

Molecular Portrait of High Alpha-Fetoprotein in Hepatocellular Carcinoma: Implications for Biomarker-Driven Clinical Trials

Authors

Robert Montal¹, Carmen Andreu-Oller¹, Laia Bassaganyas¹, Roger Esteban-Fabró¹, Sebastián Moran², Carla Montironi¹, Agrin Moeini¹, Roser Pinyol¹, Judit Peix¹, Laia Cabellos¹, Augusto Villanueva³, Daniela Sia³, Vincenzo Mazzaferro⁴, Manel Esteller^{2,5-8}, Josep M. Llovet^{1,3,6}.

Author affiliations

¹Translational Research in Hepatic Oncology, Liver Unit, IDIBAPS, CIBERehd, Hospital Clínic, University of Barcelona, Barcelona, Catalonia, Spain. ²Cancer Epigenetics and Biology Program (PEBC), Bellvitge Biomedical Research Institute (IDIBELL), L'Hospitalet, Barcelona, Catalonia, Spain. ³Liver Cancer Program, Division of Liver Diseases, Icahn School of Medicine at Mount Sinai, New York, NY, USA. ⁴University of Milan and Gastrointestinal Surgery and Liver Transplantation Unit, Fondazione IRCCS, Istituto Nazionale dei Tumori, Milan, Italy. ⁵Centro de Investigación Biomédica en Red Cancer (CIBERONC), Madrid, Spain. ⁶Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Catalonia, Spain. ⁷Physiological Sciences Department, School of Medicine and Health Sciences, University of Barcelona (UB), Barcelona, Catalonia, Spain. ⁸Josep Carreras Leukaemia Research Institute (IJC), Badalona, Barcelona, Catalonia, Spain.

Corresponding author

Josep M. Llovet, MD

Translational Research in Hepatic Oncology, Liver Unit, IDIBAPS, CIBERehd, Hospital Clínic, University of Barcelona.

Carrer Rosselló 153, Barcelona (08036); Catalonia, Spain.

E-mail: jmllovet@clinic.cat; Tel: +34 93-2279155; Fax: +34 93-3129406.

Abstract

The clinical utility of serum alpha-fetoprotein (AFP) in patients with hepatocellular carcinoma (HCC) is widely recognized. However, a clear understanding of the mechanisms of AFP overexpression and the molecular traits of patients with AFP-high tumors are not known. We assessed transcriptome data, whole-exome sequencing data and DNA methylome profiling of 520 HCC patients from two independent cohorts to identify distinct molecular traits of patients with AFP-high tumors (serum concentration >400ng/ml), which represents an accepted prognostic cut-off and predictor of response to ramucirumab. Those AFP-high tumors (18% of resected cases) were characterized by significantly lower AFP promoter methylation ($p < 0.001$), significant enrichment of progenitor-cell features (*CK19*, *EPCAM*), higher incidence of *BAP1* oncogene mutations (8,5% vs 1,6%) and lower mutational rates of *CTNNB1* (14% vs 30%). Specifically, AFP-high tumors displayed significant activation of VEGF signaling ($p < 0.001$) which might provide the rationale for the reported benefit of ramucirumab in this subgroup of patients.

Introduction

The global disease burden of hepatocellular carcinoma (HCC) is increasing worldwide, with an estimated 50% of cases receiving systemic treatments for advanced stage^{1,2}. In the last two years, several compounds have shown clinical efficacy in the first- (lenvatinib) or second-line (regorafenib, cabozantinib) setting and joined the standard of care, sorafenib, ultimately leading to a median survival of two years with sequential therapies¹. Ramucirumab, a monoclonal antibody against VEGFR2, is the first drug to demonstrate efficacy in a biomarker-driven phase III trial in HCC, showing a survival benefit as second-line treatment in those patients with alpha-fetoprotein (AFP) serum levels higher than 400ng/ml³. Nevertheless, the rationale behind the use of AFP as predictive biomarker is not fully understood.

