The tumor microenvironment in hepatocellular carcinoma (Review)

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Abstract. The tumor microenvironment has been largely studied as a dynamic system orchestrated by inflammatory cells, including cancer cells, stroma as well as the extracellular matrix. It is useful to describe and predict the phenotypic characteristics of cancer. Furthermore, a better understanding of its interplay with the various aspects of the tumor cells may be utilized for the discovery of novel molecular targets. Liver cancer is considered a model of the relation occurring between the tumor microenvironment and tumor development. The chronic inflammatory status of the liver, sustained by the infection of hepatitis viruses, as well as the production of cytokines and growth factors within the parenchyma, lead to an intricate microenvironment. The identification of novel molecular therapeutic targets may improve the outcome of patients with liver cancer as it remains the third leading cause of cancer death worldwide. In the present study, the tumor microenvironment in hepatocellular carcinoma (HCC) was explored by a review of the literature. Studies on hepatitis virus infections and the consequent chronic inflammatory status were examined. In this context, immune-mediated and/or virus-related molecular mechanisms have been hypothesized as being responsible for liver cancer development. The interlink among HCC microenvironment components, comprising cellular elements, cytokines, growth factors and several proteins is also described together with the role of matrix metalloproteinases in HCC development. Finally, the rationale for targeting tumor-stromal interface is summarized in the context of new therapeutic opportunities.

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1. Introduction

The tumor microenvironment is a changing concept that defines the behaviour of cancer not by the genetics of the tumor cells alone, but by the surrounding milieu that the tumor cells need for survival, growth, proliferation, and metastasis (1). It is a dynamic system, largely orchestrated by inflammatory cells, that includes cancer cells, stromal tissue (immune cells, fibroblasts, myofibroblasts, cytokines, and vascular tissue), as well as the surrounding extracellular matrix (2).

Although signs of 'smouldering' inflammation are present in tumors for which a firm causal relationship to inflammation has not been established (breast tumors for example), it is estimated that underlying infections (sustained by *Helicobacter pylori* and hepatitis viruses) and the resulting chronic inflammatory state, which can promote carcinogenesis, are linked to 15-20% of all deaths from cancer worldwide (3,4). Indeed, inflammatory cells and mediators are present in the microenvironment of most, if not all, tumors, irrespective of the trigger for development (5). In addition population-based studies have shown that individuals who are prone to chronic inflammatory disorders have an increased risk of cancer development (6) and, accordingly, treatment with non-steroidal anti-inflammatory agents decreases the incidence and the mortality of several tumor types (7,8).

Liver cancer is a paradigm of the relation occurring between tumor microenvironment and tumor development. The chronic inflammatory status of the liver, sustained by the infection of hepatitis viruses, as well as the production of cytokines and growth factors within the parenchyma, lead to an intricate microenvironment.

Liver cancer is the fifth most prevalent form of cancer and the third leading cause of cancer-related deaths, immediately following lung and colon cancer throughout the world. Hepatocellular carcinoma (HCC) is the most common form of adult liver cancer, representing over 90% of all cases of primary liver cancer (9).

The majority of HCC patients have an underlying chronic inflammatory liver disease and liver cirrhosis is the main risk factor for the development of HCC (10,11). Chronic liver injury is associated with dysregulated growth of hepatocytes and results in the formation of regenerative nodules, dysplastic nodules, and HCC.

In recent years, a significant amount of attention has been drawn to the concept of the tumor microenvironment in an effort to better describe and predict the phenotypic characteristics of cancer (1,2,12). Moreover, a better understanding of the unique interplay between the various aspects of the tumor cells and the microenvironment may be useful for the discovery of novel molecular therapeutic targets (13,14).

2. Viruses, inflammation and HCC

The chronic infection sustained by hepatitis viruses (hepatitis B virus, HBV, and hepatitis C virus, HCV) is a major risk factor for HCC development (15) and several clinical studies observed that more than 85% of HCCs worldwide retain markers for HBV and/or HCV (16-21). This causal association between viral infection and tumor development has been well established and supported in animal and epidemiological studies (22).

Much of the liver injury, characteristic of acute HBV, is caused by recruitment of inflammatory cells, consequent secretion of cytokines and the ultimate lysis of infected cells (23,24). The HBV infection spread is controlled by NK cells that can directly lyse infected cells and can also down-regulate HBV replication by producing IFN-γ and TNF-α (25). Moreover, IFN-γ activates macrophages and increases TNF-α-mediated liver damage (26). The cytotoxic T cells ($CD8^+$) are involved in the recognition of viral peptides derived from phagocytized and proteolytically cleaved HBV proteins, in the activation and differentiation of B cells, and secrete IFN-γ and TNF-α, which inhibit the replication and gene expression of HBV. Results from transgenic mouse studies showed that chronic HBV-specific T cell-mediated liver disease was sufficient to induce HCC in HBV *in vivo* models (27).

The immunopathology of HCV infection is largely represented by an unspecific immune response against the virus sustained by a cytokine context that recruits non-specific lymphocytes (28,29), furthermore no HCV-neutralizing antibody has been detected (30). In the absence of viral clearance, this pathway boosts itself, leading to necro-inflammatory and fibrotic liver disease.

Furthermore, hepatic viruses are able to systematically evade the immune system and persist in the host through their ability to mutate under immune pressure (31,32) and therefore produce variation within T cell epitopes that may down-regulate T cell functions leading to an inhibition of the immune response against the original epitope (33-35).

