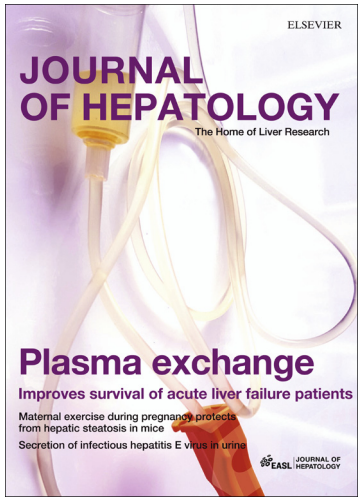
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Review

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Role of epithelial to mesenchymal transition in hepatocellular carcinoma

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Key words: HCC, EMT, MET, epithelial plasticity, tumor heterogeneity, cell invasion, therapy, patient survival

Abbreviations: BCLC, Barcelona Clinical Liver Cancer; CLDN, claudin; CTC, circulating tumor cell; CSC, cancer stem cells; ECM, extracellular matrix; EGF, epidermal growth factor; EMT, epithelial to mesenchymal transition; FSP-1, fibroblast-specific protein; GANK, gankyrin; GFP, green fluorescent protein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HNF, hepatocyte nuclear factor; HNRNP, heterogeneous ribonuclear protein; HIF, hypoxia-inducible factor; HSC, hepatic stellate cell; K, cytokeratin, Lcn, lipocalin; lncRNA, long non-coding RNA; MET, mesenchymal to epithelial transition; miR, microRNA; PI3K, phosphoinositide 3-kinase; PDGF, platelet-derived growth factor; RTK, receptor tyrosine kinase; SMA, smooth muscle actin; TACE, transarterial

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chemoembolization; TIP, Tat-interacting protein; TCF, T cell factor; TF, transcription factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor; YFP, yellow fluorescent protein.

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Abstract

The epithelial to mesenchymal transition (EMT) is a multistep biological process whereby epithelial cells change in plasticity by the transient de-differentiation into a mesenchymal phenotype. EMT and its reversal, MET, essentially occur during embryogenetic morphogenesis and have been increasingly described in fibrosis and cancer during the last decade. In carcinoma progression, EMT plays a crucial role in early steps of metastasis when cells lose cell-cell contacts due to ablation of E-cadherin and acquire increased motility to spread into surrounding or distant tissues. Epithelial plasticity has become a hot issue in hepatocellular carcinoma (HCC), as strong inducers of EMT such as transforming growth factor-β are able to orchestrate both fibrogenesis and carcinogenesis, showing rising cytokine levels in cirrhosis and late stage HCC. In this review, we consider the significance of EMT-MET in malignant hepatocytes as well as changes in the plasticity of hepatic stellate cells for cellular heterogeneity of HCC, and further aim at explaining the current limiting insights into EMT by snapshot analyses of HCC tissues. Recent advances in the identification of clinically relevant mechanisms that impinge on important EMT-transcription factors, as well as on miRNAs causing EMT signatures and HCC progression are highlighted. In addition, we draw particular attention to framing EMT in the context of potential clinical relevance for HCC patients. We conclude that some aspects of EMT are still elusive and further studies are required to better link the clinical management of HCC with biomarkers and targeted therapies related to EMT.

Key Points

- A large body of evidence shows that hepatocellular EMT is a de-differentiation of malignant hepatocytes, hepatic progenitor cells or HSCs that can be reversed to MET; a genetic proof for EMT-MET in HCC by lineage tracing transgenes is lacking
- The heterogeneity of HCC provided by EMT-MET in the tumor and its microenvironment is insufficiently displayed in HCC tissues by using available techniques (e.g. immunohistochemistry) which “only” reveal a snapshot at a given time

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- Clinically relevant mechanisms responsible for driving hepatocellular EMT have been identified that might be used to combat HCC progression
- The lack of a well-defined consensus on biomarkers for EMT-MET impedes accurate conclusions on how EMT affects the clinical outcome of HCC patients

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Introduction

Hepatocellular carcinoma (HCC) ranks sixth among the most common malignancies worldwide and shows the third highest mortality among cancer patients [1,2]. Long-term intoxication with alcohol or aflatoxin, chronic infection with hepatitis B or C virus (HBV, HCV) or fatty diets leading to obesity are well-defined etiological factors that can cause the development of HCC over decades [2]. Liver carcinogenesis develops in a sequential evolution from dysplastic lesions harboring minor genetic variations to advanced stages of HCC showing a vast molecular heterogeneity [3]. Generally, the heterogeneity derives from differences in etiology, underlying chronic liver disease, genetic and epigenetic events, expression signatures and differentiation of carcinoma cells, as well as the impact of the tumor microenvironment [4]. HCC show heterogeneity at the cellular level by the neoplastic transformation of hepatocytes and progenitor cells which are both epithelial cell types. The complexity of the cellular heterogeneity is increased by changes in the plasticity of these epithelial cells, commonly described as epithelial to mesenchymal transition (EMT). Heterogeneity is further provided at the genetic level by the multifocal development of HCC that is caused by the synchronous formation of tumor nodules or by intrahepatic metastases of the primary cancer. While multiple HCC nodules show an accumulation of different sets of genetic and epigenetic changes, few genetic alterations have been observed between primary HCC, portal vein tumor thrombi and intrahepatic metastasis [5]. The subset of metastatic HCC patients has been identified by an imbalance of Th1/Th2 cytokines associated with an increased expression of colony stimulating factor in the tissue microenvironment, suggesting that the inflammatory milieu displays a key role in promoting metastasis and affecting the clinical outcome [6].The extensive tumor heterogeneity at multiple stages of HCC development hampers the stratification of patients for effective therapy. In particular, the identification of those HCC patients that will develop disease reccurrence after curative therapy is of outmost relevance. In a recent study, a metastatic gene signature combined with α-fetoprotein has been reported as a good predictor of HCC outcome independently of etiology and ethnicity [7].

