Review

The Advances in Molecular Biology of Hepatoblastoma: Implications for Diagnostic Pathology

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As the most common pediatric liver malignancy, hepatoblastoma (HB) accounts for more than 90% of primary hepatic malignant tumors in children less than five years of age in the US, and its incidence has been increasing in the past decades. Despite extensive studies, the pathogenesis of HB remains to be elucidated. Multiple signaling pathways may be involved in the oncogenic process of HB. The best characterized pathways include the canonical Wnt/beta-catenin pathway, the hepatocyte growth factor (HGF)/c-Met signaling pathway, the Notch pathway and the Hedgehog pathway. In addition, signaling molecules associated with these signaling pathways have been shown to be potential novel tumor markers for HB. Preoperative chemotherapy is the current standard of care for HB. Highly sensitive and specific tumor markers are not only important for the accurate diagnosis of HB but are also essential for predicting its clinical behaviors and prognosis. This review summarizes the recent advances in the molecular aspects of HB with a focus on the pathogenic signaling pathways and tumor markers. Their implications for diagnostics and prognostics are also discussed from a pathologist's point of view.
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Key Words: *hepatoblastoma, pediatric liver malignancy, canonical Wnt/beta-catenin signaling pathway, hepatocyte growth factor (HGF)/c-Met signaling pathway, Notch signaling pathway, Hedgehog signaling pathway*

INTRODUCTION

Primary liver cancers account for 1.1% of all pediatric malignancies in the US, and the rate is as high as 4% in infants.[1,2] The annual incidence is 0.5-1.5 diagnoses per 1 million children younger than 15 years for Western countries. Approximately 100-150 new cases per year are diagnosed in the US alone. The predominant hepatic malignant tumor in younger children is hepatoblastoma (HB), which accounts for more than 90% of primary hepatic malignancies in children under 5 year-old age.[3] The median age at diagnosis of HB is 18 months.

The incidence of HB has been increasing in the past decades probably due to the improvements in diagnostic technology and the better outcomes for premature infants.[1] The tumorigenesis of HB is complex and not well understood; however, some important signaling pathways are becoming elucidated. Potential novel tumor markers have also been identified. These advances in the molecular biology of HB are providing promising tools for the diagnostic pathology of HB and also for the prediction of the tumor's clinical behavior, treatment outcome and prognosis.

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HISTOLOGIC FEATURES OF HEPATOBLASTOMA

The histologic classification of HB has undergone several revisions. Two types of HB, the pure epithelial type and the mixed epithelial and mesenchymal types, were proposed by Ishak and Glunz in 1967.[4] As their names suggest, the former contains only epithelial tissue. The latter consists of both epithelial and mesenchymal components. In 1994, Stocker suggested six HB histologic patterns:[5] I. Pure fetal epithelial; II. Mixed embryonal and fetal epithelial; III. Macrotrabecular; IV. Small cell undifferentiated; V. Mixed epithelial and mesenchymal type with teratoid features or VI. without teratoid features. The most common subtype is the mixed embryonal and fetal subtype, followed by the mixed epithelial and mesenchymal subtype. The other subtypes are rare (**Figure 1**).[1] Currently, disagreements remain regarding the classification of HBs, and the original two-type classification has regained its popularity.

The epithelial type is composed of fetal-type and/or embyronal-type cells (**Figure 2**). The fetal-type cells are large and polygonal with clear or granular cytoplasm. The nuclei are round to oval with a single nucleolus. Cells are arranged in irregular cords that are 2-3 cells thick. Extramedullary hematopoiesis is often present. In contrast, the cells of the embryonal component are small, elongated, hyperchromatic and with scant cytoplasm. Typically, the growth pattern is solid. However, rosette-like clusters, cords

and ribbons may be observed. The variants of the epithelial type include the small cell undifferentiated type, comprised of anaplastic small cells in sheets, and the macrotrabecular type, in which tumor cells are organized similarly to hepatocellular carcinoma (HCC).