AFP is a protein transcribed from the albuminoid genes located on chromosome 4 with a known multi-functionality (i.e. binding of hydrophobic ligands, regulation of proliferation and immunomodulation) provided by its multi-modular structure⁴. AFP is considered an oncofetal protein due to its presence during fetal development and its association with some tumor types such as liver, testes and ovary⁴. In HCC, AFP serum concentration may vary from normal (<10ng/ml) to extremely high (>100000ng/ml)^{2,4}. For this reason, AFP has been extensively explored as a biomarker. For surveillance and diagnostic purposes, AFP sensitivity and specificity depend on the established cut-off, with a global accuracy that is suboptimal for routine clinical practice². As a prognostic factor, it has been clearly demonstrated that patients with AFP >400ng/ml have poor outcomes².

Considering the prognostic and predictive capabilities of AFP in HCC, our hypothesis is that the molecular profile of high AFP tumors differs from those with low AFP and might be associated to VEGF signaling. Herein, we describe the biological traits of HCC with high serum AFP levels through a comprehensive molecular analysis that may provide the rationale for the design of future biomarker-driven clinical trials.

Methods

For the purpose of the study, we analyzed the molecular profiles of 520 HCC human samples with available baseline AFP serum concentrations, including an internal cohort of 244 surgically resected fresh frozen samples (HEPTROMIC dataset)⁵, and an external publicly available cohort of 276 primary HCC from The Cancer Genome Atlas (TCGA dataset)⁶ ([Supplementary Figure 1](#)). Differential molecular patterns of HCC patients based on serum AFP levels were obtained from whole-genome expression, DNA methylome profiling and whole-exome sequencing as described in [Supplementary Methods](#).

Results

AFP serum concentrations followed a logarithmic distribution in our internal HEPTROMIC cohort, with values ranging from 0 to 71770ng/ml ([Supplementary Figure 2A](#)). According to the well-established 400ng/ml cut-off², only 12% (29/244) of patients with early HCC presented high serum levels of AFP, which was accompanied by aberrant overexpression of the gene in the tumor (FC=40; $p < 0.001$) compared to adjacent non-tumoral tissue ([Supplementary Figure 2B](#)). In accordance with previous reports², high AFP serum concentration was found significantly associated with aggressive clinical-pathological features, poor differentiation ([Supplementary Table 1-2](#)) and poor overall survival ([Supplementary Figure 2C](#)).

Based on the oncofetal nature of AFP, we next analyzed its DNA methylation status. The AFP promoter is a low density CpG region that was found hypermethylated in the non-tumor adjacent tissues and in low AFP tumors, but hypomethylated in AFP-high tumors ($p < 0.001$) ([Supplementary Figure 2D](#)). The inverse correlation observed between AFP promoter methylation and AFP expression (HEPTROMIC/TCGA: $R = -0.56/-0.49$; $p < 0.001 / < 0.001$) suggests that this mechanism may play a key role in the aberrant overexpression of AFP in HCC. Indeed, *TET1*, an enzyme able to reverse the DNA methylation status⁷, was one of the

top genes whose expression was found significantly associated with hypomethylation of the AFP promoter ([Supplementary Table 3](#)). Whether this correlation means causation is to be determined.

In order to determine unique somatic derangements associated with AFP-high tumors, HCC samples were analyzed by whole-exome sequencing ([Figure 1A](#), [Supplementary Figure 3A](#), [Supplementary Table 4](#)). AFP-high tumors had fewer non-silent *CTNNB1* mutations (high=14.1%, low=29.9%; p=0.009), a feature that has been associated with T-cell priming failure⁸ and resistance to immune checkpoint inhibitors⁹. The lower rate of *CTNNB1* mutations is in line with the observation that AFP-high tumors fall outside of the recently described *Immune Exclusion class* of HCC⁸. Mutations statistically more prevalent in the AFP-high group included the driver gene *BAP1* (high=8.5%, low=1.6%; p=0.009), a member of the polycomb-group proteins, required for long-term silencing of genes that regulate the cell cycle and cellular differentiation¹⁰.

