Genomic Analysis of Hepatoblastoma Identifies Distinct Molecular and Prognostic Subgroups

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Despite being the most common liver cancer in children, hepatoblastoma (HB) is a rare neoplasm. Consequently, few pretreatment tumors have been molecularly profiled, and there are no validated prognostic or therapeutic biomarkers for HB patients. We report on the first large-scale effort to profile pretreatment HBs at diagnosis. Our analysis of 88 clinically annotated HBs revealed three risk-stratifying molecular subtypes that are characterized by differential activation of hepatic progenitor cell markers and metabolic pathways: high-risk tumors were characterized by up-regulated nuclear factor, erythroid 2-like 2 activity; high lin-28 homolog B, high mobility group AT-hook 2, spalt-like transcription factor 4, and alpha-fetoprotein expression; and high coordinated expression of oncofetal proteins and stem-cell markers, while low-risk tumors had low lin-28 homolog B and lethal-7 expression and high hepatic nuclear factor 1 alpha activity.

Conclusion: Analysis of immunohistochemical assays using antibodies targeting these genes in a prospective study of 35 HBs suggested that these candidate biomarkers have the potential to improve risk stratification and guide treatment decisions for HB patients at diagnosis; our results pave the way for clinical collaborative studies to validate candidate biomarkers and test their potential to improve outcome for HB patients.

Hepatoblastoma (HB) is the most common pediatric liver tumor. It has an annual incidence rate of approximately 1.8 diagnosed cases per million in the United States, and this rate is increasing at more than 4.3% annually.1 HBs are embryonal neoplasms that are most commonly diagnosed during the first 3 years of life. They are believed to arise from hepatic cell precursors and are characterized by heterogeneous histological patterns reminiscent of liver developmental stages.2 Therapeutic strategies combining surgical resection and chemotherapy have improved outcomes for children with HB, but the prognosis for patients with advanced or chemotherapy-refractory disease remains poor.1 In addition, the most effective platinum-based agents for treatment of HB often lead to serious long-term adverse effects, including ototoxicity and nephrotoxicity.1

We describe the results of a comprehensive genomic analysis of the largest set of clinically annotated HBs reported to date. Such efforts have previously identified...
prognostic groups and biomarkers for other embryonal
tumors (3) as well as adult hepatocellular malignancies. (4-6)
For example, survival-predictive and metastasis-predictive
biomarkers based on both gene and microRNA (miRNA)
expression profiles have been reported for hepatocellular
carcinoma (HCC), (7) the most common liver tumor in
adults. (4) Interestingly, a "stem-cell" gene-expression sig-
nature, involving several oncofetal, stem-cell markers, and
pluripotent stem-cell expression profiles, has been identi-
fied in a particularly aggressive type of HCC. (4,8-10)
Results from recent international HB clinical studies
suggest that underlying biological differences may be
responsible for the prognostic variability and wide range
of responses to chemotherapy seen in HB patients. (11,12)
However, there are currently no biomarkers or interna-
tional consensus regarding risk stratification for HB
patients. In North America, Children's Oncology Group
protocols have historically stratified patients by postsurgi-
cal stage and histological type, while in Europe and Japan,
HB diagnosis is often based on tumor imaging criteria
prior to therapy rather than pathologic analysis. (13)
Consequently, few pretreatment HB specimens are available
for molecular profiling outside of the United States. (11,12)
Activation of the canonical Wnt-signaling pathway
occurs in the vast majority of HBs through somatic
mutations at beta-catenin (CTNNB1) and or other
Wnt-signaling genes, but it can also be caused by
germline alterations including adenomatous polyposis
coli (APC) mutations and mutations associated with
related genetic syndromes. (14,15) It remains unclear
whether Wnt-signaling pathway dysregulation is
required for HB genesis or whether it is prognostically
significant. Studies have also identified HB-specific
expression signatures that are related to liver develop-
ment and HB histologic subtypes (16,17) as well as genes
that are differentially expressed in HB relative to HCC
and normal liver. (18) Multiple studies have speculated
about the biological and prognostic importance of spec-
cific genes and signaling pathways, but these are limit-
ed by the availability of HB tumor specimens, most of
which have been postchemotherapy samples with
incomplete clinical annotation. (19)
The goal of our study was to molecularly characterize
a large cohort of pretreatment, clinically and histopatho-
logically annotated HBs of sufficient size to identify sig-
nificantly predictive diagnostic and prognostic
biomarkers in this disease. Conclusions from previous
efforts that focused on profiles of posttreatment tumors
were limited to high-risk patients and were not able to
identify prognostic biomarkers. Here, pretreatment HBs
were profiled by whole-exome sequencing (WES) and
targeted sequencing, copy-number single-nucleotide
polymorphism (SNP) arrays, and messenger RNA (mRNA)
and miRNA expression arrays to identify bio-
markers that differentiate between low-risk and high-
risk patients. The risk-stratification potential of candi-
date biomarkers identified in our 88-tumor study was
evaluated prospectively using protein expression profiles
of 35 clinically annotated HBs. The analysis of these
data provided a more in-depth view of the landscape of
HB genomes and transcriptomes and identified molecu-
lar targets for HB diagnostics and therapeutics.