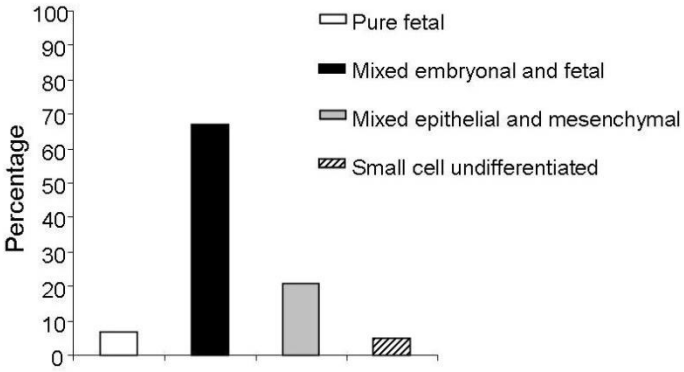

Figure 1. Distribution of major HB subtypes.

The mixed epithelial and mesenchymal type contains in addition to epithelial components, mesenchymal elements. The mesenchymal elements may be osteoid, cartilage, undifferentiated spindle cells, or, rarely, skeletal muscle or neural tissue.

Some other primary hepatic malignancies of the childhood can mimic HB. This includes HCC which is the most common liver cancer of adolescents and adults, and occurs in 12.5% of children younger than 5 years of age.[1] Among children living in Asian countries with a high viral hepatitis rate, HCC is the most common liver cancer in children.[6] Undifferentiated embryonal sarcoma (UES) is another type of tumor that can mimic HB. UES is the third most common hepatic malignancy in children (after HB and HCC). Other malignancies such as embryonal rhabdosarcoma, angiosarcoma and primary extragonadal germ cell tumors, although much rarer, have been observed in children as well. These HB mimickers are treated differently and generally show a worse prognosis when compared with HB. Therefore, it is essential to exclude them before making a definitive pathologic diagnosis of HB. Ancillary diagnostic techniques, including immunohistochemistry, electron microscopy, flow cytometry, and cytogenetics, have limited utility. Accordingly, highly sensitive and specific diagnostic tumor markers for HB are greatly desired.

