

Novel hepatocellular carcinoma molecules with prognostic and therapeutic potentials

Bruna Scaggiante, Maryam Kazemi, Gabriele Pozzato, Barbara Dapas, Rosella Farra, Mario Grassi, Fabrizio Zanconati, Gabriele Grassi

Bruna Scaggiante, Barbara Dapas, Gabriele Grassi, Department of Life Sciences, University Hospital of Cattinara, 34100 Trieste, Italy

Gabriele Pozzato, Fabrizio Zanconati, Maryam Kazemi, Department of Medical, Surgery and Health Sciences, University of Trieste, Cattinara Hospital, 34100 Trieste, Italy

Rosella Farra, Mario Grassi, Department of Engineering and Architecture, University of Trieste, 34100 Trieste, Italy

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Correspondence to: Gabriele Grassi, MD, PhD, Department of Life Sciences, University Hospital of Cattinara, Strada di Fiume 447, 34100 Trieste, Italy. ggrassi@units.it

Telephone: +39-40-3996227 Fax: +39-40-3994593

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turned out not to be of practical utility. This is the reason why active investigations are ongoing in this field. Given the huge amount of published works aimed at the identification of HCC biomarkers, in this review we mainly focused on the data published in the last year, with particular attention to the role of (1) molecular and biochemical cellular markers; (2) micro-interfering RNAs; (3) epigenetic variations; and (4) tumor stroma. It is worth mentioning that a significant number of the HCC markers described in the present review may be utilized also as targets for novel therapeutic approaches, indicating the tight relation between diagnosis and therapy. In conclusion, we believe that integrated researches among the different lines of investigation indicated above should represent the winning strategies to identify effective HCC markers and therapeutic targets.

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Key words: Hepatocellular carcinoma; Biochemical markers; Micro-interfering RNA; Epigenetic variations; Tumor stroma; Angiogenic factors

Abstract

Hepatocellular carcinoma (HCC), the predominant form of primary liver cancer, is the sixth most common cancer worldwide and the third leading cause of cancer-related death. The difficulty to diagnose early cancer stages, the aggressive behaviors of HCC, and the poor effectiveness of therapeutic treatments, represent the reasons for the quite similar deaths per year and incidence number. Considering the fact that the diagnosis of HCC typically occurs in the advanced stages of the disease when the therapeutic options have only modest efficacy, the possibility to identify early diagnostic markers could be of significant benefit. So far, a large number of biomarkers have been associated to HCC progression and aggressiveness, but many of them

Core tip: Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death. The modest efficacy of the therapeutic approaches also depends on the fact that the diagnosis often occurs in the late stages of the disease. Thus, the identification of early markers of onset and progression could be of significant benefit. Here we mainly focus on the latest data pointing the attention towards: (1) biochemical cellular markers; (2) micro-interfering RNA; (3) epigenetic variations; and (4) tumor stroma. The integration of these different lines of investigations should represent promising strategies to identify effective HCC markers.

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INTRODUCTION

Hepatocellular carcinoma (HCC), the predominant form of primary liver cancer is the sixth most common cancer worldwide and the third leading cause of cancer-related death. Almost 80 percent of cases are etiologically related to chronic hepatitis B and C virus infection. Whereas the disease is most common in Asia and Africa, its incidence in the western countries is worrisome increasing. At present, the first line of curative treatment consists of the surgical resection or liver transplantation. The worldwide number of deaths per year in HCC is quite similar to the incidence number, underlying the very aggressive behavior of this disease as well as the poor effectiveness of first and second line of curative treatments. Additionally, as HCC is characterized by fast tumor cell growth, early hepatic metastasis, high grade malignancy and multidrug resistance, the 5-year survival rate is in the range of only 5%^[1].

Effective treatment of HCC remains a big challenge also due to the lack of sensitive, early and specific diagnostic markers. Whereas the molecular mechanisms contributing to carcinogenesis and progression of HCC still need to be fully clarified, many biological molecules have been proposed as relevant players. Some of them are mutually exclusive of neoplastic transformation as in the case of eukaryotic elongation factor 1 A2, a protein normally expressed only by skeletal muscle, heart and nervous system^[2-7]. Thus, a better understanding of the mechanisms underlying these signaling cascades may be of great diagnostic and therapeutic potentials.

A large number of biomarkers associated with HCC progression and aggressiveness has been proposed so far, but most of them turned out not to be of practical utility. This is the reason why the scientific community is actively investigating this field as demonstrated by the number of papers published in 2012 (740 items in PubMed using the key words: "hepatocellular carcinoma" and "marker"). Given the fact that the diagnosis of HCC typically occurs in the advanced stages of the disease when the therapeutic options have only modest efficacy, the possibility to identify early markers could be of significant benefit. In this regard, it should be pointed out that biomarkers can be searched in fresh/paraffin-fixed tissue specimens and in serum, targeting both proteins or nucleic acids^[8].

Given the huge amount of published works aimed at the identification of HCC biomarkers, in this review we mainly focused on the data published in 2013 with particular attention to the role of (1) molecular and biochemical cellular markers; (2) micro-interfering RNAs (miRNAs); (3) epigenetic variations; and (4) tumor stroma.

MOLECULAR AND BIOCHEMICAL CELLULAR MARKERS

Table 1 resumes the most relevant findings with regard to the molecular and biochemical markers below described.

Glypican-3

Glypican-3 (GPC3) is a family member of heparan sulfate proteoglycan membrane proteins. It anchors to the cytoplasmic membrane *via* a glycosyl-phosphatidyl-inositol linkage. GPC3 can regulate in a positive or negative manner the activities of many growth factors depending on the cellular context^[9]. In the adult, the expression of GPC3 can be detected in many types of cancers, including lung carcinoma and ovary adenocarcinoma. GPC3 was first proposed as a potential marker for hepatocellular carcinoma in 1997^[10]. Nowadays it is considered as a relatively specific HCC biomarker being not detectable in hepatic para-carcinomatous and cirrhotic tissues^[11]. It is interesting to note that more than 80% of HCCs over-express GPC3^[12]; additionally, GPC3 is also an interesting therapeutic target for HCC^[9]. In this regard, a humanized monoclonal antibody against GPC3 has been recently developed^[13] and it is under evaluation in a clinical trial phase II in patients with advanced or metastatic HCC (<http://www.cancerresearchuk.org/cancer-help/trials/trial-looking-gc33-advanced-liver-cancer>). Moreover, it is worth mentioning that a vaccine GPC3-derived has been developed and tested in phase I and II clinical trials. The results of these studies are under evaluation^[14].

GPC3 has been also proposed as a marker of HCC progression as it promotes cell growth by stimulating Wnt signal transduction pathway. Wnt is known to be involved in cancer acting as an oncogene able to induce the cytosolic accumulation and nuclear translocation of the transcription factor for the β -catenin; in turn, β -catenin activates the expression of genes promoting cell proliferation and survival^[15]. The progression-associated meaning of GPC3 has been corroborated by a multivariate analysis on patients affected by HCC of different etiology where GPC3 resulted to be a predictor of poor prognosis^[16]; however, in another study, the correlation was found only for HCC developed in HCV positive patients^[17]. In a recent study of 172 patients undergoing liver resection, GPC3 was positively correlated with alpha-fetoprotein (AFP) levels and tumor node metastasis stage. Importantly, an high level of GPC3 in tissue was found to be an independent risk for a shorter post-operative disease-free survival, recurrence and for a decreased overall survival^[18].

Dickkopf-1

Dickkopf-1 (DKK1), a secreted inhibitor of the Wnt/ β -catenin pathway, is a negative regulator of bone formation and plays a role in cell proliferation and survival control. DKK1 is frequently overexpressed in many human tumors where it can promote or suppress tumor

Table 1 Molecular and biochemical markers of human hepatocellular carcinoma progression and poor prognosis