Materials and Methods
We molecularly profiled 88 HBs with corresponding
surgical pathology reports following histological review
(Supporting Table S1). Because of limited DNA and
RNA availability in some cases, not all tumors were

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profiled using the same assays. In total, 34 HBs were pro-
filed by WES and 46 by SNP arrays to identify mutations
and determine copy number; 50 and 57 HBs were profiled
by microarrays for mRNA and miRNA expression,
respectively. All tumors were profiled for genetic alter-
ations at CTNNB1, nuclear factor, erythroid 2–like 2
(NFE2L2), and the telomerase reverse transcriptase
(TERT) promoter using targeted sequencing.

HISTOLOGICAL REVIEW

Histological review of representative glass slides and/or
digital images of 94 samples was performed by M.J.F. and
D.L.-T., who confirmed diagnoses and histological sub-
types. There were no integrase interactor 1–negative
(INI1 or SMARCB1) tumors included in the study, as
recommended by the International Consensus HB Classi-
fication group.(11) Subtypes included epithelial, pure well-
differentiated fetal, pleomorphic or anaplastic fetal,
embryonal, small cell, mixed, or HB with HCC features.
Six samples—three non-HB malignant tumors, two sam-
ples with low HB content, and one sample with poor
tumor RNA viability after extraction—were excluded
from the study due to incorrect initial diagnosis or poor
tumor RNA content. A total of 88 histologically con-
firmed cases—50 males and 38 females—were selected
for profiling.

CTNNB1, NFE2L2, AND TERT
PROMOTER MUTATION
ANALYSIS

CTNNB1 exon 3 and 4 mutation analysis was complet-
ed using SuperScript One-Step reverse-transcription
polymerase chain reaction (RT-PCR) with Platinum Taq
reagents (Invitrogen) and primers BCAT-F and
BCATlong-R to generate a 507-bp PCR product.(15)
Two-directional Sanger sequencing analysis of PCR
products was completed with Mutation Surveyor v.4.0.4
(Softgenetics). TOPO TA cloning (Invitrogen) of
CTNNB1 PCR products was performed as needed when
more than one PCR product was detected by agarose gel
electrophoresis or when sequence quality was poor.

GENE AND miRNA EXPRESSION

mRNA expression was profiled by Affymetrix microarrays in a total of 50 tumors, six normal pediat-
ric liver tissues, and a pool of five normal fetal liver
samples; two of the profiled tumors (TLT-033 and
TLT-079) had insufficient follow-up or clinical
annotation and were excluded from biomarker discover-
ey efforts. miRNA expression was profiled by Agilent
microarrays in a total of 57 tumors and four normal
pediatric liver samples. RNA was isolated from
approximately 25 mg of frozen tissue specimens using
the Qiagen RNeasy Mini extraction system, followed
by deoxyribonuclease 1 treatment. miRNA was simul-
taneously isolated using the mirVana miRNA isolation
kit (Ambion). Samples extracted by both methods
were subsequently quantitated using the Nanodrop
ND-1000 spectrophotometer, and integrity was moni-
tored by the Agilent 2100 Bioanalyzer capillary elect-
rophoresis system (RNA 6000 Nano Kit). Samples
were selected for RNA integrity and profiled using
Human Genome U133P2 Affymetrix arrays and Agi-
ilent miRNA Microarray System v2.2.

DNA AND RNA EXTRACTION

DNA from frozen tumor specimens and matched
normal liver tissue or peripheral blood samples was
extracted using the QIAamp DNA Mini Kit (Qiagen).
Samples were treated with RNase A and eluted in
Buffer AE. DNA and RNA concentration and integrity
were determined with the Nanodrop ND-1000 spectrophotometer and 0.8% agarose gel electrophoresis, using
the Agilent 2100 Bioanalyzer. Paired samples demon-
strating intact genomic DNA were selected for WES;
these included 24 and 10 tumor samples paired with
normal liver and blood DNA, respectively. RNA from
frozen hepatic tumor specimens and matched normal
liver tissue specimens was isolated using the RNeasy
Mini Kit (Qiagen) or the mirVana miRNA Isolation
Kit (Ambion). Samples were treated with deoxyribonu-
clease 1 and eluted in nuclease-free water. Samples with
RNA integrity above 6.0 were used for genomic studies.