Overall, in this context an immune-mediate mechanism may be responsible of liver cancer development (Fig. 1). The chronic cell damage and regeneration processes, mediated by viral hepatitis-induced immune responses, may lead to liver cancer by promoting cell proliferation and death (24,29,36). The necrosis of hepatocytes, as a result of chronic inflammation and consequent regeneration, enhances mutagenesis in host cells, which can accumulate and culminate in HCC (23,24,28). The risk of developing HCC increases the longer the viral infection-induced inflammatory process lasts (22,37). A general well-defined consequence of chronic inflammation is the release of free radicals, such as reactive oxygen species and NO reactive species (38). The exposure of liver tissue to oxyradical injury may lead to post-translational modification at critical residues of p53 protein (39) and accumulation of DNA adducts in HBV-transgenic mouse have been associated with HCC progression (40).

Virus-related molecular mechanisms can also be identified in liver cancer development (Fig. 1). HBV and HCV are involved in the genesis of HCC through the alterations of DNA repair system and centrosome duplication mechanisms, and through the viral-encoded oncoproteins that have transforming capability by disruption of gene expression and signaling pathways (41-44). In addition, several proteins encoded by HCV and HBV are able to directly alter cytokine expression and finally directly modulate the tumor microenvironment and the immune response in the liver, contributing to HCC development. A meaningful example is HCV $p21^{core}$, a viral structural protein that has been shown to play several roles: a) to decrease the production of IFN-γ and IL-2; b) to suppress HCV-specific CTL responses (45); c) to bind the TNF receptor I (TNFRI) and the TNF-related lymphotoxin receptor modulating the signal of these cytokines (46); d) to activate the IL-2 promoter through the NFAT pathway and suppress the immunity by inhibiting IL-12 and NO production from macrophages (47); e) to down-regulate MHC class II genes in B cells and prevent B cell apoptosis (48). The HCV envelope protein, E2, inhibits NK cell function by binding CD81 on NK cells (49). In addition, the non-structural HCV phosphoprotein, NS5A, through its ability to inhibit IFN-α induced protein kinase, is implicated in mediating HCV resistance to IFN-α (50). Among HBV encoded protein, HBx can activate two transcription factors, NF-κB and NFAT, implicated in the expression of important cytokines, such as IL-6, IL-8, TNF-α, and modulates the function of the inflammatory mediators including IL-8, ICAM-1 and MHC factor (51).

In the context of hepatitis virus infections, both immune-mediate and molecular mechanisms of HCC development may also be associated with the development of extrahepatic lymphoproliferative disorders such as type II mixed cryoglobulinemia syndrome and B cell non-Hodgkin's lymphoma (52-62).

3. Components of the HCC microenvironment

Components of the HCC microenvironment comprises cellular elements, cytokines, growth factors and several proteins. Their linkage is described in Fig. 2.

Cancer-associated fibroblasts (CAFs). CAFs are a central elements of tumor microenvironment. They are the most prominent cell type within the tumor stroma of many cancers and play a critical role in tumor-stromal interactions (63-65). CAFs are involved in HCC growth and invasion, as they are able to produce epidermal growth factor (EGF), fibroblast growth factor (FGF),

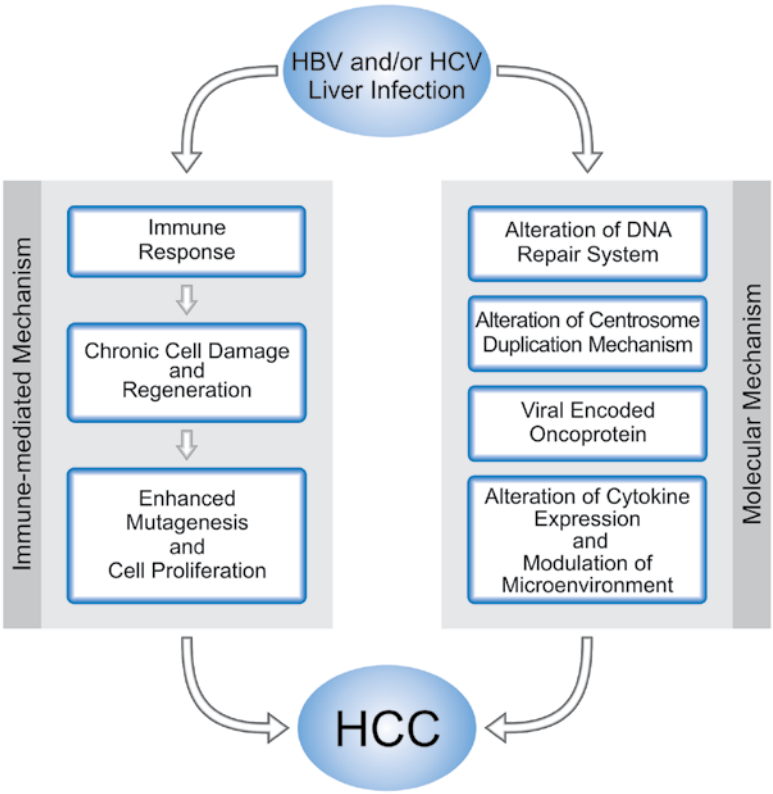

Figure 1. Representative scheme of the immune-mediate and viruses-related molecular mechanisms responsible of liver cancer development. HBV, hepatitis B virus; HCV, hepatitis C virus; HCC, hepatocellular carcinoma.

