cancer*. 2022;150(6):1053-1066. doi:10.1002/ijc.33898