WHOLE-EXOME SEQUENCING

DNA was sequenced on an Illumina Hiseq 2000
with 80 million to 100 million successful 2 × 100-bp
reads per sample. Putative mutations identified
through exome sequencing were confirmed on a sec-
ond sequencing platform. See Supporting Information
for details on library construction, sequencing, and var-
iant calling. Tumor exomes were analyzed using the
Mercury v.3 pipeline,(20) including variant calling and
annotation with a minimum variant ratio cutoff of
0.05.(21) Somatic mutations were annotated with infor-
mation from the Catalog of Somatic Mutations in
Cancer database.(22)
SNP ARRAY

Gene copy number was estimated from Affymetrix Genome-Wide Human SNP Array 6.0 profiles of 47 paired normal and HB tumor samples. DNA was digested with NspI and StyI enzymes (New England Biolabs), ligated to the respective Affymetrix adapters using T4 DNA ligase (New England Biolabs), amplified (Clontech), purified using magnetic beads (Agen-court), labeled, fragmented, and hybridized to the arrays. Following hybridization, the arrays were washed and stained with streptavidin-phycocerythrin (Invitrogen). GISTIC-normalized segments with log2 ratios >0.3 or <−0.3 were designated as copy-number gains or losses, respectively. Analysis of genes whose expression and copy-number variation profiles were significantly correlated identified a total of 174 genes with $r > 0.58$ (Pearson correlation between expression and copy-number variation), a minimum that was required to achieve both $P < 0.01$ and a false discovery rate (FDR) <0.1 for the selection. A chromosomal view of copy-number variation changes is given in Supporting Information.

STANDARDIZED EXPRESSION

Gene expression profiles, as estimated by individual probe sets, were standardized by transforming expression profiles, measured as maximum signal log ratio, to standard deviations from mean. Namely, for each probe set, expression mean and standard deviation were computed across all tumors—when comparing or aggregating expression—or across all nontumor samples—when identifying differentially expressed genes. Then, expression estimates in each sample were transformed to the number of standard deviations from the mean expression of this probe set.$^{(23)}$

RNA EXPRESSION CLUSTERING

Profiles of our HBs together with profiles of 29 samples given by Cairo et al.$^{(16)}$ were processed by robust multi-array average and quantile-normalized. To exclude genes with low expression and variability across HBs, we focused on 9,835 probe sets with a maximum signal log ratio (normalized relative expression) above 6 and standard deviation above 0.3. Hierarchical clustering based on these probe sets was performed using hcluster in R with Pearson correlation and average linkage. To select the number of tumor clusters, we evaluated results using 2-10 clusters and averaged Silhouette values, Dunn’s Index, Gap statistics, and cluster homogeneity and cluster separation.$^{(24-27)}$ Analysis results, given in the Supporting Information, suggested that the optimal number of clusters is between three and five. We chose to partition the dendrogram to four clusters; partitioning to five clusters split the low-risk group into two groups with nearly identical survival and did not improve prognostic prediction.

GENE SET ENRICHMENT ANALYSIS

Gene set enrichment analysis (GSEA) of MSigDB v5.1 set c2 was performed with GSEA 2.2.2.$^{(28)}$ using the 9,835-probe sets described above and with $P$ values estimated by 10 K gene-label permutations, signal to noise metric, and the default weighted statistic. Array probes were collapsed into genes with the HGU133P2 max_p-probe conversion. Normal liver sample expression was estimated using six samples, including normal liver samples from patients TLT-001, TLT-041, TLT-047, TLT-048, TLT-052, and TLT-061. Favorable-prognosis HB profiles included profiles from patients who lived event-free for over 2 years following diagnosis and were diagnosed with stage 1 tumors, including TLT-023, TLT-068, TLT-031, TLT-086, TLT-043, TLT-011, TLT-028, TLT-040, TLT-042, TLT-003, TLT-049, and TLT-078. Poor-prognosis profiles were taken from patients who died of disease or relapsed with stage 3-4 tumors and included TLT-048, TLT-074, TLT-036, TLT-070, TLT-038, TLT-060, TLT-058, TLT-091, TLT-004, and TLT-009.

TRANSCRIPTION FACTOR ACTIVITY INFERENCE

Transcription factor (TF) activity was estimated using average standardized expression of validated targets according to TRANSFAC.$^{(29)}$ We inferred activity for TFs with at least two targets, based on the average of their standardized profiles. TFs whose target sets overlapped by 50% or more were clustered, and a representative with the largest number of targets was selected.$^{(30)}$ Hepatic nuclear factor 1 alpha (HNF1A) activity was inferred based on expression profiles of RIPPLY1, CLDN2, CD41B, SLC22A9, and IATIL; NFE2L2 based on profiles of NQO1, CYP2A6, PREPL, BSEP, NAT2, ASL, and PRIP; and Yes-associated protein 1 (YAP1) based on profiles of CYR61, BIRC5, CTGF, and JAG1.
TESTING PREDICTIVE ACCURACY IN A VALIDATION COHORT

We quantified protein expression of alpha-fetoprotein (AFP), CTNNB1, lin-28 homolog B (LIN28B), high mobility group AT-hook 2 (HMGA2), HNF1A, and NFE2L2 by immunohistochemistry in 35 clinically annotated HB tumor specimens and matched normal liver specimens using formalin-fixed, paraffin-embedded tissue blocks available in the archives of the Department of Pathology of Texas Children’s Hospital, after institutional review board approval. Immunohistochemistry was performed using formalin-fixed, paraffin-embedded tissue sections and the automated Leica Bond system. Epitope retrieval was carried out on the automated Bond system using either ER1 (Leica; AR99641) (pH 6) or ER2 (Leica; AR9640) (pH 9). Sections were incubated for 15 minutes with the primary antibody. We used Bond Polymer Refine Detection (Leica; DS9800), incubation with postprimary for 8 minutes, polymer for 8 minutes, 3, 3'-diaminobenzidine for 10 minutes, and hematoxylin for 5 minutes. Lists of antibodies, antigen retrieval methods, working concentrations, and interpretation (scoring) guidelines for each antibody are given in Supporting Table S5.

