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New Molecular Biomarkers Candidates for the Development of Multiparametric Platforms for Hepatocellular Carcinoma Diagnosis, Prognosis and Personalised Therapy

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, showing a rapid progressive clinical course, poor response to pharmacological treatment and a severe prognosis (Colombo, 2003; Sherlock & Dooley, 2002). HCC generally develops from chronic liver injury, which leads to inflammation, hepatocyte regeneration, liver matrix remodeling, fibrosis, and finally, cirrhosis. The main risk factors for HCC are hepatitis B (HBV) or C virus (HCV) infection, alcohol-induced liver disease (ALD), non-alcoholic fatty liver disease (NAFLD), primary biliary cirrhosis and exposure to environmental carcinogens (particularly aflatoxin), and genetic metabolic diseases. (Chuang et al., 2009; Di Bisceglie, 1995; Kato et al, 1994; Malaguarnera et al., 2006; Malaguarnera et al., 2009; Seitz & Becker, 2007; Takano et al., 1995). Obesity has also been identified as an independent risk factor for developing HCC in patients with alcoholic or cryptogenic cirrhosis (Nair et al, 2002). Actually, HCV-related cirrhosis is considered the major risk factor since many HCV chronically infected patients remain asymptomatic for a long period, with liver cirrhosis developing after approximately 30 years (Yano et al., 1996; Poynard et al., 1997). The lack of predictive markers that makes unforeseeable the insurgence of liver cirrhosis in chronic HCV patients may also contribute to HCC late diagnosis, progression and poor prognosis. Currently, alpha-fetoprotein (AFP) is the most common marker for early malignancy used in clinical practice, in combination with hepatic echography, to detect HCC in patients suffering from cirrhosis. Nevertheless, most episodes of AFP elevation were transient and closely correlated with the presence of bridging hepatic necrosis, without subsequent development of HCC (Liaw et al., 1986). Since an early diagnosis of HCC is extremely important in improving the survival of patients, the identification of new and more reliable biological markers of HCC insurgence, recurrence and metastasis is essential for the proper management of this malignancy. Once hepatic cancer develops, one of the main reasons for the high mortality rate in patients with HCC is the lack of effective treatment options, especially for those with advanced disease. Although surgery and percutaneous ablation can achieve long-

term control in some patients with early HCC, recurrence rates are high, approximately 50% at 3 years (Mulcahy, 2005). Furthermore, due to the asymptomatic nature of early HCC, lack of awareness and poorly defined screening strategies, approximately 80% of patients present with advanced or unresectable disease (Thomas & Abbruzzese, 2005). These patients generally have a very poor prognosis and treatments, such as transarterial chemoembolization, intra-arterial or systemic chemotherapy, radiotherapy, immunotherapy or hormonal therapy, are mainly used as palliative, with a 5-year relative survival rate of only 7% (Bosch et al., 2004).

The lack of effective and well-tolerated treatments for advanced HCC highlights the need for innovative approaches for diagnosis, prognosis and therapy for hepatic cancer. In this context, multiparametric platforms allowing simultaneous detection of multiple serological and immunohistochemical markers for HCC insurgence, recurrence and metastasis would represent a high-performance technological tools useful not only for diagnosis and prognosis, but also for improving the clinical management of HCC patients, allowing us, in the near future, to design therapies adapted to the aggressiveness of each individual tumor.

Starting from this background, in this chapter will be collected some of the data existing in literature on the main serological and immunohistochemical biomarkers for HCC diagnosis, prognosis and target therapy, also focusing on new molecules which might be attractive candidates for improvement of the diagnostic/therapeutic approaches. In particular will be covered the following topics : 1) Some new candidates recently proposed as potential biological markers of HCC insurgence, recurrence and metastasis, that could be useful for early diagnosis of this malignancy and improve patient's prognosis; 2) Some signaling pathways which deregulation or constitutive activation have been demonstrated to have a role in HCC insurgence and progression and that could be of interest for therapeutic perspectives, since targeting them may contribute to prevent tumorigenesis or achieve tumor reversion; 3) Molecules over-expressed in late stages of cancer or in the metastatic diseases that should be considered a good targets for therapy and drug delivery.

2. Biological markers useful for early diagnosis of HCC insurgence, recurrence and metastasis

Currently, the diagnosis of HCC is mainly based on the atypical histopathology of bioptic liver tissues, combined with the laboratory screening including the index of hepatic damage (alanine aminotransferase and aspartate aminotransferase), the index of hepatic synthesis (albumin, prothrombin time, bilirubin), the index of cholestasis (alkaline phosphatase and gamma-glutamyl transpeptidase), and finally, tumor markers and instrumental analyses, including hepatic ultrasonography, computed tomography, nuclear magnetic resonance. Some of the tumor markers for HCC diagnosis, such as alpha-fetoprotein (AFP), *lens culinaris* agglutinin-reactive AFP (AFP-L3) and des-γ-carboxyprothrombin (DCP) have now been incorporated into HCC staging classification (Marrero et al., 2010) and are routinely taken into account for the screening for early malignancy (**Table 1**). Other biomarkers, including some growth factors, such as Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor β1 (TGF-β1), Hepatocyte Growth Factor EGF), Epidermal

Growth Factor receptor (EGFR), and numerous other molecules (**Table 1**), are used as diagnostic/prognostic aid for HCC and for staging (Malaguarnera et al., 2010; Mann et al., 2007; Qin & Tang, 2004). Nevertheless, each existing marker alone is poorly specific to predict the disease and most markers are not related to each other. Currently the absolute positive and negative serological and/or immunohistochemical markers for HCC are still lacking, and even those selected for high sensitivity and specificity do not exhibit an universal diagnostic/prognostic value. Therefore, in the last years, a great number of studies has been dedicated to the discovery and validation of more specific biomarkers for HCC, driven by the idea that the simultaneous screening for multiple markers should greatly reduce errors from false-negative results, which significantly contribute to an incorrect diagnosis.

2.1 Main markers used for the screening for early malignancy

Alpha-fetoprotein (AFP) is a 70 kDa glycoprotein that is physiologically synthesized by the embryonic liver cells of the yolk sac and fetal intestinal tract. The AFP is expressed in hepatocytes and endodermal cells of the yolk sac during fetal life and its expression is reduced after birth, with very low levels in adults. The AFP levels rise in hepatocyte regeneration, hepatocarcinogenesis, and embryonic carcinomas. Its biological function in embryo- and carcinogenesis and in adult organisms is still not well identified, but, due to its structural similarity with albumin, a function as a carrier for several ligands, including bilirubin, steroids, fatty acids and various drugs has been proposed (Mizejewski, 2002; Terentiev & Moldogazieva, 2006). Recognized as a tumor-associated fetal protein, AFP has long been considered the 'gold-standard' among tumor markers, and, it has been purified, characterized, cloned and sequenced for use in the clinical diagnostic. It is principally used: i) for the screening and diagnosis of hepatocarcinoma in patients at risk of developing HCC, in combination with hepatic ultrasonography; ii) as a marker of tumor progression in HCC patients with high levels of AFP; iii) for monitoring the response to treatment during the follow-up of HCC patients, with a prognostic value; iv) in HCC staging.

Lens culinaris agglutinin-reactive AFP (AFP-L3) is one of the AFP isoform which exhibits an elevated affinity for *Lens culinaris* agglutinin (LCA). This AFP isoform, that has α1→6 fucose residues on N-acetylglucosamine at reducing end, seems to be exclusively expressed by cancer cells, and is considered a more specific marker for HCC (Oka et al., 2001; Sato et al., 1993). AFP-L3 should be used as a supplemental test in patients with elevated total AFP. It has been reported as a potential indicator of a poor prognosis, since increasing AFP-L3 levels seem to correlate with progression from moderately differentiated to poorly differentiated tumors (Miyaaki et al., 2007).

Des-c-carboxy prothrombin (DCP) or prothrombin induced by vitamin K absence (PIVKA) is an abnormal prothrombin derived by an acquired defect in the post-translational carboxylation of the prothrombin precursor in HCC cells (Ono et al., 1990). DCP derives by a reduced activity of gamma-glutamyl carboxylase, highly expressed in the liver; this reduced activity is attributed to defective gene expression in HCC patients (Grizzi et al., 2007). DCP is a HCC marker more specific than AFP since other liver diseases are not

associated to an increase of DCP serum levels. Apart its diagnostic significance, increased DCP levels may also have a prognostic value, being often related to early portal vein invasion and metastatization by cancer cells.

