

Prognostic significance of hypoxic and metabolic gene profiling in hepatocellular carcinoma

Fabiola Milosa¹  | Rosina Maria Critelli¹  | Simone Lasagni¹  | Alessandra Pivetti¹  |
 Lorenza Di Marco¹  | Dante Romagnoli¹  | Lucia Carulli¹  | Francesco Dituri²  |
 Serena Mancarella²  | Gianluigi Giannelli²  | Maria-Luz Martinez-Chantar³  |
 Luca Fabris⁴  | Erica Villa¹ 

¹Gastroenterology Unit, Department of Medical Specialties, University of Modena & Reggio Emilia and Azienda Ospedaliero-Universitaria di Modena, Modena, Italy

²National Institute of Gastroenterology "Saverio de Bellis", Research Hospital, Castellana Grotte, Italy

³CIC bioGUNE, CIBERehd, Parque Tecnológico de Bizkaia, Derio, Spain

⁴Department of Molecular Medicine, University of Padua School of Medicine, Padua, Italy

Correspondence

Erica Villa, Department of Gastroenterology, University of Modena & Reggio Emilia and Azienda Ospedaliero-Universitaria di Modena, Via del Pozzo 71, Modena 41124, Italy.
 Email: erica.villa@unimore.it

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Abstract

Background & Aims: Hepatocellular carcinoma (HCC) is characterized by high clinical and biological heterogeneity, depending on the extremely variable combinations of pathways, linked with immune mechanisms, neo-angiogenesis, ECM remodeling, metabolism and/or hypoxia. We recently identified a 5-genes neo-angiogenic transcriptomic signature (TS), able to discriminate between "aggressive" HCCs (TS-positive) from "bland" HCCs (TS negative), the former having extremely poor survival. The aim of this study was to compare gene expression of our HCC cohort with gene expression of well-characterized, published signatures, which have been related with several different functions potentially relevant in carcinogenesis (ie immune control, hypoxia, metabolism, vascular invasion). We also aimed to ascertain the prognostic power for survival.

Methods: The gene expression profile of a cohort of 78 HCC patients prospectively identified were analysed according to a series of published gene expression signatures related with hypoxia, metabolism and immunity and related with the ability of the signature to predict survival.

Results: Only few genes described in the various immune-signatures analyzed were differentially expressed and were related with reduced survival in our prospective cohort, especially in TS-positive HCCs. Genes composing hypoxic, metabolic and vascular invasion signatures were instead much more deregulated both in aggressive or bland HCCs. For most of them, the level of expression related with reduced survival. This suggests their possible value as biomarker of tumor aggressiveness.

Conclusion: Altogether, our data demonstrate that in HCC, and especially in aggressive TS-positive HCC, signaling pathways related with hypoxic and metabolic/

Abbreviations: ECM, extracellular matrix; EMT, Epithelial-Mesenchymal Transition; E-S, Edmondson-Steiner; ICCA, intrahepatic cholangiocarcinoma; TCGA-LIHC, Cancer Genome Atlas-Liver Hepatocellular Carcinoma data set; TS, Transcriptomic signature; YAP, Yes-Associated Protein.

Trial registration number: ClinicalTrials ID:NCT01657695.

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glycolytic signatures are more relevant in determining a poorer outcome of HCC than immune-related pathways.

KEYWORDS

gene expression, hypoxia, inflammation, liver cancer, metabolism

1 | INTRODUCTION

In the last years, a huge mass of biologic data has unveiled the high clinical heterogeneity of hepatocellular carcinoma (HCC).¹ It is now quite clear that HCCs are characterized by the up-regulation or down-regulation of extremely different combinations of pathways, which eventually influence the course of the disease and the response to therapy.

Despite the vast number of molecular signatures identified so far,² none has entered clinical practice. Possible reasons include their complexity,³ and the applicability to only a fraction of HCC, since the HCC source is mostly represented by surgical samples,³ given the scarce propensity to perform biopsy in HCC.⁴

In a prospective study, we recently identified a neoangiogenic signature composed of 5 genes, which accurately assess HCC prognosis at first presentation.⁵ "Aggressive" HCCs defined by the presence of this signature were also shown to express unique molecular features, such as marked local up-regulation of both PD-1 and PD-L1 and concurrent FoxP3-positive lymphocytic infiltrate, a loss of E-cadherin, gain of epithelial-to-mesenchymal transition (EMT) phenotype and extreme poor differentiation at histology.⁶ From a histological point of view, the aggressive HCCs have often Edmondson-Steiner (E-D) grade 2 or 3, but the relationship between E-D score and the neoangiogenic signature is partial, as patients with aggressive HCCs harboring a high signature score can have low E-D score. Despite the neoangiogenic composition of the signature, at presentation typical wash-in/wash-out radiological features do not characterize aggressive HCCs, suggesting relevant influence of hypoxia on the HCC course. Furthermore, in a small percentage of these aggressive cases, features reminiscent of intrahepatic cholangiocarcinoma (iCCA) can be highlighted.

Starting from these considerations, the aim of this study was to investigate the impact of microenvironment-related genes on the clinical outcome of patients with HCC.

2 | MATERIAL AND METHODS

2.1 | Patients

We prospectively collected a cohort of patients with Child-Pugh class A liver cirrhosis of any etiology, undergoing ultrasound (US) surveillance at 6-months interval in our Unit. Clinical features of the cohort were previously reported in detail.⁵ Briefly, patients with a new CT-confirmed HCC diagnosis underwent US-guided liver biopsy both inside the lesion and in the surrounding tissue. Tumor (T)

Lay Summary/Key Points

Hepatocellular carcinoma (HCC) is a highly heterogeneous cancer in which is implicated extremely different combinations of pathways that influence course of disease and response to therapy.

