Towards a Therapeutically Pragmatic Molecular Definition of Hepatoblastoma

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Abstract
Hepatoblastoma (HB) is the most common pediatric liver cancer while also holding the distinction of being the least genomically altered of all malignancies. Three major oncogenic pathways have been identified as underlying the pathogenesis of this disease. Mouse models have demonstrated that deregulating any two is the minimal requirement for generating a tumor and that deregulating all three generates particularly aggressive ones. The terminal mediators of these three pathways, namely β-catenin of the Wnt/β-catenin, YAP of the Hippo pathway and NFE2L2/NRF of the NRF2 pathway are all transcription factors (TFs) that directly regulate hundreds-thousands of both unique and common downstream targets. Different pairwise combinations of these three TFs generate HBs with distinct growth patterns, histologies and gene expression patterns. Moreover, different patient-derived mutant forms of β-catenin also generate HBs with similarly distinct profiles. Based on the analyses of numerous tumors with these various combinations of TFs, and in different genetic backgrounds, it has been possible to identify a key set of 22 so-called “BYN genes” genes that are always similarly deregulated across all experimental HB types. A subset of these can be used to classify human HBs into those with long-term favorable and unfavorable outcomes and another subset is predictive of survival in hepatocellular carcinomas and over a dozen other human cancers. None of the BYN genes encode TFs; rather they mostly encode secreted or extracellular membrane proteins or enzymes. They therefore make more appealing targets than do the more refractory upstream TFs.

Keywords: β-Catenin, Hepatocellular Carcinoma, Hippo, Myc, NRF2, Wnt, YAP/TAZ

1. Introduction
Although hepatoblastoma (HB) is by far the most common liver cancer in children, it is nonetheless rare, with only about 100-200 cases per year being diagnosed in the United States, thus accounting for an annual incidence of about 1-1.5 per million in both the US and Europe [1,2]. Nearly all cases arise is children prior to the age of 5 years and even neonatal and in utero cases have been reported [3].

Four major HB histologic sup-types have been described (epithelial, small cell undifferentiated, macrotrabecular and mixed/epithelial/mesenchymal although each category contains additional sub-groups and individual tumors may display areas with different histologies [1]. Accurate pathologic classification is important as it is one of the factors that impacts survival, with others including clinical stage, a low alpha fetoprotein level at the time of diagnosis and age >2.5 years [4,5].

Overall long-term-survival/cure of HB is generally quite high (ca. 70-80%), with lower stage cases being particularly amenable to surgery and chemotherapy [4,6,7]. In contrast, cures in late-stage or recurrent disease, which accounts for the vast majority of HB-related mortality, can sometimes only be achieved following liver transplantation, a procedure that is limited by cost, the availability of appropriately matched donor organs, the proximity of centers with the necessary expertise and resources to perform the procedure and the morbidities associated with life-long immunosuppression [8].

2. Molecular Features of HB
Despite its intricate histologic classification scheme, HB is actually a fairly simple tumor at the molecular level. Indeed, it is the least complex of all pediatric cancers and retains this distinction even when adult cancers are included [9,10]. Over the past several years, three major signaling pathways have been identified whose deregulation is most frequently associated with HB in humans as well as being causally associated in mouse models of HB. These pathways, all converging on transcription factors (TFs), include those for Wnt/β-catenin, NRF2 (also known as NFE2L2) and Hippo [11-17].
β-catenin mutations were among the first recurrent ones to be identified in HB and are associated with as many as 70% of cases [11,12,14,15,18]. Unlike other major oncogenes such as RAS, BRAF, EGFR and PIK3A in which recurrent mutations tend to be highly restricted, those involving β-catenin show marked variability, ranging from single point mutations to large in-frame deletions [14,17-19]. Despite their heterogeneity, these mutations are functionally similar in that they inhibit β-catenin’s ability to interact with the APC tumor suppressor complex [14,19]. APC is essential to maintain β-catenin in an inactive and highly unstable form within the cytoplasm where its release, stabilization and nuclear entry are subject to regulation by members of the extracellular Wnt ligand family [20]. Recent studies have shown that patient-derived non-HB tumor-associated β-catenin mutations, never previously identified in HBs, can nonetheless induce HBs in mice. Indeed, even wild-type β-catenin can drive HB pathogenesis under conditions in which its over-expression exceeds its capacity to be sequestered by APC, thereby allowing the excess to escape cytoplasmic regulation, enter the nucleus and dysregulate transcription [21,22]. These findings are entirely consistent with the findings in individuals with familial adenomatous polyposis (FAP), who have inherited inactivating APC gene mutations and, as a result, have an extremely high incidence of early onset colorectal cancer. This is driven by the excessive nuclear accumulation of β-catenin, thus providing a constitutively active proliferative signal [23]. Individuals with FAP also have as much as a 5000-fold greater relative risk of developing HB as young children [24]. In keeping with this theme, other rare forms of inherited HB are more than 50% of human HBs and other cancers although precisely how is unclear [13,15,31,32]. Like β-catenin and NFE2L2/NRF2, the levels of the Hippo pathway terminal effector Yes-Associated Protein (YAP) are normally maintained under scrupulous nuclear/cytoplasmic balance, whereas in cancers, and HB in particular, the protein is stabilized and permanently localized to the nucleus where transcription of its target genes now becomes constitutively deregulated [15,31,33]*.