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Characteristics of EMT

EMT is the mechanism that drives a transient and reversible de-differentiation of epithelial cells to a mesenchymal-like or a mesenchymal phenotype, depending on how the completion of de-differentiation is. EMT-induced changes in epithelial plasticity are evidenced by the loss of epithelial markers such as the adherence junction component E-cadherin and cytokeratins of the intermediate filament system (K8, K18, K19), as well as the increasing expression of the mesenchymal proteins such as N-cadherin, α-smooth muscle actin (α-SMA), fibroblast-specific protein (FSP-1) and the EMT-transcription factors (EMT-TFs) Snail (SNA1), Slug (SNA2), Twist and ZEB. Some markers, such as FSP-1, lack specificity for the EMT of parenchymal liver cells as they are not expressed in myofibroblasts [8] or fail to show specificity, like vimentin, which is expressed in injured hepatocytes [9]. Epithelial cells with an induced but not complete EMT are referred to as "partial" EMT, and cells at this stage of de-differentiation co-express epithelial as well as mesenchymal markers. Both partial and complete EMT can be reversed by a mesenchymal to epithelial transition (MET), allowing the recovery of epithelial traits. Notably, the EMT-MET is essentially required for the metastatic colonization as demonstrated in breast and colon carcinoma progression [10,11], and may play a crucial role in intra-and extrahepatic metastasis. On the other hand, the EMT is crucial for tumor chemosensitivity, as in the case of pancreatic ductal adenocarcinoma, in which the deletion of Snail or Twist does not prevent invasion and metastasis but does sensitize pancreatic cancer cells to gemcitabine treatment, leading to an increased survival of mice [12]. However, genetic evidence by lineage tracing transgenes showing the relevance of EMT in the dissemination of HCC cells is lacking.

Evaluating the various EMT phenotypes of malignant epithelial liver cells by partial and complete EMT and the reversal to MET, we can estimate the complexity and cellular heterogeneity of HCC, which is difficult to assess by cell imaging in vivo. In this scenario, neoplastic hepatocytes are either (i) epithelial or (ii) mesenchymal-like after induction to partial EMT or (iii) mesenchymal after completion of EMT and indistinguishable from hepatic

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stellate cell (HSC)-derived myofibroblasts in fibrotic tissue. These phenotypes can be reversed at any stage by MET (Fig. 1). Thus, snapshots of EMT markers by expression analyses in transitional tissues - in the absence or presence of cell tracking - reveal only partial insights into the EMT process and provide a small window of the EMT-MET status at a given time. In addition, the complexity of epithelial cell plasticity in the liver is reinforced by the chimeric epithelial/mesenchymal phenotype of HSCs which are derived from mesothelial cells of the epiblast during embryonic development. They express both epithelial and mesenchymal markers. HSCs undergo “EMT-like” processes via (de)-differentiation to either more mesenchymal cells which still express epithelial markers, or activation to a “MET-like” transformation by canonical Hedgehog signalling without complete ablation of mesenchymal markers [13-15].

EMT in fibrosis and HCC

Injured epithelial cells have been suggested to be an important source of fibroblasts which are essentially involved in tissue fibrosis of lung and kidney [16,17]. Similarly, the contribution of parenchymal cells to liver fibrosis by EMT has stimulated animated debate during the past decade. While primary hepatocytes and cholangiocytes can be induced to EMT in vitro by transforming growth factor (TGF)-β or Hedgehog [18-20], the role of EMT in vivo during hepatic fibrosis is controversial. Initially, FSP-1-positive myofibroblasts were detected in an albumin promoter-driven LacZ transgene during CCl_4-induced fibrosis, suggesting changes in epithelial plasticity of hepatocytes. Yet, this study showed incomplete Cre-dependent recombination of the Rosa26-floxstop-LacZ cassette in hepatocytes, and importantly, co-expression of β-gal/FSP-1 failed to detect myofibroblasts [21]. However, multiple studies further addressed this issue by lineage tracing transgenes using markers which more faithfully monitor the hepatocyte-dependent transformation to myofibroblasts. In this context, the expression of α-SMA and deposition of fibrillar collagen were considered as relevant markers of hepatic myofibroblasts. Therefore, the collagen1α1-f/f-green fluorescent protein (GFP) reporter strain was crossed with albumin promoter-driven LacZ transgenic mice to

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monitor the fate of albumin/GFP-positive hepatocytes after liver injury. Yet, no conversion of hepatocytes to myofibroblasts expressing collagen1/GFP or FSP-1/GFP was detected during CCl_4-induced fibrosis, refuting hepatocyte-derived generation of myofibroblasts by the EMT [22]. In the same line, the breeding of tamoxifen-inducible K19-Cre mice with Rosa26-f/f-yellow fluorescent protein (YFP) mice failed to show the occurrence of K19/YFP-positive myofibroblasts derived from cholangiocytes during bile duct ligation and CCl_4-induced fibrosis [23]. These data were further confirmed by lineage tracing of all liver epithelial cells, i.e. hepatocytes, cholangiocytes and oval cells in α-fetoprotein-Cre mice crossed to Rosa26-YFP mice, as no YFP could be detected in myofibroblasts [24].