Molecule	Molecular category	Biological function	Expression levels in human HCC tissue or serum	Clinical pathological indications	Target for therapy	Ref.
GPC3	Member of heparan sulfate proteoglycan membrane proteins family	Regulates the signalling pathways of several growth factors, including Wnts	Over-expression; In tissue or serum (protein)	Metastasis, overall survival decrease, recurrence, prognostic value in early diagnosis	Yes	[12,14,16-18]
DKK1	Member of dickkopf family of secreted protein with two cysteine rich regions	Cell proliferation and survival control, differentiation, and cell motility; negative regulator of Wnt/ β -catenin pathway	Over-expression in tissue (mRNA and protein) and in serum (protein)	Vascular invasion and metastasis, early diagnosis in AFP-negative HCC, distinguish between malignant and non-malignant liver diseases	Potential	[22]
S100A4	Member of S100 calcium-binding protein family	Target gene of Wnt/ β -catenin pathway	Over-expression in tissue	Poorly differentiated HCC, vascular invasion and recurrence, overall survival decrease, up to now limited to HBV + HCC	Potential	[24]
S100 calcium-binding protein A14	Member of S100 calcium-binding protein family	Role not yet defined	Over-expression in tissue (mRNA and protein)	HCC vascular invasion and metastasis, disease-free and overall survival decrease	No data	[25]
Sex determining region Y (SRY) related high-mobility group box 6 (SOX6)	Member of the SRY box gene superfamily of transcription factors containing a DNA-binding high mobility group (HMG) domain	Involved in cell embryogenesis, differentiation and proliferation	Decreased expression in tissue (mRNA and protein)	Overall survival decrease, advance tumor stage	No data	[27]
SUOX	Mitochondria member of molybdenum oxotransferases superfamily	Involved in oxidative phosphorylation for ATP generation <i>via</i> electron transport chain	Decreased expression in tissue (protein)	Overall survival decrease in serum AFP positive, with increase of AKR1B10 and CD34 early diagnosis: high grade dysplastic nodules and HCC progression	No data	[28]
xCT	Component of cystine/glutamate transporter designated as system x _c ⁻	In mammalian cells involved in redox balance by mediating cystine entry in exchange for intracellular glutamate	Over-expression in tissue (mRNA)	Overall survival decrease related to the mRNA levels	Potential	[31]
GRK6	Member of the G protein-coupled receptor kinase of the Ser/Thr protein kinase family	Phosphorylates the activated forms of G protein-coupled receptors. It is involved in migration and invasion	Over-expression in tissue (protein)	Aggressiveness related to Ki-67, overall survival decrease	No data	[34]
GPR87	Member of the family of G protein-coupled cell surface receptor related to lysophosphatidic acid receptor family	Antiapoptotic role sustaining p53-mediated pro-survival of cells, involved in cell communication	Over-expression in tissue (protein)	Aggressiveness, intrahepatic metastasis related to CD133 up-regulation	Potential	[37]
MT-1 and MT-2	Members of the cysteine-rich, low molecular weight membrane proteins, localized on Golgi apparatus	Protect from oxidative damage and regulate metals homeostasis (storage, transport and detoxification). Involved in angiogenesis, apoptosis, cell cycle progression and differentiation	Decreased expression in tissue (protein)	Aggressiveness, vascular invasion, recurrence free survival time reduced and overall survival decrease	No data	[39]
RAI3	Member of G-coupled cell surface receptor	Involved in the regulation of cell proliferation	Over-expression in tissue (mRNA and protein)	Aggressiveness, vascular invasion, increased tumor recurrence, shorter overall survival	Potential	[42]
SSX2IP	Spindle protein	Helps centrosome prepare for mitosis. Essential for centrosome maturation	Over-expression in tissue (mRNA)	Larger tumor size and metastasis, overall post-operative survival time reduced	Potential	[44]

Protein Phosphatase magnesium-magnesium-Ppm1d	Member of PP2C family of Ser/Thr protein phosphatases	Inhibits p53 signalling, down-regulates p38 mitogen-activated protein kinase and thus is a putative oncogenetic protein	Over-expression in tissue (mRNA and protein), correlates with high AFP level	Larger tumor size, metastasis, shorter overall survival	No data	[46]
BCL9	Protein associated with B-cell acute lymphoblastic leukemia	Involved in signal transduction through Wnt pathway, promotes β -catenin's transcription activity	Over-expression in tissue (protein)	Aggressiveness, vascular invasion and metastasis. Shorter disease free status after hepatectomy	No data	[47]
IRF-1 and IRF-2	Belongs to transcription factors, member of the interferon regulatory factor family	IRF-1 and its functional antagonist IRF-2 are involved in the modulation of IFNs	IRF-1 decreased expression or IRF-2 over-expression in tissue (protein)	Aggressiveness, shorter overall survival and early recurrence	No data	[49]
CDK4	Member of the Ser/Thr kinase family	Controls the G1-S phase by phosphorylation of retinoblastoma protein	Over-expression in tissue (mRNA and protein)	Larger tumor size, HBV and shorter median survival	potential	[50]
LASP-1	Not yet elucidated	Involved in actin assembly	Positive cytosolic expression in tissue (protein)	Poorer survival	No data	[51]
PTP4A3	Member of PRL subgroup of protein tyrosine phosphatases	Involved in signaling pathways modulating cell proliferation, growth and motility	Over-expression in tissue (protein)	Overall survival decrease and shorter recurrence free survival	Potential	[52]
Steroyl-CoA-desaturase	Endoplasmic reticulum enzyme that catalyzed the biosynthesis of monounsaturated fatty acids	Catalyzed the D ⁹ -cis desaturation of a range of fatty acyl-CoA substrates such as palmitoyl- and stearoyl-CoA (converted into palmitoleoyl- and oleyl-CoA, respectively)	Over-expression in tissue (mRNA)	Distinguishes between high and low risk HCC, positively related to AFP levels, tumor size and staging	Yes	[54]
PAK5	Member of the p21-activated kinase (Pak) family that serves as target for GTP-binding proteins involved in modulation of cell shape, movement, proliferation and survival	A mediator of filopodia formation	Over-expression in tissue (mRNA)	Early diagnosis and possibly indicator of HCC progression	Potential	[56]
hnRNP-L	Member of the hnRNPs that are involved in the processing of pre-mRNAs	Regulator of alternative splicing with both activator and repressor functions (retention and skipping of the exons)	Over-expressed (serum and tissue)	Progression in HBV-HCC patients, tumor size increase and overall survival decrease that correlates with high serum levels	Potential	[57]
CYLD	Protease, displaying endodeubiquitinase activity. Specifically cleaves 'Lys-63'-linked polyubiquitin chains.	Involved in the regulation of cell survival, proliferation, and differentiation. Plays an important role in pathways activating NF- κ B and is a negative Wnt regulator. Involved in modulation of cell polarization, migration and angiogenesis	Downregulation at mRNA and protein levels (tissue)	Relates to resistance to chemotherapeutic agents	Yes	[58]
MAGE-D4	Component of the MAGE family protein	Expressed during development and involved in regulation of cell survival, cell cycle progression and apoptosis	Over-expression at mRNA and protein levels (tissue)	Relates to HCC poor differentiation and invasion. Marks early recurrence and decrease overall survival	No data	[59]

EphA3	Member of the receptor of tyrosine kinase family	Involved in development, particularly of the nervous system. Binds to ephrin-A ligands (especially EFNA5). Regulates cell-cell adhesion, cytoskeletal organization, cell differentiation and migration	Over-expression at protein level (tissue)	Decrease in overall survival	potential	[60]
Flot-1	Member of the Flotillin subfamily	Acts as a scaffolding protein within caveolar membranes and mediates cell signalings. Involves in the formation of caveolae and caveolae-like vesicles to regulate membrane trafficking. Participates to cytoskeleton organization, cell adhesion and invasion	Over-expressed at mRNA and protein levels (tissue)	High levels relates to increase tumor size, invasion and short disease-free time and overall survival	No data	[61]

GPC3: Glypican-3; DKK1: Dickkopf-1; S100A4: S100 calcium-binding protein A4; SUOX: Sulfite oxidase; xCT: Cystine/ glutamate transporter; GRK6: G protein-coupled receptor kinase 6; GPR87: G protein-coupled receptor 87; MT-1: Metallothionein 1; MT-2: Metallothionein 2; RAI3: Retinoic acid-induced protein 3; SSX2IP: Synovial sarcoma X break point 2 interacting protein; Ppm1d: Protein phosphatase magnesium-dependent 1d; BCL9: B-cell CLL/lymphoma 9 protein; IRF-1: Interferon regulatory factor-1; CDK4: Cyclin-dependent kinase 4; LASP-1: LIM and SH3 protein 1; PTP4A3: Protein tyrosine phosphatase type IVA member 3; PAK5: P21-activated kinase 5; hnRNP-L: Heterogeneous nuclear ribonucleo protein L; CYLD: Cylindromatosis (turban tumor syndrome); MAGE-D4: Melanoma-associated antigen D4; EphA3: Ephrin receptor A3; Flot-1: Flotillin-1.

growth, depending on the tumor type^[19]. In HCC, DKK1 expression was found to be significantly up-regulated. The identification of DKK1 in the serum allowed the detection of early stages of HCC development^[20] and the identification of HCC in patients negative for the tumor marker AFP or to distinguish, in AFP positive patients, between HCC and chronic hepatitis B or liver cirrhosis^[21]. In HCC tissue samples, the increased expression of DKK1 correlated with poor overall and disease-free survival, indicating its prognostic value^[20]. Finally, as DKK1 up-regulation promotes invasion and metastasis in HCC, probably *via* a non-canonical Wnt pathway, it has been proposed as a useful therapeutic target^[22].

S100A4

The up-regulation of S100 calcium binding protein A4 (S100A4), a member of the calcium-binding protein family, has been related to the metastatic cancer phenotype in a wide spectrum of cancers^[23]. In HCC, a retrospective study showed that the expression levels of S100A4 correlated with tumor differentiation, invasion, recurrence, and overall survival. Notably, undifferentiated HCC tumors expressed higher levels of S100A4 compared to well-differentiated tumor forms. Additionally, increased levels of S100A4 were detected in HCC tumors with vascular invasion and recurrence^[24]. It is worth mentioning that in this study the majority of the samples derived from patients with HBV-induced HCC; thus it has to be pointed out that the usefulness of S100A4 as a marker for HCC progression needs to be confirmed in other HCC types non promoted by HBV infection.