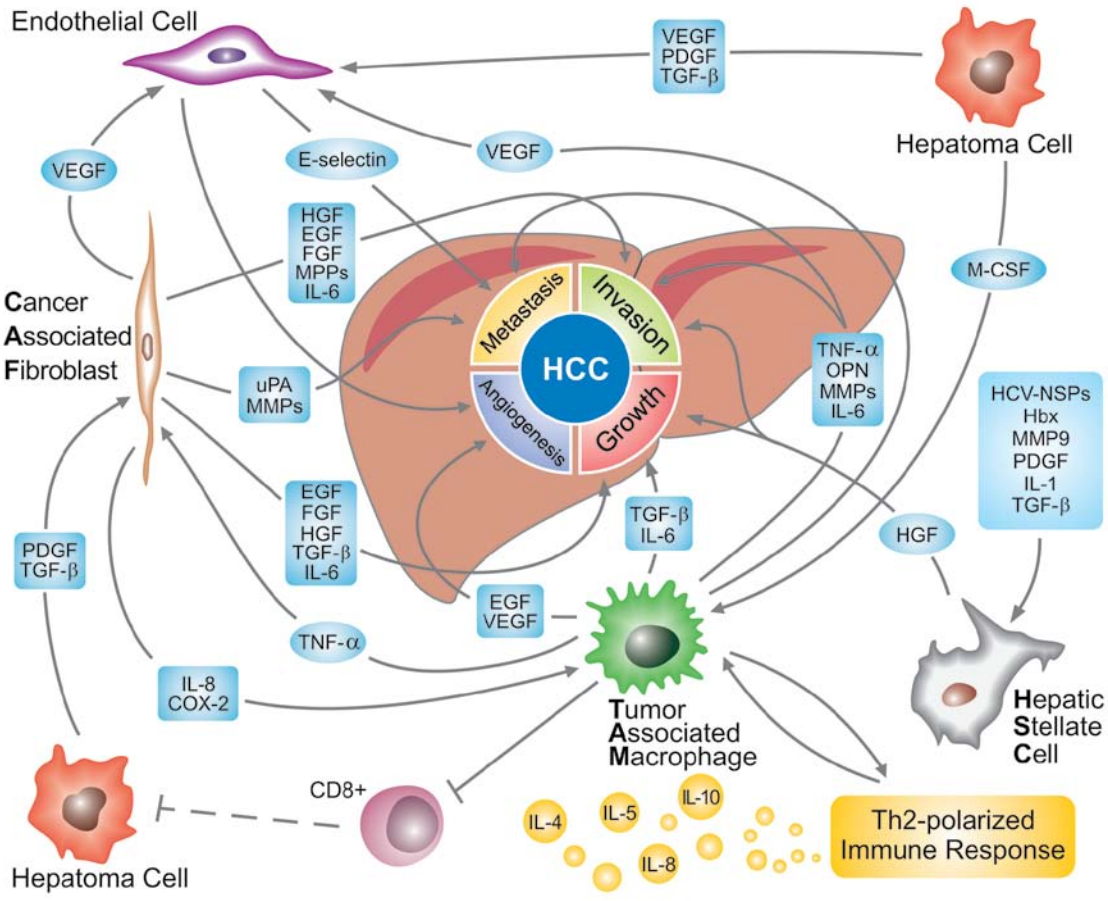

Figure 2. Hepatocellular carcinoma (HCC) microenvironment components and their linkage. Refer to the text for abbreviations.

hepatocyte growth factor (HGF), cytokines (IL-6), chemokines (stroma-derived SDF-1/CXCL12) and metalloproteases (MMP-3 and MMP-9) (65-67). CAFs secrete also interleukin-8, COX-2 and secreted protein acidic rich in cysteine (SPARC) to recruit and stimulate macrophage production, which can increase the activation of CAFs through secretion of TNF-α and PDGF (68,69). Experiments on invading co-cultures of cancer cells and CAFs revealed that the leading cells to tumor progression are always CAFs and that cancer cells would move into the ECM behind the CAF (70). Thus, HCC cell growth and metastatic spread are dependent upon the presence of CAFs and HCC cells reciprocally stimulate their proliferation (71).

Hepatic stellate cells (HSCs). HSCs, or Ito cells, are perisinusoidal cells whose activation is responsible for collagen synthesis in the liver (72). In response to a repeated liver injury, HSCs are activated and thus they trans-differentiate into myofibroblast-like cells. This phenotypical transformation is recognized as a central event in the development of hepatic fibrosis (73) in which activated HSCs are responsible for the production of cytokines, chemokines, growth factors and an extensive ECM (74,75). HSC/myofibroblasts also infiltrate the stroma of liver tumors and localize around tumor sinusoids, fibrous septa and capsules (11,76). Specifically, the conditioned media collected from HSCs induces proliferation and migration of HCC cells in culture, moreover through the activation of NF-κB and extracellular-regulated kinase (ERK) pathways, HCSs promote HCC growth and reduce the extent of central necrosis (11). Xia *et al* showed how HCC cells are able to promote HSC activation in rat cell culture (77). Accordingly, co-culture of HSCs with HCC cells stimulated HSC proliferation, migration and expression of proangiogenic genes such as VEGF-A and MMP-2 (78). Hepatitis B virus X protein, and HCV non-structural proteins, MMP-9, PDGF, TGF-β1, JNK, insulin-like growth factor binding protein 5, cathepsins B and D, are potent inducers of Ito cell activation, proliferation and therefore enhance liver fibrosis and carcinogenesis (79-87).

Tumor-infiltrating leukocytes. A leukocyte infiltrate, varying in size, composition and distribution, is present in most tumors. Its components include TAMs and related cell types, mast cells and T cells. Evidence, based on adoptive-transfer studies, cell-depletion studies, clinical correlations and gene-manipulation studies, showed that each of these bone-marrow derived components can be involved in carcinogenesis and/or tumor invasion and metastasis (88-90).