MULTIPLE TESTING CORRECTIONS

All P values were corrected for multiple testing using Bonferroni correction. See Supporting Information for the number of tests used for each statistic.

Results

We begin by describing results from our profiling effort. Molecular profiles were used to identify prognostic biomarkers and to construct a predictive function, which was then tested prospectively using protein expression from 35 additional HB patients.

WHOLE-EXOME SEQUENCING

WES revealed that HB tumors contain few coding mutations: a total of 131 somatic mutations occurring in 22 of 34 profiled tumors (3.9 mutations per tumor, range 0-24 mutations), resulting in a somatic mutation rate of <0.2 mutations/Mb of sequenced DNA (Fig. 1A). Supporting Table S2 lists all mutations identified by WES. Interestingly, younger patients were likely to have tumors with fewer somatic mutations (P < 1E-04; r = 0.62, Pearson correlation). Somatic alterations at CTNNB1 were detected in 13 tumors, and somatic mutations were discovered in genes related to regulation of oxidative stress, including recurrent point mutations at NFE2L2 (NRF2) in 3/34 HBs. NFE2L2 mutations (p.R34G × 2; p.D29N × 1) occurred in hot spots that have been described in a variety of cancer types, including HCC and cancers of the lung, endometrium, and urinary tract. Somatic and germline mutations were identified in other genes that have been implicated in both pediatric and adult cancers, including APC and the chromatin-remodeling genes MLL2 and ARID1A. WES profiles are available at European Nucleotide Archive under project PRJEB11805.

TARGETED SEQUENCING

Identified alterations are given in Supporting Table S1. RT-PCR and sequencing of CTNNB1 exons 3 and 4 identified point mutations and/or in-frame deletions in the ubiquitination domain of CTNNB1 in 78 of 88 HBs (89%; Fig. 1B); TLT-022 carried both a point mutation and a deletion. Three HBs were not successfully tested for alterations at CTNNB1 by RT-PCR due to a lack of viable RNA (TLT-029, TLT-037, and TLT-094); however, long PCR and sequencing of tumor DNA in these cases identified a p.D32Y point mutation in TLT-029 and a p.S22_G34del deletion in TLT-094. Among the 10 wild-type CTNNB1 cases, one (TLT-028) harbored a germline mutation at APC (identified by WES). Targeted sequencing of NFE2L2 exon 2 confirmed the three mutations identified by WES. In total, we identified mutations at NFE2L2 in four of 88 tumors (5%). NFE2L2 mutations (p.D29N in one patient and p.R34G in three patients; Supporting Fig. S1) clustered within the neh2 domain of the protein and were detected in tumors that also carried mutations at CTNNB1, as has been observed in adult HCC. NFE2L2 p.R34G has been shown to increase NFE2L2 activity. Two HBs—from patients 6 and 8 years old, the two oldest profiled—had somatic point mutations at the TERT promoter.

SNP ARRAY-BASED COPY-NUMBER CALLING

Comparative genomic hybridization/SNP array analysis detected abnormalities in 44/46 profiled tumors,
demonstrating characteristic whole copy-number alterations in described chromosomes or chromosomal regions of the HB genome, including distinctive gains at 1q (34%), 2 (17%), 8q (10%), and 12 (10%) and chromosomal losses at 1p (12%), 4q (2%), and 20 (19%), as well as other focal alterations (Supporting Fig. S2). A recurrent gain at 20q13.2 was recorded in poor-prognosis tumors, suggesting the presence of prognostic biomarkers.
RNA AND miRNA EXPRESSION PROFILING

Analyses of genes that were highly expressed in HB tumors relative to normal liver tissue and of TFs and their targets across HB tumors revealed significant activation of Wnt-signaling genes, activation and up-regulation of oncogenes and genes previously associated with HB and HCC prognosis, and cholangiocytic lineage differentiation genes, including genes whose expression or inferred activity was prognostically predictive (Supporting Table S3). Differential expression analysis, comparing miRNA expression profiles for HB tumors and normal liver samples, identified frequent down-regulation of lethal-7 (let-7) family members. Few signaling pathways were enriched between HB and normal liver profiles; however, multiple gene sets showed enrichment in tumors and for prognosis (Supporting Table S8).

