1. ABSTRACT

Hepatoblastoma (HB) is the most common type of pediatric liver cancer. This tumor is thought to derive from hepatic progenitor cells that are arrested at various stages of liver development, as illustrated by a variety of histologic subtypes. Recent genomic studies have led to better understand the molecular pathogenesis of HB, to point out the crucial roles of the Wnt Myc signaling pathways in malignant transformation of liver progenitor cells. Molecular classification of HB based on genome-wide studies, as well as identification of reliable diagnostic prognostic markers, open the way to the development of new personalized targeted therapies for the management of aggressive lethal childhood tumors.

2. INTRODUCTION

Pediatric tumors are thought to derive from malignant transformation of immature cells and thus, to require less oncogenic events than tumors that develop in adulthood (1). Hepatoblastoma was first recognized as a cancer associated to developmental disorders by histopathological examination that revealed common morphological features with embryonic and fetal liver, and by strong expression of the oncofetal marker alpha-fetoprotein (AFP) (2-4).

While a variety of signaling pathways including growth factor and developmental pathways have been implicated in HB pathogenesis, a major driver appears to be
the canonical Wnt pathway, which is activated by oncogenic mutations of the β-catenin gene in a majority of tumors (5, 6). Importantly, the Wnt signals control stem cell functions, progenitor cell expansion and lineage decisions during embryogenesis, and their disruption may lead to developmental defects and different diseases, including cancer (7).

Recent studies of gene expression profiles in HB have allowed identifying specific expression patterns in different HB histotypes, and revealed strong similarities with different steps of normal hepatic development (8, 9). Evidence has been provided for two HB molecular subclasses that reflect early and late phases of liver differentiation. Importantly, these subclasses are associated with different clinicopathological characters and disease outcome. A Myc signature has been detected in poorly differentiated, highly proliferating tumors and it has been linked to features of aggressive tumors and poor prognosis (8). This review will be focused on the distinctive features linked to the activation of Wnt/β-catenin and Myc signaling in HB, and will seek at providing some clues for potential therapeutic options that could warrant clinical validation.

2. MUTATIONAL ACTIVATION OF β-CATENIN IN LIVER CANCER

Wnt/β-catenin signaling plays pivotal roles in animal life by regulating the genetic programs of embryonic development and adult homeostasis. This pathway is essential for stem cell maintenance, and for the control of cell fate and proliferation in a variety of cancers (10, 11). These effects are achieved through the stabilization of β-catenin and its translocation to the nucleus as a coactivator for transcription factors of the Tcf/Lef family (12). In the absence of Wnt signaling, β-catenin is phosphorylated at N-terminal serine and threonine residues in a multiprotein complex including the kinases GSK3β and CK1, adenomatous polyposis coli (APC) and Axin, and phosphorylated β-catenin is directed towards proteasome-mediated degradation (13). Activation of Wnt abrogates the degradation pathway, leading to elevated levels of transcriptionally active β-catenin. The selective activation of distinct target genes in proper context is strictly controlled by the interplay of positive and negative regulatory signals on Wnt-responsive promoters. Recent studies have outlined the importance of Wnt/β-catenin signaling in liver development and physiology, by regulating hepatoblast proliferation and differentiation, and by governing the metabolic zonation in adult liver (14-16).

In cancer, nuclear accumulation of β-catenin is mostly a consequence of loss-of-function mutations in the APC or Axin genes, or activating mutations in the N-terminal domain of β-catenin, which interfere with the degradation control of the protein (17). Wnt/β-catenin signaling can also be activated by alterations in other components of the Wnt pathway, such as mutations in the sFRP1 or TCF4 gene, or changes in the expression levels of Wnts, Frizzled receptors or Wnt antagonists (18). As such, aberrant reactivation of Wnt signaling has been implicated as a major mechanism of liver tumorigenesis (for review, (19)). In human liver cancer, APC is rarely mutated, and activating mutations in the N-terminal domain of β-catenin or deleterious mutations of Axin genes are predominant. It has been shown that β-catenin mutations are associated with increased risk of malignant transformation in a subset of hepatic adenomas (20). In hepatocellular carcinoma (HCC), mutations of β-catenin have been reported in up to 40% of cases, and AXIN1 mutations in less than 10% of cases (21, 22). In animal models however, spontaneous liver carcinogenesis was observed only in APC-null mice, but not in β-catenin transgenic mice, suggesting that β-catenin requires the cooperation of other signaling pathways for triggering fully malignant conversion (23, 24).