While Brenner's group concluded that myofibroblasts derive from HSC in hepatotoxic injury or from portal fibroblasts in cholestatic liver injury rather than from the EMT of hepatocytes [25-27], Diehl's group avoided premature conclusions and suggested to perform more reliable fate map studies of EMT-MET during liver damage [9]. In particular, different detection techniques must be used in combination to overcome the limitations of double-labelling in immunohistochemistry. In addition, different types of injury models and different time points of analysis in larger cohorts of animals must be employed. Notably, Pinzani suggested a more flexible interpretation of EMT-MET, as observed via lineage tracing, by proposing a model of "escape reaction". In this concept, non-transformed hepatocytes react by an adaptive response to a hostile inflammatory, hypoxic and redox-activated microenvironment by acquiring a motile, mesenchymal phenotype. This allows hepatocyte derivatives to move and escape from damage and apoptosis under more benign conditions, and finally to restore the epithelial phenotype through a MET-like process [28]. In this model, the plasticity of hepatocytes counteracts fibrogenesis rather than being pro-fibrogenic during chronic liver injury.

Upon HCC development, the centers of tumors are hostile for cancer cells due to oxygen depletion and necrosis as compared to tumor-stroma borders, showing comparable conditions for EMT to those hypothesized in the "escape reaction" during chronic liver injury ([29]). Hence, EMT-transformation of malignant hepatocytes or progenitor cells essentially

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equips mesenchymal offspring with anti-apoptotic and migratory traits to resist cell death stimuli and to move towards a cytokine/chemokine-enriched microenvironment [30]. In addition, EMT associates with the production of pro-angiogenic factors, allowing HCC cells to generate new blood vessels for better support with nutrients and oxygen. The reversibility of EMT to MET after cell dissemination via the circulation is considered a further hallmark for colonization at distal sites.

In HCC, EMT was initially described in murine hepatocytes through the functional collaboration of oncogenic H-Ras and TGF-β [31-34] and then in HCC patients through activation of the laminin-5/TGF-β axis [35]. Importantly, TGF-β has been identified as one of the most potent inducer of EMT ([36,37]. In HCC, TGF-β has a dual role by acting anti-oncogenic at the early stage of tumor development but being pro-oncogenic at later stages which promotes EMT and cancer dissemination [38,39]. Yet, the molecular mechanisms underlying the switch from tumor-suppressive to tumor-promotive TGF-β functions are still poorly understood [40]. Notably, TGF-β regulates Wnt signaling and both define a subgroup of HCC patients with a poor prognosis [41]. Furthermore, the “late TGF-β signature” of TGF-β-positve HCC patients associates with an invasive phenoptype and HCC metastasis [42]. Therefore, TGF-β represents a crucial pathway of EMT leading to HCC progression.

During the past decade, much solid evidence has accumulated showing the relevance of EMT for HCC progression. In the sections below, we focus on the regulatory pathways that impinge on EMT-TFs such Snail, Twist and ZEB for executing EMT and discuss the important role of microRNAs (miRs) in regulating EMT-MET, as it has emerged between 2010 and 2015.

Mechanisms of EMT in HCC

EMT requires the re-programming of epithelial gene expression which predominantly involves the activation of EMT-TFs. Here we address the role of the SNAI family with its members Snail (SNA1) and Slug (SNAI2), the Twist family comprising Twist1, Twist2, E12, E47 and ID as well as the ZEB family with ZEB1 and ZEB2 [43] [44]. Snail is the most

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prominent inducer of EMT in HCC. Interestingly, the Tat-interacting protein (TIP)30 correlates with a sustained epithelial phenotype and prevents HCC cells from undergoing EMT by blocking the nuclear import of Snail via interaction with importin-β2, whereas TIP30 deficiency leads to nuclear Snail accumulation and E-cadherin repression (Fig. 2) [45]. Upon EMT and HCC progression, upregulation of the receptor tyrosine kinase (RTK) Axl induces Snail expression by binding to 14-3-3ζ and molecularly collaborating with TGF-β signalling [46]. Particularly, Gas6/Axl phosphorylates the Smad3 linker region leading to the activation of TGF-β target genes such as Snail and MMP9, causing enhanced cell invasion and transendothelial migration. Axl overexpression is strongly correlated with poor patient survival, suggesting Axl as a promising therapeutic target in TGF-β-positive HCC patients. Another study showed that the heterogeneous ribonuclear protein AB (HNRNPAB) is overexpressed in metastatic HCC cells and induces EMT by transactivation of Snail [47]. Intervention with Snail reduces HNRNPAB expression and reverses HNRNPAB-promoted HCC metastasis. Wnt/β-catenin signalling is well known to induce EMT through the competitive mechanism of β-catenin action in complex with T cell factor (TCF)4, leading to Snail and Slug expression and EMT. Yet, hepatocyte nuclear factor (HNF)4α can bind to TCF4, thereby competing with β-catenin [48]. The HNF4α/TCF4 complex abolishes the transcriptional activity of β-catenin/TCF4, thus downregulating Snail/Slug expression and maintaining an epithelial phenotype. Epigenetic silencing through promoter hypermethylation of claudin (CLDN)3 is another means whereby HCC cells can undergo EMT. Silencing of CLDN3 is associated with HCC invasion through activation of Wnt/β-catenin signalling and Slug, indicating CLDN3 as a suppressor of Wnt/β-catenin-dependent EMT and HCC progression [27].