2.2 Some growth factors used as diagnostic/prognostic aid

Vascular Endothelial Growth Factor (VEGF), plays an crucial role in angiogenesis and is highly expressed in various human cancers (Brown et al., 1993; Mattern et al., 1996; Toi et al., 1994), including HCC (Mise et al., 1996; Suzuki et al., 1996). Specifically, VEGF levels are higher in HCC patients than in patients suffering from chronic hepatitis, and its expression is more elevated in advanced HCC as compared to early HCC. High serum VEGF levels are associated with tumors with portal vein emboli, poor-encapsulated tumors, microscopic vein invasion, and recurrence in HCC patients (Li et al., 1999). It is considered as a possible marker for predicting invasion and metastatization of HCC, and in general, of tumor aggressiveness.

Transforming Growth Factor β1 (TGF-β1), is a polypeptide member of the transforming growth factor beta superfamily of cytokines. It is an important mediator of control of liver cell proliferation and replication. In normal liver tissues, TGF-β1 is produced by non-parenchymal cells (Kupffer cells, storing cells, and endothelial cells), but not by hepatocytes. Conversely, transcription of TGF-β1 gene is activated in human HCC tissues and is higher in patients with advancing histological aggressiveness (Ito et al., 1990). Moreover, TGF-β1 serum levels are reported to be increased in HCC patients (Grizzi et al., 2007). TGF-β1 has been proposed as a possible prognostic factor for reduced survival in patients with HCC (Mann et al., 2007; Okumoto et al., 2004; Tsai et al., 1997)

Hepatocyte growth factor (HGF) is a cytokine with a wide range of effects, including liver regeneration for protection and/or repair of different organs, including kidney, lung, and cardiovascular system (Birchmeier et al., 1998). It promotes proliferation in normal hepatocyte and in hepatocellular carcinoma cells (Breuhan et al., 2006) through expression of its high-affinity tyrosine kinase receptor (Met/*HGF*-R). HGF is detected in the serum from patients suffering from hepatic chronic disease and its serum values seems to be correlated with a worsening of liver disease (Breuhan et al., 2006). Increased HGF serum levels in cirrhotic patients is an indicator of HCC development (Yamagamim et al., 2002). It is considered a prognostic marker since elevated HGF serum levels, are predictive of HCC recurrence and metastasis after hepatic resection (Wu et al., 2006).

Epidermal growth factor receptor (EGFR) is a member of the ErbB family of receptors, a subfamily of four closely related receptor tyrosine kinases, EGFR (ErbB-1), HER2/c-neu (ErbB-2), Her 3 (ErbB-3) and Her 4 (ErbB-4). These receptors bind ligands of the EGF family, including EGF, TGF-α and heparin-binding EGF. EGFR has been found to be overexpressed in poorly differentiated HCC and primarily in patients with early tumor recurrence (Daveau et al., 2003; Ito et al., 2001) EGFR tissue overexpression is also correlated with high proliferating activity, advanced stage, the presence of intrahepatic metastasis and poor disease-free survival following resection (Ito et al., 2001). EGFR strongly reflects the biological aggressiveness of HCC and might be considered a possible prognostic factor of reduced survival of HCC patients.

HCC biomarker	Biological material mainly analyzed	Main use/s	References
Alpha-fetoprotein (AFP)	Serum	Early diagnosis; HCC staging; monitoring the response to treatment during the follow-up of patients with HCC	Terentiev & Moldogazieva, 2006; Mizejewski, 2002; Malaguarnera et al., 2010; Marrero et al., 2010
Lens culinaris agglutinin-reactive AFP (AFP-L3)	Serum	Early diagnosis and prognosis; progression from moderately differentiated to poorly differentiated tumors	Oka et al., 2001; Sato et al., 1993; Malaguarnera et al., 2010; Marrero et al., 2004
Des-c-carboxy prothrombin (DCP)	Serum	Early diagnosis and prognosis (more specific than AFP); related to early portal vein invasion and metastasis	Grizzi et al., 2007; Malaguarnera et al., 2010; Marrero et al., 2004
Golgi protein-73	Serum	HCC early diagnosis	Malaguarnera et al., 2010
Squamous cell carcinoma antigen (SCCA)	Tissue/Serum	Early diagnosis; detection of micro-metastasis in tissues; large-scale screening of serum in patients at risk	Malaguarnera et al., 2010
Glypican-3	Tissue/Serum	HCC early diagnosis; useful for discriminating malignant from benign hepatic lesions	Malaguarnera et al., 2010
Vascular Endothelial Growth Factor (VEGF)	Tissue/Serum	HCC prognosis; predictive of invasion and metastatization of HCC cells	Suzuki et al., 1996; Mise et al., 1996; Li et al., 1999; Qin &Tang, 2004; Mann et al., 2007; Malaguarnera et al., 2010
Transforming Growth Factor β1 (TGF-β1)	Tissue/Serum	HCC progression; prognostic factor for reduced survival in patients with HCC	Ito et al., 1990; Grizzi et al., 2007; Mann et al., 2007; Okumoto et al., 2004; Tsai et al., 1997
Hepatocyte Growth Factor (HGF)	Serum	HCC prognosis; predictive of HCC recurrence and metastasis after hepatic resection	Breuhan et al., 2006; Yamagamim et al., 2002; Wu et al., 2006; Malaguarnera et al., 2010
Epidermal Growth Factor Receptor (EGFR)	Tissue	HCC prognosis; predictive of reduced survival of HCC patients	Daveau et al., 2003; Ito et al., 2001; Mann et al., 2007
p53 antibodies	Serum	HCC prognosis (poor differentiation); associated with a poor prognosis of HCC patients	Malaguarnera et al., 2010
Survivine	Tissue	HCC prognosis; poor prognosis following resection of HCC; associated with reduced disease-free survival.	Fields et al., 2004; Mann et al., 2007
Nerve Growth Factor (NGF) and its high-affinity receptor trkA^{NGF}	Tissue/Serum	HCC prognosis and progression; predictive of progression of liver fibrosis towards HCC	Rasi et al, 2007; Malaguarnera et al., 2010

Table 1. List of the main biomarkers useful for HCC diagnosis/prognosis

2.3 The Nerve Growth Factor (NGF): A new candidate proposed as potential histological/serum marker for HCC diagnosis and prognosis

In the last years, some new candidates have been proposed as potential biological markers of HCC insurgence, recurrence and metastasis, that could be therefore useful for early diagnosis of this malignancy and improve patient's prognosis. In particular we focus on our recently published data that suggested an involvement of Nerve Growth Factor (NGF) in liver tissue remodelling processes and HCC progression, describing the correlation between NGF tissue distribution and serum levels in patients suffering from cirrhosis and/or HCC (Rasi et al, 2007).

NGF is a prototypical member of neurotrophin family essential for survival, differentiation, and maintenance of neuronal cells in the central and peripheral nervous system (Levi-Montalcini, 1987). In recent years, many findings have indicated that NGF could also have a role outside the central and peripheral nervous system. In particular, it may be involved in lung and skin tissue repair (Micera et al., 2001) as well as in allergic inflammation and fibrosis (Micera et al., 2003). Increased levels of circulating NGF were observed in several autoimmune, chronic inflammatory and fibrotic disorders (Aloe & Tuveri, 1997; Bonini et al., 1999). Numerous data also indicate that NGF is involved in tumor growth, invasion and metastasis (Bold et al., 1995; Descamps et al., 1998; Djakiew et al., 1991; Koizumi et al., 1998; McGregor et al., 1999; Oelmann et al., 1995; Pflug et al., 1992; Revoltella & Butler, 1980; Sortino et al., 2000).The NGF effects are mediated by two types of receptor: the high-affinity receptor trkA^{NGF}, specific for NGF, and the low-affinity glycoprotein receptor p75^{NTR}, also binding other neurotrophins (Meakin & Shooter, 1992). Most of the biological activities elicited by NGF are mediated by binding to the trkA^{NGF} receptor (Sofroniew et al., 2001).