Several studies have demonstrated a high rate of β-catenin mutations in HB, providing evidence for abnormal reactivation of the developmental Wnt pathway in this tumor (5, 6, 25-27). Sequence analysis of the β-catenin N-terminal domain revealed interstitial deletions or missense mutations in the GSK3β phosphorylation motif in 50 to 90% of cases. This is among the highest mutation rate described so far in human neoplasms. The finding of identical β-catenin gene alterations within different histotypes of the same tumor suggested that mutational activation of the oncogene occurred in a common precursor cell. Because β-catenin mutations were found at similar rates in all HB histotypes and at different tumoral stages, they were not endowed with prognostic value. Besides, loss-of-function mutations of AXIN have been reported in about 5-10% of cases (28, 29). Moreover, the increased incidence of HB in familial adenomatous polyposis (FAP) has led to evaluate sporadic tumors for alteration in the APC gene. While different studies failed to find mutations in the APC mutation cluster region (5, 6), others found a mutation rate around 10% (30, 31). Thus, genetic alterations in the Wnt/β-catenin pathway have been detected in a vast majority of the HB cases analyzed worldwide, leading to consider this pathway as an essential player in HB pathogenesis.

The downstream effects of constitutive β-catenin activation on gene expression play a key role in malignant neoplasms. The identification of Wnt target genes activated in HB tumors may provide important clues on underlying mechanisms. Different reports converge to demonstrate that c-myc, one of the critical targets of Wnt, is not activated in response to Wnt in the hepatic context (23, 32). However, activation of MYC and MYCN has been associated to genetic alterations on chromosomes 2p and 8q in immature and aggressive HBs harboring β-catenin gene mutations (8). Other targets such as cyclin D1 and the transcriptional repressor TBX3 might be involved in the growth-promoting and anti-apoptotic effects of Wnt in the liver, and both factors have been associated with unfavorable prognosis (27, 33, 34). While general upregulation of target genes implicated in feedback regulation of the Wnt/β-catenin signaling has been found in most HBs (8, 29, 35), differential activation of specific Wnt-target genes has recently been described in two HB subclasses identified by gene expression profiling (8). In this sense, the tumor
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subclass displaying mainly fetal histotype showed a profile similar to human and murine HCCs with β-catenin mutation and high expression of hepatic perivenous genes involved in metabolic pathways such as GLUL and CYP450 genes (36, 37). The other subclass consisted of immature HBs that exhibited a predominant expression of genes related to stemness, cell proliferation and survival such as SUZ12, SOX9, EpCAM and Survivin. Differential expression of Wnt-target genes might be due to differences in the intracellular localization of β-catenin, since poorly differentiated HBs showed intense nuclear accumulation and decreased membranous localization of the protein (6, 8). This can be related to the absence of membranous E-cadherin and/or to cross talks with growth-stimulating pathways in poorly differentiated and highly proliferating tumor cells (6, 38).

Given the central role of this pathway in HB, better understanding of the oncogenic events triggered by activated Wnt/β-catenin signaling will have a broad impact on both biology and medicine.

3. MOLECULAR CLASSIFICATION OF HEPATOBLASTOMA AND ACTIVATION OF MYC SIGNALING

During the last decade, the development of gene expression profiling has permitted detailed examination of the biological phenotypes of human cancers. This methodology, frequently combined with genome-wide genetic screens, has demonstrated strong power for identifying new malignant subtypes and for predicting clinical outcome. Moreover, this approach has shown high efficiency for predicting sensitivity or resistance to specific chemotherapeutic drugs.