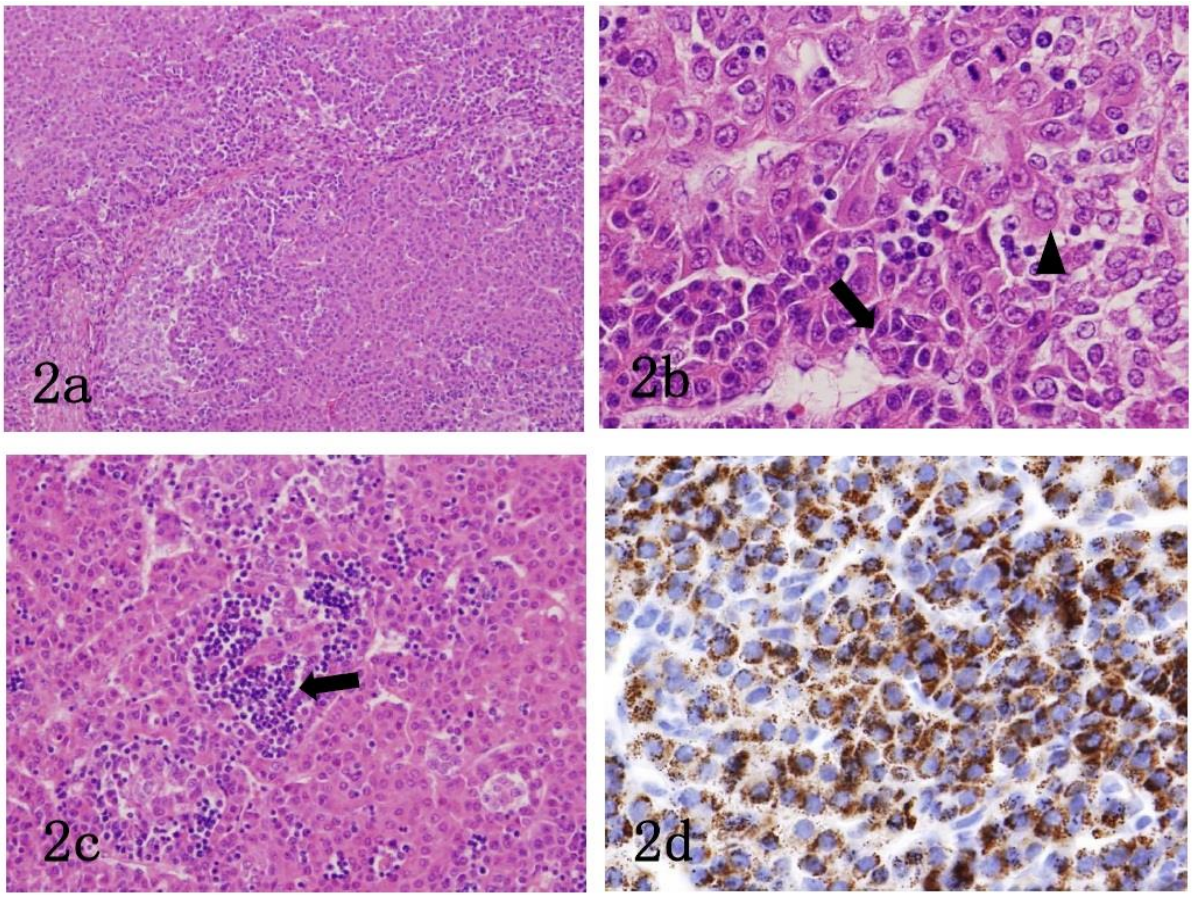

Figure 2. Epithelial type HB. In this case, the mixed embryonal and fetal pattern is present (**2a**). The embryonal (arrow, **2b**) and fetal type cells (arrowhead, **2b**) show distinct morphologies (see text). Extramedullary hematopoiesis is evident (arrow, **2c**). Immunohistochemically, both types of tumor cells are positive for Hep Par 1 (**2d**).

MOLECULAR SIGNALING PATHWAYS OF HEPATOBLASTOMA

HB is an embryonal tumor, and the tumor cells are derived from pluripotent hepatic stem cells. Gene expression profiling has shown HB to express hepatic progenitor markers. Immunohistochemistry has also demonstrated protein expression of these cancer stem cell markers.[7,8] Multiple signaling pathways have been implicated in HB carcinogenesis. These include pathways implicated in a wide variety of tumors, such as the phosphatidylinositol 3 kinase-Akt (PI3K-Akt) and the insulin-like growth factor 2 (IGF2) pathways.[9,10] Other pathways considered to be more specific to HB, which are the focus of this review.

1. Wnt/beta-catenin signaling pathway. The evolutionally conserved Wnt signaling pathway is essential to the normal biological processes of development and self-renewal.[11,12] Studies have also implicated genetic and epigenetic abnormalities in the Wnt pathway leading to various cancers, including hepatic malignancies.[13,14]

The Wnt signaling pathway has been very well characterized, especially the canonical beta-catenin-dependent Wnt pathway (**Figure 3**)[15-18]. Wnt ligands bind to Frizzled (Fzd), a seven-transmembrane receptor, and its co-receptor, the lipoprotein receptor-related protein (LRP) 5/6. This in turn inhibits serine/threonine phosphorylation of beta-catenin by glycogen synthase kinase 3β (GSK3β). The hypo-phosphorylated beta-catenin cannot be degraded, resulting in its cytoplasmic accumulation and nuclear translocation. The nuclear localization of beta-catenin enables its binding to HMG-box transcription factors, such as T-cell factors (Tcf) and lymphoid enhancer factor (Lef), which eventually activates its final effectors. Multiple Wnt/beta-catenin target genes have been identified,[8,19-21] including c-myc, cyclin D1, matrix metalloproteinase-7, FRA-1, c-Jun, urokinase plasminogen activator receptor, immunoglobulin transcription factor 2, EGF receptor and VEGF receptor. These genes are key regulators of a variety of biological processes including cell proliferation, anti-apoptosis and angiogenesis.

The Wnt/beta-catenin signaling pathway can be turned off by the absence of Wnt ligand. In the absence of Wnt ligand, beta-catenin becomes phosphorylated by GSK3β on serine/threonine residues. Phosphorylated beta-catenin forms a multi-protein degradation complex with axins (axin1 and axin2) and adenomatous polyposis coli (APC), which binds to the ubiquitin ligase receptor β-transducin repeat-containing protein (β-TrCP) and undergoes ubiquitin-mediated degradation.[22,23]

A large proportion of HBs contain beta-catenin gene (CTNNB1) mutations,[24,29] and these mutations prevent beta-catenin from being degraded. As a result, beta-catenin accumulates aberrantly in the cytoplasm, and then translocates to the nucleus. Nearly all HBs show increased beta-catenin levels in the cytoplasm and nucleus.[25,37] It is believed that the nuclear localization of beta-catenin leads to uncontrolled hepatoblast proliferation (**Figure 3**).[26]