In the 2007 (Rasi et al., 2007), we provided immunohistochemical evidence that NGF and its high-affinity receptor trkA^{NGF} are over expressed in patients suffering from HCC (**Fig. 1**) and to a greater extent from HCC with cirrhosis (**Fig. 2B, C**). Specifically, in HCC tissues NGF was detectable in a high number of cells (**Table 2**), at different levels of intensities depending on the patient, but never in normal liver tissue. Interestingly NGF and trkA^{NGF} were negative in liver specimens from patients with cirrhosis undergoing transplantation (Child-C) but without HCC (**Fig. 2A**), while they were markedly positive in patients with cirrhosis that had evolved into HCC, already at early staging (Child- Pugh A, **Fig. 2B**).

Transmission electron microscopy, after immunogold labeling, showed that in hepatocytes of HCC tissue and, at higher extent, of cirrhotic tissue from the same liver, NGF mainly localized on cytoplasmic vesicles, free in the cytoplasm and along endoplasmic reticulum (**Fig. 3**), indicating that it might be actively produced by the hepatocytes constituting the cirrhotic/HCC tissues. The evidence that hepatocytes in HCC and cirrhotic tissues from the same liver produce NGF and express its receptor suggested that NGF may act by both autocrine and paracrine mechanisms, as a messenger molecule in the cross-talk between different cell types. Moreover, in sera obtained from patients with documented cirrhosis, HCC, or both, circulating NGF levels elevated 25-fold over the normal (range 73-520pg/ml, compared to a mean of 20pg/ml in healthy donors) were recorded (**Fig. 4**). These elevated circulating NGF levels, as well as the tissue distribution of NGF and its receptor strongly support a correlation between NGF activity and the progression of liver fibrosis towards HCC. This open up an interesting perspective for the

possible use of NGF, not only as a marker of progression and transformation, but also as an attractive target for a new therapeutic approach.
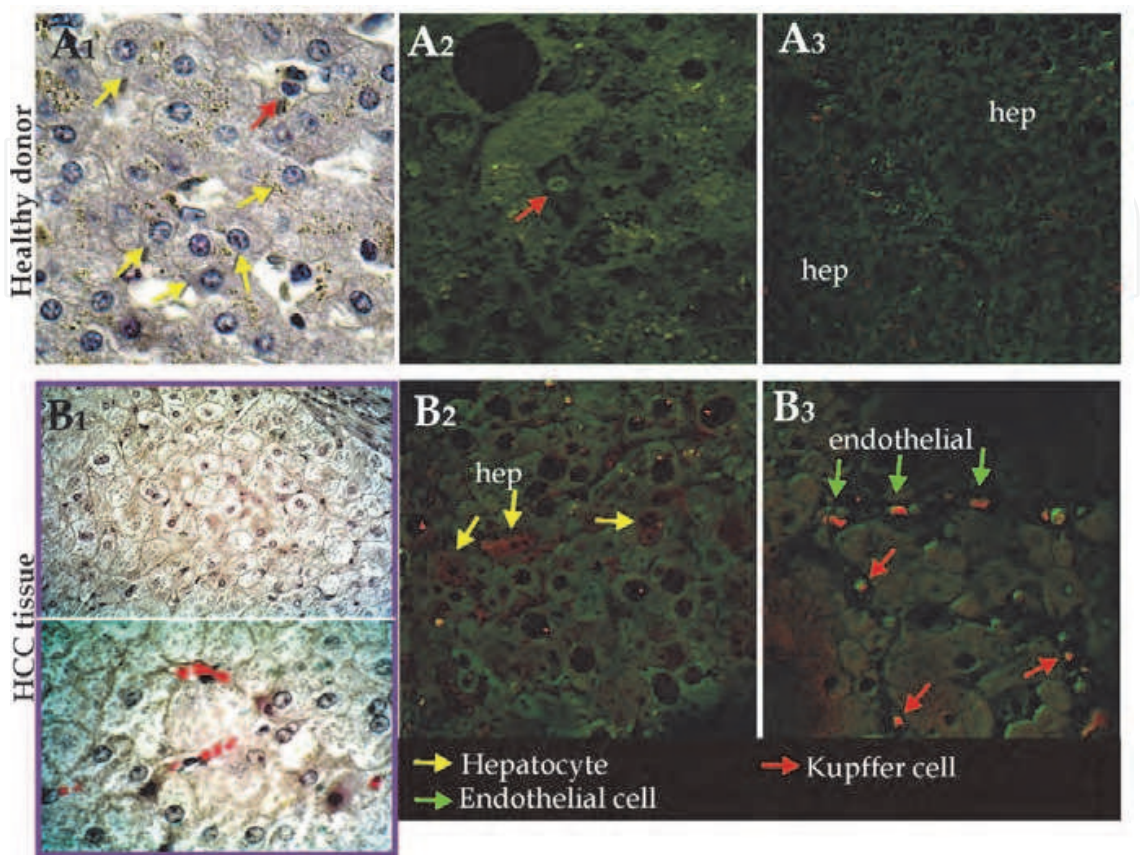

Fig. 1. NGF distribution (red hue) in tissues from healthy donors (**A**) and from patients suffering from HCC (**B**). Green hue represents the auto-fluorescence used to visualize liver tissue morphology. **A1** and **B1**: Images of H&E stained sections close to that used for immunohistochemistry. Differently coloured arrows indicate the different cell types (see legend). hep: hepatocytes.

	Marker	Hep	Bec	Ec	Ssc	Lymph	Kpf
Health	NGF	–	–	–	nd	+	–
	trkA	–	–	–	nd	nd	–
HCC	NGF	+*	±	+	nd	+	+
	trkA	±	±	±	nd	nd	±
CIRR	NGF	++*	++**	++	+	+	±
	trkA	++	++**	+	+	+	+

IF: immunofluorescence labelling; IG: immunogold labelling; nd: not determined.
*Immunoreaction mainly localized on cytoplasmic vesicles and endoplasmic reticulum.
**Immunoreaction mainly localized in the portion of cells near the ductal lumen.