In different studies, specific gene expression profiles have been recognized in HB tumors compared to normal livers, fetal livers or HCCs (8, 9, 39, 40). Major differences were found in genes regulating cell cycle and apoptosis, and strong deregulation of genes involved in the Wnt and BMP/TGF-β pathways was observed, consistent with the view that common mechanisms may underlie organ development and tumor formation. Genome-wide analysis of gene expression profiles in HB has led to identify two different HB subclasses with specific expression patterns (8). The two molecular subclasses could be distinguished by a genomic signature of 16 genes that demonstrated strong prognostic relevance both in diagnostic biopsies and in post-treatment tumor specimens. These subclasses differed not only by liver developmental stage and proliferative rate, but also by the extent of genetic instability and by clinical presentation. As immature cells are frequently endowed with intrinsic capacities of proliferation, motility and mobility, poorly differentiated tumors usually display aggressive and invasive phenotypes. Accordingly, the HB molecular subclasses were tightly associated with features of tumor stage, such as vascular invasion and extrahepatic metastasis, with strong impact on patient survival rates. Despite marked morphological heterogeneity in a majority of tumors, these two subclasses were mostly associated with the main epithelial component (fetal histotype versus other less differentiated patterns such as embryonal, crowded-fetal and macrotrabecular types) and with proliferative rate. Importantly, genetic instability associated with gains of chromosomes 2p and 8q was found to correlate with immature and proliferative tumors. Two oncogenes of the Myc family, MYCN and MYC are localized on these chromosomal arms, and activation of Myc signaling and the MAPK cascade have been specifically linked to poorly differentiated HBs (8, 9).

Myc activation has been observed in about 50% of human cancers, frequently associated with invasive phenotype and poor prognosis (41). Both MYC and MYCN are transcriptional targets of the transforming growth factor-β (TGF-β) pathway (42, 43). Among genes differentially expressed in HB, overexpression of several genes of the TGF-β pathway, such as BMP4 and genes of the SMAD family, has been observed in HBs associated with good prognosis (8, 9), suggesting that the pathway is activated in these tumors but not in the aggressive HB type. Also, Myc is considered to mediate the loss of TGF-β anti-proliferative responses induced by Wnt signaling (44). Accordingly, hyperactive β-catenin/LEF/TCF signaling in colon and breast cancers leads to sustained Myc expression, which renders cells unable to undergo growth arrest in response to TGF-β. During embryonic development, β-catenin and LEF/TCF form complexes with Smads for regulating common target genes (45, 46). A similar crosstalk of the Wnt and TGF-β signaling pathways might be found in aggressive HBs, due to their molecular and morphological analogies with the early developing liver. In addition, abrogation of Myc transcriptional control by TGF-β is associated with advanced tumor stages. Therefore, given the tight association of aggressive HB phenotype with gain of chromosomes 2p and 8q, chromosomal gain could allow MYCN and MYC escape TGF-β-mediated repression in aggressive HBs.