HCC with increased p28(GANK) (gankyrin) shows EMT by activating phosphoinositide 3-kinase (PI3K)/Akt/hypoxia-inducible factor (HIF)-1α signalling to promote Twist1, which increases vascular endothelial growth factor (VEGF) expression leading to enhanced tumor angiogenesis, vascular invasion and metastasis [49]. The transcription factor RUNX3 positively correlates with E-cadherin and claudin in HCC cells, resulting in a less aggressive

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HCC phenotype. However, loss of RUNX3 in HCC leads to increased cell motility and Twist1-mediated EMT via activation of Notch 2 signalling by jagged-1 [50]. Another mechanism of Twist-induced EMT activation depends on the downregulation of lipocalin-(Lcn)-2 expression through TGF-β1 or epidermal growth factor (EGF) [51]. Moreover, acquired drug resistance potentiates EMT in HCC cells as gemcitabine treatment leads to enhanced expression of platelet-derived growth factor (PDGF)-D and Twist1 [52].

A recent report showed that forkhead box Q1 (FoxQ1) induces EMT by direct binding to the ZEB2 promoter [53]. Together with the activation of another FoxQ1 target, VersicanV1, ZEB2 promotes HCC metastasis and leads to increased infiltration with tumor-associated macrophages. Interestingly, long non-coding RNAs (lncRNAs) come into field of EMT as LncRNA-ATB upregulates ZEB1/ZEB2 by the competitive binding to the miR-200 family which normally sustain the epithelial phenotype, thus promoting EMT and HCC invasion [54]. A further study revealed that lncRNA ZEB1-AS1 is increased in HCC by promoter hypomethylation and that ZEB1-AS1 positively modulates ZEB1 transcription through physical contiguity and enhancer activity [55]. A further investigation showed that p53 upregulates miR-200 and miR-192 family members, which repress ZEB1/2 expression [56]. Yet, p53 action is often impaired during HCC progression, releasing ZEB1/2 from miR-200-mediated repressive control and inducing EMT.

Among the closely investigated miR-200 family members, several miRs were discovered to be important during HCC progression. The expression of miR-216a/217 correlates with EMT, a cancer stem cell (CSC) phenotype and poor survival of patients by targeting PTEN and SMAD7, causing activation of PI3K/Akt and TGF-β signalling [57]. Another example of miR involvement is miR-612 that directly targets Akt2 and is expressed in reverse correlation to EMT and metastasis in HCC patients [58]. The RTK c-Met is frequently overexpressed or mutated in HCC correlating with progression and metastasis. MiR-148 directly targets c-Met and inhibits Met/Snail signalling, thus acting as a tumor suppressor in HCC [59]. Similarly, miR-451 increases chemo- and radiosensitivity and reverses EMT by targeting c-Myc [60]. A further study of TGF-β-induced EMT showed a significant decrease of miR-125b [61].

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Overexpression of miR-125b attenuates EMT and associates with chemoresistance, migration and a CSC phenotype by targeting SMAD2/SMAD4 which disrupts canonical TGF-β signalling.

EMT progression involves MUC15 expression which is downregulated in HCC patients, correlating with vascular invasion and poor differentiation [62]. Interaction between MUC15 and EGF-R attenuates EGF-induced dimerization of EGF-R and promotes receptor degradation, leading to an inhibited PI3K/Akt signalling and decreased MMP2/MMP7 expression. Another study showed upregulation of the protein deacetylase Sirtuin (Sirt)2 in HCC, which contributes to EMT by deacetylation and activation of PKB, affecting the glycogen synthase kinase-3β/β-catenin pathway [63]. Cellular stress is another important aspect promoting EMT during HCC. Starvation-induced autophagy of HCC cells leads to increased EMT-driven invasion, accompanied by MMP9 expression and TGF-β/Smad3-mediated downregulation of epithelial markers [64]. A recent investigation identified a role of EGF-Like Repeats and Discoidin I-Like Domains 3 gene (EDIL3) in HCC. EDIL3 interacts with αvβ3 integrin to promote EMT, metastasis and angiogenesis through the activation of MAPK/Erk and TGF-β signalling [65]. Studies focusing on HCV-associated HCC revealed that the transcriptional E-cadherin repressors E12 and E47 are increased upon expression of the HCV core protein, which blocks their degradation and contributes to EMT progression [66].

Clinical role of EMT

Studies devoted to investigating how EMT affects the clinical outcome of patients with HCC are hampered by two main technical limitations. Firstly, the lack of a well-defined consensus on how to define EMT, so that each study reports only some of the recognized EMT markers, often using different techniques, reagents and tissue preparations. For example, the expression of β-catenin, an important EMT marker, changes according to whether the tissue is frozen or paraffin-embedded. This is likely due to the different techniques used to fix the tissues which crucially affect protein blocking and

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immunotargeting. For these reasons, most studies have focused on the expression, regulation and function of E-cadherin, reported to be involved in tumor progression in transgenic experimental models [67-73]. Many studies in the literature have estimated the blood levels of E-cadherin, another aspect that warrants further debate. To further complicate the situation, new EMT markers have been described in HCC, such as metadherin that has been reported in a cohort of 323 patients to be correlated with the down-regulation of E-cadherin and nuclear translocation of β-catenin, leading to microvascular invasion and metastasis [74].