Our data demonstrate that in HCC, and especially in more aggressive HCC (with worse survival), signalling pathways related with hypoxic and metabolic/glycolytic signatures are more relevant in determining a poorer outcome of HCC than immune-related pathways.

These findings can also offer an interpretation for the somewhat disappointing results of immune-based therapeutic strategies and lead the way to new pre-clinical studies on mechanisms really involved in the progression of HCC.

and non-tumor (NT) liver samples were collected in cold RNAlater (Qiagen) and immediately processed for gene expression analysis. For each biopsy, a portion was also fixed in 10% formaldehyde, paraffin-embedded, and stained with H&E. The diagnosis of HCC was based on established histological criteria.⁷

The Ethics Committee of Azienda Ospedaliero-Universitaria, Modena approved the study protocol (IRB10/08_CE_UniRer; ClinicalTrials ID: NCT01657695).

2.2 | Methods

2.2.1 | Analysis of gene expression

Total RNA was isolated from T and NT liver tissues using Trizol (Invitrogen), according to the manufacturer's instructions. After evaluation of the quality and quantity of the RNA obtained, the samples were processed using 4 × 44 K whole genome oligonucleotide-based gene expression microarrays (Agilent Technologies; Genomics Service Department of Miltenyi Biotec GmbH Bergisch Gladbach, Germany) as reported in Villa et al.⁵ Briefly, in a first step, total RNA was converted into cDNA and then into cRNA and labelled with Cy3-CTP. After purification, labelled cRNAs were hybridized to Agilent Whole Human Genome Oligo Microarrays 4 × 44 K, using standard reagents and protocols. Fluorescent signals of the hybridized Agilent Microarrays were detected using Agilent's Microarray Scanner System (Agilent Technologies) and converted in gene expression data.

2.2.2 | Discriminatory gene analysis (DGA)

To determine if there were genes differentially expressed across all tumor samples considered, we performed a discriminatory gene analysis (DGA), as previously described.⁵ Briefly, each T sample was compared individually with the combined group of NT samples. We identified 243 genes able to discriminate two types of tumor with different aggressiveness, herein indicated as 'fast-growing' and 'slow-growing' tumor samples. Details of the statistical analysis were previously described in reference (5), and are reported in brief in Supplemental Methods. We included the most discriminatory genes, *ANGPT2*, *ESM1*, *NETO2*, *NR4A1* and *DLL4*, known to be involved in angiogenesis, in a novel neoangiogenic transcriptomic signature (TS), able to accurately identify fast growing tumors with gloomy prognosis.

In the current study we have tested different existing signatures described by literature in HCC and in other solid tumors, involving immune, hypoxic, metabolic and vascular pathways, against the prospective cohort cited above. We have indeed investigated if these genes were differently expressed in our cohort of patients characterized by presence/absence of neoangiogenic TS and which genes were related with survival and outcome of patients.

2.2.3 | Statistical analysis

Patients in the original study⁵ were censored at the time of liver transplant (LT), death, or last available follow-up. We used the non-parametric Mann-Whitney U test to evaluate differences in expression levels of the genes of the various signatures examined.

The Kaplan-Meier method was used to estimate the cumulative probability of overall survival; the factors evaluated to estimate survival were the median gene expression levels of the different signatures. Differences in observed probability were assessed using the log-rank test.

The Cox proportional hazards model was used to identify risk factors for mortality. Candidate risk factors for mortality were the different signatures evaluated for comparison⁸⁻²² vs. the transcriptomic signature.⁵

The PASW Statistics 26 program (IBM Corp.) was used for statistical analyses.

3 | RESULTS

A total of 78 patients were enrolled in the Microarray study. Clinical characteristics are detailed in Table S1. Data were censored in December 2012 (mean follow-up 28.1 ± 13.2 months).

3.1 | Expression profile of HCC tissues

Expression data of T and NT tissue samples for genes relevant to immunity, extracellular matrix (ECM), profibrotic growth factors, proliferation and hypoxia, were analyzed according to different histopathologic (severity of fibrosis, intensity of inflammation, grading

according to Edmondson-Steiner score) and molecular (presence of the neoangiogenic transcriptomic signature) features.

3.2 | Severity of fibrosis and inflammation

Several genes were altered in both T and NT tissues (Table 1). Among them, *KRT19* was the only down regulated gene, while all the others were up regulated.

When analyzing gene expression in relation with intensity of inflammation, only few genes were found altered, all but Notch3 linked with collagen and ECM expression (Table 1).

3.2.1 | Edmondson-Steiner score (E-S score)

Few genes (*JAG1*, *TGFb2*) were found altered in tumor tissue when analyzed in relation with HCC grading according to E-S score. None of the genes were found significantly altered in non-tumor tissue (Table 1).

3.2.2 | Neoangiogenic transcriptomic signature

When analyzing data according to transcriptomic signature (TS), only *COL4A2* gene was found hyper-expressed in tumor tissue of aggressive HCC while *COL22A1* was down regulated in non-tumor tissue. Interestingly, *BGN* gene was down regulated in both tumor and non-tumor tissue (Table 1).