ANALYSIS OF RNA AND miRNA EXPRESSION PROFILES: CLUSTERING OF mRNA PROFILES

We hierarchically clustered mRNA expression profiles (5,823 genes, 9,835 probe sets) of our HB tumors together with HBs reported by Cairo et al. (16)—a total of 73 tumor samples—in addition to 13 profiles of normal liver samples. Averaged Silhouette values (26) and Dunn’s Index (25) suggested the presence of two to four distinct HB molecular clusters together with a cluster of normal liver profiles. We chose to represent HBs in three clusters (Fig. 2A; Supporting Information). Survival analysis using our 51 tumor samples supported the predictive value of the resulting inferred molecular subtypes ($P < 0.01$), suggesting that the three clusters represent low-risk (HB1), high-risk (HB2), and intermediate-risk (HB3) groups (Fig. 2B). We found significant correlation between our molecular classification and that developed by Cairo et al. using profiles of post-chemotherapy HBs (16): six of seven of their poor-prognosis tumors (C2 HBs) were included in our HB2 high-risk cluster. Moreover, while subtype prediction using the 16-gene signature of Cairo et al. was not prognostically predictive ($P > 0.1$), other genes that were differentially expressed in their profiled tumors relative to normal samples were differentially expressed across prognostic groups (GSEA analysis; Supporting Table S8).

DIFFERENTIALLY EXPRESSED GENES

Differential expression analysis revealed genes that were consistently dysregulated in HB tumors. The eight most up-regulated genes were previously linked to Wnt signaling (Fig. 2C). Dickkopf-1, a bellwether of Wnt-signaling and beta-catenin/T-cell factor pathway activation, (15,16,33-35) was the most up-regulated, 267-fold on average. We used average standardized expression profiles of Dickkopf-1, APC down-regulated 1, axis inhibitor 2, and cyclin D1 to infer Wnt-signaling activity. (36) Five HBs showed significant increases in both Dickkopf-1 expression and inferred Wnt-signaling activity, and the remaining HBs showed exceptional increases in both (Supporting Fig. S3); we refer to these five HBs as tumors exhibiting mild Wnt-signaling activation (Fig. 2C; Supporting Table S3). GSEA identified multiple sets of differentially expressed genes identified by Cairo et al. but not their 16-gene signature, as enriched in differentially expressed genes across both HB and normal liver profiles and across favorable and poor prognosis. The most down-regulated genes, when compared to normal liver, were involved in metabolic and oxidation-reduction pathways, including metabolism of xenobiotics by cytochrome P450, as reported (see Supporting Fig. S4). (16,17)

VARIABLY EXPRESSED miRNAs

A total of 19 miRNAs were dysregulated ($P < 0.01$ by t test comparing expression in HB and normal, after multiple testing correction) and variably expressed (top 5% in terms of variability) in HB tumors. Hierarchical clustering suggested the presence of two clusters, including one cluster composed of let-7 family members in addition to miR-99a and miR-199a-3p (Fig. 2D; Supporting Table S4); miR-99a is often coexpressed with let-7 miRNAs. (37) Low let-7 expression correlated with high LIN28B expression ($P < 1E-3$, Pearson correlation). LIN28B is a known regulator of let-7 miRNAs (38) and its expression is inversely correlated with inferred HNF1A activity. In addition, let-7 expression directly correlated with inferred NFE2L2 activity (Fig. 2D; $P < 1e-8$, Pearson correlation) and survival ($P < 0.01$ for let-7b by survival analysis).

GSEA

We identified gene sets that were enriched with dysregulated genes in HB tumors and in poor versus
FIG. 2. Analysis of RNA and miRNA expression. (A) Hierarchical clustering of mRNA profiles of our seven normal liver samples and 48 HBs, together with four normal samples and 25 HB tumors profiled by Cairo et al.—73 HB and 11 normal samples in total—suggested the presence of three distinct prognostically predictive molecular HB clusters in addition to a cluster of normal liver expression profiles. The three clusters are annotated as HB1, HB2, and HB3 and are compared to clusters C1 and C2, which were proposed by Cairo et al. (B) The HB2 survival rate was significantly lower than that of HB1. (C) The 20 most up-regulated genes in the 48 HB tumors compared to normal pediatric liver included genes associated with Wnt signaling (in bold) as well as hepatic progenitor/stem-cell markers (*). (D) Hierarchical clustering of miRNA profiles suggested the presence of two clusters, including one cluster composed of let-7 family members. High let-7 expression directly correlated with normal or low LIN28B expression (in green), and low let-7 expression correlated with inferred increase in NFE2L2 activity (red).
favorable prognosis tumors. Genes identified downstream from CTNNB1(39) were among the strongest enriched in both poor-prognosis tumors and tumors versus normal tissue. In addition, sets of differentially expressed genes identified by Cairo et al., but not their 16-gene signature, were differentially expressed in both HB and prognosis. These included a 188-gene set identified as down-regulated in HB and a 676-gene set identified as up-regulated in HB. Other significantly enriched gene sets in poor-prognosis tumors include sets of differentially expressed genes in HCC by Boyault et al.(40) and Chiang et al.(39) In total, we identified 351 gene sets that were enriched in HB and/or or poor-prognosis HB at \( P < 0.01 \) after multiple testing correction. These are given in Supporting Table S8.