4. POTENTIAL THERAPEUTIC TARGETS IN HB

Given the critical importance of Wnt/β-catenin and Myc signaling in HB, these pathways are likely to represent major therapeutic targets for killing or sensitizing cancer cells to conventional chemotherapy and radiation. Mutations of the β-catenin, Axin and APC genes are key molecular lesions leading to aberrant activation of the Wnt pathway in many cancer types. It is generally assumed that targeting different steps of the Wnt cascade might convey therapeutic benefit. A variety of small molecules targeting one or more components of Wnt signaling or the β-catenin/Tcf transcriptional complex have been described (7, 47-49). Moreover, activation of Wnt signaling associated with up-regulation of the progenitor cell marker EpCAM has been shown to induce chemoresistance in HCC (50). It has been shown recently that EpCAM has oncosgenic potential, and that it can signal into the cell nucleus where it interacts with the Wnt pathway (51). The trifunctional monoclonal antibody catumaxomab with bispecificity for EpCAM and the T-cell antigen CD3 is currently evaluated for immunotherapy of a variety of epithelial cancers (52). Deregulation of Myc family genes
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Figure 1. Activation of multiple pathways and potential therapeutic targets in HB. (a) Comparative analysis of protein profiles obtained by a proteomic approach (Pathway ProfilerTM, Millipore) and gene expression profiles obtained by Affymetrix microarray mining. (b) Scatter plot of total and phosphorylated CREB1 and Hsp27 protein levels in 12 HB samples compared to 4 nontumor livers. (c) Schematic representation of the protein network that could be targeted for therapeutic intervention. Red boxes indicate proteins significantly overexpressed in HB as well as hyper-phosphorylation (p < 0.05). Light pink boxes denote p-values between 0.05 and 0.1. Green boxes correspond to proteins significantly underexpressed in HB as well as hypo-phosphorylation. White boxes indicate proteins that were not assessed in our proteomic analysis (straight line) or that showed no significant difference between HBs and normal livers (dashed line). Abbreviations: HB, hepatoblastoma samples; NL, nontumor liver samples; GPCR, G-protein-coupled receptor; P, phosphorylation.

has been frequently associated with poor differentiation and unfavorable outcome in childhood tumors, including also medulloblastoma and neuroblastoma. Different strategies that seek to either inhibit the proliferation-promoting effect of Myc or activate its pro-apoptotic function are under current investigation (53). Furthermore, therapeutic delivery of specific microRNAs has been shown to efficiently suppress liver tumorigenesis induced by Myc in a murine model (54). Among other genes overexpressed in HB, two members of the Melanoma-associated antigen family (MAGE) represent potential targets of immunotherapy (55).

To gain further insight into deregulated pathways in HB, we have recently integrated transcriptome analysis with a bead-based multiplex analysis of proteins involved in signaling cascades such as receptor tyrosine kinases, MAPK, NF-kB, and STAT pathways (Figure 1a). Comparison of 12 tumors and 4 non-tumor liver samples showed marked over-representation of total and phosphorylated CREB1, a transcription factor involved in cell growth, metabolism and angiogenesis (56). We also observed consistent up-regulation and hypo-phosphorylation of Hsp27, a heat shock protein that triggers resistance to stress-induced apoptosis via the formation of hypo-phosphorylated homo-multimers (57) (Figure 1b). Increased amount of c-Kit, Erk1/2 and p38 protein kinases correlated with increased phosphorylation of JUN and of p70S6K, a protein that acts downstream of mTOR and ERK to regulate mRNA translation, and a potential therapeutic target (58). By contrast, EGFR was downregulated both at protein and phosphorylation level. Analysis of proteins that transduce signals associated to inflammatory stimuli revealed significant hypo-phosphorylation of STAT6, and overexpression of the NF-kB antagonist IkBa. A schematic view of functional
relationships among potentially druggable pathways is presented in Figure 1c.

Because current treatments are ineffective in 20-30% of HB cases, specific targeting of active oncogenic pathways could represent a novel therapeutic option, particularly for poorly differentiated, aggressive and metastatic tumors. Further work integrating molecular and genetic data with clinical information in larger tumor sets will be crucial for accurate classification of tumors, risk stratification, and successful development of new therapies for pediatric HB patients. Despite the complexity of oncogenic mechanisms, combined efforts in biological and pharmacological studies might help fight liver cancer in childhood.

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**Send correspondence to**: Marie Annick Buendia, Institut Pasteur, Department of Virology, 28 rue du Dr Roux, 75015 Paris, France, Tel: 33-145688823, Fax: 33-145688943, E-mail: marie-annick.buendia@pasteur.fr

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