Another limit is that the findings observed in liver specimens correspond to the clinical conditions “frozen” at the moment when the liver biopsy was executed, which precludes any pursuit of the dynamic multistep process of the EMT. Furthermore, it is difficult to interpret and compare data from different papers because each study deals with only some of the EMT biomarkers, often investigated using different techniques including qPCR, immunohistochemistry and immunofluorescence. The correlation with clinical outcomes is also poorly comparable. Moreover, the limited tissue specimens available when obtained by fine needle biopsy precludes a wider analysis of different tumor areas within the context of the same tumor. This also presumably contributes to underestimations of EMT in HCC.

Few studies have investigated several EMT markers in a large number of tissues. For instance, an ample analysis of EMT markers in 123 HCC patients demonstrated the presence of EMT markers in tissues at the moment of biopsy in 56% of patients. In particular, in this cohort of patients Snail, Slug and Twist overexpression was confirmed to be correlated with a downregulation of E-cadherin expression, and hence a more aggressive disease [75]. On the other hand, this study also emphasized the difficulties in recognizing EMT in a snapshot investigation like analyzing a biopsy sample. Nevertheless, considering the large bulk of evidence it seems that a stronger expression of EMT markers, in particular E-cadherin, the most reliable and closely investigated, is directly correlated with a worse prognosis and shorter survival [76,77]. Four EMT genes, including E-cadherin, were found to be predictive of clinical outcome at univariate Cox analysis in a cohort of 128 HCC patients,

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and this was further confirmed in a study involving three different centers [78]. Similar conclusions were suggested in a cohort of 150 patients in whom a down-regulation of E-cadherin regulated by TGF-β was correlated with vascular invasion and extrahepatic recurrence [79].

A new approach to investigating EMT in cancer including HCC could be the detection and analysis of circulating tumor cells (CTC). By definition, these cells have the potential to metastasize and since EMT triggers a more aggressive and malignant phenotype, this source of biological specimens could be very important to make a more dynamic inquiry into EMT of HCC patients. However, the detection of CTCs is extremely difficult owing to a number of issues, including the sensitivity of the technique. Nevertheless, it has been reported that CTCs expressing EMT markers have been isolated in 40/60 (66.7%) patients, correlating with local or distant spread through blood vessels [80]. While CTCs clearly represent a subpopulation of cells in the tumor context, further studies are needed to clarify whether CTCs express EMT markers and if a similar pattern could be observed in the primary tumor. While CTCs clearly represent a subpopulation of cells in the tumor context, further studies are needed to clarify whether CTCs express EMT markers and if a similar pattern could be observed in the primary tumor. However, it should also be considered that, by definition, in CTCs there is already a down-regulation of some epithelial markers i.e. E-cadherin, so the EMT could be over-interpreted.

Role of EMT in HCC stemness and tumor-stroma

The microenvironment surrounding the tumor communicates with cancer cells, leading to the growth and progression of several tumors including HCC. Such interactions are mediated by a number of cytokines, growth factors, ECM proteins which are mainly released by fibroblasts/myofibroblasts, macrophages and immune cells. For instance, TGF-β is stored in the tissue microenvironment in latent form, and activated after proteolytic cleavage by plasmin, MMP2 and MMP9 that are highly expressed during the tissue re-modelling which commonly occurs as a consequence of the tumor growth [81,82]. After activation, TGF-β

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orchestrates several biological activities in HCC, triggering EMT and strongly boosting the expression of the integrin subunit α3 in those cells lacking its expression. Thus, HCC cells expressing the integrin receptor α3β1 acquire motility, efficiently migrating on Laminin-5 (recently renamed Laminin-332), while the migration of HCC cells expressing α3β1 can be inhibited by anti-α3 blocking antibodies [83]. Another study reported that the α6 integrin subunit is associated with an overexpression of the tetraspanin member CD151, which induces EMT and drives a more invasive and aggressive phenotype of HCC through phosphatidylinositol-3-kinase (PI3K) signalling. A high α6 integrin expression has been correlated with a worse clinical outcome, poor survival and early cancer recurrence [84]. It is evident that integrins play a key role in HCC progression, engaging with the different ECM proteins. These molecules, present in the microenvironment, are responsible for cancer cell dissemination and growth, like Laminin-332 which is expressed at the leading edge of several malignancies including HCC and cholangiocarcinoma [85]. The presence of Laminin-332, in addition to TGF-β previously discussed as a major inducer of the EMT, is important to complete the EMT multistep transformation process of the constitutionally non-invasive HCC cells [35]. Laminin-332 is produced by HSCs and stimulates the proliferation of HCC cells via interactions with α3β1 and α6β4 integrin receptors (Fig. 3) [86,87]. [25]

HCC cells acquire a more invasive and aggressive phenotype as they undergo de-differentiation, gaining stemness properties like cancer stem cells (CSCs) [18,88,89]. This observation is arousing great interest because CSCs are considered to be responsible for a higher capability for tumor re-initiation and chemoresistance [90]. In preclinical models, HCC cells that were EMT-transformed upon TGF-β stimulation and expressed high levels of CD44 were resistant to sorafenib-induced apoptosis. This observation may contribute to explain chemoresistance in patients [91]. K19, a marker of cholangiocytes and hepatocellular stemness in HCC, has been reported to be associated with the EMT and with other stemness markers including EpCam and CD133. In a large cohort of 274 patients, K19-positive HCC patients showed a shorter survival, and K19 expression was an independent predictive factor of a worse clinical outcome and poorer survival even after resection, being