**PROGNOSTIC BIOMARKERS**

Unsupervised clustering identified three distinct prognostically predictive molecular clusters of HB tumors (Fig. 2A,B), suggesting a potential for specific prognostic biomarkers and predictive classifiers based on RNA expression. In total, we profiled RNA expression in 47 tumors with known clinical staging and survival state, including 24 tumors from patients who lived 2 or more years after diagnosis with no evidence of disease (favorable prognosis) and two and eight tumors diagnosed in patients who went on to relapse and/or died of disease (poor prognosis), respectively.

**NFE2L2 ACTIVATION**

\( NFE2L2 \) was recurrently mutated (5% of tumors), and NFE2L2 involved activity was predictive of prognosis at \( P < 1E-03 \), by fluoroethyl-L-tyrosine and by survival analysis. In total, nine high-risk tumors exhibited significant increases in inferred \( NFE2L2 \) activity (>2 standard deviations from mean), including three tumors with identified \( NFE2L2 \) mutations. Note that only three of the four tumors with mutations at \( NFE2L2 \) were profiled for RNA expression and that inferred NFE2L2 activity, but not its expression, was prognostically predictive.

**SALL4 UP-REGULATION**

There were 174 genes that showed significant correlation between expression and copy number at \( r > 0.58 \) (\( P < 0.01 \), FDR <0.1); however, copy number at the loci of these genes was not significantly predictive of prognosis, and these genes showed no significant pathway enrichment. The only prognosis-predictive copy-number change was a recurrent gain at 20q13.2, a region that includes four genes. Analysis of RNA expression of these genes identified spalt-like transcription factor 4 (\( SALL4 \)) as a candidate prognostic biomarker. No favorable and seven poor-prognosis HB tumors exhibited high \( SALL4 \) expression (TLT-009, TLT-021, TLT-036, TLT-058, and TLT-088) or \( SALL4 \) copy gain (TLT-009, TLT-012, TLT-056, and TLT-058). Amplifications at \( SALL4 \) (at least one extra copy) and its up-regulation (2-fold change and 7 standard deviations from normal) were significantly predictive of poor prognosis (\( P < 0.01 \) by survival analysis).

**PROGNOSTICALLY PREDICTIVE mRNA EXPRESSION**

Given the size of our data set, in order to identify prognostic biomarkers, we had to limit our pool of candidates before testing their association with prognosis to 50 or fewer. We focused on the analysis of a small subset of genes with evidence for HB-specific activity, including \( NFE2L2 \) and \( SALL4 \), identified as candidates based on DNA evidence, and a panel of 20 highly expressed genes in HB relative to normal liver samples (Fig. 2C; Supporting Table S3). These included the prognostically predictive genes \( LIN28B \), \( HMGA2 \), and \( AFP \). Mild Wnt-signaling activation, identified in five tumors, was predictive of favorable prognosis (5/5 patients) but not significantly (\( P < 0.2 \)); however, because Wnt-signaling-associated genes were significantly up-regulated in our data, we included Wnt-signaling activation in our predictive function. Low \( LIN28B \) and high \( HMGA2 \) expression predicted favorable and poor prognosis (\( P < 5E-03 \)), respectively. Higher \( AFP \) expression correlated with poor prognosis: 7 patients with tumors expressing \( AFP \) at \( 2 \times (6 \) standard deviations away from normal) died of the disease, and only one survived disease-free for more than 2 years (\( P < 5E-03 \)). LIN28B expression levels were highly correlated, with increased expression of other oncofetal genes (\( AFP \), glypican 3 [\( GPC3 \)]) and stem-cell/precursor markers (epithelial cell adhesion molecule [\( EPCAM \)], delta-like 1 homolog [\( DLK1 \)], and inversely correlated with NOTCH and phosphatase and tensin homolog signaling (Supporting Fig. S5). In addition, while DLK1 and GPC3 expression were significantly increased in HB, they were not prognostically predictive (Supporting Table S3). Interestingly, while c-MYC expression was previously implicated in
determining HB risk, most of our samples were negative for both c-MYC mRNA and protein expression (Supporting Fig. S6).

PROGNOSIS-PREDICTIVE FUNCTION

When combined, mRNA expression profiles of candidate biomarkers produced significant HB risk prediction. High NFE2L2 activity and high expression of LIN28B, HMGA2, SALL4, and AFP were predictive of poor prognosis and marked our high-risk group; all 10 HB patients who were profiled for mRNA expression and died of disease or relapsed were markedly high for at least one of these biomarkers. Low Wnt-signaling activity and low expression of LIN28B and HMGA2 were predictive of favorable prognosis and marked our low-risk group, identifying 11 of our favorable-prognosis patients and none of our poor-prognosis patients \( (P < 0.05) \). Patients who were not predicted to be at low or high risk were placed in the intermediate-risk group (Fig. 3). The resulting classification function was predictive at \( P < 0.05 \) by survival analysis.