3.3 | Relationship between transcriptomic signature of aggressiveness and hyper-expressed genes in different prognostic signatures

Genes included in several different well-characterized signatures, related to functions potentially relevant in carcinogenesis (ie immune control,²³ hypoxia,²⁴ proliferation/fibrosis²⁵), were analyzed with respect to HCC aggressiveness, defined by the presence/absence of the neoangiogenic TS as TS-positive and TS-negative HCC.⁵

3.3.1 | Immune signatures

We have examined six different immune signatures.⁸⁻¹³ Among the various signatures, only ten genes were differentially expressed in neoangiogenic TS-positive HCCs, six being up regulated and 4 down regulated (Table 2).

3.3.2 | Hypoxic signatures

Hu et al¹⁴ have described a hypoxia-related prognostic signature for HCC. Among 13 genes, *PSRC1*, *MEX3A*, *PLOD2*, *KPNA2* and *CDCA8* were upregulated in aggressive HCC (Table 2).

TABLE 1 List of differentially expressed genes according to higher intensity of fibrosis, higher severity of inflammation, higher Edmondson-Steiner score or aggressive neoangiogenic signature. Gene expression was analyzed by Mann-Whitney test. Red: up regulated genes; Blue: down regulated genes (fold change; *P* value)

Category	Intensity of fibrosis		Severity of inflammation		Edmondson-Steiner score		Neoangiogenic signature	
	T	NT	T	NT	T	NT	T	NT
Collagen	COL1A1 (10.9; 0.001)	COL1A1 (9.7; 0.003)		COL1A1 (9.7; 0.024)				
	COL1A2 (4.9; 0.002)	COL1A2 (4.7; 0.004)		COL1A2 (4.8; 0.012)				
	COL3A1 (8.7; 0.000)	COL3A1 (8.7; 0.012)						
	COL4A2 (12.9; 0.002)	COL4A2 (12.3; 0.018)	COL4A2 (13.0; 0.049)				COL4A2 (12.9; 0.036)	
	COL5A1 (8.6; 0.000)	COL5A1 (7.9; 0.016)						COL22A1 (-1.65; 0.021)
ECM	SPARC (13.3; 0.001)	SPARC (12.9; 0.069)						
	BGN (7.4; 0.087)						BGN (-1.5; 0.011)	BGN (-1.7; 0.011)
	ELN (5.4; 0.090)	ELN (5.2; 0.070)						
	FBN1 (9.7; 0.001)	FBN1 (9.1; 0.023)						
	KRT19 (-0.95; 0.029)	KRT19 (0.0; 0.042)	KRT19 (-0.3; 0.061)					
	FNDC1 (6.3; 0.003)	FNDC1 (5.6; 0.033)						
Notch			NOTCH3 (4.7; 0.012)					
	JAG1 (8.9; 0.002)	JAG1 (8.6; 0.110)	JAG1 (9.1; 0.070)		JAG1 (8.8; 0.061)			
	MAMLD1 (7.8; 0.146)							
Hedgehog	HEYL (9.4; 0.001)	HEYL (9.3; 0.014)						
	GLI2 (5.3; 0.001)	GLI2 (5.8; 0.001)						
	GLI3 (6.2; 0.001)	GLI3 (6.7; 0.001)						
	TGFB2 (7.6; 0.002)				TGFB2 (7.6; 0.058)			
	PDGFRB (10.8; 0.004)	PDGFRB (10.5; 0.004)						
		IGFBP5 (13.2; 0.896)						

Ten out of 26 genes from the hypoxic signature described by Eustace et al¹⁵ were either up regulated (7 genes) or down regulated (3 genes) in aggressive HCC (Table 2). They were mostly involved in metabolism/glycolysis, and in vascular and ECM remodeling.

Chang et al¹⁶ described two different hypoxic signatures, common to several solid cancers, one associated with poor prognosis and one with good prognosis. In the 'good prognosis' signature, one gene (*P4HTM*) was up regulated in neoangiogenic TS-positive HCC, while

TABLE 2 Genes included in the hypoxic, immune, metabolic, and vascular invasion signatures found altered in the prospective cohort of 78 hepatocellular carcinoma (HCC) patients at first HCC diagnosis in relation with presence of the aggressive phenotype according to the neoangiogenic transcriptomic signature.⁵ Different levels of gene expression were analyzed by Mann-Whitney test (P value *** $P < .001$, ** $P < .01$, * $P < .05$, #borderline)

	Up-regulated genes	Down-regulated genes	Author
Immune signature	PLCG1*, VDR# AXIN2* IRF1* NDRG1**, SEMA3F*** IL12A*, GLMN**	ADAMTS2** PTEN*, MLL3*	Jiang et al ⁸ Okrah et al ⁹ Sia et al ¹⁰ Lal et al ¹¹ Wang et al ¹² Xu et al ¹³
Hypoxic signature	PSRC1**, MEX3A*, PLOD2*, KPNA2***, CDCA8*** ALDOA*, HIG2***, KRT17**, SLC16A3**, VEGFA***, ENO1*, DCBLD1* P4HTM* PLOD2 [†]	P4HA1***, SDC1*, DAPK1** KDM8***, ALKBH4**	Hu et al ¹⁴ Eustace et al ¹⁵ Chang et al (good prognosis) ¹⁶ Chang et al (bad prognosis) ¹⁶ Zhu et al ¹⁷
Metabolic signature	G6PD*, PRIM1***, RRM2***, TXNRD1*, UCK2***, CAD**, DTYMK*** HMMR***, SLC16A3**, VEGFA***, SOD1** ALDOA**, PFKFB4**, PKM2* UCK2***, GOT2*, ACAT1 [#]	SLC22A1*** ENO3**, PFKFB1*, PFKI*, ALDOB***	Zhang et al ¹⁸ Cassim et al ¹⁹ Liu et al ²⁰
Vascular invasion signature	SCIN**, MMP12**, TRIM45 [#] , HOXD10*** PON1***, PIK3R1*, MAT1A***, RCL1**, DPYS*, AASS**, UGT2B15**, DEPDC7***, GLYATL1**, MYLK*, PAH**, PCK1**, PPARGC1A**, E2C**, CDKN3***, XPOT**, UBE2C** GORASP2*, YY1AP1**, NARF*, SLC38A4***, TYMS**	HSD17B13** GLYAT***, ADH4***	Yi et al ²¹ Minguez et al ²²

two (*KDM6B*, *ALKBH4*) were down regulated. In the 'poor prognosis' signature, only *PLOD2* was up regulated in T ($P = .034$) and had borderline significance in NT ($P = .063$) tissue.