S100A14

A novel member of S100 protein is S100A14. In fresh

HCC tissues with and without vascular invasion, the mRNA and protein levels of S100A4 were found to correlate with multiple tumor nodes and vascular invasion. Moreover, S100A14 detection correlated with both shortened disease-free survival and overall survival in HCC patients, thus proving its potential as prognostic factor. The role of S100A14 in sustaining HCC proliferation, migration and invasion was confirmed in HCC cell culture and *in vivo* (mice) analysis, thus supporting the role of S100A14 in sustaining HCC metastasis^[25].

SOX6

SOX6 belongs to the high mobility group (HMG)-containing domain proteins whose biological role has been poorly investigated in humans so far. SOX proteins play part in embryogenesis regulating cell differentiation and proliferation. In particular SOX6 has a primary role in erythropoiesis and it seems to contribute to stem cell phenotype^[26]. The decreased expression of SOX6 in HCC tissue samples, has been correlated with advanced tumor stage and less than 5-year-disease free overall survival in HCC patients. Notably, whereas in fresh and paraffin-embedded HCC human tissues SOX6 expression is down-regulated, in adjacent non-neoplastic tissues its level is un-affected. Therefore, reduced SOX6 expression is now considered a poor prognostic factor in HCC^[27].

SUOX, AKR1B10 and CD34

Sulfite oxidase (SUOX), aldo-ketoreductase family 1 member B10 (AKR1B10) and CD34 markers, alone or in combination, have been very recently used for the differential diagnosis between high-grade dysplastic nodules and well-differentiated HCC. During hepatocarcino-

genic progression, SUOX levels were decreased, while AKR1B10 and CD34 showed elevated levels. Moreover, when combined with serum AFP, SUOX can independently predict post-surgical outcome, as well as the risk of tumor recurrence^[28].

Cystine/glutamic acid transporter

Cystine/glutamic acid transporter (xCT) regulates the redox state in the cell by acting as an exchange system for cysteine/glutamine to produce glutathione (GSH), a major cellular antioxidant. GSH availability is limited by the amount of the sulfhydryl amino acid cysteine, which is readily oxidized to cystine and taken up by the Na⁺-independent cystine/glutamate exchange transporter (system x_c⁻). System x_c⁻ is composed of a light-chain subunit named xCT, which confers substrate specificity, and a glycosylated heavy-chain subunit (4F2hc or rBAT) common to the transporter family. xCT has been demonstrated to play a role in many cancers regulating the redox state of the tumor cells^[29]. Recently, xCT has been proved to sustain the viability of the cancer stem cells^[30]. In HCC fresh tissues it was found that xCT expression was higher than in normal liver tissues. Moreover, poor prognosis of HCC patients correlated with tumor size and the amount of xCT mRNA, indicating that xCT expression level can be used as a marker of HCC aggressiveness. Additionally, xCT has been proposed as a novel potential molecule for targeted therapy^[31].

GRK6

G protein-coupled receptors belong to a superfamily of cell surface molecules known to regulate cell proliferation, migration and survival. G protein-coupled receptor kinase 6 (GRK6) is a member of the guanine nucleotide-binding protein (G protein); it is bound to the receptor kinase subfamily belonging to the Ser/Thr protein kinase family^[32]. GRK6 regulates the activity of chemokine receptor and it is implicated in the modulation of cell adhesion^[33]. A study performed using fresh and paraffin-embedded human tissue samples from HCC patients, demonstrated that GRK6 expression is higher in cancerous cells compared to the peri-tumor cells. Additionally, GRK6 expression level was positively related to the aggressiveness of the tumor and with the cell proliferation marker Ki-67. Finally, GRK6 expression level resulted to be negatively correlated with the survival rate. Thus GRK6 is considered a potential useful marker to predict prognosis of HCC patients^[34].

GPR87

G protein-coupled receptor 87 (GPR87) can, among its functions, mediate p53 pro-survival effect^[35]; additionally, it is frequently overexpressed in cancer^[36]. GPR87 was found to be up-regulated in CD133⁺ HCC cancer stem cells (CSCs) and to promote their survival *in vitro*. Notably, GPR87 favors CSCs migration and invasion as shown by the fact that GPR87 knock down significantly reduced these properties in CSCs. CSCs are proposed

as key players in the development of different tumors, including HCC. For example, the knocking down of GPR87 expression resulted in the decrease of CSCs cell migration and invasion in mice^[37]. Together, these data suggest a strong connection between GPR87 expression and CSCs, thus indicating GPR87 as a potential marker of HCC tumor development.

Metallothioneins

A group of intracellular metal binding proteins, called metallothioneins (MTs)-1 and -2 play distinct roles in metal homeostasis and in different cellular events including carcinogenesis^[38]. The presence of MT-1 and -2 in the nuclei of rapidly growing cells is mainly correlated with their functions to provide zinc to the enzymes involved in the synthesis of nucleic acids or to chelate zinc from transcriptional factors, respectively. The expression of these two proteins is up-regulated in a variety of cancers, contributing to aggressiveness, poor prognosis, as well as to radiation and drug-resistance. In contrast to many other cancers, in HCC tissues, MT-1 and MT-2 expression, evaluated by microarray, has been found to be reduced, a fact correlated with a poor prognosis^[39]. Additionally, reduced expression levels have been correlated to higher HCC grade and tumor vascular invasion^[40]. Together these data indicate that the down-regulation of MT-1 and MT-2 may be undertaken as a poor prognostic factor for HCC patients.

Retinoic acid-induced protein 3

Retinoic acid-induced protein 3 (RAI3) is a member of G-protein-coupled receptors (GPCRs). It acts as a pivotal tumor suppressor or oncogene, depending on the human cancer type^[41] where its expression can be either down or up-regulated. In HCC, increased expression of RAI3 has been associated with malignant progression and poor outcome after hepatic resection. Moreover, it has been considered an attractive candidate for targeted therapies^[42].

Synovial sarcoma X breakpoint 2

Synovial sarcoma X breakpoint 2 interacting protein (SSX2IP), initially identified as an acute myeloid leukemia associated antigen, has been recently proved to play an important role in centromere maturation and thus in the correct spindle formation^[43]. In HCC cell cultures and in mice, overexpression of SSX2IP promoted migration and invasion as well as drug-resistance. Additionally, the expression levels of SSX2IP in tumor tissues correlated with tumor size, tumor thrombosis and reduced survival of HCC patients. Thus SSX2IP may represent an interesting marker for HCC progression and a new target for therapeutic treatments^[44].

Protein phosphatase magnesium-dependent 1 delta

Protein phosphatase magnesium-dependent 1 delta isoform (Ppm1d) also known as Wip 1 (wild type p53 induced protein phosphatase 1) belongs to Ser/Thr protein phosphatase 2C (PP2C) family. Ppm1d is involved in G₂/

M transition and acts as a regulator of cell proliferation. In particular it inhibits p53 and p38 functions thus being recognized as a putative oncogene^[45]. Ppm1d is overexpressed in fresh HCC tissues compared to non-cancerous liver tissues. Notably, high levels of PPMD1 mRNA correlated with tumor size and stage and with poor patient's prognosis. Thus PPMD1 can be considered an interesting potential marker of HCC progression^[46].

BCL9

BCL9, a gene derived from the translocation t(1;14) (q21;q32), is overexpressed in B-cell acute lymphoblastic leukemia and plays a role in Wnt/ β -catenin signaling pathway thus contributing to cancer development and progression. Immuno-histochemical staining of primary HCC tissues indicated that BCL9 protein overexpression is related to younger age, higher Edmondson grade, tumor microvascular invasion and intrahepatic metastasis. Moreover, increased expression levels of BCL9 correlated with a lower five-year disease free survival rate, thus making of this protein a novel potential marker of HCC prognosis^[47].

Interferon regulatory factor-1 and 2

Interferon regulatory factor-1 and 2 (IRF-1, IRF-2) are transcription factors that mediate interferon (IFN) functions. As these two transcription factors compete for the same DNA sequence, they are each other functional antagonist. Many evidences confer to IRF-1 and 2 a role in tumor onset and progression: in particular, the first is considered a tumor suppressor while the second an oncogene^[48]. In HCC tissues both IRF-1 and 2 proteins localize in the nucleus. Whereas low levels of nuclear IRF-1 correlate with a poor outcome, low levels of IRF-2 correlate with a favorable outcome. Moreover, analyzing HCC cancer cell lines with low, moderate or high metastatic potential, correlations between the ratio IRF-2/IRF-1 and cell phenotype were observed. Thus IRF-1 and IRF-2 expression and their ratio may be considered relevant with regard to HCC prognosis^[49].