Tumor-associated macrophages (TAMs) form the basis for the model that leukocyte infiltrates are involved in tumor progression. They are located in tumor stroma and can undertake a wide spectrum of polarized activation states. Interleukin-4, interleukin-10, transforming growth factor-β (TGF-β) found within the tumor microenvironment promotes TAM polarization towards M2 activated cells (91,92). M2-like TAM expresses a distinctive set of cytokines, such as IL-10 and TGF-β (93,94) and chemokines including CCL17, CCL22 and CCL24 favouring regulatory T cell (Treg) recruitment and development of an ineffective Th2 polarized immune response (93,95). Moreover, M2 macrophages support tissue repair and remodelling, as well as angiogenesis through the production of VEGF or EGF (93). It has also been found that, Kupffer cells, that are liver-specific TAMs, are able to impair T cell $CD8^+$ dependent immune response through the interaction between programmed death 1 (PD1) on T cell $CD8^+$ and programmed death ligand-1 (PD-L1) produced by Kupffer-TAMs causing the deficiency of $TCD8^+$ cytotoxic function in HCC (96). Accordingly blocking the interaction between PD-L1 and PD1 restores $CD8^+$ activity (97). Moreover, Kupffer cells, as well as stellate cells, when stimulated by inflammatory cytokines (IL-1, TNF-α, PDGF), produce excessive osteopontin that plays a pivotal role in various cell signaling pathways that promote inflammation, tumor progression and metastasis (98,99).

Lymphocytes can be consistently observed in a variety of human cancers, and in some cases these infiltrating lymphocytes correlate with a favourable prognosis (100). However, not all T cells are anti-tumor effector immune cells. A subpopulation of T cells $CD4^+CD25^+Foxp3^+$, called Tregs (regulatory T cell), plays a pivotal role in promoting tumor growth and progress by inhibiting the immune response against cancer (101). Regarding HCC, it has been demonstrated that Tregs were more predominant than T $CD8^+$ in HCC tissues in comparison with nearby benign tissues and that Tregs prejudice T $CD8^+$ proliferation, activation, degranulation and production of granzymes and perforin (102). Moreover, low intratumoral T $CD8^+$ and high regulatory T cells are associated with a worse prognosis in HCC (102,103). In a recent study by Shen *et al* it was demonstrated that Treg level and function is related with TNM stages in HCC patients and that SDF-1 may be responsible for the increased recruitment of Tregs to HCC tumor sites (104).

Th17 cells are $CD4^+$ lymphocytes producing IL-17. Recently, they both have been found with increased frequencies within certain tumors (105). However, the relationship between Th17 cells and tumor immunopathology has been controversial (106,107). In HCC, Th17 cells have been found in increased numbers within tumors and correlate with poor survival and increased postoperative recurrence, indicating that Th17 cells and IL-17 may promote tumor progression in HCC (108).

Endothelial cells. Endothelial cells play an essential role in blood vessel formation and its migration contributes to the creation of the tumor neovasculature. Endothelial cells in HCC tissues and normal tissues have molecular and functional differences. They express a variety of angiogenic receptors, including vascular endothelial growth factor receptors (VEGFR), epidermal growth factor homology domains-2 (Tie-2), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and C-X-C chemokine receptors (CXCRs). Several signaling pathways connected with survival, proliferation, mobilization and invasion of endothelial cells are regulated by the interaction between ligands and their corresponding receptors (109-112). Moreover, tumor-associated endothelial cells have a high expression of TGF-β1 and CD105. TGF-β1 plays a function of chemo-attractant for CD105 expressing endothelial cells and promotes tumor angiogenesis (113). Of note, it has been shown that $CD105^+$ endothelial cells from HCC, had features of increased angiogenesis activity with higher resistance to chemotherapeutic agents and to angiogenesis inhibitors (114).

Hepatoma cells. Hepatoma cells are not just passive observers of the tumor microenvironment because it has been suggested

that they directly alter the surrounding milieu. Hepatoma cells are able to produce VEGF, PDGF, TGF-β, or monocyte colony stimulating factor (MCSF) leading to the recruitment and the activation of CAFs, TAMs, and endothelial cells (113,115,116). Hepatoma cells can also inhibit differentiation and maturation of DCs by down-regulation of protein kinase C β II expression and an increase in regulatory T cell production (117,118). Another study shows that expression of glypican-3 on the hepatoma cell membrane is involved in macrophage recruitment (119).

Extracellular matrix. ECM includes the interstitial matrix and the basement membrane and is composed of many different glycoproteins, proteoglycans and hyaluronan (HA). The main roles of proteoglycans are to maintain the structural framework of the tissue and to store growth factors within the ECM. Heparan sulfate, chondroitin sulfate, and keratan sulfate are the major types of proteoglycans in the ECM. Of these, heparan sulfate proteoglycans (HSPGs) are known to play an important role in the pathogenesis of HCC as key growth factors such as FGF, HGF, PDGF, and VEGF are either stored in HSPGs or utilize HSPGs as co-receptors for binding to their tyrosine kinase receptors (80,120,121). Several previous studies have shown that the heparin-degrading endosulfatases, sulfatase 1 (SULF1) and sulfatase 2 (SULF2), play important roles in modulating these heparin-binding growth signaling pathways (122,123). Although SULF1 and SULF2 are structurally very similar, they play an opposite role in FGF signaling and its downstream AKT/mitogen-activated protein kinase pathway. Particularly, desulfation of co-receptor type HSPGs, SULF1-dependent, inhibits binding of the growth factor to its receptor, abrogating growth factor signaling and producing a tumor suppressing effect. Desulfation of HSPGs, SULF2-dependent, releases growth factors from the storage subtype of HSPGs and increases binding of growth factors to their receptors, leading to the activation of growth signaling (122,124,125). A heparan sulfate mimetic, PI-88, synthesized for targeting heparanases in cancer, has been shown to inhibit SULFs activity and its safety and efficacy, as an adjuvant therapy for hepatocellular carcinoma after curative resection, was shown recently in a phase II clinical trial (126,127). Several cell surface adhesion receptors and various ECM components (such as fibronectin, laminin, collagens, and elastin) are involved in extensive and complicated interactions through chemotaxis. Collagens are the most abundant protein in the ECM and provide a structural support for cells. As mentioned above, myofibroblasts/activated HSCs are the main source of collagen production in the HCC stroma (128,129). Collagens also promote cell migration and proliferation in HCC. Let-7g, a known tumor suppressor miRNA, down-regulates COL1A2 and inhibits HCC cell migration and growth (130). Laminin is an important ECM protein involved in various biological activities, including assembly of the basement membrane, cell attachment, cell migration, cell growth and differentiation, and angiogenesis (131). Of the different subtypes of laminins, laminin-5 is expressed in HCC nodules, and its expression is associated with the metastatic phenotype of HCC (132). Moreover, Laminin-5 (Ln-5), together with TGF-β1, was reported to promote epithelial to mesenchymal transition (133). Integrins are surface receptor proteins that mediate cell-matrix and cell-cell adhesion (134). The overexpression of β1 integrin inhibits HCC cell proliferation by preventing Skp-2 dependent degradation of p27 via PI3K pathways (135). On the other hand, the overexpression of α3β1 and α6β4 integrin is associated with increased migration and invasion of HCC cells in an Ln-5 dependent condition (136-139).