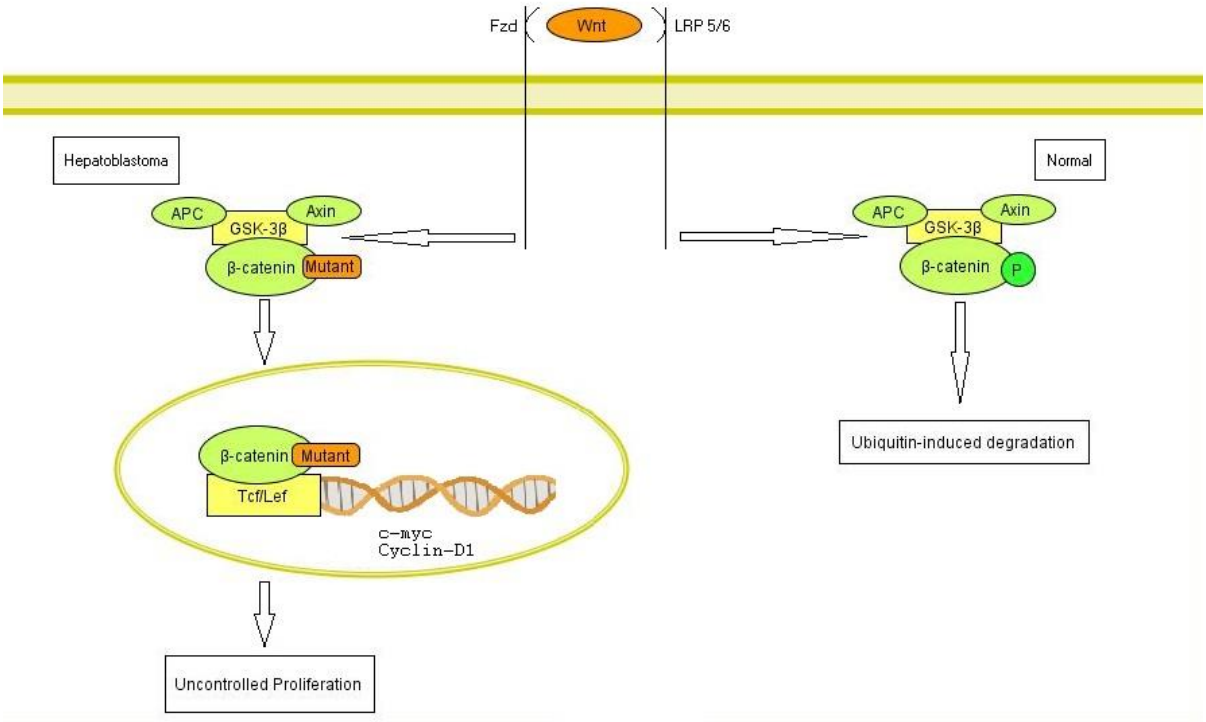

Figure 3. Schematic diagram depicts the Wnt/beta-catenin signaling pathway. Mutations in beta-catenin inhibits its ubiquitin-induced degradation, resulting in cytoplasmic accumulation and nuclear translocation. Nuclear beta-catenin activates c-myc, cyclin D1 and other target genes. This culminates in uncontrolled cell proliferation.

Interestingly, some HBs without beta-catenin mutations still display beta-catenin accumulation. This is probably because of other aberrant components in the pathway. Two thirds of sporadic HBs have been observed to possess APC gene alterations.[27,28] APC is a co-factor of beta-catenin degradation complex; therefore, mutations in APC may prevent beta-catenin from degradation and lead to its accumulation in the cells. Similarly, axin mutations have been shown in HB, which may also hinder the degradation complex from functioning.[29,30] In addition, in the absence of beta-catenin mutations, HBs with overexpression of telomerase reverse transcriptase (TERT) also demonstrate beta-catenin accumulation.[31] How TERT affects the beta-catenin levels is not well understood.