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predictive of early recurrence [92,93]. Consistently, in another study, K19 was significantly correlated to tumor de-differentiation, metastasis, microvascular invasion, and overall with a shorter survival. The same authors also showed in a cohort of 75 patients that highly invasive HCC cells were K19-positive and expressed a number of invasion-related genes. These included LAMC2 which encodes the Laminin-332 γ2 chain and was previously associated with a worse clinical outcome [94,95]. This was further confirmed by a recent study demonstrating that the γ2 chain is a component of the CSC niche, inducing a high expression of K19 and a low proliferation index. It seems to be related to a more quiescent and more biliary phenotype, but it is also more resistant to doxorubicin and sorafenib therapy owing to an increased proliferation [96]. Also, in a cohort of 87 cholangiocarcinoma patients, TGF-β and LAMC2 genes present in the surrounding stroma were independent prognostic factors in an analysis of 1,073 non-redundant genes [97]. In conclusion, an investigation of the microenvironment is beginning to explain a number of apparent discrepancies among different studies and different clinical outcomes, while it is also helping to dissect the role of each component of the microenvironment, such as Laminin-332.

Role of the EMT in therapeutic effectiveness

Understanding the molecular basis responsible for chemoresistance and for the highly different clinical outcome in patients with HCC is currently one of the most urgent priorities. EMT is likely implicated in affecting therapeutic responsiveness in HCC, while therapies can also trigger EMT as a consequence of inducing tissue remodelling. This occurs, for instance, in hypoxia following transarterial chemoembolization (TACE) therapy leading to the up-regulation of HIF-1α which is a strong inducer of EMT via Snail regulation [63]. The increase of hypoxia by TACE was confirmed in another study using a mouse model [98], and is consistent with previous data showing the role of VEGF-R as a potential therapeutic target [99]. This mechanism could contribute to explain, at least in part, the variable and unpredictable outcome of patients receiving TACE, and could also orient drug-based

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therapies supporting TACE in order to prevent or limit hypoxia-derived pro-tumorigenic effects.

Hepatocyte growth factor (HGF) has been described to promote HCC progression through EMT [100,101]. Its main receptor, c-Met, is known to be increased in patients with more aggressive disease and shorter survival [102,103]. The c-Met inhibitor tivantinib has been demonstrated to improve overall survival in a phase II clinical trial in patients with HCC expressing higher levels of c-Met [104]. Another important pathway is TGF-β, the most potent EMT inducer and therefore an ideal target for therapy directed against the EMT [105]. While several different approaches have failed to demonstrate a therapeutic effectiveness in inhibiting TGF-β, galunisterib is currently being tested in an ongoing phase II clinical trial in patients with advanced HCC [106]. Galunisertib is a small molecule inhibitor of the TGF-β receptor I kinase, proven to block the canonical and non-canonical pathways of TGF-β according to different dosages and timing of administration on HCC cells [107]. In a number of different preclinical experimental models, galunisertib showed an antitumoral effect via reversion of the EMT process. Meanwhile, it also improved the overall homeostasis of the surrounding tissue microenvironment, including regulation of the desmoplastic response around the tumor and so interrupting the stroma-tumor crosstalk where HCC commonly develops [38]. However, it is likely that not all HCC patients will be suitable for treatment with galunisertib. In particular, patients showing the “late TGF-β signature” as described by Coulouarn, or belonging to the ”S1 subgroup” according to Hoshida are theoretically those most liekly to benefit from this therapy [41,42]. The results of this clinical trail are highly relevant not only in view of the therapeutic relevance of developing a new systemic therapy, but also for gaining a better understanding of the role of EMT in HCC progression.

It seems evident that EMT is an important aspect to be taken into account in order to improve current clinical classifications, including the Barcelona Clinical Liver Cancer (BCLC) system, and to better stratify patients for prognosis and treatment. Yet, the lack of reliable biomarkers still limits its use. This brings us back to the previously debated issue of the biomarkers needed to assess the occurrence of the EMT. In a cohort of 149 patients

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stratified according to the BCLC classification, E-cadherin was inversely correlated to TGF-β1, both measured in the blood, but they were not useful to predict survival [108]. This is not surprising since therapies obviously change the natural history of the disease, and this was not a longitudinal study. On the other hand, it also underlined the fact that patients in the same BCLC stage have a very different tumor biology that can affect clinical outcome and long-term prognosis. Therefore, EMT biomarkers could be very important to help select patients receiving systemic targeted therapy, like c-Met. Longitudinal studies are required to explore the role of such biomarkers in terms of prognostic values, although the meaning of consistent circulating and local tissue concentrations of EMT biomarkers warrants further exploration.