Cytokines. The liver hosts many cell types that are susceptible to the actions of cytokines. Hepatocytes bear a variety of cytokine receptors such as IL-1, TNF-α, and IL-6. Non-parenchymal cells, such as the resident liver macrophages (Kupffer cells), not only synthesize many cytokines, but the cytokine environment can also affect the actions of these immune cells. Liver sinusoidal endothelial cells are also targets and producers of various cytokines. Mounting evidence indicates the involvement of cytokines in hepatocarcinogenesis.

IL-6 is produced by Kupffer cells at high levels in response to hepatocyte death and thus it contributes to compensatory hepatocyte proliferation (140). Several studies investigated the interaction between IL-6 and HBV. It has been shown that HBx can induce IL-6 release in HBV infected patients (141). In addition, HBx induced an increase in IL-6 transactivation (142). Indeed, HBx may play a role in hepatic inflammation and disease by up-regulating IL-6, leading to cirhossis and HCC (143). Serum IL-6 is increased in cirrhosis and high serum IL-6 is associated with increased risk for HCC and a poor prognosis in patients with HCC (144-146). It is a key cytokine, encouraging cancer cell proliferation while also inhibiting their apoptosis through activation of signal transducer and activator of transcription 3 (Stat3) (147). IL-6 signaling can also influence T cell subset differentiation, particularly in the presence of other cytokines such as TGF-β (148). Estrogen suppresses IL-6 production in Kupffer cells, partly explaining the gender discrepancy in HCC development (149). Moreover, it has been recently shown that IL-6 is a link between obesity and HCC as increased expression of IL-6 and TNF in obese mice leads to the activation of the IL-6 signaling pathway via the downstream STAT3 and ERK pathways, thus promoting tumorigenesis in the liver (150). Some studies have correlated changes in cytokine expression with HCC metastasis and/or recurrence. In a rat model, IL-6 has been implicated in HCC metastasis, as highly metastatic HCC (metastatic to the abdominal cavity) has been shown to release more IL-6 in serum (151). Exogenous addition of IL-6 did not affect primary tumor formation but did affect the metastatic potential of tumor cells when compared with tumor cells expressing endogenous IL-6 (151). Furthermore, Coskun *et al* showed that in breast cancer patients, higher serum levels of IL-6 could be used to distinguish primary or metastatic liver tumors from benign HCC lesions (152). Studies focusing on the association between IL-6 polymorphisms and HCC risk are still controversial, further studies are warrented to clarify these differing findings (153-155).

TNF-α is produced by Kupffer cells and other immune cells in response to tissue injury. It modulates NF-κB and Akt pathways and is involved in several tumor models (37,156,157). It is associated with an increase in cell cycle progression and oxidative stress through the formation of 8-oxo-deoxyguanosine, an established marker of DNA damage associated with chronic hepatitis in human livers (158). Cytokine stimulation of TNF-α, IL-1β, or IL-18 has also been shown to induce expression of TRAIL in HCC cell lines (HepG2, Hep3B, Huh7). The expression of TRAIL on the HCC cell surface might contribute to tumor cell immune evasion by inducing apoptosis of activated human lymphocytes (159). Studies on TNF-α expression are still contro-

versial, 2 reported high levels in HCC patients, especially those with recurrence (160,161). In addition, the levels of the TNF-αRs, TNF-αRI and TNF-αRII, were higher in HCC patients when compared with healthy individuals (162). However, in other studies, TNF-α levels were lower in HCC tumor tissue versus the tissue surrounding the tumor and in HCC patients versus healthy individuals (163,164). In a study by Kakumu *et al* TNF-αRs correlated with disease progression and IFN-α treatment did not affect their level (162). Regardin TNF-α polymorphism, the TNF-α (-308) SNP (single nucleotide polymorphisms) in the promoter region of the gene, which includes TNF-α1 (-308G) and TNF-α2 (-308A) alleles, is associated with cancer susceptibility and induced expression of TNF-α (153,165-167).

IL-1 is a pro-inflammatory cytokine that promotes MyD88 adaptor protein-dependent compensatory proliferation of hepatocytes (168). IL-1 also promotes HSC proliferation, activation, and transdifferentiation into the myofibroblastic phenotype in addition to activating HSCs to produce and activate MMPs, particularly MMP-9 (169). As mentioned, IL-1β has also been shown to induce expression of TRAIL in HCC cell lines (HepG2, Hep3B, Huh7) (159). In HCC patients, proinflammatory IL-1β was shown to be elevated compared with healthy individuals (160). However, in the analysis performed by Bortolami *et al* IL-1β mRNA was lower in tumors versus the tissue microenvironment (163). Polymorphism analyses of proinflammatory IL-1β related to hepatotropic viruses infection (HCV and HBV) have been reported, suggesting that IL-1β polymorphism may be a genetic marker for the development of hepatitis-related HCC (170-172).