CDK4

CDK4 is a member of the cyclin - dependent Ser/Thr protein kinase family that plays an important role in G₁-S cell cycle phase progression by phosphorylating the retinoblastoma protein. CDK4 overexpression has been observed in many cancers. In HCC tissues, the analysis of mRNA and protein expression showed that CDK4 is frequently (68%) overexpressed. Notably, CDK4 expression directly correlates with tumor stage, size and poor survival rate^[50].

LASP-1

LIM and SH3 protein 1 (LASP-1) is a protein of not yet well-characterized functions. It is known to participate in cell motility and its up-regulation has been associated with invasive cancers. The immune-reactivity of cytosolic LASP-1 in HCC tissues correlates with overall patients'

poor survival. *In vitro* LASP-1 was found to promote migration of HCC cell lines and its silencing strongly reduced migration. Thus, these findings suggest that the cytosolic elevation of LASP-1 levels may represent a marker of poor outcome and a potential target for therapeutic approaches^[51].

PTP4A3

Protein tyrosine phosphatase type IVA member 3 (PTP4A3) is also known as phosphatase of regenerating liver-3 (PRL-3). It belongs to the PRL family of enzymes that regulates the signaling cascade for the maintenance of cellular viability and growth. By microarray gene expression assay, PTP4A3/PRL-3 was found to be overexpressed in poorly differentiated HCC tissues; additionally, its overexpression was related to a reduced overall survival. The close association between PTP4A3/PRL-3 expression and HCC progression, invasion and metastasis suggests that PTP4A3/PRL-3 could be a prognostic marker of poor outcome^[52].

Fatty acid

The lipid metabolites derived from the activity of the stearoyl-CoA-desaturase (SCD) enzyme, a modulator of fatty acid flux, were independently associated with HCC outcome. The potential of SCD as a biomarker of poor outcome was demonstrated in a panel of HCC cell lines with increasing aggressive features. Additionally, high levels of SCD were detected in HCC specimens and were correlated with a short disease-free survival. Notably SCD expression was found to predict the prognosis both in HCC associated and HCC non-associated hepatitis B infection^[53]. How SCD contributes to HCC aggressiveness remains to be defined. It has been suggested that the increased levels of the mono-unsaturated palmitoleate (MUPA), derived from the SCD enzymatic conversion of saturated palmitic acid (SPA), negatively affect Kupffer cell activation, cytokine expression and increase liver fibrosis. Finally an increased MUPA level promotes and sustains metabolic syndrome and inflammation, factors favoring tumor growth and progression^[54,55].

PAK5

P21-activated kinase 5 (PAK5) represents the latest family member of P21-activated kinases (PAKs) with Ser/Thr kinase activity. Expression of *PAK5* gene in 25 out 30 HCC tissues has been demonstrated to be highly elevated with respect to the surrounding paraneoplastic tissue, making this gene an interesting diagnostic/prognostic marker for HCC^[56].

hnRNPL

Humoral immunity response to tumor antigens (autoantibodies), known as tumor-associated antigens (TAAs), can occur in cancers and can be used to detect tumor presence. Heterogeneous ribonuclear protein L (hnRNP L) is a component of the hnRNP complexes involved in the processing of the pre-mRNA. By immune-proteomic

screening of HBV-HCC sera, hnRNP L has been recognized as a TAA in HCC. TAAs against the N-terminal glycine-rich region of hnRNP L (polypeptide sequence 67-88) were detectable in 60% of the serum samples from patients with HBV-related HCC but neither in normal controls nor in HBV-related liver cirrhosis serum samples. Interestingly, the titer of autoantibodies against hnRNP L 67-88 epitope, positively correlated with the increase in tumor size and with a reduced patients' survival. Moreover, in HCC cell cultures hnRNP L knocking down demonstrated that this protein sustains the growth, migration and invasion of the cancer cells. Thus the N-terminal epitope of hnRNP L is a potential biomarker for HCC progression at least in HBV-associated HCC and a potential candidate for HCC therapeutics^[57].

Cylindromatosis gene

The tumor suppressor cylindromatosis gene (CYLD) codifies an ubiquitin carboxyl terminal hydrolase, a protease able to cleave Lys-63-linked polyubiquitin chains in proteins. CYLD plays important roles in the regulation of cell survival/proliferation/differentiation, as a proapoptotic factor and as a regulator of the pathways connected to NF- κ B activation. In immuno-histochemical and tissue microarray analysis of HCC tissues, CYLD mRNA and protein levels were found to be considerably down-regulated in tumor sections with respect to surrounding non-malignant tissue. Interestingly, in cell cultures, the down-regulation of CYLD by siRNA was found to relate to an increase of HCC cells resistance to chemotherapy and to TNF- α -induced apoptosis. Moreover, in HCC cells, CYLD was found to be a negative modulator of NF- κ B activity; importantly, CYLD expression can be restored by inhibition of EGFR-Raf-MEK-ERK signaling cascade as demonstrated by the use of EGFR tyrosine kinase inhibitor AG1478. Thus, CYLD could be a promising marker for HCC aggressiveness and progression, as well as an interesting target for HCC therapy^[58].

Melanoma-associated antigen family protein D-4

Melanoma-associated antigen family protein D-4 (MAGE D-4), originally termed MAGE-E1, is a MAGE family gene expressed at high levels in malignant tumors. Overexpression of MAGE D-4 mRNA was found in 34 out of 94 HCC tissue samples; additionally, the expression levels of MAGE D-4 as well as of the MAGE D4b protein were found to be directly related to HCC aggressiveness. Moreover, MAGE D-4 expression in combination with the AFP level, correlates with a poor tumor differentiation and vascular invasion, thus underlying the potential of MAGE-D4 as marker for early tumor recurrence and poor survival outcome^[59].

EphA3

Ephrin receptors, the largest family of tyrosine kinases interacting with ephrins, are involved in a variety of cell functions including cell motility and adhesion. The eph-

rin receptor EphA3 is mainly localized in the cytoplasm and plasma membrane of cells. The down-regulation of EphA3 expression in HCC cell lines determines a decrease in the cell invasion capacity, suggesting its possible role as a therapeutic target. Finally, the overexpression of EphA3 in HCC tissues correlates with a decrease in the overall survival of patients^[60].

Flotillin-1

Flotillin-1 (Flot-1) is a member of the membrane proteins belonging to the ubiquitarily expressed lipid raft family. These proteins take part in the regulation of many cellular processes as membrane receptor signaling factors and regulate membrane trafficking, cytoskeleton organization, cell adhesion and migration. Flot-1 acts as a scaffolding protein within caveolae and is involved in the formation of caveolae; its de-regulation has been documented in many human cancers. Flot-1 was found overexpressed both at mRNA and protein levels in HCC cell lines. In HCC archive tissue samples, high level of Flot-1 protein correlates with an increase of tumor size, invasion, recurrence and shorter patients' survival time. Flot-1 levels thus could mark the aggressive HCC status and the poor prognosis of HCC patients^[61].

MICRORNAS

In the last seven years the role of miRNAs in HCC development has become evident. MicroRNAs are small (approx 18-25 nt) non-coding double-stranded RNAs with the capacity to regulate the expression of target genes mainly by impairing mRNA translation, but also by inducing mRNA degradation^[62,63] and by affecting chromatin remodeling^[64]. The miRNA pathway starts with the transcription of a long precursor defined primary miRNA (pri-miRNA), which is subsequently processed (pre-miRNA) in the nucleus by a cellular enzyme called Drosha. The pre-miRNA is then exported from the nucleus to the cytoplasm where it is processed by the DICER enzyme complex to generate an approximately 22 bp long RNA duplex. The mature miRNA is then loaded onto RNA-induced silencing complexes (RISC) whose best-known component is the Argonaute (Ago2) protein. Within the two miRNA strands, the "sense" is discarded while the other, "antisense", is selected and used by RISC to bind complementary target RNA determining the so called "interference process". It should be noted that miRNA action tends to be highly redundant as multiple miRNAs can regulate the same transcript and a single miRNA can regulate several transcripts.

Due to their capacities to control gene expression behaving like tumor suppressors or oncogenes, miRNAs are considered to play a crucial role in carcinogenesis. The key role of many miRNAs in the control of cell proliferation, apoptosis and invasion underlies the relevance of their de-regulation in promoting cancer^[65]. The frequent de-regulation of miRNAs in various human cancers, has lifted these small molecules to the ranks of attracting

pharmaceutical targets^[66]. miRNAs are also considered amenable anticancer targets because of the wide spectrum of oncogenic targets they can control. There are two strategies to develop miRNA-based therapies: (1) microRNA antagonists, typically represented by antisense DNA oligonucleotides, which inhibit microRNAs by binding to the antisense strand of the miRNA; and (2) miRNA mimics, represented by short double stranded RNA molecules mimicking the miRNA of interest, which are used to simulate miRNAs function^[67]. A relevant number of miRNAs have been proved to be down-regulated in HCC (see^[68] for a comprehensive review). Here we focused on some new discoveries (Table 2) of miRNAs involved in HCC progression.

miRNA-122 is the best studied miRNA in liver; not only it plays an important role in maintaining the normal liver phenotype, its deregulation has been related to an increased risk to develop HCC^[69]. Another HCC related miRNA is miR-181b. Upon HCC cells exposure to transforming growth factor (TGF)- β 1, the expression of miR-181b is up-regulated with the consequent promotion of cell growth, survival, migration and invasion^[70]. TGF- β 1 can also induce the expression of miR-23a, 27a, and 24, which are involved in HCC cell growth and survival too^[71].