Although mutation, amplification and deregulation of B, N and Y represent the most common molecular abnormalities in HB, there remains a smattering of others, two of which involve the TSs APC and AXIN1 already mentioned above. Other gene mutations or pathway disruption include those involving TP53 and Hedgehog, JAK/STAT and TGF β/SMAD signaling (22, 34-36). Epigenetic and histone modifications and changes in the expression of long non-coding RNAs have also been identified in HBs although their direct relationship to HB causation remain to be determined (35,36).

3. A Molecular Ménage a Trois (or Deux) in HB
None of the patient-derived mutants of B or N nor an engineered mutant form of Y (YAP127A) that constitutively localizes to the nucleus are oncogenic when delivered individually via HDTVI to the livers of mice [15-37,37]. However, any pairwise combination of these vectors is oncogenic with the triple combination (B+Y+N) being particularly so and capable of generating large tumors within 2-3 weeks [15,16,37]. Moreover, the tumors arising from these different combinations grow at distinct rates and possess distinct histologic features that closely mimic those of certain human HB subtypes [16,17]. Subsequent work showed that different patient-derived mutants of B, including some not previously associated with HB, when delivered together with YAP127 also gave rise to tumors with distinct growth rates, histologic features and metabolic properties [17]. Taken together, these findings as well as those obtained with N mutants answered several questions that had long confronted the HB research community [16]. These included how to reconcile the histologic and growth rate variability of HB with its mutational simplicity, how to explain why some HBs arose by seemingly deregulating only B or the Hippo pathways.

*Hereafter, oncogenic forms of β-catenin, NFE2L2/NRF2 and YAP will be referred to as “B”, “N” and “Y”, respectively.
(many had previously unappreciated deregulation or mutation of N) and whether B mutants not previously identified in HBs would produce HBs if expressed in the liver (they could, as could wild-type B itself) [17].

One possibility to explain why HBs containing different B mutations differed from one another in appearance, growth rates and gene expression profiles was suggested by the finding that each B mutant was expressed at distinct levels and localized to the nucleus with somewhat different efficiencies [17]. Depending upon the location and identity of the mutation within the B molecule, it might be expected to retain different residual capacities to engage with the APC complex and thus be subject to its normal Wnt ligand-dependent regulatory control. Independently, its ability to enter the nucleus, to interact with its various transcriptional cofactors and to activate its target genes might also be differentially impacted by different B mutations [17].

Myc is among the most highly up-regulated target genes in both human and murine HBs [11,12,17,37]. This is not surprising given that the Wnt/β-catenin, NFE2L2/NRF2 and Hippo pathways all converge upon Myc and upregulate its expression, most likely in a cooperative manner [38-40]. The prominence of this additional and wide-ranging oncogenic TF, with literally thousands of its own targets sets up a transcriptional cascade that lends additional and overwhelming complexity to the gene expression profiles of all HBs [41,42]. Although mice bearing a hepatocyte-specific deletion of the Myc gene remain capable of generating tumors in response to the oncogenic B+Y combination, their growth rate is markedly impaired, thus attesting to the importance of Myc’s contribution [37]. Similarly, the hepatocyte-specific deletion of Chreb, a glucose-responsive TF that is distantly related to and communicates with Myc and shares numerous common target genes, is also detrimental to HB growth in response to B+Y overexpression [43,44]. The combined knockout of Myc and Chreb exerts an even more pronounced inhibitory effect on HB growth thus attesting to the redundancy of the two TFs [44]. Interestingly, in none of these cases did the loss of Myc and/or Chreb expression affect tumor initiation. Thus, both Myc and Chreb, neither of which is required for tumor initiation, appear to regulate genes that are largely supportive of and maximize HB growth initiated by any combination of its primary driver oncogenes. Computational analyses of the Myc and Chreb target genes both in liver and other cell types have shown substantial overlap, with the largest functional categories being involved in mitochondrial and ribosomal structure and function, DNA damage recognition and repair, cell cycle, aging, senescence and cholesterol metabolism [44,45].