Table 2. Expression of NGF and trkA^{NGF} in liver cell types from healthy donors and from patients with cirrhosis and HCC. Hep: Hepatocyte; Bec, Biliary epithelial cells; Ec, Endothelial cells; Ssc, Spindle-shaped cells; Lymph, Lymphocytes; Kpf, Kupffer cells.
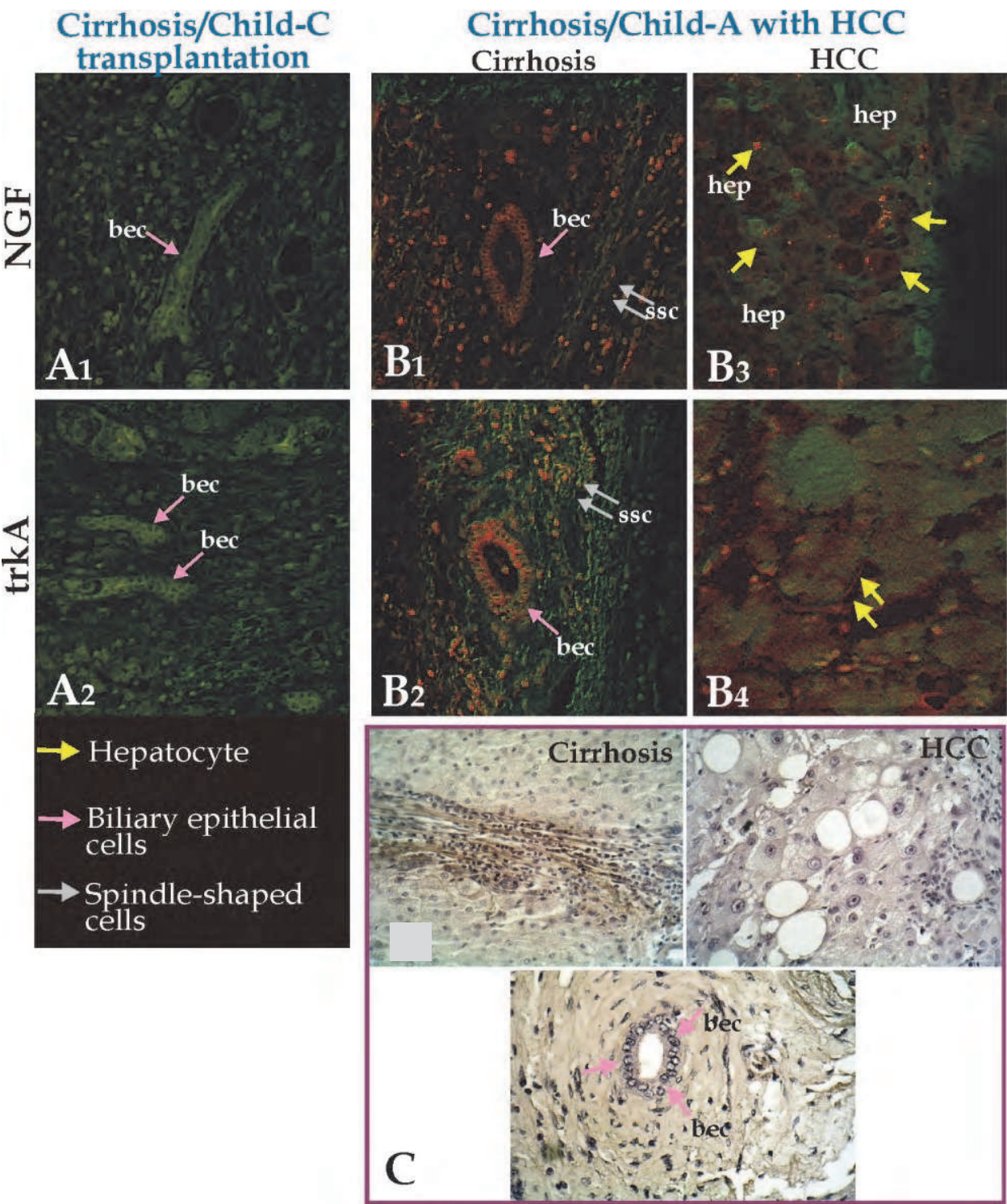
Fig. 2. NGF and trkA^{NGF} distribution in liver specimens. **A**: tissues obtained before transplantation from patients with cirrhosis but without HCC (Child-Pugh C)**. B:** tissue from patient also suffering from cirrhosis with HCC. Red hue represents the NGF or trkA immunostaining; green hue represents the auto-fluorescence used to visualize liver tissue morphology. **C**: Images of H&E stained sections close to that used for immunohistochemistry. Differently coloured arrows indicate the different cell types (see legend). hep: hepatocytes; bec: biliary epithelial cells; ssc: spindle-shaped cells
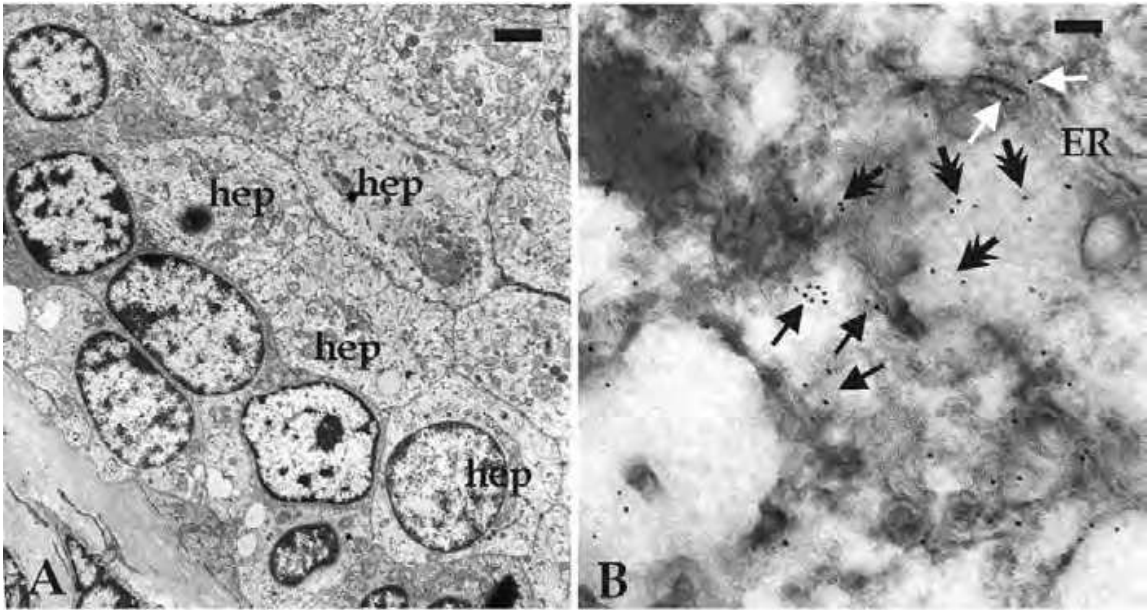
Fig. 3. NGF distribution in cirrhotic tissue from patient with HCC by immunogold labelling.
Transmission electron micrographs of hepatocytes showing positive immunogold reaction
on cytoplasmic vesicles (black arrows), free in the cytoplasm (double pointed arrows) and
along endoplasmic reticulum (white arrows). hep: hepatocytes; ER: endoplasmic reticulum.
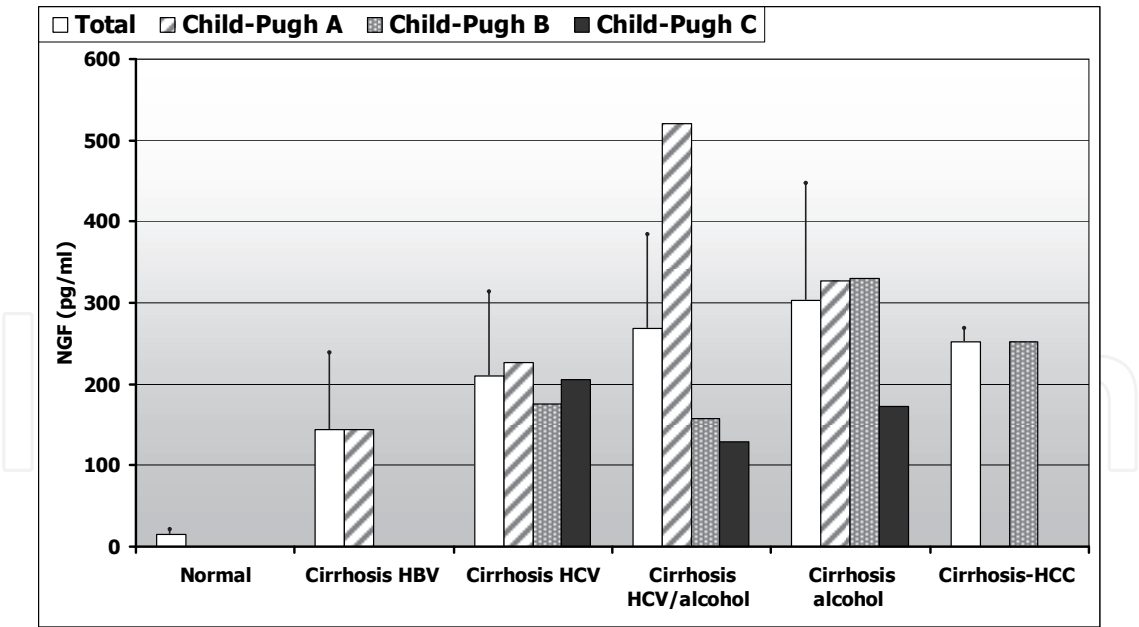Scale bars = **A**: 2μm; **B**: 100nm.

Fig. 4. Bar diagram illustrating the circulating NGF levels, determined by ELISA test , in
patients with documented cirrhosis/HCC. NGF amounts, reported with regard to the
etiology, is calculated either as total mean values ± SD (all patients examined) or as mean
values for Child-Pugh class A (score = 5-6), for Child-Pugh class B (score = 7-9) and for
Child-Pugh class C (score = 10-15). As a control, mean value ± SD of circulating NGF levels
from some healthy individuals is also reported

3. Components of signaling pathways involved in HCC insurgence and progression as innovative biomarkers for diagnosis, prognosis and drug targeting

In the last years, great attention has been given to some signaling pathways which deregulation or constitutive activation have been demonstrated to have a role in cancer insurgence and progression. These pathways could be of interest for therapeutic perspectives, because targeting them may contribute to prevent tumorigenesis or achieve tumor reversion. Drugs directly acting on components of the signaling pathways implicated in tumorigenesis have exhibited clinical benefit in patients with various tumor types, including colorectal, renal, breast and lung cancers, and more recently, HCC (Whittaker et al., 2010). Thus, deepening of knowledge on the molecular pathways actively involved in HCC insurgence and progression could potentially provide new targets for drug delivery and therapy, allowing to overcome the poor response to the current therapeutic strategies. Moreover, owing the role of these pathways in the carcinogenetic process, crucial molecules of this signaling should be validate as new HCC-related biomarkers for the improvement of the current diagnostic/prognostic tools.