In HCC cell lines and in HCC human tissue samples the overexpression of miR-18b is related to tumor progression and metastatic potential. Additionally, miR-18b overexpression is associated with a poor HCC prognosis. The tumorigenic effect of this miRNA seems to be related to the down-modulation of its target gene trinucleotide repeat containing 6B (TNRC6B), whose reduction promotes the metastatic potential of HCC cells. TNRC6B is a RRM-protein that mediates miRNA-guided mRNA cleavage^[72]. Recently TNRC6B has been indicated to contribute to breast cancer metastasis probably acting like a transcription factor^[73].

miR-372 regulates cell cycle, pro-survival signals and proliferation in cancer cells. In retrospective HCC human tissue samples, it was observed that miR-372 is overexpressed compared to the peri-tumor tissue; this observation was confirmed in HCC cell line compared to normal human hepatocytes. From the clinical point of view, miR-372 overexpression correlates with tumor aggressiveness and overall shorter patients survival. Because of all these observations, miR-372 has been suggested as a potential therapeutic target for HCC^[74].

miR-650, a novel miRNA overexpressed in gastric cancer, promotes cell proliferation by binding to the inhibitor of growth family 4 (ING4)^[75,76]. miR-650 was also found to be overexpressed in HCC tissues; notably, the highest levels were found in younger patients and in poor differentiated tumors.

miR-100 functions as a tumor suppressor and is often de-regulated in many cancers. Down-regulation of miR-100 in HCC tissues was found to positively correlate with higher tumor grade, metastasis and recurrence; it also represents an independent factor of poor prognosis.

Moreover, *in vitro* up regulation of miR-100 was proved to inhibit HCC cell growth and to promote cell apoptosis *via* down-regulation of the polo like kinase-1. Thus it could be also an interesting new target for HCC therapy^[77].

Recently it has been proposed that miRNAs may not only promote HCC when over-expressed in the cellular environment, but also when released into the extracellular environment by the producing cells. Thus, HCC cells can also condition the tumor microenvironment^[78] (for a comprehensive review see^[79]).

A mechanism through which miRNA expression can be de-regulated in HCC cells includes the modification of the methylation pattern of the miRNA promoters. For example, it was demonstrated that HBx, the oncoprotein encoded by the hepatitis B virus (HBV), causes the hyper-methylation of the promoter of miR-132 thus down-modulating its expression; this in turn results in the activation of Akt-signaling pathway which promotes HCC development in HBV positive patients^[80].

The effects of miRNAs on HCC biology are not limited to miRNA up/down regulation. It is indeed possible that in the presence of physiological miRNA levels, the specific miRNA cannot control the expression of its target. This condition takes place when nucleotide polymorphisms/deletions/insertions/mutations occur either in the miRNA or in the target sequence. Since the binding of a miRNA to its target mRNA depends on a certain degree of complementarity, the presence of nucleotide polymorphisms/deletions/insertions/mutations may alter the binding capacity thus preventing/minimizing miRNA function. For example, a "TTCA" insertion disrupts the binding site for miR-122 and miR-378m to interleukin (IL)-1 α leading to the up-regulation of IL-1 α expression and the promotion of HCC development^[81].

Recent studies indicate that among the molecular mechanisms de-regulating miRNA functions in tumors, a relevant role is played by the miRNA biogenesis-related genes (miRBir genes). These genes codify for proteins involved in miRNA processing and miRNA-induced silencing. Among these, it should be mentioned the Ago2 protein. Ago2 plays a paramount role in RNA-silencing as it cleaves the miRNA targeted mRNA^[82]. *In vitro* and *in vivo* studies showed that Ago2 overexpression promotes HCC cell proliferation and migration. Moreover, mice injected by HCC cells with different aggressive phenotypes and containing the recombinant adenovirus harboring Ago2, developed tumors and metastasis. In contrast, non-visible or smaller tumors were found in control mice injected with the same HCC cells but containing an Ago2 empty adenovirus. Notably, the knocking down of Ago2 significantly reduced and in some cases suppressed cell tumor migration and metastasis. To further point towards a role of Ago2 in HCC, there is the observation that Ago2 is frequently found to be overexpressed in HCC tissues^[83]. It seems that Ago2 tumorigenic potential may be due to the concomitant overexpression of the focal adhesion kinase (FAK), a protein tightly involved in tumor metastasis^[83].

Ago2 is not the only miRBir genes involved in HCC.

Table 2 Micro-interfering RNA, miRBir and epigenetic markers of human hepatocellular carcinoma progression and poor prognosis

Molecule	Molecular category	Biological function	Expression levels in human HCC tissue	Clinical pathological indications	Target for therapy	Ref.
miRNA-18b	Belongs to Oncomir-1 or miR17-92 cluster	Involved in embryogenesis and spermatogenesis	Over-expressed	Poor differentiated HCC, high levels positively correlate with lower survival time	Potential	[70]
miRNA-372	Oncomir	Not well defined: it can act as an oncomir or as an anti-oncomir depending on the malignancy	Over-expressed	High levels positively correlate with advance TNM stage, shorter recurrence-free survival and overall survival	Potential	[74]
miRNA-650	Oncomir	Targets protein involved in cell proliferation and survival	Over-expressed	Higher levels in less than 60-year-old patients; levels positively correlate with HCC differentiation status	Potential	[75,76]
miRNA-100	Tumor suppressor	Regulates cell differentiation and survival	Down-regulated	Levels negatively correlate with tumor grade, metastasis and recurrence	Potential	[77]
miRNA-132	Tumor suppressor	Involved in the repression and control of inflammation and in neuronal morphogenesis	In HBV-HCC down-modulated by HBx protein causing promoter methylation	Levels of miR-132 inversely correlate with those of HBx in HBV-HCC patients. Early diagnosis of HBV-HCC	Potential	[80]
Ago2	Belongs to eIF2C/AGO subfamily	One of the effectors of RNA interference either at initiation or elongation phases, endowed with PIWI domain (endonuclease activity)	Overexpressed (mRNA and protein levels) and gene amplification	Can signed metastasis	No data	[83]
Dicer	Belongs to the RNase III family	Cleaves double-stranded RNA and pre-miRNA in the short (20-25 nt) active miRNA	Down-regulated	Shorter recurrence-free survival time	No data	[84]
TFPI2 gene	Member of the Kunitz-type serine protease inhibitors gene	The encoded protein inhibits plasmin-and trypsin-mediated activation of matrix metallo-proteinases.	Up-regulation of gene promoter methylation (HCC tissues and serum)	TFPI2 methylation levels positively correlate with high grade TNM, improved detection of HCC in patients with AFP levels less than 400 µg/L	No data	[87]
Ten-eleven translocation protein 1 gene	Member of the TET family of methylcytosine dioxygenase enzymes gene	The encoded protein convert 5-methylcytosine to 5-hydroxymethylcytosine (5hmC) in various embryonic and adult tissues	Up-regulation of promoter methylation	Low levels of TET 1 and 5hmC relates to shorter overall survival	No data	[89]
MAT1A, MAT2A genes	Member of the SAM synthesizing isozymes genes	The encoded enzymes are essential to SAM biosynthesis. MAT1A is expressed in mature liver, whereas MAT2A is expressed by fetal liver, extrahepatic tissues or in HCC.	Up-regulation of MAT1A promoter methylation and down-regulation of MAT2A promoter methylation	Based on in cell culture and animal models studies, decreased MAT1A/MAT2A expression ratio has been proposed as a poor prognostic factor in HCC patients	No data	[90]

TFPI2: Tissue factor pathway inhibitor-2; Ago2: Argonaute-2; MAT1A: Methionine adenosyltransferase; HCC: Hepatocellular carcinoma; HBx: Hepatitis B virus x; SAM: S-adenosylmethionine.

In this regard, it has been observed that Dicer and Ago3/Ago4 down-regulation is related to HCC promoting risks. In particular, their down-regulation was observed in non-cancerous samples derived from patients with smoking or alcohol intake habits. Finally, a decreased expression of Dicer genes was of prognostic significance of shorter recurrence-free survival time^[84].