TF ACTIVITY

TF activity was inferred for a total of 88 TFs with validated targets in TRANSFAC. It was evaluated as a predictor of age at diagnosis, survival years following diagnosis, survival state (deceased, relapsed, or free of disease), and clinical stage. Inferred HNF1A activity was the most significantly (anti) correlated with tumor stage, FDR \( <9\times10^{-5} \) (Supporting Fig. S7). NOTCH1 activity and the expression of its target, phosphatase and tensin homolog, were predictive of survival. NFE2L2 activity was also highly correlated with age at diagnosis (FDR \( <0.05 \)), suggesting that high NFE2L2 activity may be a feature in older children. Interestingly, inferred YAP1 activity, based on the expression of targets identified by Tao et al.,\(^{36}\) suggests that YAP1 is differentially activated in high-risk HB \( (P < 0.001; \) Supporting Fig. S8).

VALIDATION SET TO TEST PREDICTIVE ACCURACY

To test whether our RNA-based predictive function is also predictive on the protein level in the prospective setting, we assembled a panel of 35 HBs (validation set), including 15 tumors from patients who died of disease or had relapsed at their last follow-up visit (poor-prognosis tumors) and 20 tumors from patients who survived at least 2 years after diagnosis with no evidence of disease (favorable-prognosis tumors). Of the 35 tumors, 15 were pretreatment and 20 were obtained after chemotherapy. Each pretreatment and posttreatment tumor sample was stained with antibodies for HNF1A, SALL4, LIN28B, AFP, HMGA2, and NFE2L2; and tumor stains were evaluated by reviewing pathologists (D.L.-T., M.J.F.) as exhibiting focal or diffuse expression and having negative or weakly or strongly positive expression of each of the proteins; see Supporting Table S6 for classifications and Fig. 4 for representative examples. We note that HBs may be highly heterogeneous and that biomarker expression can vary greatly across cellular compartments (Supporting Figs. S9 and S10). HNF1A was not a prognostic biomarker candidate, and its expression was used to predict clinical staging. In addition, tumors were tested for CTNNB1 and GPC3 protein expression as a part of standard diagnostic protocols.

A total of five tumors in this validation set were identified to exhibit low HNF1A expression, all high-stage tumors; this was not significant because 90% of our validation-set tumors were high-stage. CTNNB1 expression was uniformly high and provided no predictive value. SALL4, HMGA2, and LIN28B expression were high in six, four, and three tumors, respectively, all poor prognoses. AFP expression was high, and NFE2L2 was expressed in five and six posttreatment tumors, respectively, all with poor prognoses; targeted sequencing revealed no mutations at \( NFE2L2 \).

Our classifier was permitted to select features independently for pretreatment and posttreatment tumors, and it limited use of AFP and NFE2L2 expression to posttreatment tumors only; we conjecture that this was due to successful treatment of high-risk pretreatment tumors with high expression for the two proteins. In total, two patients in the favorable-prognosis set, HBTH28 and HBTH8, had pretreatment tumors with high AFP and NFE2L2 expression, respectively. Both patients were cured by complete resection. Interestingly, even weak expression of NFE2L2 suggested high-risk disease: 9/9 tumors (including HBTH8) with positive NFE2L2 expression were either poor prognosis or fully resected.

In total, 13/15 (87%) poor-prognosis tumors and 0/20 of the favorable-prognosis tumors were flagged by high protein expression of at least one of our five proposed prognostic biomarkers (Supporting Table S6).
These results mirror conclusions from RNA-profile analysis, suggesting that our antibody panel is useful for predicting risk (FDR $<0.006$). In total, two patients died of disease but were not predicted to have poor prognosis by our classifier. The first case, HBTC1725, was a pretreatment macrotubular HB that shared histological features with HCC and had elevated NFE2L2 expression but was unclassified because NFE2L2 was used to flag posttreatment tumors only. The second case was one of two patients with tumors that were negative for GPC3; both of these patients were diagnosed with mixed HB with a mesenchymal component postchemotherapy.

**Discussion**

Genomic and transcriptomic analyses of 88 clinically annotated HBs facilitated the identification of distinct molecular risk groups with characteristic genomic and signaling-pathway alterations. Our analysis demonstrated that HB represents a biological spectrum of disease, ranging from tumors with few aberrations that exclusively target Wnt-pathway genes to tumors with high genomic instability and somatic alterations observed in HCC.

**HALLMARKS OF HB TUMORIGENESIS**

Our analysis confirmed that WNT-pathway activation is nearly universal in HB, with exceptionally high levels seen in poor-prognosis cases. Mutations and deletions of CTNNB1 were found in 78/88 (89%) tumors tested. Of the 10 CTNNB1 wild-type HBs, one had a germline APC mutation and six others showed strong Wnt-pathway activation (Figs. 1 and 3). In total, 85/88 (97%) patients had evidence from Wnt-pathway activation. Its universality may be indicative of stage of maturation arrest as the highest levels of Wnt-pathway activation in our samples were accompanied by high expression of hepatic stem-cell and progenitor markers LIN28B, EpCAM, AFP, and SALL4.$^{[41-45]}$ However, in contrast to what has been reported for HCC, EpCAM and DLK1 levels in our study were elevated in a large proportion of HBs and not clearly associated with prognosis.