Conclusions

There is no doubt that the EMT plays a key role in HCC development, while this issue is still controversial in underlying liver fibrosis. Thus, EMT regulators are potential therapeutic target in HCC. The difficulty in recognizing the process at the morphological level has so far hampered the identification of EMT-related biomarkers in clinical practice. However, new therapies in clinical validation phases are investigating molecules inducing EMT, like c-Met and TGF-β. These studies will provide important evidence that may help to shed light upon the process and understand the clinical role of EMT. In the personalized medicine setting, c-Met has proven to be important in better predicting a successful clinical outcome. According to the preclinical data, we expect patients with high TGF-β and low E-cadherin levels will have a worse prognosis. This patient subset is the most likely to benefit from therapy inhibiting the TGF-β pathway. Finally, a general consensus on the biomolecular/histological phenotype denoting the EMT will be a crucial step toward a better detection of circulating biomarkers.

Acknowledgments

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Author names in bold designate shared co-first authorship.

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Figure Legends

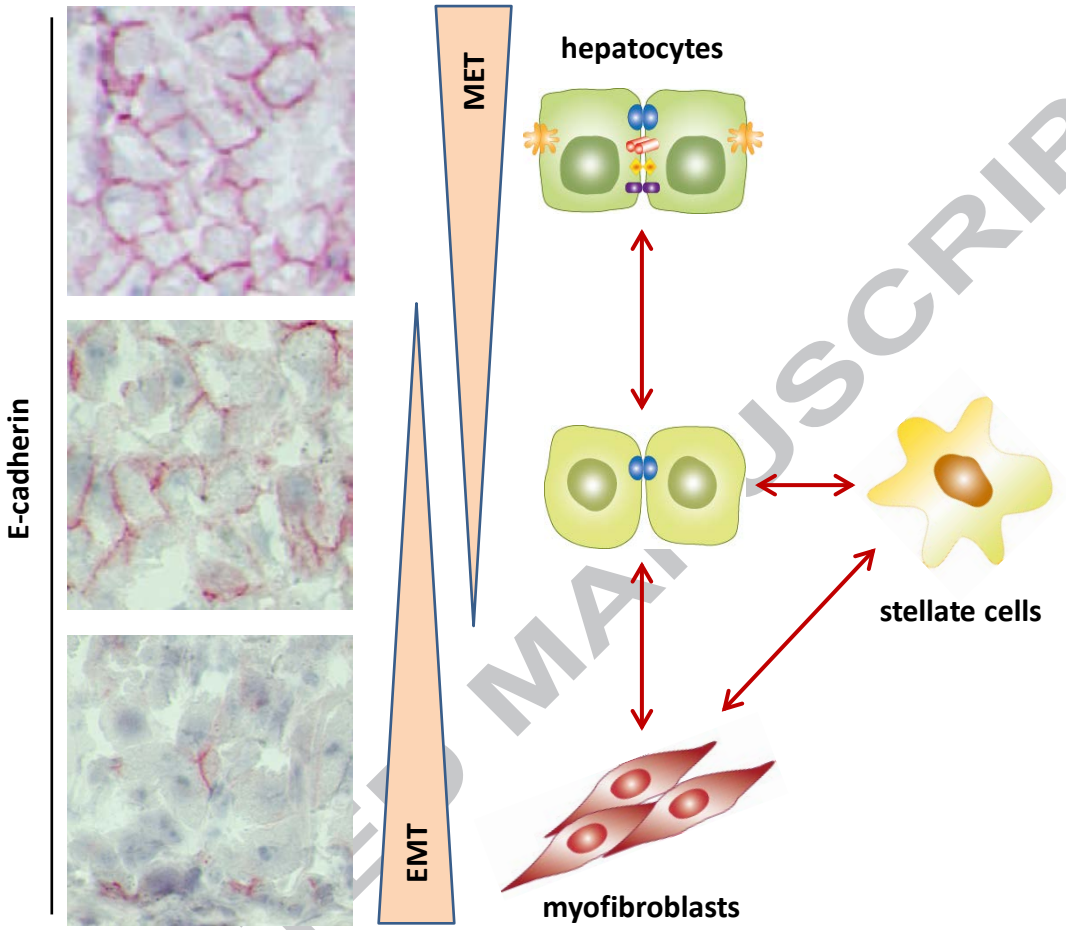
Fig. 1: The cellular heterogeneity of HCC by EMT-MET. The differentiation repertoire of malignant hepatocytes or progenitor cells contributes to the mosaic phenotype of HCC cells via partial or complete EMT-MET, as well as changes in the cell plasticity of HSCs in chronic liver disease. Left panel: Human HCC samples stained with anti-E-cadherin (E-cadherin) antibody. E-cadherin localizes at adhering cell-cell junctions in differentiated HCC while poorly differentiated HCC show either redistribution of E-cadherin to the cytoplasm or loss of its expression. Right panel: Scheme depicting the differentiation potential of hepatocytes and HSCs. Epitheloid HSCs provide an even higher complexity of HCC heterogeneity through a possible “liver cell panplasticity” in the underlying fibrosis [28].

Fig. 2: Key regulators impinging on EMT-TFs in hepatocellular EMT and HCC progression. Multiple regulatory components either activate or repress Snail, Twist or ZEB transcription factors which are involved in the downregulation of E-cadherin, resulting in EMT.

Fig. 3: Triggers of EMT in HCC. Schematic representation of molecular pathways triggering EMT in HCC development and progression [105,109-115].