EPIGENETIC VARIATIONS

Modification of accessibility of the transcription machinery to gene promoters is one of the mechanisms regulating gene expression in mammalian cells. Although several mechanisms concur to regulate this process, the status of DNA methylation/de-methylation is of major relevance. DNA methylation is mediated by a class of enzymes

called DNA methyltransferases which covalently link a methyl group to the cytosine residue within 5'-CpG-3' palindromes. While 5'-CpG-3' palindromes cluster (CpG islands) in the promoters of about half of all human genes, they are rare in other regions of the human genome, indicating their role in the regulation of gene expression. Following methylation, particular proteins containing a methylcytosine binding domain are recruited and linked to the methylated DNA, thus repressing gene transcription by blocking the access of transcription factors to the gene promoter. Thus, the preservation of the physiological methylation pattern of gene promoters is critical for the homeostasis of the normal cells. In contrast, alteration of promoter methylation patterns is commonly found in tumor cells including HCC cells^[85] (Table 2). For example, the tissue factor pathway inhibitor-2

(TFPI2) gene encodes a serine protease inhibitor that is secreted into the extracellular matrix by specialized cells (*i.e.*, endothelial, smooth muscle, fibroblasts, keratinocytes and urothelium). Once secreted, it inhibits plasmin and trypsin-mediated activation of the matrix metalloproteinases (MMPs, see section 5.3 for more MMPs biological details). Inhibition of MMPs activation in turn results in a reduced invasion capacity of the tumor cell. Thus, the down-regulation of TFPI2 expression by promoter methylation reduces the inhibition of MMP activation favoring cell migration and metastasis. Notably, aberrant *TFPI2* promoter methylation has been detected in circulating cells of patients with colorectal cancer but not after complete surgical resection of the tumor^[86]. In HCC serum samples, the degree of *TFPI2* promoter methylation in circulating cell-free tumor DNA was found to positively relate to tumor stage^[87]. Thus, *TFPI2* promoter methylation is becoming to be considered a potential marker for HCC diagnosis and progression.

In normal mammalian cells, the methylation pattern can be reversible; this phenomenon, although not yet elucidated in detail, depends on three enzymes ten-eleven translocation (TET)1, TET2 and TET3, which belong to the TET family. TET1-3 is necessary to convert the 5-methyl-cytosine (5mC) into 5-hydroxy-methyl-Cytosine (5hmC) to favor promoter de-methylation. Thus, promoter methylation status is based on the balance between 5mC and 5hmC^[85]. In cancer cells 5hmC is frequently decreased compared to 5mC, indicating a hyper-methylated status of gene promoter^[88]. The reduction of 5hmC in HCC tissue was observed in a rat model of diethyl-nitrosamine-induced liver cancer. Even more interestingly, in 146 human HCC tissue specimens, 5hmC was found to be significantly decreased in 69% of the samples compared to non-tumor tissues. Notably, patients in the 69% group were found to be associated with a shorter overall survival^[89]. The fact that TET1 but not TET2 or TET3 proteins, was significantly decreased in HCC fresh human tissue specimens compared to non-tumor tissues, indicates a major role of TET1 over the other two members in regulating 5hmC/5mC balancing.

Methionine adenosyltransferase 1 alpha (MAT1A) gene encodes for an enzyme able to transfer the adenosyl moiety of ATP to methionine to form *S*-adenosyl-methionin (SAM). The liver is the most relevant source of SAM that in turn is the main source of methyl groups for biological methylations. The *MAT1A* gene products are iso-enzymes organized either in homo-tetramer (MAT I) or in homo-dimer (MAT III); a third iso-enzyme, MAT II, is encoded by another gene named MAT2A. Both *MAT1A* and *MAT2A* genes can be epigenetically modulated. Down-regulation of MAT1A expression with the consequent reduction of the SAM levels induces HCC cell proliferation and promotes neo-vascularization. Since MAT I:MAT II ratio can significantly predict patient's survival, it has been proposed that expression ratio of MAT1A:MAT2A can be used as a potential prognostic marker for HCC^[90]. Finally, liver

MAT2A expression is substituted in adult life by MAT1A and the induction of the non-liver specific *MAT2A* gene is believed to contribute to hepato-carcinogenesis by stimulating liver cell proliferation^[91].

TUMOR STROMA

During recent years, a large body of literature has considered the cross-talk between tumor cells and their surrounding stroma as a fundamental step in the process of HCC progression, epithelial-to-mesenchymal transition (EMT), tumor invasion and metastasis^[92-95]. Tumor microenvironment (TME) is a dynamic system orchestrated by inflammatory and cancer cells, as well as stromal tissue (immune cells, fibroblasts, myofibroblasts, cytokines, and vascular cells), and extracellular matrix^[96]. TME can affect various aspects of tumor biology, including development, progression and therapy response. Stromal cells contribute to the hallmarks of cancer by preserving proliferative signaling, preventing growth suppressors, arresting apoptosis, inducing angiogenesis, stimulating invasion and minimizing immune destruction^[97,98]. Cell components of the TME can be originated either from nearby or distant tissues; this last event seems to be due to the fact that in the case of rapid cancer development, resident cells may be unable or numerically insufficient to support the requirements of tumor growth. In line with these findings, improving the knowledge on the cross-talk between cancer cells and their neighboring environment seems an emerging challenge for identifying novel molecular targets with diagnostic and prognostic potentials for many tumors including HCC.

TME, which plays a crucial role in the initiation and progression of HCC, mainly consists of: (1) a cellular component which includes hepatic stellate cells, fibroblasts, a vast range of immune cells such as tumor-associated macrophages and endothelial cells embedded into the extracellular matrix; (2) secreted factors including cytokines and growth factors such as TGF- β 1; and (3) proteolytic enzymes such as MMPs.

Cellular components

Among the components of tumor stroma, cancer-associated fibroblasts (CAFs), an heterogeneous population of stromal cells, are the most abundant cell type. During cancer progression, stromal fibroblasts, the major source of CAFs, undergo a process called "fibroblast to myofibroblast trans-differentiation"^[99]. This event is triggered by growth factors such as TGF- β , platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF), mainly secreted by malignant cells^[100]. Recent findings indicate that CAFs can arise from mesenchymal stem cells (MSCs), which are recruited to the tumor milieu from both adjacent and distant tissues (either bone marrow or adipose tissue^[101,102]). CAFs exhibit completely different morphological and functional characteristics compared to their normal counterparts: they contribute to diverse mechanistic aspects of cancer progression including ex-

tracellular matrix remodeling, suppression of immune responses and the secretion of growth factors and cytokines. Notably CAFs are very resistant against apoptosis^[103]. Plenty of studies indicate the significance of numerous signaling pathways involved in the modulation of cancer-promoting characteristics of CAFs^[100,104,105]. In this respect, targeting signaling pathways which modulate the cross-talk between CAFs and other cellular components of tumor milieu can be considered as a promising therapeutic strategy for cancer treatment^[106].

Under physiological conditions, macrophages play a leading role in the process of wound healing. They also take a relevant part in tumors which can be considered as wounds that never heal^[107]. Tumor-associated macrophages (TAMs), another relevant class of the TME cellular component, secrete cytokines and growth factors necessary for tumor tropism. Moreover, they have a fundamental role in the degradation of extracellular matrix, a fact that leads to the release of additional growth factors from the matrix and to the promotion of tumor cell invasion. Factors released by TAM not only influence tumor cells, but also TAM themselves. In this regard, cytokines such as IL-4^[108] and IL-10, as well as TGF- β , found in the tumor milieu, can promote polarization of TAMs towards M2-activated cells. This last cell type, defined as M2 in comparison to the classically activated M1-type macrophages, are activated by glucocorticoid, IL-10 and cytokines such as IL-4/IL-13 and participate in HCC progression^[109,110]. TAMs showing characteristics of M2 cells can further secrete a cocktail of cytokines and chemokines, which influence tumor cells and the surrounding environment. For example, M2 macrophages can secrete vascular endothelial growth factor (VEGF) or endothelial growth factor (EGF) inducing tumor angiogenesis. Additionally, by favoring the degradation of the extracellular matrix, TAMs contribute to the generation of an inflammatory microenvironment, which promotes tumor progression. Together, the above reported observations allow the conclusion that the presence of TAMs represents a marker of poor prognosis in cancers, including HCC^[111,112]. Additionally, TAMs targeting can open a novel avenue towards innovative therapeutic approaches in various cancers, including HCC^[113].

At the tumor site, the role of macrophages and monocytes is not limited to the generation and maintenance of a tumor inflammatory micro-environment, it also extends to a pro-angiogenic contribution. HCC is a highly vascularized neoplastic disease with the neo-tumor vessels functioning abnormally. Angiogenesis-promoting cell types, belonging to myeloid lineage (such as macrophages and monocytes), play multifaceted roles in the process of pathological angiogenesis. Angiogenic factors, among which we can nominate angiopoietin-2 (ANG-2), are secreted from HCC cells to stimulate tumor vascularization. Tie-2, a tyrosine kinase receptor of angiopoietins capable of binding to all members of angiopoietin family, is involved in the transduction of angiogenic signals. Immunofluorescence staining has

revealed that Tie2 expressing monocytes/macrophages (TEMs) are actively present in human HCC tissues when compared to non-HCC tissues. Notably, the amount of TEMs is definitively superior at the HCC tumor site than in the peripheral blood. Moreover, significantly higher micro-vessel density at the site of HCC tumor correlated with the presence of TEMs, proposing a unique role for this population of tumor-infiltrating bone marrow-derived cells in the process of angiogenesis. It should be noted that TEMs are not peculiar of HCC; they are also detectable in a wide range of cancers, where angiogenesis is a determinant factor in cancer progression^[114,115]. Together, this information makes the presence of TEMs an indicator of active angiogenesis in human HCC. Thus TEMs may be undertaken as a promising diagnostic marker for HCC^[116].