3.1 Main signaling pathways implicated in HCC

During hepatocarcinogenesis, two main pathogenic mechanisms predominate: 1) cirrhosis associated with hepatic regeneration after tissue damage caused by hepatitis infection, toxins such as alcohol or aflatoxin, or metabolic syndromes such as insulin resistance, obesity, type 2 diabetes or dyslipidemia in non-alcoholic steatohepatitis (Bugianesi, 2005); 2) mutations occurring in single or multiple oncogenes or tumor suppressor genes (Thorgeirsson & Grisham, 2002; Villanueva et al., 2007; Wang et al., 2002). These two mechanisms have been related to aberrations in various critical molecular signaling pathways that participate to the carcinogenic process. The most important of these pathways include the growth factor-mediated angiogenic signaling (mainly the VEGF receptor signaling), the epidermal growth factor receptor (EGFR), the insulin growth factor receptor (IGFR), the hepatocyte growth factor receptor HGF/c-MET signaling, and the platelet-derived growth factor receptor (PDGFR) signaling (**Fig. 5**) (Whittaker et al., 2010).

Since liver is a highly vascular organ, HCC growth and invasion is highly dependent on dysregualtion of angiogenensis (Semela & Dufour, 2004), and targeting molecular components of pathway signaling involved in the angiogenetic process are currently the main therapeutic strategy exploited for HCC treatment. Actually, targeted drug selectively hitting the VEGF/VEGFR and PDGFR signaling (Sunitinib, Bevacizumab, Cediranib and Vatalanib) or the EGF/EGFR and IGFR signaling (Lapatinib, Cetuximab, Erlotinib Gefitinib, Everolimus, Sirolimus) (Fig. 5) are under evaluation in phase I-III clinical trials as monotherapy or in combination with other chemotherapeutics (see for review, Whittaker et al., 2010). Sorafenib (Nexavar; Bayer HealthCare Pharmaceuticals Inc., Wayne, NJ, USA), a potent inhibitor of VEGFR and PDGFR, has been approved for treatment of HCC and is the only option of effective systemic treatment currently available for management of the advanced malignancy (Llovet et al., 2008).
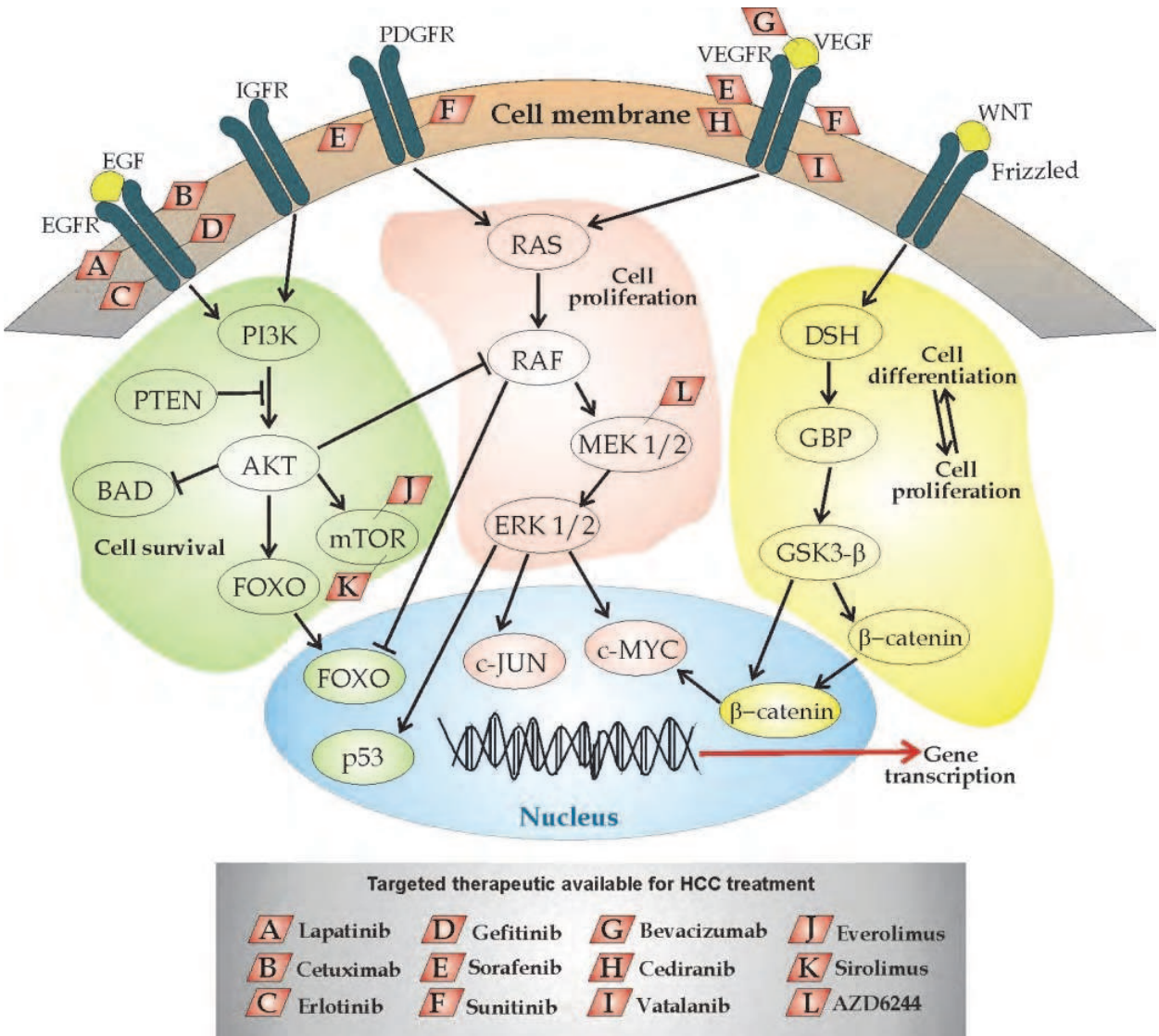
Fig. 5. Cellular signaling pathways implicated in the pathogenesis of HCC and therapeutics targeting molecular components of these pathway, useful for HCC treatment. EGF, epidermal growth factor; EGFR, EGF receptor; IGFR, insulin-like growth factor receptor; PDGFR, platelet-derived growth factor receptor; WNT, family of secreted glycoproteins that act as ligands of the Frizzled receptor; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor. Details and main function/s of AKT, BAD, c-JUN, c-MYC, DSH, ERK 1/2, FOXO, GSK-3β, GBP, MEK 1/2, mTOR, PI3K, PTEN, p53, RAF, RAS, β-catenin, are reported in **Table 3**. Adapted from Whittaker et al., 2010

Besides the mentioned pathways, directly or indirectly involved in the angiogenic signaling, in the last years numerous studies demonstrated that the WNT/β-catenin pathway is actively involved in initiation and progression of several kinds of human cancers, including HCC (De La et al., 1998; Polakis, 1999; Waltzer & Bienz, 1999) and growing attention has been given to new anti-tumor therapeutic approaches targeting components of this signaling pathway (Gonsalves et al., 2011; Luu et al., 2004; Moon et al., 2004).