Aiming to explore the putative link between high AFP levels and targetable phenotypic traits, we evaluated the enrichment of signaling pathways and previously reported molecular classes of HCCs¹ ([Figure 1A](#), [Supplementary Figure 3A](#)). High AFP tumors were particularly associated with the proliferation and the S2 classes, with a consistent enrichment of gene signatures defining progenitor features and overexpression of the known epidermal driver *IGF2*¹¹ ([Supplementary Table 5](#)) when compared with AFP-low tumors. Moreover, the targetable signaling pathways IGF1R, NOTCH, and mTOR were upregulated in AFP-high tumors. On the other hand, the RB1 loss of function signature (designed to predict absence of benefit to CDK4/6 inhibitors) was also a key characteristic of AFP-high tumors. Finally, we identified VEGF pathway enrichment in AFP-high tumors ([Figure 1B](#), [Supplementary Figure 3B](#)). While analyzing the RNA expression of *VEGF* receptor ligands, we observed overexpression of *VEGFB* and *PGF*, but not *VEGFA*. As previously reported^{12,13}, *VEGFB* and *PGF* compete with *VEGFA* for the binding of *VEGFR1*.

Discussion

In the present study, we confirm the aberrant tumor overexpression of *AFP* in those patients with serum concentrations above 400ng/ml and propose DNA methylation of its promoter as the driving mechanism of such overexpression. AFP-high tumors show a distinct phenotype characterized by poor differentiation, enrichment of progenitor features and enhanced proliferation. All these aggressive characteristics are in-line with its known prognostic capacity and explain why the percentage of AFP >400ng/ml tumors increases with disease progression (from 9% in BCLC-A to 42% in BCLC-C) ([Supplementary Table 1](#)). This is relevant since patients at advanced stages are the ones treated with systemic therapies. In this regard, the inclusion in the present study of mostly early-stage HCCs treated with surgical resection may partially hamper to understand the complex biological properties of advanced HCC. Nevertheless, we propose the VEGF ligands/receptors interplay^{12,13} (unbalanced in AFP-high tumors due to VEGFB/PGF overexpression) as rationale for the enhanced activation of VEGF pathway and thus the efficacy of ramucirumab in AFP-high HCC³ ([Figure 1C](#)). Other signaling pathways significantly deregulated in AFP-high tumors and worthy of further analysis include IGF2-IGFR, mTOR, NOTCH and BAP1.

In conclusion, the aberrant over-expression of targetable molecular signaling pathways in HCC patients with high AFP suggests that the measurement of its serum level might serve as a non-invasive predictive tool for biomarker-based clinical trials with targeted therapies.

Additional information

Supplementary information is available at the British Journal of Cancer's website.

Ethics approval and consent to participate

The institutional review boards of the participating centers (IRCCS Istituto Nazionale Tumori [Milan] and Hospital Clínic [Barcelona]) approved the study. The study was conducted in accordance with the International Standards of Good Clinical Practice.

Availability of data and material

Data from the internal HEPTROMIC cohort is stored in the Gene Expression Omnibus (GEO) repository (GSE63898) and (GSE56588).

Conflict of Interest

Josep M Llovet is receiving research support from Bayer HealthCare Pharmaceuticals, Eisai Inc, Bristol-Myers Squibb and Ipsen, and consulting fees from Bayer HealthCare Pharmaceuticals, Eli Lilly, Bristol-Myers Squibb, Eisai Inc, Celsion Corporation, Exelixis, Merck, Ipsen, Glycotest, Navigant, Leerink Swann LLC, Midatech Ltd, Fortress Biotech, Sprink Pharmaceuticals and Nucleix. Manel Esteller reports grants from Ferrer International SA and Quimatryx. Augusto Villanueva reports personal fees from Exelixis, Health Advances LLC, GroupH, Gerson Lehrman Group and Exact Sciences.