3.3.3 | Metabolic signatures

Zhu et al¹⁷ described a metabolic ten-gene signature in HCC with excellent ability for predicting survival prognosis. Among these genes, 7 were up regulated in aggressive HCC (Table 2). Among the 9 genes reported by Zhang et al¹⁸ as significantly associated with metastasis and shorter overall survival in lung carcinoma, five were differentially expressed in neoangiogenic TS-positive HCCs (Table 2).

Cassim et al¹⁹ found a pro-invasive metabolic signature in HCC composed of 27 genes. Among them, seven were differentially expressed in aggressive HCC, 4 down regulated and the others up regulated compared with neoangiogenic TS-negative HCCs (Table 2).

In the 4-genes signature described by Liu et al,²⁰ only *UCK2* ($P < .000$) and *GOT2* ($P = .023$) were differentially expressed

in aggressive HCC, while significance of *ACAT1* was borderline ($P = .064$). Liu et al²⁶ also evaluated a series of metabolic genes derived from the Cancer Genome Atlas-Liver Hepatocellular Carcinoma data set (TCGA-LIHC). We evaluated the 172 most differentially expressed of this series. We identified 92 genes, out of 172 tested that were up regulated (33 genes) or down regulated (59 genes) in aggressive HCC (Table S2).

3.3.4 | Vascular invasion signature

Few predictive signatures of vascular invasion have been found in HCC. Among them, Yi et al²¹ proposed a classification model composed of 14 genes able to discriminate patients with and without vascular invasion. Five genes of this signature were differentially expressed in aggressive HCCs, all but *HSD17B13* up regulated (Table 2).

In the 35-gene signature predictive of vascular invasion described by Minguez et al,²² 13 genes were up regulated in bland tumors and 9 in aggressive tumors (Table 2). Two genes (*GLYAT* and *ADH4*) were significantly down regulated in aggressive tumors.

TABLE 3 Genes included in the hypoxic, immune, metabolic, and vascular invasion signatures, which were found altered when tested in the gene expression database of the prospective cohort of 78 hepatocellular carcinoma (HCC) patients at first HCC diagnosis described in reference (5). The relationship with worst survival of up regulated or down regulated genes was evaluated by Kaplan-Meier analysis (Level of significance: *** $P < .001$, ** $P < .01$, * $P < .05$, #borderline significance)

	Up-regulated genes	Down-regulated genes	Author
Immune signature	PLCG1**, VDR# KCNE4#, JAG1**, IGFBP5** AXIN2*, WNT10B# IRF1# ANGPT1#, NDRG1*** CKLF*	ADAMTS2** PTEN**, MLL3* OSGIN1*	Jiang et al ⁸ Okrah et al ⁹ Sia et al ¹⁰ Lal et al ¹¹ Wang et al ¹² Xu et al ¹³
Hypoxic signature	PSRC1*, PLOD2**, KPNA2**, CDCA8**, ADAMTS5* CDKN3*, ALDOA**, HIG2#, KRT17**, SLC16A3***, VEGFA*, P4HTM** PLOD2**	DAPK1* KDM8**, ALKBH7*	Hu et al ¹⁴ Eustace et al ¹⁵ Chang et al (good prognosis) ¹⁶ Chang et al (bad prognosis) ¹⁷
Metabolic signature	LPCAT1**, RRM2** HMMR**, SLC16A3***, VEGFA* ALDOA**, PFKFB4**, PGK1**, PKM2**, ENO2# TAT**, ME2**, TK1#, SL41A3, ACOT7#, ACOT9_A**, ACOT9#, CHILA_A#, PGK1_B**, SLC6A13_A***, CA12_A**, CA12_B**, CA12_C**, ACACA_A*, PPAT_A**	SLC22A1** ALDOB*** ACAT1***, GOT2*, CYP2C19*, EHHADH*, FTCD*, CYP411***, CYP411_A**, CA2**, HAO1*, GNE***, SLC27A5*, ABAT*, ARG1*, MUT***, CYP26A1*, ACADS**, ADA*, SRD5A1***, ADK*, ACAA1*, GOT2_B*, ACAT1_A**, GPD1_A*, GPD1_B*, GPD1**, PON1A***, PON1***, STARD5*, STARD5_A**, TAT_B***, TAT_A**, TAT*, SULT1A1***, SLC16A2*, ALDOB_A**, PPAT_A*, FBP1**, GLS2**, CBR4**, DPYS_A*, DBT*, SORD_A**, SORD_B*, ACADM**, PDK4*	Zhu et al ¹⁸ Zhang et al ¹⁹ Cassim et al ²⁰ Liu et al ²¹
Vascular invasion signature	MMPI2*, SLC35F3**, HOXD10***, PPP2R2C** CDKN3**, E2C**, PAH#, PCK1**, TYMS**, UBE2C*	SCIN** MAT1A#, RCL1**, UGT2B15***, DEPDC7*, PCK1***, PPARGC1A*, SLC38A4***, SLC38A2**, GLYAT**, PON1***, PIK3R1***	Yi et al ²² Minguez et al ²³

3.4 | Relationship between hyper-expressed genes of different prognostic signatures with survival and relationship with the neoangiogenic transcriptomic signature

Relationship between level of gene expression and survival was evaluated by Kaplan-Meier analysis.