4. Untangling the Ménages

The fact that B, Y and N, along with Myc and ChREBP, each regulates hundreds-thousands of both unique and overlapping target genes reveals little as to the actual underlying mechanisms by which these TFs rewire hepatocytes to survive, expand and become bona fide HBs. Indeed, depending upon the combination of B, Y and N used, the resulting tumors can differ from normal livers by as many as 6748 individual gene expression differences and among themselves by over 4600 [16]. Even HBs generated by the combination of Y and different B mutants can differ by >1500 transcripts [17]. How does one determine which of these truly contributes directly to transformation as opposed to merely being a non-contributing bystander or even a secondary response to the transformed state?

We took advantage of numerous RNAseq studies obtained from each of the above B,Y and N combinations and different B mutations while also comparing the transcriptomes of HBs generated by B+Y in wild-type, Myc-/-, Chreb-/- or Myc-/- x Chreb-/- “double knockout” genetic backgrounds [16,17,44]. We sought to identify transcripts that, irrespective of these different drivers or genetic backgrounds, were always de-regulated in the same direction. Although the degree of deregulation of these transcripts tended to be related to growth rates of the different tumor groups, 22 members of what we termed “BYN” genes were identified. In the most rapidly growing tumors, i.e. those generated by the triple combination of B+Y+N, 14 BYN transcripts were down-regulated by an average of >20-fold relative to normal livers (range 3-132-fold) and 8 were up-regulated by an average of nearly 25-fold (range 3.5-110-fold) [16].

Most BYN genes were also deregulated in human HBs and 10 could be used to distinguish those with favorable from unfavorable outcomes. Moreover, they did so independently of other small combinations that have been previously reported [11,12]. Fourteen of the BYN genes also correlated with survival in over a dozen other human cancers, 18 correlated with survival in human hepatocellular carcinoma (HCC) and nearly three-quarters of BYN genes were associated with the canonical Cancer Hallmarks [46]. At least seven also mapped to chromosomal loci that are subject to recurrent amplification or deletion in specific cancer types Collectively, these findings suggest that BYN genes play fundamental roles in tumor initiation and/or maintenance.

Unlike B, Y and N, none of the BYN genes encode TFs, which, despite their theoretical therapeutic appeal, are notoriously difficult to inhibit [47]. Rather most BYN genes encode enzymes, secreted proteins or membrane-associated proteins or receptors that are potentially more amenable to inhibition with monoclonal antibodies or small molecule enzyme inhibitors. Because this group is small, relatively labor intensive but direct approaches can be employed to test the hypothesis that individual BYN members are essential for tumorigenesis before embarking upon therapeutic campaigns. Such approaches could include the direct in vivo determination of whether normalizing the expressing of these genes in tumors, either by direct over-expression or via various knock-down-based strategies can reveal their essentiality. The potential success of this approach has already been demonstrated by showing that enforcing the over-expression of the potent TS p14ARF, which is down-regulated in most HBs, completely abrogates HB tumorigenesis mediated by B+Y [48]. The questions that can be addressed by this type of approach include not only whether a particular BYN gene is effective at inhibiting tumor growth but whether it does so irrespective of the TF combination
that generates the HB in the first place. Because HDTVI in an extremely efficient means of delivering plasmids to hepatocytes, another question is whether modulating the expression of more than one BYN gene can have additive or synergistic effects on tumor initiation and/or growth. Understanding the workings of this compelling group of genes will open the door to future studies that explore their functions and interrelationships in greater detail with the intention of developing the novel gene-specific and low-toxicity therapies mentioned above.

5. Conclusions
The last several years have witnessed the revelation of the major molecular factors that drive the development of the vast majority of HBs. There are several of these although the most prominent ones, namely, B,Y and N are TFs that deregulate hundreds-thousands of target genes, many of which are regulated in common by two or even all three TFs [14,16]. Although none of these are individually transforming, different combinations are and generate tumors with distinct growth rates, histologic appearance and gene expression profiles that mimic to a large degree those of their human HB counterparts. Underlying these tumors is a set of 22 “BYN” genes, the deregulation of which is a constant feature of all HBs. Different subsets of BYN genes are also highly predictive of survival, not only in HB but in well over a dozen other human cancers. Because this gene set is relatively small, and because straight-forward techniques for doing so exist, it should be relatively easy to determine the necessity of these for supporting HB initiation and growth with the outlook that these potentially represent significant and novel therapeutic targets.

References


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