Molecular components	Main Cellular signaling	Main role and function
AKT	EGF/EGFR, IGFR	serine/threonine protein kinase involved in regulating cell survival
BAD	EGF/EGFR, IGFR	BCL-2-associated death promoter, involved in regulating apoptosis
c-JUN	VEGF/VEGFR, PDGFR	in combination with c-FOS, forms the activator protein-1 (AP-1) early-response transcription factor; involved in cell proliferation and apoptosis.
c-MYC	EGF/EGFR , IGFR, WNT	Encodes for a transcription factor that regulates the expression of many genes involved in cell proliferation; overexpression of c-MYC is associated with carcinogenesis.
DSH (Dishevelled)	WNT	downstream effector of WNT sinaling
ERK 1/2	VEGF/VEGFR, PDGFR	extracellular signal-regulated kinases
FOXO (Forkhead box subclass O)	EGF/EGFR , IGFR	transcription factor regulating the expression of genes involved in cell survival and proliferation
GSK-3β	WNT	glycogen synthase kinase-3β, component of β-catenin destruction complex
GBP	WNT	GSK3-binding protein
MEK 1/2	VEGF/VEGFR, PDGFR	kinases that phosphorylate mitogen-activated protein (MAP) kinase (MAPK)
mTOR	EGF/EGFR , IGFR	mammalian target of rapamycin, a serine/threonine protein kinase that regulates cell growth, proliferation, motility, and survival
PI3K	EGF/EGFR , IGFR	phosphatidylinositol-3-kinase
PTEN	EGF/EGFR , IGFR	phosphatase and tensin homolog that regulates cell-survival pathway
p53	VEGF/VEGFR, PDGFR, EGF/EGFR, IGFR	tumor suppressor protein, regulates the cell cycle
RAF	VEGF/VEGFR, PDGFR	MAP kinase kinase kinase (MAP3K); functions in the MAPK/ERK signal transduction pathway
RAS		prototypical member of the RAS superfamily of proteins; RAS signaling causes cell growth, differentiation and survival
β-catenin	WNT	integral component of the WNT/β-catenin signaling

Table 3. List of the main molecular component of cellular signaling pathways implicated in the pathogenesis of HCC.

3.2 Molecular component of the WNT/β-catenin signaling as innovative diagnostic biomarkers and therapeutic targets

Wnts are secreted glycoproteins that act as ligands to stimulate receptor-mediated signal transduction pathways in both vertebrates and invertebrates. Activation of Wnt pathways can modulate cell proliferation, survival, cell behavior, and cell fate in both embryos and adults. Wnt signaling pathway, and its signaling cascade is one of the core signal transduction pathway driving tissue morphogenesis during both development and progression of human cancers (see for reviews on Wnt: Moon et al., 2004; Nelson et al., 2004). Wnt signaling also plays a critical role in regulating liver cell proliferation during development (Monga et al., 2003; Suksaweang et al., 2004) and in controlling crucial functions of the adult liver (Sekine et al., 2006).

β-catenin was originally identified as a protein interacting with the cell adhesion molecule E-cadherin (E-cad) at the cell-cell junction (Ozawa et al., 1989; Vestweber & Kemler, 1984), but in the last few years has gained growing interest as one of the most important mediators of the Wnt signaling pathway (Moon et al., 2004; Nelson et al., 2004), specifically in respect to the role of this pathway in tumorigenesis (**Fig. 6**). In non normal condition, β-catenin exists in a cadherin-bound form that regulates adhesion, and the β-catenin excess, not segregated by E-cad on the cell membrane, is rapidly phosphorylated by glycogen synthetase kinase-3β(GSK-3β) in the adenomatous polyposis coli (APC)/axin/GSK-3β complex (destruction complex) and is subsequently degraded by the ubiquitin-proteosome pathway. Conversely, in tumor cells, Wnt signaling, through the Frizzled serpentine receptor and the low-density lipoprotein receptor-related protein-5 or -6 (LRP5 or 6) coreceptors, activates the cytoplasmic phosphoprotein Dishevelled, which blocks the degradation of β-catenin that accumulates in the cytosol and is translocated into the nuclei. Here, through the binding with transcription factors, T-cell factor (TCF)/lymphoid enhancer factor (LEF), β-catenin activates transcription of genes such as cyclinD1 and c-MYC, thus modulating cell proliferation and invasion.

Many of the molecular component of the WNT/β-catenin signaling have been reported to be modified in HCC, and are proposed as HCC diagnostic/prognostic markers or as therapeutic target for treatment of the primary or metastatic malignancy (**Table 4**).

Mutations of Axin or stabilizing mutations of β-catenin genes, leading to constitutive activation of the Wnt/β-catenin pathway, have been recovered in various cancers, including hepatoblastoma and HCC (Buendia, 2000; De La et al., 1998; Whittaker et al., 2010).

Conversely, inactivating mutations of the APC gene, frequently implicated in other tumor and particularly in colorectal cancer, have not been described in HCC. However, loss of APC function activating the WNT/β-catenin signaling seems to be implicated in liver carcinogenesis (Colnot et al., 2004). Moreover, aberrant reactivation of Wnt signaling due to accumulation of β-catenin is evident in many different tumors of the liver (Colnot et al., 2004). Frequent overexpression of the Wnt receptor Frizzled-7 has been detected in HCC and mainly in hepatitis B virus–related HCCs, and this overexpression seems to be an early event in hepatocarcinogenesis (Merle et al., 2004). It has been recently reported that serum β-catenin levels were significantly elevated in patients with HCC compared to those with chronic hepatitis or healthy controls, and it has been proposed as a potential marker for early diagnosis of HCC in HCV infected patients (Zekri et al., 2011). Moreover, in human

HCC tissues, higher levels of β-catenin expression was found in the tumor area compared to the non-tumor area and the level of expression and nuclear translocation of β-catenin was increased in HCC late-stage. Thus, β-catenin have been proposed as a suitable diagnostic marker of metastasis in human HCC (Lai et al., 2011).
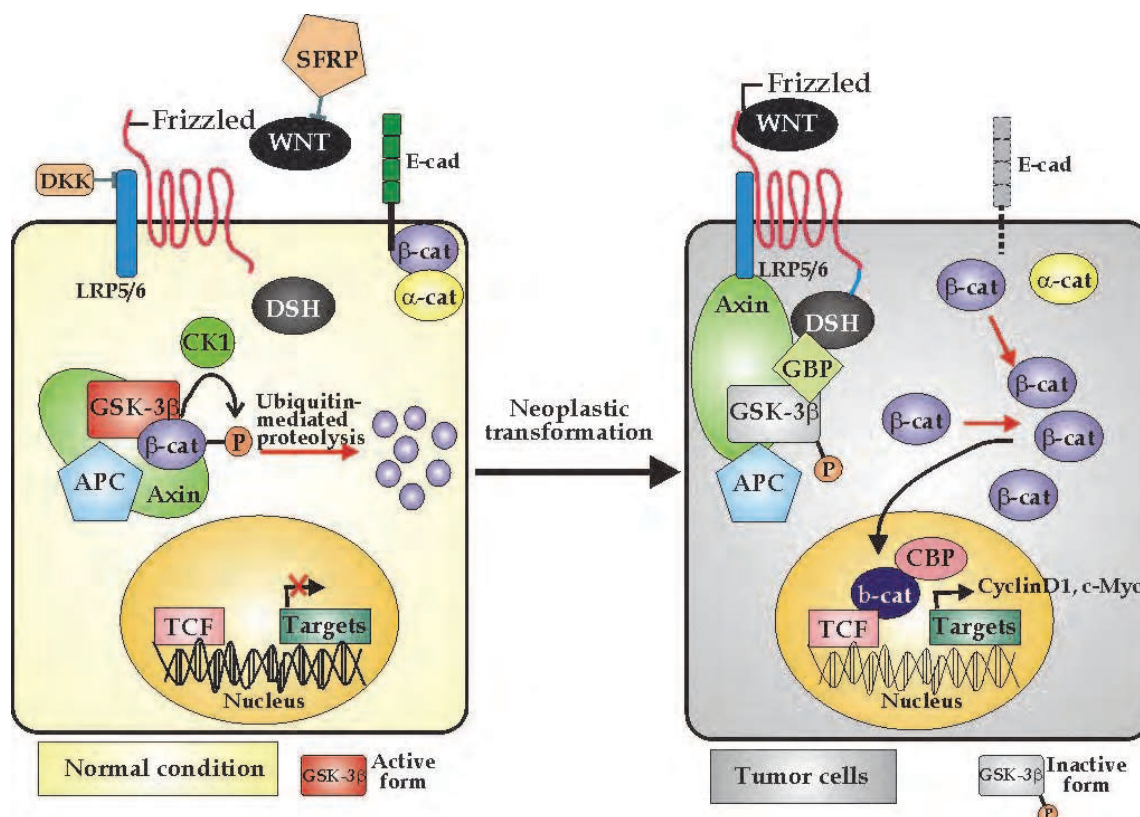

Fig. 6. Schematic representation of the Wnt/β-catenin signaling activation. APC, adenomatous polyposis coli; β-cat, β-catenin; CBP, CREB-binding protein; CK, casein kinase; DKK, Dickkopf; DSH, Dishevelled; GBP, GSK3-binding protein; GSK-3β, glycogen synthase kinase 3β; LRP, LDL receptor-related protein; P, phosphorylation; sFRP, secreted Frizzled-related protein; TCF, T-cell factor.