Secreted factors

A wide spectrum of cytokines including various growth factors and tumor proliferating molecules has been demonstrated to contribute to HCC development and progression (Table 3). Hyeon *et al.*^[117] have shown that fibroblast growth factor 19 (FGF19) expression is correlated with early recurrence and poor prognosis in HCC. It has been previously demonstrated that FGF family members play key roles in development, angiogenesis, and cancer^[118]. In HCC, over-expression of FGF19 contributes to the occurrence and progression of the disease; this is mainly due to the de-regulation of Wnt/ β -catenin signaling which promotes tumor invasion and metastasis^[119]. Notably, inactivation of FGF19 or its receptor, FGFR4, has been shown to reduce the risk of tumor development^[120-122].

TGF β -1, the most potent pro-fibrotic cytokine, is in large part produced by cancer cells. TGF β enhances HCC progression through a paracrine mechanism. Under normal conditions, TGF β -1 inhibits cell proliferation, induces cell differentiation or triggers apoptosis. However, during cellular transformation, inhibition of cell growth by TGF β -1 is hampered, resulting in an excessive cell proliferation. During tumor development, TGF β -1 plays a pivotal role in processes such as fibrogenesis, angiogenesis, immunosuppression, and invasiveness of neoplasia. In HCC patients, elevated plasma concentration of TGF β -1 is correlated with shorter survival^[123]. It is believed that TGF β -mediated EMT plays a crucial role in the aggressiveness of HCC. In this regard, the TGF β concentration has been considered as a potent negative prognosticator in un-resectable HCC patients^[124]. TGF β can induce EMT of malignant hepatocytes by stimulating CAFs proliferation. Once activated, CAFs modulate growth, intra-vasation and metastatic spread of HCC cells. Interestingly, TGF β receptor inhibition can significantly reduce the stromal component of the tumors and the metastatic dissemination of HCC cells *via* the reduction of CAF proliferation^[125]. TGF β activation of CAFs seems to be mediated by the down-regulation of E-cadherin and by the up-regulation of Snail/PDGF

Table 3 Some secreted factors from cellular components of tumor microenvironment and their contribution to hepatocellular carcinoma prognosis

Molecule	Molecular category	Biological function	Expression in human HCC	Clinico-pathological indications	Target for therapy	Ref.
Interleukin-4	Cytokine	Alternative activation of macrophages into M2 cells	Over-expressed (elevated level in serum)	Associated with extrahepatic metastasis	Not presented	[108]
TGF- β 1	Multifunctional cytokine	Proto-oncogene/tumor suppressor	Over-expressed (elevated level in serum)	Decreased overall survival, increased risk of metastasis and recurrence, positively correlated with vascular invasion, higher PVT, higher overall invasiveness	Potential	[123]
FGF19	Growth factor	Activation of mitogen-activated protein kinase; activation of Wnt/ β -catenin pathway	Over-expressed in tissue	Decreased disease-free and overall survival, increased risk of metastasis, positively correlated with vascular invasion	Potential	[117]
Fox C1	FOX transcription factor family member	Induction of EMT, promotion of epithelial cell migration <i>via</i> inhibition of E-Cadherin transcription through transactivation of SNAI1 expression	Over-expressed in tissue	Increased risk of metastasis and recurrence, increased tumor size and number, poorer tumor differentiation, poorer TNM stage, higher vascular invasion	Potential	[130,131]
Lcn2	Member of lipocalin protein family	Inhibition of epithelial-to-mesenchymal transition; suppression of JNK and PI3K/Akt signaling pathways, down regulation of Twist1	Over-expressed in tissue	Positively correlated with poor prognosis and high risk HCC, decreased overall survival, however, elevated levels of Lcn2 is correlated with the inhibition of EMT <i>in vitro</i> and <i>in vivo</i>	Not presented	[134,135]
uPA	Serine protease	Plasminogen activation, extracellular proteolytic activity	Over-expressed in tissue	Decreased disease-free and overall survival, increased HCC invasion, poorer pathological grade	Potential	[144,146]
uPAR	Urokinase receptor	ECM degradation, migration, invasion and metastasis	Over-expressed in tissue	Decreased disease-free and overall survival, higher risk of HCC invasion, portal cancer embolus and tumor metastasis, poorer tumor cell differentiation	Potential	[145,146]
PAI-1	Protease inhibitor	Regulating extracellular matrix degradation	Over-expressed in tissue	Decreased disease-free and overall survival, increased risk of metastasis and recurrence, increased regional invasion, poorer pathological grade	Not presented	[144,146]
VEGF	Growth factor	Regulation of vascularization and angiogenesis	Over-expressed in tissue, elevated level in serum	Poorer overall survival, increased risk of metastasis and recurrence, higher vascular invasion	Potential	[156]
HIF-1 α	Subunit of HIF-1 transcription factor	Activating the transcription of a variety of genes involved in angiogenesis, survival pathways, immune evasion, invasion, and metastasis	Over-expressed in tissue	Decreased disease-free (and overall) survival, increased risk of metastasis, higher vascular invasion	Potential	[162,163]

PVT: Portal vein thrombosis; EMT: Epithelial-to-mesenchymal transition; TNM: Tumor node metastasis; FGF19: Fibroblast growth factor 19; Fox C1: Forkhead box C1; Lcn2: Lipocalin-2; uPA: Urokinase plasminogen activator; uPAR: Urokinase plasminogen activator receptor; PAI-1: Plasminogen activator inhibitor type-1; VEGF: Vascular endothelial growth factor; HIF-1 α : Hypoxia-inducible factor-1 α .

signaling pathways^[126,127]. Notably, the reduced expression of E-cadherin has been associated with poor HCC tumor outcome and shorter disease-free survival^[128].

Other transcriptional regulators play a role in EMT modulation. For example, forkhead box C1 (FoxC1), a member of the FOX transcription factor family^[129,130], is one of these. Fox proteins are key regulators of epithelial-to-mesenchymal transition. Accordingly, the fundamental role of EMT in the incidence of HCC invasiveness and metastasis strongly indicates the involvement of these transcriptional factors in HCC metastasis. Xia *et al.*^[131] have demonstrated that the expression of FoxC1 in

HCC patients is associated with shorter overall survival time and higher risk of recurrence, thus representing a negative prognostic factor for HCC patients. FoxC1 contributes to EMT induction through the inhibition of E-Cadherin expression *via* the transactivation of Snai1 expression; alternatively, FoxC1 induces the up-regulation of a protein named “neural precursor cell expressed, developmentally down-regulated 9 (NEDD9)”, a member of Cas family of signal transduction factors. In turn, NEDD9 contributes to integrin-dependent cell migration and invasion through the activation of FAK- and Src-signaling cascades^[132]. In HCC patients, NEDD9 over-

expression is significantly correlated with shorter overall survival and higher rate of metastases. FoxC1 can also favor EMT *via* the interaction with Notch and VEGF pathways, thus facilitating tumor neovascularization and vessel maturation processes^[133]. Together these observations indicate FoxC1 as an interesting prognostic biomarker and potential target for novel therapeutic strategies for HCC.

In HCC, lipocalin-2 (Lcn2), also known as neutrophil gelatinase-associated lipocalin (NGAL), negatively modulates EMT acting on the EGF or TGF β /Lcn2/Twist1 pathway^[134,135]. Up to now, conflicting data have been reported for the biological functions of elevated Lcn2, stressing its neoplasia-specific effects. However, in HCC an increasing body of literature indicates Lcn2 as a prognostic marker. Wang *et al.*^[134] have demonstrated that HCC specimens express significantly higher levels of Lcn2 compared to the healthy adjacent liver tissues. In this study, Lcn2 expression was significantly associated with a worse differentiation tumor grade even though it negatively correlated with EMT. In line with this last observation, adenoviral transduction of Lcn2 into sarcomatoid hepatocellular carcinoma (SH-J1) cells reversed EMT *in vitro*, inhibited cell proliferation and invasion and suppressed tumor growth and metastasis in a mouse model of HCC, further stressing the anti EMT function of Lcn2. In contrast, Lcn2 knock-down in HKK-2, a cholangio-carcinoma cell line, promoted EMT. In fact, a large body of evidence indicates the role of Lcn2 in preventing invasion and metastasis *in vitro*^[136-138]. However, elevated expression level of Lcn2 has been significantly correlated with a poor prognosis and shorter survival time. Whereas more knowledge about Lcn2 in HCC needs to be gained, the above observations propose Lcn2 as a possible metastasis suppressor and a potential therapeutic target in HCC^[139].

Together, the above reported data support the concept that targeting the cross-talk between HCC and its TME can represent a promising approach for the development of novel prognostic and therapeutic strategies against HCC.