3.4.1 | Immune signatures

Only few genes obtained from the different immune signatures evaluated (Jiang et al,⁸ Okrah et al,⁹ Sia et al,¹⁰ Lal et al,¹¹ Wang et al¹² and Xu et al¹³) were significantly related to survival (Table 3).

None of the genes identified by Jiang et al⁸ but *PLCG1* ($P = .005$) was significantly related with survival, the relationship,

after stratification according to TS, being with aggressive tumors only.

In the T cell signatures described by Okrah et al,⁹ 3 genes were up regulated; for all of them, increased expression was related with reduced survival. Only ADAMTS2 was down regulated and significantly associated with lower survival (Table 3). Evaluating their expression in HCC according to neoangiogenic TS, only JAG1 was up-regulated in aggressive HCCs ($P = .042$; Table 4). The opposite was found for IGFBP5 (bland: $P = .010$; aggressive: NS; Table 4). Of note, 3 genes, whose expression was not different in the unstratified cohort, were up regulated specifically in aggressive HCCs (KRT19, $P = .032$; EMILIN1 ($P = .038$), GLI2 ($P = .032$; Table 4).

Among the genes described by Sia et al,¹⁰ PTEN (decreased expression, $P = .006$), AXIN2 (increased expression, $P = .036$), MLL3

(decreased expression, $P = .039$), WNT10B (increased expression, $P = .073$) were related to decreased survival. Interestingly, when evaluating them in accordance to the neoangiogenic TS, the relationship with PTEN and WNT10B reached significance only in bland tumors (decreased survival for lower levels of PTEN [$P = .025$] and WNT10B [$P = .031$] expression)) (Table 4).

Among the 8 genes identified by Xu et al,¹³ only CKLF_C ($P = .031$) was significantly related with survival, the relationship holding true only for aggressive HCC after stratification according to TS (Tables 3 and 4). Wang et al¹² developed a 9-gene prognostic model for survival. Among them, only 2 genes were up-regulated (ANGPT1 $P = .053$ and NDRG1 $P < .0001$) and one was down-regulated (OSGIN1, $P = .040$; Table 3). When evaluating them in accordance to the neoangiogenic TS, NDRG1 was significant in bland HCC ($P = .002$) and borderline in

TABLE 4 Genes included in the hypoxic, immune, metabolic, and vascular invasion signatures, which were found altered when tested in the gene expression database of the prospective cohort of 78 hepatocellular carcinoma (HCC) patients at first HCC diagnosis described in reference (5). The relationship with worst survival of up- or down regulated genes according to the aggressive phenotype, defined by the neoangiogenic transcriptomic signature, was evaluated by Kaplan-Meier analysis (Level of significance: *** $P < .001$, ** $P < .01$, * $P < .05$, #borderline significance)

	Up-regulated genes		Down-regulated genes		Author
	TS-	TS+	TS-	TS+	
Immune signature		PLCG1 [#]			Jiang et al ⁸
	IGFBP5**	JAG1*, KRT19*, EMILIN1*, GLI2*	ADAMTS2*		Okrah et al ⁹
	WNT10B*, PTEN*		OSGIN1**		Sia et al ¹⁰
	NDRG1**	NDRG1 [#] CKLF_C**			Wang et al ¹² Xu et al ¹³
Hypoxic signature	KPNA2*	PSRC1*, MEX3A**, PLOD2*	SLC1A7 [#]		Hu et al ¹⁴
	SLC16A3*	SLC16A3*, ALDOA [#]	KDM8*		Eustace et al ¹⁵
	P4HTM*			ALKBH7***, KDM3A*	Chang et al (good prognosis) ¹⁶
		PLOD2*			Chang et al (bad prognosis) ¹⁶
Metabolic signature	LPCAT1*	G6PD*			Zhu et al ¹⁷
	SLC16A3*	SLC16A3*, RBCK1*, AGRN*		SLC22A1 [#]	
	PGK1**, ALDOB**, PKM2*	ALDOA*, PKM2*, PPP2CA [#] , PFKFB4*, PGK1*, ENO2**			Cassim et al ¹⁹
	SL41A3*, ACOT9_A**, ACOT9*	KCNJ16*, CYP26A1 [#] , CA12_A [#] , CA12_B [#] , GPD1_B*, TRPC1*	ACAT1**, CYP20A1*, CYP411_A*, CYP411**, CHST4 [#] , SULT1A1*, ALDOB_A*, SRD5A1*, PON1A**	ABAT_A*, ACADM**, SORD_A*, SORD_B [#] , CBR4_A*, PON1**, PON1A***, HAO1*, GNE**, SLC27A5 [#] , MUT [#] , ACAT1_A*, GPD1*, SRD5A1*	Liu et al ²⁰
Vascular invasion signature	SLC35F3**, SCIN*, PPP2R2C*	MMPI2**, SLC35F3*			Yi et al ²¹
	SLC38A4*	SLC38A2 [#]	PCK1 [#] , PIK3R1*, UGT2B15**	RCL1*, DEPDC7*, KLF9*, PON1**, PIK3R1*	Minguez et al ²²