IL-10 expression is, among the many anti-inflammatory cytokines, the most studied with regard to HCC. Several groups have shown that IL-10 is highly expressed in HCC tumors and individuals with HCC versus non-tumorous-surrounding tissue controls or healthy cohorts (162,164,173-175). These studies suggest that increases in IL-10 and perhaps other Th2 cytokines correlate with progression. The clinical significance of post-operative IL-10 levels in patients with HCC resection was tested by Chau *et al* (176). They demonstrated how IL-10 levels were significantly higher in HCC than in healthy individuals and how patients with high IL-10 had a worse disease-free survival. A multivariate analysis implied that IL-10 might be a predictor of the postresection outcome of HCC patients (176). The association between IL-10 and the risk for developing HCC is still not clear (153,166,177-179). In a HCC mouse model, high angiogenic activity was associated with attenuated lymphocyte extravasation and correlated with the expression of anti-inflammatory IL-10 (109). Budhu *et al* analysed the tumor microenvironment in HCC metastasis from patients with HBV-positive metastatic HCC and found that a dominant, Th2-like cytokine profile (IL-4, IL-8, IL-10, and IL-5) and a decrease in Th1-like cytokines (IL-1α, IL-1β, IL-2, IL-12p35, IL-12p40, IL-15, TNF-α, and IFN-γ) was associated with the metastatic phenotype (180).

IL-12 plays a tumor suppressor role as it induces the production of interferon-γ from NK cells or naïve T cells, promotes helper T cell differentiation, enhances cell-mediated immune responses, and activates cytotoxic lymphocytes (181). This effect is supposed to be mediated by the activation of tumor specific cytotoxic T lymphocytes and NK cells, and inhibition of angiogenesis (181). High level of IL-12 has been found in HCC patients (173). In a mouse model, intra-tumoral injection of IL-12 gene therapy induced lymphocyte infiltration into the tumor and inhibited tumor growth and angiogenesis (96,182). However, the clinical use of IL-12 is limited due to the severe systemic toxicity resulting from high interferon-γ levels in large doses and the minimal efficacy of low doses (183,184).

IL-17 is produced by Th17 cells. Previous studies have shown that IL-17 is related to tumor progression via effects on immune cells, vascular endothelial cells and stromal cells (108,185). In a recent study by Gu *et al* it has been suggested that the IL-17-mediated tumor-promoting role involved a direct effect on tumor cells through IL-6 induction by activating the AKT pathway; IL-6 in turn activated JAK2/STAT3 and up-regulated pro-invasive factors (IL-8, MMP-2, and VEGF both *in vitro* and *in vivo*) (186).

Growth-factors. TGF-β exerts an indispensible and complex role in carcinogenesis and progression of tumors and particularly in liver fibrogenesis and hepatocarcinogenesis (187-189). It is up-regulated in HCC tissues and peri-neoplastic stroma (189). In HCC pathogenesis TGF-β has a dual role. In the premalignant state it plays as a tumor suppressor through anti-proliferative effects and activation of apoptosis signals. The inhibition of cell proliferation is mediated by the mobilization of cyclin-dependent kinase inhibitors and suppression of c-Myc while the proapoptotic mechanisms of TGF-β1 are mediated by down-regulation of anti-apoptotic proteins (187,190). The tumor suppressor effect of TGF-β acts also through the suppression of tumor stroma mitogens and tumorigenic inflammation (187). In addition, a study by Murata *et al* reported that TGF-β suppressed viral RNA replication and protein expression from the HCV replicon and was also associated with a Smad-dependent cellular growth arrest (191). However, TGF-β may function to enhance tumorigenicity and plays as an oncogenic growth factor via several different mechanisms (187). Matsuzaki *et al* and Murata *et al* showed, respectively, that HBx and HCV could shift hepatocytic TGF-β signaling from the tumor-suppressive pSmad3C pathway to the oncogenic pSmad3L pathway through the activation of c-Jun N-terminal kinase (JNK) (192,193). Sohn *et al* proposed that promoter methylation of tristetrapolin (TTP), a negative post-transcriptional regulator of C-Myc, shifts TGF-β1 signaling in HCC tumorigenesis (194). Moreover, TGF-β1 increases migration, vascular invasion (via α3 integrin expression), angiogenesis (via VEGF production) and metastasis (via connective tissue growth factor) (71,195-199). TGF-β is also involved in the EMT through the down-regulation of E-cadherin and the up-regulation of Snail and the PDGF signaling pathway (133,200,201). In a recent study, Wang *et al* reported that exposure of hepatocytes to TGF-β1 increases miR-181b expression, which promotes cell growth, survival, migration and invasion of HCC cells (202). Similarly, TGF-β induces miR-23a, 27a, and 24, which promotes growth and survival of HCC cells (203). Okumoto *et al* studied the relationship of plasma TGF-β to anti-tumor immunity and prognosis. HCC patients with high TGF-β concentration had a shorter survival period than those with concentrations below that of healthy individuals. Therefore, the concentration of TGF-β was shown to be a predictor of outcome of patients with unresectable HCC (204). Single nucleotide polymorphism studies show that TGF-β SNPs may be associated with reduced risk of developing viral hepatitis-mediated HCC but further studies are needed to clarify this association (153,179,205-207).