Funding

Robert Montal is supported by a FSEOM-Boehringer Ingelheim Grant. Carmen Andreu-Oller has received financial support through the "la Caixa" INPhINIT Fellowship Grant for Doctoral studies at Spanish Research Centres of Excellence, from "la Caixa" Banking Foundation (European Union's Horizon 2020 under the Marie Skłodowska-Curie grant agreement No. 713673). Laia Bassaganyas was supported by Beatriu de Pinós grant from Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR, Generalitat de Catalunya). Roger Esteban-Fabró is supported by MICINN/MINECO (BES-2017-081286). Carla Montironi is a

recipient of Josep Font grant from Hospital Clinic de Barcelona. Roser Pinyol is funded by European Commission/Horizon 2020 Program (HEPCAR, Ref. 667273-2). Augusto Villanueva is supported by U.S. Department of Defense (CA150272P3) and Tisch Cancer Institute (Cancer Center Grant P30 CA196521). Daniela Sia is supported by the Gilead Sciences Research Scholar Program in Liver Disease. Vincenzo Mazzaferro is supported by grants from Associazione Italiana per la Ricerca sul Cancro and the Oncology Research Project of the Italian Ministry of Health. Manel Esteller is supported by the Department of Health PERIS project SLT/002/16/00374 and AGAUR projects 2017SGR1080, 2014SGR633 and 2009SGR1315 of the Catalan Government (Generalitat de Catalunya); the Spanish Institute of Health Carlos III (ISCIII) with project DTS16/00153 and the Integrated Project of Excellence PIE13/00022 (ONCOPROFILE), and the Ministerio de Economía y Competitividad (MINECO) grant SAF2014-55000-R, co-financed by the European Development Regional Fund 'A way to achieve Europe' (ERDF); CIBER 2016 CB16/12/00312 (CIBERONC); the Cellex Foundation; 'la Caixa' Banking Foundation (LCF/PR/PR15/11100003). Josep M. Llovet is supported by National Cancer Institute (P30-CA196521), U.S. Department of Defense (CA150272P3), European Commission/Horizon 2020 Program (HEPCAR, Ref. 667273-2), EIT Health (CRISH2, Ref. 18053), Accelerator Award (CRUCK, AECC, AIRC) (HUNTER, Ref. C9380/A26813), Samuel Waxman Cancer Research Foundation, Centro de Investigacion Biomedica en Red de Enfermedades Hepaticas y Digestivas (CIBERehd) - ISCIII, Spanish National Health Institute (SAF2016-76390) and the Generalitat de Catalunya/AGAUR (SGR-1358).

Author's Contributions

Study concept and design: RM, RP, DS, JML.

Acquisition, analysis, or interpretation of data: RM, CAO, LB, REF, SM, CM, AM, RP, JP, LC, AV, DS.

Drafting of the manuscript: RM, CAO, LB, REF, AM, RP, DS.

Critical revision of the manuscript for important intellectual content: RP, DS, VM, ME, JML.

Acknowledgements

This study has been in part developed at the building Centre Esther Koplowitz from IDIBAPS/CERCA Programme/Generalitat de Catalunya.

References

1. Llovet JM, Montal R, Sia D, Finn RS. Molecular therapies and precision medicine for hepatocellular carcinoma. *Nat Rev Clin Oncol.* 2018;15(10):599–616.
2. EASL Clinical Practice Guidelines: Management of hepatocellular carcinoma. *J Hepatol.* 2018;69:182–236.
3. Zhu AX, Kang Y, Yen C, Finn RS, Galle PR, Llovet JM, 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;2045(18):1–15.
4. Mizejewski GJ. Biological role of alpha-fetoprotein in cancer: prospects for anticancer therapy. *Expert Rev Anticancer Ther.* 2002;2(6):709–35.
5. Villanueva A, Portela A, Sayols S, Battiston C, Hoshida Y, Méndez-González J, et al. DNA methylation-based prognosis and epidrivers in hepatocellular carcinoma. *Hepatology.* 2015;61(6):1945–56.
6. Ally A, Balasundaram M, Carlsen R, Chuah E, Clarke A, Dhalla N, et al. Comprehensive and Integrative Genomic Characterization of Hepatocellular Carcinoma. *Cell.* 2017;169(7):1327–1341.e23.
7. Pastor WA, Aravind L, Rao A. TETonic shift: biological roles of TET proteins in DNA demethylation and transcription. *Nat Rev Mol Cell Biol.* 2013;14(6):341–56.