It is recently shown that the molecular signature of Wnt/catenin signaling in HB is dependent on the liver context. It may contribute more to the genesis of the embryonal than the fetal component of HB.[32] Of note, the non-canonical Wnt pathway, which uses Wnt11 as a ligand and activates the protein kinase C pathway, may function to antagonize the canonical Wnt pathway.[33]

2. Hepatocyte growth factor (HGF)/c-met signaling pathway. HGF/c-met signaling may contribute to the pathogenesis of HB because it also leads to aberrant beta-catenin accumulation in hepatoblasts.[34-37]

HGF is the natural ligand for c-met receptors. C-met is a cell surface receptor tyrosine kinase. Upon HGF binding, c-met undergoes autophosphorylation on tyrosine residues.[38] Phosphorylation of tyrosine residues creates a docking site for intracellular adapters via SH-2 domains and other recognition motifs, leading to further downstream signaling. Beta-catenin is one of this tyrosine kinase's substrates. Tyrosine phosphorylation of beta-catenin shields beta-catenin from serine/threonine phosphorylation, subsequent degradation, and leads to beta-catenin accumulation in the tumor cells.[39] This accumulation is independent of Wnt but the consequences are the same.

Besides beta-catenin, other important substrates for the c-met tyrosine kinase include PI3K and Ras/MAPK,[40] which are involved in many tumorigenic pathways.

3. Notch signaling pathway. The Notch signaling is an important pathway in stem cell renewal, differentiation, angiogenesis and endothelial sprouting.[41,42] It has been shown to play a critical role in both hepatocyte embryogenesis and cholangiocyte differentiation.[43]

The Notch ligands belong to the Delta-Serrate-Lag 2 (DSL) family of ligands. In mammals, there are five Notch ligands (three Delta and two Jagged proteins), which signal through four Notch receptors.[44] The binding of Notch ligands to their receptors initiates the proteolytic cleavage of the Notch receptor by γ-secretase presenilin, which in turn releases the Notch intracellular domain (NICD).[45] NICD then translocates to the nucleus and binds to a transactivation complex known as CSL, which consists of C promoter binding factor 1 (CBF-1), suppressor of hairless and Lag-1. This interaction activates target genes such as Hairy and Enhancer of Split (Hes1, Hes5 and Hes7), Hes-related proteins (HERP1 and HERP2) and Deltex1.[46]

Deregulation of Notch signaling in HB has been well documented.[47,48] Notch activation is more associated with the pure fetal subtype of HB,[24] as compared to the Wnt/beta-catenin signaling. The role of Notch signaling in tumorigenesis appears to depend on the cellular context.[49] The crosstalk between Notch and Ras, a cell survival pathway, or the death receptor 5, an apoptotic pathway, may determine whether Notch functions as an oncogene or a tumor suppressor, respectively.[50,51]

4. Hedgehog signaling pathway. The Hedgehog signaling pathway was first delineated in Drosophila. It is conserved in humans and plays a crucial role in controlling cell specification and pattern formation. It is essential for embryonic development and mature tissue homeostasis.[52]

In mammals, there are three Hedgehog ligands, namely Sonic Hedgehog, Indian Hedgehog and Desert Hedgehog. They bind to two receptors, Patched (Ptc) 1 and Ptc2.[53] Ptc is an inhibitor of Smoothend (Smo), a protein related to the Frizzled family of Wnt receptors and to other 7-transmembrane G protein-coupled receptors. In the absence of Hedgehog ligand, Ptc represses Smo and prevents the activation of Hedgehog signaling.[54,55] Upon binding to Hedgehog ligands, Ptc relieves this inhibition and activates Smo signaling. Glioblastoma (Gli) family transcriptors (Gli1-3) are activated by Smo and translocate into nucleus. This induces target genes, including beta-catenin, cyclins, and insulin-like growth factor 2 (IGF-2).[56,57] Gli1 and Gli2 predominantly act as transcriptional activators, and Gli3 may function as a repressor.[58]

The Hedgehog pathway has been intensely studied. Activation of Hedgehog signaling induces a variety of tumors, including hepatic malignancies.[59] In the case of HB, many signaling molecules of the Hedgehog pathway, Sonic Hedgehog, Ptc, Smo and Gli1, have been shown to be overexpressed in HB.[60,61] Specific blockade of Hedgehog signal transduction inhibits the growth of HB, highlightling the importance of this pathway in the oncogenesis of HB.[60]

TUMOR MARKERS FOR HEPATOBLASTOMA

The need to obtain an accurate pathologic diagnosis on biopsy tissue is of great clinical importance, as the current standard treatment modality for HB involves pre-surgical chemotherapy.[62,63] A diagnostic biopsy is now mandatory before any chemotherapy can commence. With limited tissue available and several HB mimickers to exclude, highly sensitive and specific HB markers would facilitate HB diagnostics.