TABLE 5 Prediction of survival for the different signatures reported in hepatocellular carcinoma (HCC) (Cox regression analysis)

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P Value	HR (95% CI)	P Value
Neoangiogenic signature ^{5a,b}	4.168 (2.194-7.992)	<.0001	3.758 (1.927-7.330)^a 2.207 (1.049-4.640)^b	<.0001 .030
Immune signature				
Okrah et al ⁹	0.675 (0.348-1.312)	.246		
Sia et al ^{10a}	1.858 (0.979-3.530)	.058	1.728 (0.885-3.374)	.109
Wang et al ¹²	1.189 (0.648-2.181)	.576		
Xu et al ^{13b}	2.531 (1.357-4.719)	.003	1.431 (0.647-3.166)	.376
Hypoxic signature				
Hu et al ^{14b}	4.928 (2.407-10.092)	<.0001	3.053 (1.313-7.101)	.010
Chang et al (good prognosis) ^{16a}	2.862 (1.516-5.401)	.001	1.341 (0.626-2.871)	.450
Chang et al (bad prognosis) ¹⁶	2.179 (1.125-4.221)	.020		
Metabolic signature				
Zhu et al ^{17b}	3.702 (1.871-7.326)	.000	1.669 (0.766-3.635)	.197
Cassim et al ^{19a}	4.376 (2.293-8.352)	.000	2.345 (1.125-4.889)	.023
Liu et al ²⁰	2.539 (1.154-5.582)	.020		
Vascular invasion				
Yi et al ^{21a}	4.350 (2.338-8.090)	.000	2.541 (1.254-5.148)	.010
Minguez et al ^{22b}	1.731 (1.175-2.548)	.005	1.233 (0.775-1.961)	.376

The bold character identifies the statistically significant data.

^aModel 1.

^bModel 2.

aggressive HCC ($P = .063$; Table 4). OSGIN1 was significantly down regulated in bland HCCs only.

3.4.2 | Hypoxic signatures

Expression in the tumor tissue of seven genes present in the signature by Eustace et al¹⁵ was significantly related with decreased survival (six of them being up regulated, one down regulated; Table 3).

Among 13 genes in the signature by Hu et al,¹⁴ only 5 were related to survival (Table 3). Only one, *SLC1A7* ($P = .036$), was found significant down regulated in the neoangiogenic TS- (Table 4).

Of the genes reported by Chang et al,¹⁶ down regulated *KDM8* and *ALKBH7* and up regulated *P4HTM* (indicated in the Chang's study as being related with better prognosis) were instead related with decreased survival as well as up regulated *PLOD2* when tested in our series (Table 3). Following stratification by neoangiogenic TS, we found altered *P4HTM* in TS- but not in TS+ HCC, while *PLOD2* was found up regulated in the TS+ HCC only (Table 4).²⁶ *KDM8* was down regulated in TS- only and *ALKBH7* down regulated in TS+ only (Table 4).

3.4.3 | Metabolic signatures

Within the 10-gene signature by Zhu et al,¹⁷ only *LPCAT1* ($P = .007$) and *RRM2* ($P = .007$) were related to survival (Table 3). Analyzing

survival in relation to the presence of neoangiogenic TS, a significant difference in survival was present for 2 genes of the Zhu et al signature (*G6PD* and *LPCAT1*) in TS+ and TS- HCC respectively; Table 4). Four genes from the 9-gene Zhang's signature (3 up regulated, one down regulated)¹⁸ were significantly related with survival (Table 3). Among those not significantly related with survival in the unstratified cohort, *AGRN* and *RBCK1* were up regulated in the 'aggressive' tumors with significantly lower survival ($P = .015$; Table 4).

The original 4-gene signature described by Liu et al²⁰ in our cohort was unable to discriminate between patients with good and bad prognosis. Only patients belonging to the highest quartile of gene expression had significantly lower survival. Among the 172 metabolic genes from a TCGA-LIHC data set also tested by Liu et al,²⁰ that we verified in our series, 50 showed strong relationship with survival at Kaplan-Meier analysis (Table S3). When survival was analyzed in relation to the presence of neoangiogenic TS, a significant difference in survival was maintained for 14 of these genes in TS+ HCC only (Table 4). For other 11 genes, survival was significantly different for bland tumors only (Table 4).

3.4.4 | Vascular invasion signature

Several genes included in the Yi et al²¹ and Minguez et al²² signatures were found to be significantly related with survival (Table 3). In the Yi's signature, *SLC35F3* was up regulated both in bland and

aggressive HCC *MMP12* were up regulated in 'aggressive' HCC, while *SCIN* and *PPP2R2C* were up regulated in 'bland' HCC only. The evaluation of the Minguez signature in relation to neoangiogenic TS showed that some altered genes in the unstratified cohort, were found to be deregulated in 'aggressive' or in 'bland' HCC alternatively (Tables 3 and 4).

3.4.5 | Cox regression analysis

The different signatures (neoangiogenic, immune, hypoxic, metabolic, vascular invasion) reported in HCC at univariate analysis were then tested to identify in each category, which was more powerful in predicting survival (Table 5). Signatures were tested in different combinations at multivariate analysis (Table 5). The two best fitting models are reported in Table 5. In one, the neoangiogenic transcriptomic signature, the vascular signature described by Yi et al²¹ and the metabolic signature described by Cassim et al¹⁹ were found to be independently associated with mortality. In the other, the neoangiogenic and the hypoxic signature described by Hu et al¹⁴ were independently related with survival. In none of the models, the immune signature was independently related with survival.