Other growth factors play important roles in HCC pathogenesis. FGFs are involved in angiogenesis, tissue regeneration and wound healing (208,209). It has been found that aberrant expression of FGFs promotes HCC and endothelial cell proliferation through the activation of downstream Erk and AKT pathways (124) and facilitates invasion and metastasis of HCC (210,211). HGF is expressed in hepatic stellate cells or myofibroblasts and is thought to be a mediator of tumor-stromal interactions through which myofibroblasts increase the proliferation and invasion of HCC cells (121). The HGF ligand exerts its effect by binding the high-affinity tyrosine kinase receptor c-MET, which is predominantly expressed on the surface of epithelial and endothelial cells. c-MET overexpression, is observed in 20-48% of HCC samples (212-214). Deregulation of c-MET is associated with various molecular-genetic factors, and overexpression has been linked with decreased 5-year survival in patients with HCC (215). Moreover, a c-MET-regulated expression signature defines a subset of HCC in humans; these patients have a poor prognosis and an aggressive phenotype (216). PDGF plays an important role in the trans-differentiation of HSC into myofibroblast-like cells, thus stimulating fibrogenesis in the liver and increasing cell proliferation. It has been showed by Campbell *et al* that overexpression of PDGFC in the liver of the transgenic mouse results in HSC activation, proliferation, tissue fibrosis and subsequent development of hepatocellular carcinoma through the activation of the ERK-1/-2 and PKB/Akt signaling pathways (80). PDGF is also involved in neoangiogenesis, as it is believed to stabilize new blood vessels (217). Angiogenesis is a critical step in HCC progression and the VEGF is a major growth factor that stimulates angiogenesis in normal and tumor tissues (218). Overexpression of VEGF may be induced by the hypoxic tumor environment (mediated by hypoxia-inducible factor 2-a), activation of EGFR and cyclo-oxygenase-2 signaling (219,220). The inflammatory condition via NF-κB signaling pathway boosts VEGF expression that acts not only on the proliferation of endothelial cells in the vasculature but also on the proliferation of cancer cells expressing VEGF-A receptor through downstream Akt/mTOR signaling (221,222). Increased VEGF and VEGF receptors (VEGFRs; which include VEGFR-1, -2 and -3) expression has been observed in HCC cell lines and tissues, as well as in the serum of patients with HCC (223-226). The hepatitis Bx antigen has also been associated with the up-regulation of VEGFR-3 (227). VEGF clearly has an important regulatory role in HCC. High levels of VEGF expression have been linked with HCC tumor grade, poor outcome after resection, disease recurrence, poor disease-free and overall survival, vascular invasion, and portal vein emboli (228-232).

Matrix metalloproteinases (MMPs). MMPs lead to tissue remodeling, inflammation, tumor cell growth, migration, invasion and metastasis in many cancers. They are major modulators of the tumor microenvironment, playing key roles in tumorigenesis (99,233-235). Different stromal and cancer cells produce various types of MMPs whose main subtypes are collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), matrylisins (MMP-7, -26), membrane type MMPs (MMP-14, -15, -16, -17, -24, -25) and stromelysins (MMP-3, -10, -11) (236). As MMPs are released in inactivated forms they should be first triggered to exert their effect. Twist 1, focal adhesion kinase (FAK), claudin-1, HBV X protein, plasmin, furin, or other MMPs are well recognised activator of MMPs function (237-240). The role of MMPs in the microenvironment is not only limited to its proteolytic activity on the surrounding stroma, but it is also involved in modulating cancer signaling pathways (99,234,241). For example, Mitsiades *et al* reported that Fas ligand is cleaved by MMP-7 and is then unable to induce apoptosis connecting MMPs to the inhibition of apoptosis signals in tumor cells (242). It has been also demonstrated that MMPs are involved in the modulation of the inflammatory response by regulating inflammatory cytokines and chemokines, which promote cancer progression (233,243,244). MMP-2, -9, and -14 are involved in VEGF bioavailability and angiogenesis in HCC (245,246) and also activate the TGF-β that is a key modulator of epithelial-mesenchymal transition in HCC (241). Moreover, TGF-β1 reciprocally activates MMPs via miR-181b, that is up-regulated by TGF-β1 and up-regulates MMP-2 and -9 promoting migration and invasion of HCC cells (202). High expression of MMP-9 is associated with activation of the PI3K/PTEN/AKT/mTOR pathways (247,248), invasion and metastasis by cleaving the osteopontin precursor into an active form (249) and capsular infiltration (250). Recent evidence suggests that MMP-9, linked with lipocalin-2 or neutrophil-associated lipocalin (NGAL), may enhance its role in cancer development (251-254). Accordingly, our preliminary studies indicate that both mRNA transcript and protein levels of NGAL were higher in HCC than in normal liver tissue; furthermore a correlation between NGAL and MMP-9 in HCC has been shown (Pezzino FM, *et al*, 15th World Congress on Advances in Oncology and 13th International Symposium on Molecular Medicine, abs. 349, S67, 2010 and Candido S, *et al*, 15th World Congress on Advances in Oncology and 13th International Symposium on Molecular Medicine, abs. 343, S65, 2011).

Tissue inhibitors of metalloproteinases (TIMPs) are natural inhibitors of MMPs and play complex roles in preventing the excessive degradation of ECM and in regulating cell proliferation, apoptosis, angiogenesis and MMPs activation. The enzymatic activities of MMP and TIMP are tightly balanced. It has been shown that high expression of TIMP-1 suppresses the proliferative and invasive potential of HCC cell lines (255,256) and that TIMP-3 is a negative regulator of MMPs able to inhibit tumor progression, invasion, and metastasis in HCC (257,258). Gene expression profiles showed that MMP-14, -1 and TIMP-1 are gene signatures linked to poor prognosis in HCC (259,260).