HB staging is found to correlate with its clinical outcome.[64] Neither age, sex, size of tumor, or histologic type is a reliable prognostic indicator. Several staging systems for HB have been developed by different professional organizations,[65-68]

and the utility of these systems vary.[69] HB prognostic biomarkers could complement clinical staging or serve as an independent factor for predicting its clinical outcome. It is proposed that in the future, a new classification system for HB, with particular emphasis on prognostics, should be developed based on the molecular make-up of the tumor.[70]

1. Current common markers used in diagnostic pathology. Similar to HCC, immunoreactivity for AFP and Hep Par 1 (**Figure 2d**) has been used to distinguish HB from non-hepatic tumors.[71,72] The usefulness of these markers is limited when other primary liver cell malignancies are in the differential diagnosis. Interestingly, it has been demonstrated that patients with low serum AFP levels are less likely to achieve curative resections, and hence lower levels of AFP may indicate a poorer prognosis.[73] Most recent data indicate that during preoperative chemotherapy, a significant decrease in serum AFP level may serve as a survival predictor.[74] Other antigens expressed in HB include polyclonal CEA, cytokeratins, glypican 3, alpha-antitrypsin, CD99, CD56, neuroendocrine markers, NB84, Bcl-2 and Desmin.[72,76,77] The specificities of these antigens vary, and none of them can provide a definitive pathologic diagnosis.

2. Markers associated with Wnt signaling pathway. The Wnt/beta-catenin pathway plays a pivotal role in HB oncogenesis; therefore, the effectors of this pathway may have both diagnostic and prognostic values. Beta-catenin is the common final effector of several HB oncogenic pathways; therefore, it is not surprising that its accumulation is detected in almost all HB cases.[25,37] This fact may render beta-catenin a highly sensitive marker for HB. However, it is not specific to HB, as 20-40% HCC also have beta-catenin accumulation.[78,79] Beta-catenin localized to the nucleus (as opposed to the cytoplasm) correlates with shorter survival time in HB patients. This raises the possibility of using nuclear beta-catenin as a prognostic marker for HB.[26] But this is still controversial,[80] and further studies are needed to address the discrepancies between these observations. As for cyclin D1, one of the first identified Wnt-regulated target genes, previous work has yielded contradictory data,[81,82] but a more recent large scale study suggests that it is a potential prognostic indicator.[83] The C-myc oncogene is upregulated in many tumors, and its expression is of little value for the specific pathologic diagnosis of HB. However, the overexpression of some microRNAs driven by c-myc expression may account for the aggressive behavior of HB, and hence these microRNAs may be useful as markers for poor prognosis of HB.[84] TERT overexpression may also predict an unfavorable clinical course.[31]

3. Markers associated with other signaling pathways. It has been shown that c-met level decreases significantly in HB following chemotherapy, but beta-catenin nuclear localization remains unaffected. Therefore, c-met level may predict the response of HB to chemotherapy.[37] Delta-like protein (DLK) is a poorly understood membrane protein that functions as a negative regulator of Notch signaling. DLK may prove to be a highly sensitive and specific immunohistochemical marker for HB.[85] Notch2 is overexpressed in HB and downregulated in HCC.[47,86] Notch2 immunoreactivity may aid in differentiating HB from HCC on biopsy specimens. As far as the Hedgehog pathway is concerned, recent research by Li et al suggests Gli1 expression may be an independent prognostic marker for HB.[87]

4. Other markers. Expression profiling and differential screening have discovered multiple other tumor markers which may be of potential diagnostic or prognostic value for HB. As a member of the polo-like kinase (PLK) family, PLK1 is significantly upregulated in HB and the level of PLK1 expression may predict the prognosis of HB. In contrast, PLK4 downregulation is considered to be associated with poor prognosis in HCC.[88] Loss of expression of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) has been demonstrated in tumors with a high metastatic potential and is considered to be a marker for poor prognosis in patients with HB.[89] DNA methylation is an important mechanism of gene regulation. Hypermethylation of the HB associated genes, such as Ras association domain family 1A (RASSF1A) and metallothionein 1G (MT1G), may predict the clinical outcome of HB.[90-92]

CONCLUSIONS

The molecular aspects of HB have been an area of interest of researchers and clinicians for years. Advances in this field have allowed a better understanding of the pathogenesis and clinical behavior of this tumor and have also uncovered many potential HB tumor markers. Further characterization of these molecular markers will provide tools for both pathologic diagnostics and prognostics of HB.

CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

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