4 | DISCUSSION

Recent therapeutic advances in inoperable HCC should have been expected by targeting the multifaceted functions of the immune tumor microenvironment. Unfortunately, results of the first controlled studies have been disappointing.²⁶ This has prompted a careful re-evaluation of the intricate role played by the different signaling pathways in order to identify the most prominent ones. Several pathways have been described as linked with the development of HCC, involving immune mechanisms, neoangiogenesis, ECM remodeling, metabolism and/or hypoxia. All of these studies investigating gene and molecular expression of HCCs reported discordant modification of the same genes or pathways, making their role difficult to decipher. This discordance may be due to several factors, in particular the large heterogeneity of cohorts enrolled in the different studies. Notably, the cohorts studied were only rarely prospectively collected. Tissue samples were most often derived from archival collections. In one recent paper, we prospectively characterized 78 patients with HCC for biological HCC aggressiveness based on a transcriptomic signature identified by means of an extensive microarray study.⁵ In the current study, we have exploited the above mentioned microarray analysis to challenge some of the principal signatures reported in HCC and in other solid cancers, to evaluate whether some of the key features related to the microenvironment, such as immune system, hypoxia, metabolism, ECM, and vascular invasion, were indeed relevant for HCC. More specifically, we investigated their prognostic relationship with survival and the possible differences with respect to tumor aggressiveness, defined by the expression of the transcriptomic signature.

Several signatures recently described in HCC patients, focused on gene expressions related to immune system^{9,10} for its potential druggability in HCC derived from the availability of new immunotherapeutic strategies.²⁷ Alterations of the immune system (ie number of immune cells or cytokine levels) were shown to contribute to progression of HCC by regulating tumor tolerance and tumor surveillance.²⁸ In these studies, the immunophenotype of HCC was related to patient survival, suggesting that a strong immune activation within the tumor could be able to hamper HCC progression with a beneficial effect on survival.²⁹ Starting from these observations, we analyzed the relevance of four immune signatures in our cohort of patients. For all of them, we found only few differentially expressed genes (not classically considered as immune-related genes) and a low correlation with survival. By evaluating the expression of these genes with respect to the presence/absence of neoangiogenic TS, only *JAG1*, *GLI2*, *EMILIN1* and *KRT19* were up-regulated in TS-positive while in TS-negative *IGFBP5*, *PTEN* and *WNT10B* were up regulated while *ADAMTS2* was down regulated.

These levels of expression correlated with reduced survival, in accordance with several lines of evidence outlining a significant correlation of these molecular signatures with HCC prognosis. Several studies have found an increased *JAG1* expression in tumoral tissue of HCC patients compared to adjacent non-tumor hepatocytes.³⁰ Jagged1 is one of the 2 ligands of Notch receptors and activates Notch signaling upon binding implying direct cell-to-cell contact. Notch signaling has been reported to be involved in the regulation of immune cell functioning during inflammatory response.³¹ However, the role played in HCC might be even more complex, probably linked with other, immune-independent, mechanisms. Villanueva et al³² in a genetically engineered mouse model suggested a cooperative oncogenic role between Notch and other pathways such as RAS, and a role as *bona fide* oncogene for Notch1. Notch1 has been also shown to directly transcriptionally regulate *GLI2*, downstream effector of the Hedgehog signaling. *GLI2*, which we found overexpressed in 'aggressive' TS+ HCC, has been significantly linked with aggressive HCC features, namely vascular invasion, early recurrence, intrahepatic metastasis, and significantly shorter overall survival.³³ The other gene found overexpressed in aggressive TS+ HCC was *KRT19* (also known as Cyfra21-1). High expression levels of *KRT19* were related to high levels of ERK activation and to a gloomy prognosis.³⁴ Several other findings indicate a direct promoting effect of *KRT19* on cancer cell survival, invasion, and angiogenesis.³⁵ Not surprisingly, in this same cohort, we previously reported that significantly higher levels of circulating Cyfra21-1 were a feature of aggressive HCCs.⁶ Overall, these genes and pathways found overexpressed in HCC, despite being involved in some of the immune-related signatures described in HCC, are more representative of other signaling pathways regulating mechanisms underpinning fibrogenesis, angiogenesis and metastasis.

We found other immune-related genes deregulated in TS-negative HCC only. *ADAMTS2* down regulation in TS-negative HCC is consistent with previous reports demonstrating its inhibitory action on angiogenesis.³⁶ *PTEN*, according to the well-established feature

of tumor suppressor, was similarly down regulated in TS-negative HCCs.³⁷ Thus, it is tempting to speculate that *PTEN* pathway could have a role in 'bland' HCC, while in 'aggressive' HCCs other activated pathways could become preponderant and thus, overcome *PTEN* effects.

Based on the analysis of our prospective cohort, it is apparent that compared with immune-related modifications, alterations of other pathways (hypoxic, glyco-metabolic, ECM remodeling, vascular invasion) were definitely more conspicuous. Several genes belonging to these pathways were found over- or down-modulated in HCC and, not surprisingly, were closely related to survival. The particularly relevant role as predictor of survival of the neoangiogenic, metabolic and hypoxic signatures, appears evident at multivariate analysis. The predominance of the latter two pathways was further confirmed, using different models, where different combinations of signatures were tested. Immune-related signatures, despite being associated with survival at univariate analysis, had less power in predicting survival compared with angiogenic, hypoxic and metabolic signatures.

Hypoxia is associated with activation of HIF-1 α that plays important roles in many critical aspects of HCC tumorigenesis, progression and metastasis, and is also an indicator of poor outcome.³⁸ The induction of angiogenesis in hypoxic conditions is relevant for tumor growth by stimulating expression of angiogenic factors.³⁹ In our study, several genes associated with hypoxia were found deregulated (described in reference [15,16]), especially in aggressive TS+ HCCs, and also associated with decreased survival.