8. Sia D, Jiao Y, Martinez-Quetglas I, Kuchuk O, Villacorta-Martin C, Castro de Moura M, et al. Identification of an Immune-specific Class of Hepatocellular Carcinoma, Based on Molecular Features. *Gastroenterology*. 2017;153(3):812–26.
9. Harding JJ, Nandakumar S, Armenia J, Khalil DN, Albano M, Ly M, et al. Prospective Genotyping of Hepatocellular Carcinoma: Clinical Implications of Next Generation Sequencing for Matching Patients to Targeted and Immune Therapies. *Clin Cancer Res*. 2019;25(7):2116–26.
10. Carbone M, Yang H, Pass HI, Krausz T, Testa JR, Gaudino G. BAP1 and cancer. *Nat Rev Cancer*. 2013;13(3):153–9.
11. Martinez-Quetglas I, Pinyol R, Dauch D, Torrecilla S, Tovar V, Moeini A, et al. IGF2 Is Up-regulated by Epigenetic Mechanisms in Hepatocellular Carcinomas and Is an Actionable Oncogene Product in Experimental Models. *Gastroenterology*. 2016;151(6):1192–205.
12. Lal N, Puri K, Rodrigues B. Vascular Endothelial Growth Factor B and Its Signaling. *Front Cardiovasc Med*. 2018;5:39.
13. Fischer C, Mazzone M, Jonckx B, Carmeliet P. FLT1 and its ligands VEGFB and PlGF: drug targets for anti-angiogenic therapy? *Nat Rev Cancer*. 2008;8(12):942–56.

Figure legends

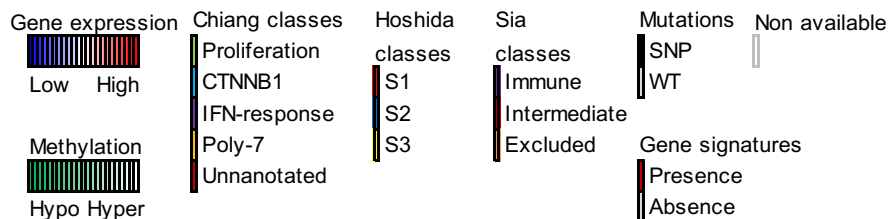
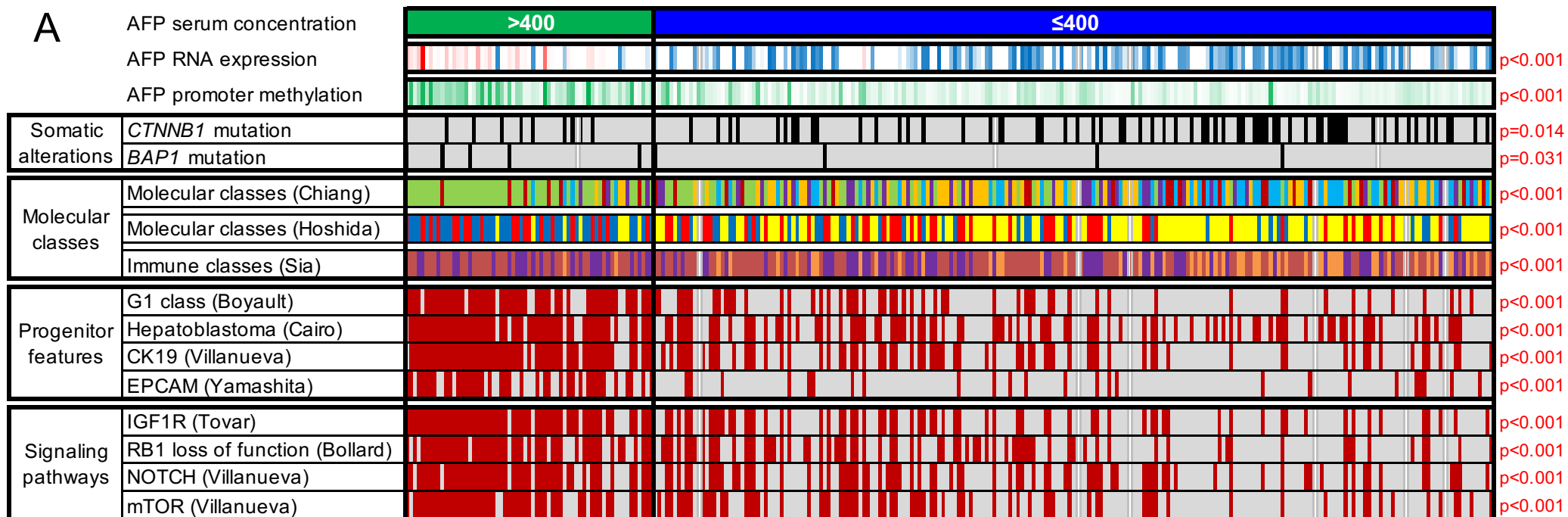
Figure 1. AFP high HCCs show a distinct molecular profile.