Finally, due to the tight interaction of β-catenin with E-cad at the cell-cell junction, activation of WNT signaling has also been related to dysregulation of cadherin expression, which is often associated with dysplasia, tumor formation, and metastasis. This causal relationship between E-cad and Wnt signaling makes E-cad an additional molecular marker that should be taken into account in the setting up a multiparametric diagnostic/prognostic platform for HCC. The E-cad expression levels have been reported to inversely correlate with histological grade and prognosis, and might be a prognostic marker of early recurrence of HCC after hepatic resection (Huang et al. 1999; Matsumura et al. 2001). Since E-cad expression is higher in well-differentiated tumors compared to poorly differentiated cancers, that exhibit lost of the intercellular junction integrity and development of metastasis (Shiozaki et al., 1996; Wijnhoven et al., 2000), it may also be predictive of invasion and metastatization of HCC cells.

While several drugs targeting the VEGF/VEGFR, PDGFR , EGF/EGFR and IGFR signaling have been approved or are in late-stage clinical trials (**Fig. 5**), clinically useful agents that

specifically inhibit Wnt signaling cascade are not currently available. However, owing the crucial role in cancer ascribed to this pathway, in the last years the researches on the molecular mechanisms driving this signaling are in increasing and conspicuous funds are invested by several pharmaceutical and biotech companies for the development of innovative drugs targeting its molecular components. The main therapeutic strategies currently explored include:

1. The use of small-molecules able to regulate the catenin responsive transcription (Chen et al., 2009a; Lepourcelet et al., 2004; Thorne et al., 2010; Vo & Goodman, 2001).
2. Compounds that inhibit Wnt signaling by influencing the stability and expression levels of β-catenin (Chen et al., 2009a; Huang et al., 2009; Thorne et al., 2010)
3. Molecules that inhibit Wnt signaling by acting on events upstream of the axin/APC/GSK-3β complex, such as the secretion or reception of Wnt ligands at the plasma membrane (Chen et al., 2009a; Chen et al., 2009b) or transduction of the Wnt signal by Dishevelled (Dvl) (Chen et al., 2009b, Shan et al., 2005)

Molecular component of the WNT signaling	Biological material analyzed	Trends found in HCC	Main possible use/s
β-catenin	Tissue	Gene mutation recovered in pre-cancerous lesion and increased in tumor; increased mRNA expression compared to normal liver; nuclear translocation in the early stages; increased protein expression in the late stage	Indicative of WNT signaling activation (early diagnosis); diagnostic marker of metastasis ; therapeutic target
β-catenin	Serum	Elevated in patients with HCC compared to those with chronic hepatitis (CH) and healthy controls	Early diagnosis of HCV-associated HCC
APC	Tissue	Gene mutation not frequently evidenced; loss of function implicated in liver carcinogenesis;	Early diagnosis; therapeutic target
Frizzled receptor	Tissue	Overexpressed in HCC compared to normal liver, already at early stages	Early diagnosis; therapeutic target
E-cadherin	Tissue	Expression increased in well-differentiated tumors compared to poorly differentiated cancers; Expression levels inversely correlated with histological grade and prognosis	Predictive of early recurrence after hepatic resection and metastatization of HCC cells; marker of tumor differentiation

Table 4. List of the main molecular component of the WNT signaling useful for HCC diagnosis/therapy

Moreover, it has been recently reported that microRNA-181s (miR-181s) are transcriptionally activated by the Wnt/β-catenin signaling in HCC and these miRs have been proposed as attractive molecular target to eradicate liver cancer stem cells (Ji et al., 2011)

4. CD44 as a multifunctional marker of HCC late stages and metastatic disease, also useful for targeted therapy and drug delivery

The high mortality rate in patients with HCC is mainly due the lack of effective treatment options, especially for those with advanced or unresectable disease. These patients generally have a very poor prognosis and treatments, such as transarterial chemo-embolization, intra-arterial or systemic chemotherapy, radiotherapy, immunotherapy or hormonal therapy, are mainly used as palliative. Thus, the development of more effective therapeutic tools and strategies is much needed. The conventional chemotherapy, implying the use of systemic administration or non-targeted distribution of the drug, has numerous drawback such as the limited accessibility of drug to the tumor tissue, that reduces its therapeutic efficacy, the requirement of high doses, and undesirable side effects, primarily the high mielotoxicity and the development of multidrug resistance. To overcome these problems, in the last decades numerous researches focused on developing cancer-specific drugs or systems of antitumor drug delivery (Allen, 2002; Gabizon, 2002; Gabizon et al., 2003; Mohanty et al., 2011; Sapra & Allen, 2003). This therapeutic strategy may allow a controlled release of the drug and a high targeting selectivity on tumor cells, increasing drug cytotoxicity and decreasing its undesirable side effects. In this context, targeted drug delivery involving the use of drugs covalently conjugated to macromolecular carriers, that are able to specifically link to over-expressed molecules on tumor cells, is one the most promising approach in developing innovative therapies against cancer.

CD44, the receptor for hyaluronic acid-mediated cell motility, is a highly glycosylated transmembrane protein involved in cell–cell and cell–matrix interactions. The standard isoform (CD44s), participates to several functions including lymphocyte homing, tissue regeneration, signal transmission involved in cell proliferation, migration and apoptosis (Goodison et al., 1999; Ponta et al., 2003). Besides its involvement in physiological activities of normal cells, CD44 is associated with pathologic functions of tumor cells. Increased expression of CD44 (the standard isoform CD44s and the splice variant CD44v) has been associated to advanced stages not only of hepatocellular carcinoma (Endo & Terada, 2000) but also of breast cancer, colorectal cancer, thyroid carcinoma, lung cancer, renal cell carcinoma, gallbladder carcinoma, ovarian carcinoma, endometrial cancer and melanoma (Akisik et al., 2002; Bendardaf et al., 2006; Jothy , 2003; Naor et al., 2002; Seiter et al., 1996). For this reason, CD44 is emerging as a valuable metastatic tumor marker, also associated with an unfavorable prognosis for a variety of cancers, including HCC (Beckebaum et al., 2008). Therefore, agents specifically targeting CD44 should be promising drug for inhibiting tumor spread and for treatment of metastatic disease. It has been demonstrated that targeting CD44 with specific anti-CD44 monoclonal antibodies is able to inhibit proliferation and to induce terminal differentiation or apoptosis in leukemic cell lines (Charrad et al., 2002; Jin et al., 2006). Furthermore, inhibition of CD44 expression by CD44 antisense oligonucleotide significantly induced apoptosis, decreased tumorigenesis and invasion, and increased chemosensitivity in a CD44 over-expressing human HCC cell line (Xie et al., 2008).