Proteolytic enzymes

The local microenvironment of a tumor cell has a fundamental role in cancer development. Extracellular matrix (ECM), the main component of this milieu, is composed of a complex mixture of macromolecules, each with unique multifaceted properties. ECM is the fundamental scaffold of liver architecture and is in constant interaction with its environment, playing key roles in signal transduction and gene expression variations^[140]. Notably, ECM abnormalities affect the behavior of stromal cells in favor of tumor-associated angiogenesis, thus establishing a tumorigenic microenvironment. In cancer, ECM is de-regulated, leading to cancer progression by facilitating cellular transformation and metastasis. In order a tumor to develop and spread, cancer cells produce certain proteases responsible for the degradation of ECM. The components of ECM (collagen, elastin, and gelatin) are

degraded by various proteolytic enzymes, which include MMPs^[141]. MMPs are a family of enzymes characterized by their proteolytic ability to degrade the ECM. There are six main families of MMPs: collagenases (MMP-1, MMP-8 and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10 and MMP-11), matrilysins (MMP-7 and MMP-26), membrane-type MMPs (MT-MMPs: MMP-14, -15, -16, -17, -24 and -25), and other MMPs, which are not categorized in any of the previous groups. The MMPs may act either as secreted enzymes or as trans-membrane pro-enzymes that require activation after secretion. Accelerated breakdown of ECM occurs in various pathological processes, including inflammation, chronic degenerative diseases and tumor invasion.

Overexpression of MMP family members alone or together, has been shown to be associated with the recurrence, invasion and metastasis of HCC^[142]. For instance, plasma levels of MMP-9 can predict the probability of vascular invasion in HCC patients^[143]. Notably, urokinase plasminogen activator (uPA) is able to degrade ECM and, by converting plasminogen to plasmin, to activate MMPs. Increased activity of uPA has been shown to act as a sensitive and reliable marker, affecting HCC invasion, disease free survival and recurrence of HCC^[144]. Additionally, elevated levels of the uPA receptor (uPAR)^[145] and uPA inhibitor also known as plasminogen activator inhibitor (PAI-1) factor^[146], correlate with tumor aggressiveness and poorer outcome in HCC patients, especially when all these three markers are overexpressed^[144]. This finding, in contrast with the inhibitory effect of PAI-1 on uPA and thus on MMPs, indicates the complexity of the relation between matrix degradation/remodeling and tumor progression.

Angiogenic factors

Angiogenesis is the process of formation of new blood vessels from pre-existing ones. Since metabolically active cancer cells need oxygen and nutrients to survive, they secrete large amounts of pro-angiogenic factors that promote tumor neovascularization^[98,147]. In the past, angiogenesis has been considered an important manifestation for cancer development only after the formation of macroscopically detectable tumors. However, recent evidence indicates that this process also significantly contributes to the microscopic phase of neoplastic formation, as observed in pre-malignant and non-invasive lesions of *in situ* carcinomas^[148,149].

Rapid growth of tumor mass leads to increased oxygen consumption. Under such a condition, tumor cells face hypoxia. Cancer cells overcome the hypoxia imposed to the tumor microenvironment by two different strategies: promoting angiogenesis, or developing metastatic behavior as a key tool to escape from hypoxic condition^[150]. A remarkable feature of most HCCs is hyper-vascularization, emphasizing the fact that HCC generates various angiogenic elements with a high tendency to invade the vasculature. Angiogenic response in HCC is regulated by an intricate network of growth factors acting

on both tumor and endothelial cell populations. So far, a wide range of angiogenic factors and their receptors have been considered as therapeutic targets for HCC. In particular, VEGFs, the most studied and potent pro-angiogenic factors, together with their receptors (VEGFRs), have been the main focus of anti-angiogenic cancer therapies for the last decades. Indeed, among key signal transduction pathways, VEGF/VEGFR system plays a crucial role in the pathogenesis of HCC and a variety of molecular targeted agents have been developed to target the process of angiogenesis by inhibitory molecules controlling VEGF pathway. For example, Sorafenib, the first oral multikinase inhibitor agent, has been demonstrated to improve the survival of patients by affecting tumor angiogenesis and proliferation^[151]. In particular, Sorafenib inhibits cancer growth by targeting tyrosine kinase receptors including VEGFR-2 and VEGFR-3. It also inhibits various kinases in the MAPK pathway. However, the benefits of VEGF-targeted therapeutics in the treatment of advanced neoplastic diseases, including HCC, is hampered by multiple escape mechanisms^[152].

Not only VEGF has been considered a potential interesting target for novel therapeutic approaches, it is also considered to have a prognostic value. Elevated plasma levels of VEGF have been considered a negative prognostic biomarker in patients with advanced HCC^[153-156]. Higher expression level of tissue VEGF, a prominent characteristic of HCC, corresponds to neovascularization at the site of tumor, associates with poor outcome and disease free survival, and correlates with higher tumor grade and vascular invasion^[155]. Additionally, increased expression of the VEGF₁₆₅ isoform has been demonstrated to be an independent prognostic marker of recurrence development^[157]. High levels of VEGF in the serum of HCC patients are significantly associated with the presence of intrahepatic metastasis and microscopic venous invasion, and thus can be considered also a biomarker of tumor invasiveness^[158,159].

In addition to VEGF, low oxygen concentration at the site of tumor, also induces the expression of hypoxia inducible factor 1 (HIF-1), a transcription factor composed by two subunits: HIF-1 α , and HIF-1 β . The expression of HIF-1 α subunit is up-regulated under hypoxic conditions^[160]. In contrast, HIF-1 β is constitutively expressed. HIF-1 α over-expression is a typical characteristic of malignant cells. This transcription factor, as a master mediator of hypoxic adaptation, activates the transcription of a variety of genes including those involved in angiogenesis, survival pathways, immune evasion, invasion, and metastasis. It has been demonstrated that both mRNA and protein levels of HIF-1 α , can be used as potential prognostic factors for survival and recurrence in HCC patients^[161-163].

In HCC, hypoxia triggers invasion and metastasis also by inducing EMT, a phenotypic modification that confers to tumor cells the ability to migrate and to invade other organs. In HCC, one of the mechanisms by which EMT is induced occurs *via* HIF-1 α . This factor can promote

tumor growth and dissemination through the regulation of some EMT modulators, such as E-cadherin, a protein known to stimulate anti-growth signals. More in detail, it has been proposed that HIF-1 α mediates repression of E-cadherin expression through the up-regulation of E-cadherin-specific repressor SNAI1. In animal models of HCC, tumor progression was associated with the up-regulation of HIF-1 α and sequential production of VEGF. Thus, blockade of HIF-1 might be considered as a promising therapeutic approach in cancer treatment^[164]. In a study by Zhang *et al.*^[165], the expression levels of HIF-1 α , together with SNAI1 and other EMT modulators, were assessed in a cohort of HCC patients. The authors demonstrated a significant correlation between the dual over-expression of HIF-1 α and SNAI1 and the reduced disease-free survival and poor prognosis. Notably, shRNA-mediated suppression of HIF-1 α reverses the process of EMT. Even more interestingly, EMT phenotype can be reversed by re-oxygenation^[165]. This phenomenon occurs also in HCC cells cultured in normoxic, hypoxic and re-oxygenated conditions.

A large body of evidence suggests that hypoxia induced by anti-angiogenic therapy can stimulate the escape of cancer cells from oxygen-deprived areas to well-nourished distant organs. It has been proposed that the more hypoxic tumor environment induced by inhibitors of VEGF and its receptors may trigger an invasive metastatic switch^[152,166]. However, this hypothesis needs further investigation^[167]. Finally, in some cases, drug induced hypoxia to the tumor site may be responsible for the development of drug resistance behavior. In this regard, it has been observed that the intra-tumor hypoxia induced by Sorafenib therapy can cause Sorafenib resistance in HCC patients; interestingly, the concomitant inhibition of HIF-1 α can enhance the antitumor effects of this drug^[168].

CONCLUSION

The diagnosis of HCC typically occurs in the advanced stages of the disease when the therapeutic options have poor efficacy. Thus, the identification of specific markers for HCC diagnosis and prognosis is thought to improve the effectiveness of the therapeutic approach to HCC. Based on this, much effort has been put in the selection of reliable HCC markers. In addition to the study of the biochemical markers, of the angiogenic mediators and of the epigenetic variations, which have obtained promising results, alternative lines of research have been proposed. In particular, miRNAs, due to their capacities to control gene expression, behaving like tumor suppressors or oncogenes, are arising as very attractive molecules. Also very interesting and novel is the consideration dedicated to the tumor microenvironment, a crucial regulator of HCC initiation and progression. Improving the knowledge about the cross-talk between cancer cells and the neighboring environment may be very useful to design novel molecular targets with diagnostic and prognostic potentials in HCC.

In conclusion, the evaluation of a broad spectrum of different HCC markers can reasonably identify a range of effective diagnostic, prognostic and therapeutic targets for HCC in the near future. Whereas it is difficult to predict the timing required to reach the goal, we believe that an integrated multidisciplinary research among the different lines of investigation highlighted in this review should represent the winning strategy.

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