- A) Heatmap representation of the most relevant molecular features of AFP high tumors (>400ng/ml) in comparison to AFP low tumors in the TCGA cohort. AFP high HCCs show higher *AFP* RNA expression and *AFP* promoter (TSS1500) hypomethylation. In terms of somatic alterations, AFP high tumors are associated with less *CTNNB1*

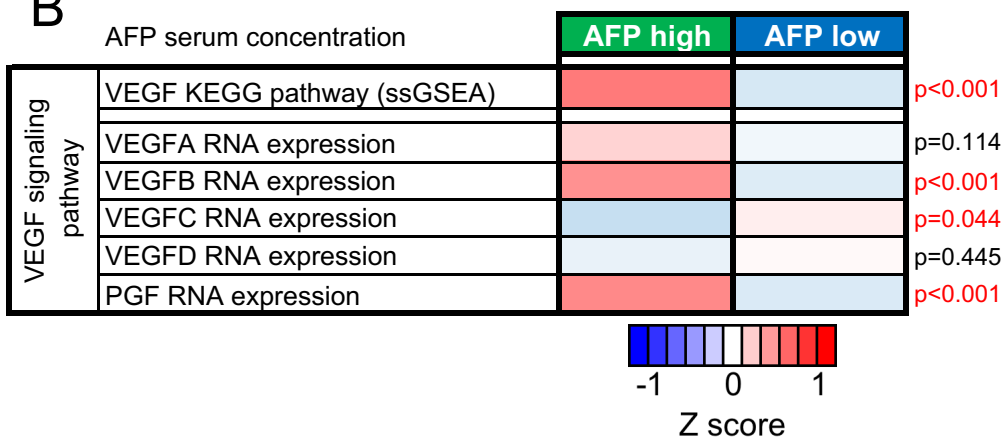
mutations and higher rate of *BAP1* mutations. High AFP tumors are predicted to belong to the proliferation (Chiang) and S2 (Hoshida) classes and show a significant enrichment of signatures of HCC with progenitor features (G1 Boyault, Hepatoblastoma Cairo, CK19 Villanueva and EPCAM Villanueva). Finally, AFP high tumors do not present the immune excluded phenotype (Sia) and present overexpression of HCC signaling pathways (IGF1R Tovar, RB1 loss of function Bollard, NOTCH Villanueva and mTOR Villanueva). Continuous variables (AFP RNA expression and AFP promoter methylation) and categorical variables (the rest) were analyzed by T-Test and Fisher's exact test, respectively.

- B) Heatmap representation of the VEGF KEGG pathway activation (inferred by single sample Gene Set Enrichment Analysis) and VEGF ligands RNA expression according to AFP serum concentration in the TCGA cohort. AFP high tumors show higher enrichment of VEGF signaling and overexpression of *VEGFB* and *PGF*. The mean values of each phenotype (AFP high and low) have been normalized and represented as Z score.
- C) Schematic representation of VEGF pathway in HCC according to AFP serum concentration. The overexpression of *VEGFB* and *PGF* ligands observed in AFP high tumors might result in an enhanced activation of VEGFR1 and, at the same time, prevent VEGFA from binding VEGFR1. The competition of VEGFA with the other ligands could favor its binding to VEGFR2 ultimately leading to its subsequent activation and release of pro-angiogenic signals. The administration of ramucirumab (monoclonal antibody against VEGFR2) might misbalance VEGFA signaling towards a preferential binding of VEGFR1, where it has limited biological activity. Purple and orange lines represent VEGFB/PGF and VEGFA signaling, respectively.

A



B



C