Physical environment. Hypoxia enhances proliferation, angiogenesis, metastasis, chemo-, and radio-resistance of HCC. Increasing evidence suggests that hypoxia exerts profound effects on the development and evolution of the tumor microenvironment by regulating differentiation of both tumor and stromal cells. Hypoxia induced factor-1 (HIF-1) is a major transcription factor induced and activated during hypoxia environment (261). Hypoxia can also induce β-catenin overexpression and intracellular accumulation in four different HCC cell lines through down-regulation of the endogenous degradation machinery (262). Activation of the HIF-1α-regulated glycolysis was closely related to HCC metastasis via ENO1, a glycolysis-related gene (263).

4. Signaling pathways in HCC

Several abnormal molecular signaling pathways are implicated in HCC development and sheding light on these mechanisms will improve the understanding of molecular hepatocarcinogenesis. In this respect, the most important elements are growth factors signaling pathways (VEGF, PDGF, EGF, HGF), the mitogen-activated protein kinase (MAPK), phosphatidylinositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin and WNT/β-catenin pathways (264). These signaling cascades are of interest from a therapeutic perspective, because targeting them may help to reverse, delay or prevent hepatocarcinogenesis.

The ERK/MAPK pathway (also known as the RAF/MEK/ERK pathway) is a ubiquitous signal transduction pathway that regulates crucial cellular processes, including proliferation, differentiation, angiogenesis and survival (265). Importantly, the overexpression or activation of components of this pathway is believed to contribute to tumorigenesis, tumor progression and disease metastasis in a variety of solid tumors (266). The ERK/MAPK pathway lies downstream of various growth factors described in HCC and it has been shown that it is constitutively activated in this type of tumor (267-274). Constitutive activation of the PI3K/AKT/mTOR signaling pathway has been firmly established as a major determinant of tumor cell growth and survival in a multitude of solid tumors (248). In HCC PI3K/AKT/mTOR signaling pathway is overboosted by different mechanisms suggesting that it may play a critical role in the pathogenesis of HCC (275-278). Alterations of the WNT/β-catenin pathway are an early carcinogenic event in the development of HCC (279). The accumulation of β-catenin stimulates the expression of genes involved in cell proliferation (for example, MYC, MYB, CJUN and CYCD1), angiogenesis, anti-apoptosis and the formation of extracellular matrix (229). Giles *et al* showed that β-catenin is frequently mutated in HCC and this leads to the accumulation and stabilization of this glycoprotein (280). Additionally, these mutations seem to be particularly common in HCCs associated with chronic HCV infection (280). Hoshida *et al* performed a gene expression profile analysis of 603 HCC patients in an effort to define molecular drivers of the disease. Three subclasses of HCC were characterized, two of which showed either increased WNT pathway activity or increased MYC/AKT pathway activity. The mechanism through which WNT pathway activation may occur was determined to be mediated by TGF-β (281).

5. New therapeutic opportunities

The basic rationale for targeting tumor-stromal interface is to suppress the effect of surrounding tissues or cell types that stimulate hepatocarcinogenesis, tumor progression, invasion, and metastasis while minimizing systemic toxicity by delivering drug effects specifically to tumors and their microenvironment. Current drugs mostly target the tumor-stromal interaction by inhibiting receptors and their downstream signaling pathways, thereby abolishing the cancer-promoting signaling provided by the tumor stroma rather than directly targeting specific components.

The TGF-β receptor inhibitor (LY2109761) is a clarifying example of this therapeutical approach. It acts through inhibition of CAFs proliferation and consequently suppresses synthesis and release of connective tissue growth factor, reduces tumor cell growth, intravasation, and metastatic dissemination of HCC cells (199). Phase I clinical trials targeting TGF-β signaling for the treatment of HCC have not yet been performed. The recombinant monoclonal antibodies, bevacizumab, cetuximab, ramucirumab, whose efficacy in HCC is under evaluation in clinical trials, represent important goals in terms of target therapies. However, the class of kinase inhibitors is showing to have great potential, as sorafenib, an oral multi-kinase inhibitor, is the most successful medication of this kind. It inhibits VEGFR-2/-3 and PDGFR as well as Raf kinase, disrupting tumor-stromal interactions and resulting in decreased cell proliferation and angiogenesis. The efficacy and safety of sorafenib have been demonstrated in phase III clinical trials, and it is currently the standard of care for patients with advanced stage HCC (282). Similarly, brivanib, which targets VEGFR-2 and FGFR, sunitinib, which targets PDGFR, VEGFR, C-KIT and FLT-3, erlotinib, which targets EGFR, linifanib, which targets VEGFR and PDGFR, which targets VEGFR-2, and PI-88, which targets heparanase as well as sulfatases, are now in phase III clinical trials for the treatment of HCC.

The generation of tumor-associated fibronectin isoforms allows the development of specific ligands to be used for selective delivery of therapeutic agents (283). Additionally, Liu *et al* and WeiXing *et al* showed that targeting HIF-1 might be used as an effective gene therapy for HCC (284,285).

6. Conclusions

Liver cancer is an intersting model to investigate the relationship between tumor microenvironment and tumor development. Improving the knowledge on this relationship may be crucial for the design of novel molecular targets. In this critical review of the literature, immune-mediate and/or viruses-related molecular mechanisms have been hypothesized as responsible for liver cancer development. The elucidation of these mechanisms regulating the interlink among HCC microenvironment components, comprising cellular elements, cytokines, growth factors and several proteins along with matrix metalloproteinases, is expected to contribute strongly to identifying the altered signaling pathways which are suitable targets for therapy. Growth factor and/or matrix metalloproteinase inhibitors and immunomodulator drugs may represent a future prospective for the treatment of HCC. Accordingly, targeting tumor-stromal interface should also be considered in the therapeutic strategy.

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