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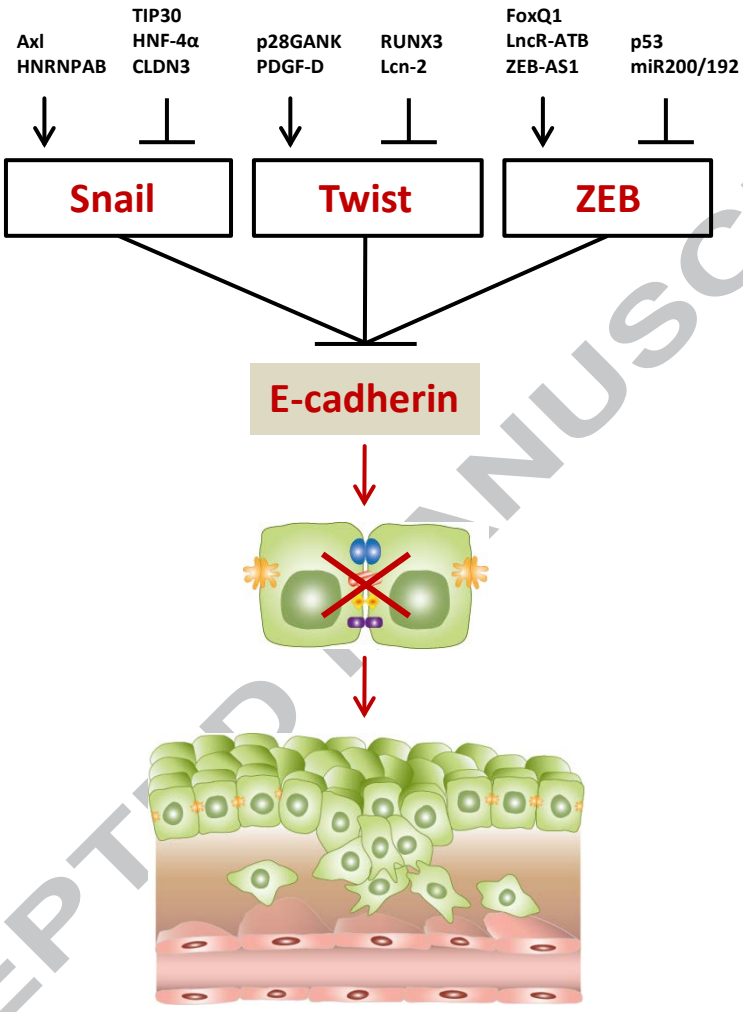
Fig. 1; Giannelli et al



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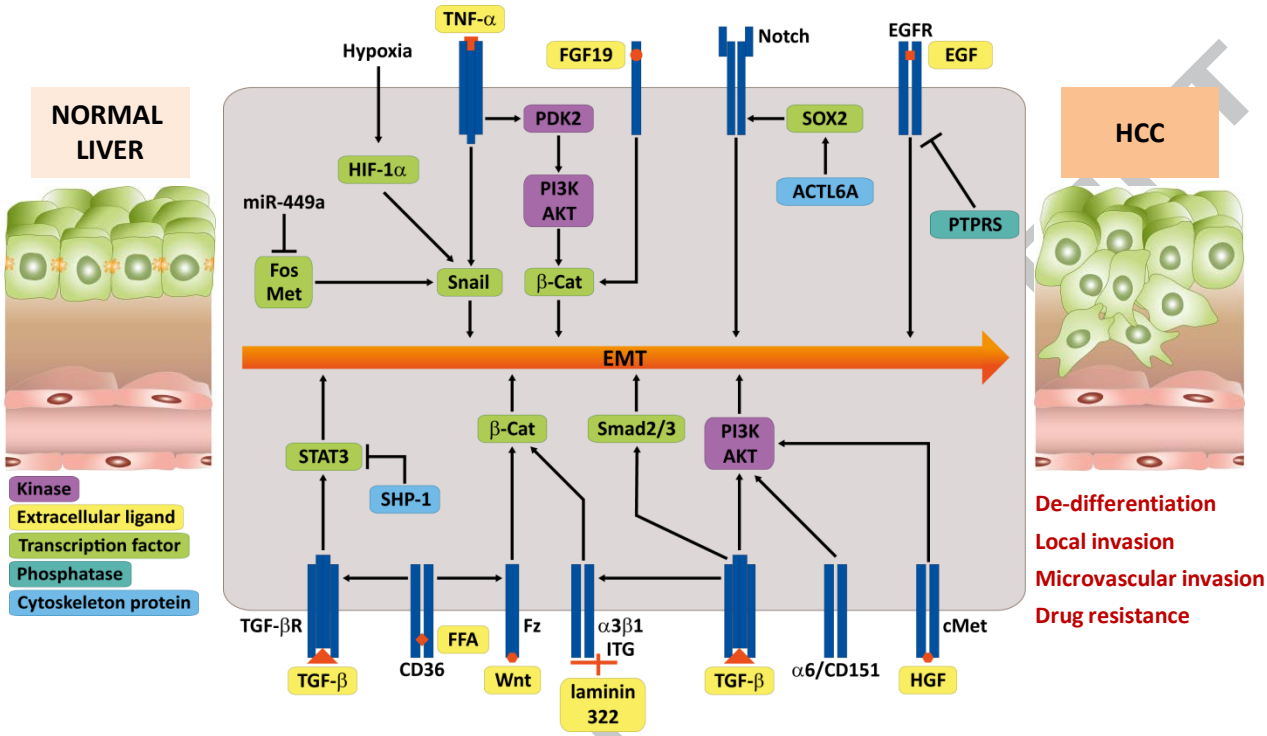
Fig. 2; Giannelli et al



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Fig. 3; Giannelli et al



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