The most striking feature of these tumors, however, was the abnormal metabolism. The liver plays a central role in metabolism owed to the hepatocyte capacity to maintain energy production and metabolic homeostasis. In HCC, altered metabolism also affects the tumor microenvironment in order to sustain cellular proliferation and/or escape from apoptosis, in particular lipid metabolism, with consequent protective effects on tumor growth, proliferation and survival.⁴⁰ For these reasons, metabolic changes can provide yet neglected but hopefully promising therapeutic targets in HCC treatment.⁴¹

By the analysis of metabolic signatures, we have found a major correspondence of genes differentially expressed in our cohort of patients (Tables 3 and 4). Among up-regulated genes, *SLC16A3*, alias *MCT4*, was up regulated in both 'bland' TS-negative and 'aggressive' TS-positive HCCs. *SLC16A3* plays a role in the glycolytic process and it is able to induce HIF1 α expression in the microenvironment of large tumors.⁴² In HCC, its expression was found at higher levels in tumor than in non-tumor tissue, and was correlated with tumor size and poor prognosis.⁴³

SLC22A1 is also known as *OCT1*, one of the organic cation transporters that were constitutively expressed in liver. It plays metabolic functions of uptake, intracellular inactivation, and biliary or urinary excretion of a broad spectrum of endogenous and exogenous compounds, including anticancer drugs.⁴⁴ It is also commonly regarded as a marker of 'cancer stemness'. In HCC and CCA, a strong down-regulation of *SLC22A1* mRNA expression has been described. This

finding was related to advanced tumor stages, tumor progression and to a significantly reduced overall survival.⁴⁵ In our cases, *SLC22A1* down-regulation was a specific feature of aggressive tumors, where it was significantly associated with worst survival.

AGRN and *PGK1* were two other up-regulated genes in TS+ patients. *AGRN* encodes for a proteoglycan (Agrin) that represent an important component of remodeled ECM. It is important for neoangiogenesis in HCC tissues, and it is incorporated into newly formed vasculature.⁴⁶ Chakraborty et al,⁴⁷ have revealed that Agrin was an important factor activating and coordinating cellular adhesion, migration and invasiveness of HCC cancer cells. In particular, Agrin behaves as mechano-activator of yes-associated protein (YAP), and cooperation between Agrin and YAP leads to liver cancer development, HCC in particular. In HCCs, up-regulation of Agrin was related to decreased survival time and presence of tumor metastasis, likely indicating a prognostic role.⁴⁸ A similar value as potential biomarker of enhanced invasiveness was reported for *PGK1*, known to be over-expressed in several carcinomas, even including pancreatic and gastric carcinoma beside liver.⁴⁹

5 | CONCLUSIONS

Altogether, our data demonstrate that in HCC, and especially in aggressive TS-positive HCC, signaling pathways related with hypoxic and metabolic/glycolytic signatures are more relevant in determining a poorer outcome of HCC than immune-related pathways. This has profound implications for therapeutic choice and also can offer an interpretation for the somewhat disappointing results of immune-based therapeutic intervention.²⁶ Indeed, therapeutic protocols involving checkpoints inhibitors have reached the endpoints only when coupled with inhibitors of angiogenesis.⁵⁰ On the other hand, the striking relevance of hypoxic and metabolic/glycolytic signatures, especially in 'aggressive' HCCs, gives an account of the grim natural history of these cancers. Unfortunately, there are no effective tools so far to efficiently counteract the activation of these signaling pathways. This means that it is still difficult to interfere with the end product of their activation, ie neoangiogenesis. As the activation of many key pathways seems to be related with processes (like fibrosis establishment and progression) that are unleashed by chronic liver injury, these observations lend support to the notion that most efforts should be put in preventing and/or curing chronic liver disease before events eventually leading to carcinogenesis are kindled.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conception and design of the study, EV and LF; acquisition of data, LC, DR, AP, LDM; statistical analysis, EV; interpretation of data and drafting manuscript, E.V, FM; manuscript revision RMC, SL, FD, SM.; study supervision, LF, GG and M.MC. All authors have read and agreed to the published version of the manuscript.

ETHICS APPROVAL STATEMENT

The Ethics Committee of Azienda Ospedaliero-Universitaria, Modena approved the study protocol (IRB10/08_CE_UniRer).

ORCID

Fabiola Milosa  <https://orcid.org/0000-0002-9869-8819>
 Rosina Maria Critelli  <https://orcid.org/0000-0002-2313-2642>
 Simone Lasagni  <https://orcid.org/0000-0002-8525-0898>
 Alessandra Pivetti  <https://orcid.org/0000-0001-8271-846X>
 Lorenza Di Marco  <https://orcid.org/0000-0002-3716-7010>
 Dante Romagnoli  <https://orcid.org/0000-0001-6960-1267>
 Lucia Carulli  <https://orcid.org/0000-0001-7792-8542>
 Francesco Dituri  <https://orcid.org/0000-0002-7943-3698>
 Serena Mancarella  <https://orcid.org/0000-0003-2692-1169>
 Gianluigi Giannelli  <https://orcid.org/0000-0002-5625-2499>
 Maria-Luz Martinez-Chantar  <https://orcid.org/0000-0002-6446-9911>
 Luca Fabris  <https://orcid.org/0000-0001-5799-9001>
 Erica Villa  <https://orcid.org/0000-0001-6388-7022>

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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