The described overexpression of CD44 in advanced stages of several kinds of cancer including HCC, makes hyaluronic acid (HA), the well-known component of the extracellular matrix to which CD44 binds for driving the cell motility, an excellent macromolecular carrier for anticancer drug delivery. HA is a natural and biodegradable polysaccharide formed by D-glucuronic acid and *N*-acetyl-D-glucosamine repetitive units (**Fig. 7A**), used for the development of pharmaceutical carriers and biomedical systems. HA plays crucial roles in cell adhesion, growth, and migration, by interacting with specific cellular receptors (CD44, RHAMM, ICAM), and acts as a signaling molecule in cell motility, inflammation, wound healing, and cancer metastasis (Marhaba & Zoller, 2004; Nedvetzkiet al., 2004; Toole, 2004; Weigel et al., 2003). In this context, HA-drug bioconjugates inherently show a marked selectivity for cancer cells, also providing advantages in drug solubilization, stabilization, localization, and controlled release. Bioconjugates of hyaluronic acid with different antineoplastic drugs, such as paclitaxel, doxorubicin and SN-38 (the active metabolite of Irinotecan) have been reported to posses promising anti-tumor effects both *in vitro* and *in vivo* (Banzato et al., 2008; Luo & Prestwich, 1999; Luo et al., 2000; Luo et al., 2002; Rosato et al., 2006; Serafino et al., 2011).
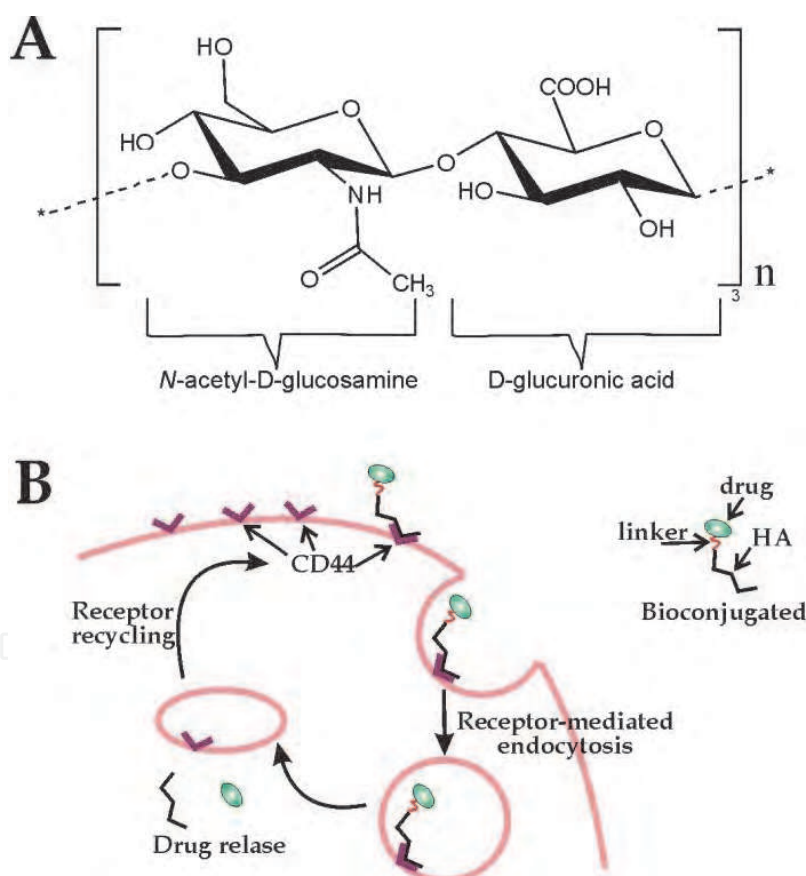

Fig. 7. **A**: Molecular structure of hyaluronic acid. **B**: mechanism of HA-drug biocojugate internalization by cancer cells.

In our recent paper (Serafino et al., 2011) we showed that the HA-drug bioconjugates, after interaction of the HA backbone with CD44, enter the tumor cells through a receptor-mediated endocytosis, followed by release of the active drug in the cytosol, where it directly reach its site of action. The internalized bioconjugate/CD44 complex has been recovered on

cytoplasmic vesicles-like and lysosome-like structures, suggesting that processing of the HA-drug molecules might be coupled with a recycling of the CD44 receptor (**Fig. 7B**). We have also demonstrated that, using this drug delivery strategy, the delivered drug exerts a strong and irreversible *in vitro* inhibitory effect on growth of CD44 over-expressing cancer cells, higher than that exerted by free drug.

As mentioned above, the effectiveness of the drug delivery strategy using HA as a carrier targeting its CD44 receptor has been demonstrated in several kinds of CD44 over-expressing tumors, including colorectal, ovarian, bladder, gastric, breast, oesophageal and lung cancers, not only *in vitro* but also *in vivo* (Banzato et al., 2008; Luo & Prestwich, 1999; Luo et al., 2000; Luo et al., 2002; Rosato et al., 2006; Serafino et al., 2011). Due the association of increased expression of CD44 to advanced stages of hepatocellular carcinoma, this therapeutic approach might be also applicable for treatment of metastatic HCC. In addition, the growing evidences concerning the CD44 expression on liver cancer stem cells (Liu et al., 2011) not only improve the prognostic significance of CD44 but also make the drug delivery strategy through CD44/HA binding interaction useful for eradicating hepatic cancer stem cells.

5. Conclusion

Hepatocellular carcinoma is a malignancy having multifactorial etiology and a very complex pathogenesis, that make difficult the clinical management of HCC patients. Similarly to the other kinds of cancer, HCC insurgence, progression and recurrence involve gene mutations, that might be different depending on the individual genotype profiling and tumor stage, and different signaling pathways, which often share some crucial molecules/steps and are subjected to additional post-transductional regulation. To overcome the complex network of signaling pathways and gene mutations underlying hepatic cancer, innovative diagnostic, prognostic and therapeutic strategies are needed. Nowadays, each existing biomarker used or proposed for HCC early diagnosis, staging and prognosis alone is poorly specific and the absolute positive and negative serological and/or immunohistochemical markers are still lacking. Even those markers selected for high sensitivity and specificity do not exhibit an universal diagnostic/prognostic value, also due to the individual genotype variability. The more promising approach for developing more specific diagnostic/prognostic tools might be to combine several positive or negative indicators in multiparametric platforms, that allow simultaneous detection of multiple serological or immunohistochemical markers for HCC. These platforms might be used to design "specific maps" for HCC early diagnosis, staging and prognosis, also taking into account the individual variability of each patient. Detecting expression patterns of combined biomarkers may also be a new method useful for identifying new and unique markers.

Moreover, since target-specific cancer therapy has remarkably improved the outcomes of patients and represents the frontline approach for cancer treatment, the development of such multiparametric platforms would also represent a high-performance technological tools useful for designing personalized therapies, adapted to the aggressiveness of each individual tumor. The final goal should be to discriminate, for each target-specific therapy and on the basis of the "biomarker profiling" of each patient, the "responder" to the "not responder", thus increasing the therapeutic effectiveness, improving patient outcomes, and resulting in saving healthcare costs. Thus, the discovery and validation of new HCC

biomarkers useful for early diagnosis and prognosis, such as the NGF, and for target therapy and drug delivery, such as the CD44, as well as the deepening of knowledge on pathways actively involved in hepatocarcinogenesis, helpful for HCC staging and target-specific cancer therapy, such as the WNT/β-catenin signaling, are essential steps to achieve this goal.

6. Acknowledgment

7. References

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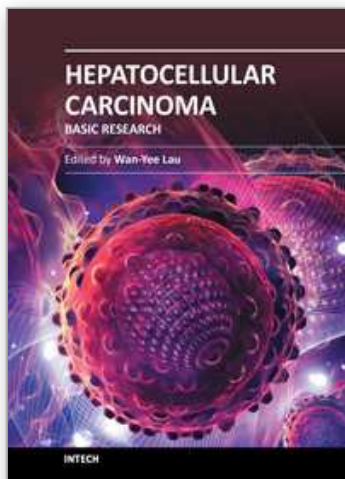
Hepatocellular Carcinoma - Basic Research

Edited by Dr. Joseph W.Y. Lau

Hepatocellular Carcinoma represents a leading cause of cancer death and a major health problem in developing countries where hepatitis B infection is prevalent. It has also become increasingly important with the increase in hepatitis C infection in developed countries. Knowledge of hepatocellular carcinoma has progressed rapidly. This book is a compendium of papers written by experts to present the most up-to-date knowledge on hepatocellular carcinoma. This book deals mainly with the basic research aspect of hepatocellular carcinoma. The book is divided into three sections: (I) Biomarkers / Therapeutic Target; (II) Carcinogenesis / Invasion / Metastasis; and (III) Detection / Prevention / Prevalence. There are 18 chapters in this book. This book is an important contribution to the basic research of hepatocellular carcinoma. The intended readers of this book are scientists and clinicians who are interested in research on hepatocellular carcinoma. Epidemiologists, pathologists, hospital administrators and drug manufacturers will also find this book useful.

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