**PROGNOSTIC BIOMARKERS**

We identified recurrent hot spot mutations at NFE2L2 as well as copy–number gains at SALL4—both potential therapeutic targets.$^{(9,46)}$ NFE2L2 mutations and pathway activation were found in 11% of our HB patients, all with high-risk tumors. These data are consistent with a previous report that identified mutations in CTNNB1 and NFE2L2 in 12/15 (80%) and 2/15 (13%) profiled HBs, respectively.$^{(32)}$ CTNNB1 alterations were identified in all four NFE2L2 mutated tumors, as is typical of NFE2L2-mutated adult HCC, suggesting a biological link between NFE2L2-mutated HBs and HCC.$^{(31)}$ All patients with tumors carrying NFE2L2 mutations in our study died of their disease, with the exception of one patient who underwent an early complete tumor resection.

Our analysis revealed three prognosis-predictive HB molecular subtypes that were characterized by differential expression of hepatic stem/progenitor markers (LIN28B, SALL4, AFP) hepatobiliary (HNF, NOTCH1), metabolic (NFE2L2), and cancer-related pathways (P53, TERT). Tumors with favorable prognosis were characterized by canonical Wnt-pathway activation, low levels of genetic instability, and low LIN28B and SALL4 expression and NFE2L2 activity. HNF1A activity levels directly correlated with the clinical tumor stage in our patient cohort (Fig. 3; Supporting Fig. S7). HNF1A is known to control liver-specific transcriptional programs$^{[47]}$ and is associated

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**FIG. 3.** Prognostic biomarkers: Our analysis of RNA expression profiles in 48 HBs revealed three risk-stratifying HB molecular subtypes that were characterized by the degree of Wnt-pathway activation; LIN28B, HMGA2, SALL4, and AFP expression; and NFE2L2 activation. Low-risk tumors were characterized by lower Wnt-pathway activity and low LIN28 and HMGA2 expression, while high-risk tumors were characterized by high HMGA2, SALL4, and AFP expression and NFE2L2 activation. The remaining tumors were classified as intermediate. Inferred HNF1 activity was predictive of tumor stage. Mutations were identified in the TERT promoter in tumor samples taken from the two oldest children in the study. These subtypes were largely in agreement with molecular classification obtained from unsupervised hierarchical clustering. For each tumor, we present survival years after diagnosis and survival status; clinical stage, age at diagnosis, and histological classification; the degree of Wnt-pathway activation (high or very high), as estimated based on target expression; expression, activation status, or the presence of a mutation in our predictive biomarkers; and the associated molecular classification obtained from unsupervised hierarchical clustering (HB1, HB2, or HB3). Tumor profiles are ordered by patient age. Stage 1 and stage 2 tumors are considered low grade, stage 3 tumors are high-grade, and stage 4 tumors are high-grade metastatic. Abbreviations: m, mesenchymal; M, mutation; SC, small cell.
FIG. 4
with hepatic progenitor cell specification\(^{(48)}\) and favorable prognosis in hepatic adenomas.\(^{(5,49)}\)

Poor-prognosis HBs expressed high levels of AFP, which is consistent with data regarding the adverse prognosis of high or very low serum AFP. Retrospective histological review of a small number of tumors from patients with low-AFP suggested that these may have been erroneously classified as HBs. Consequently, these types of tumors are currently under active consensus review.\(^{(50)}\) Histologically, HBs with high AFP expression demonstrated embryonal, small cell component, anaplasia, macrotrabecular patterns or “transitional” features and were more often seen in children older than 6 years. These have been associated with greater genomic instability.\(^{(32)}\) In contrast, HBs with low hepatic progenitor marker expression and high activation of differentiation pathways demonstrated fetal histology and clustered in the low-risk group.\(^{(51)}\)

**ASSOCIATION WITH DEVELOPMENTAL STAGES**

Many of the key genes linked to prognosis by expression profiling are known to be related to liver development and disease. LIN28B and HMGA2 expression and NFE2L2 activity were predictive of prognosis, and their profiles correlated with let-7 expression (Fig. 2D). LIN28B regulates stem-cell growth and metabolism and is known to inhibit let-7 miRNA maturation; together with HMGA2, LIN28B and let-7 are known to control stem-cell self-renewal potential.\(^{(52)}\) HBs in the high-risk group were also characterized by increased expression of other cancer stem/precursor cells, including EpCAM, DLK1, and oncofetal markers AFP, GPC3, and SALL4, and by increased activity of YAP1.\(^{(36)}\) In HCC, embryonic stem-cell and progenitor-cell gene expression, including expression of EpCAM and SALL4, are associated with poor prognosis.\(^{(6)}\) Similarly, EpCAM and stem cell–like genes including AFP and Wnt-pathway genes\(^{(4)}\) have been reported to define a subtype of HCC that is associated with poor prognosis.\(^{(53)}\) All patients with tumors demonstrating high levels of AFP were clustered in our high-risk group (Fig. 3), which is of particular interest given that high serum AFP is one of the few clinical parameters known to be associated with poor clinical prognosis in HB.\(^{(12)}\)

**GENOMIC INSTABILITY**

SNP array data revealed copy number alterations in previously described altered regions of the HB genome, including chromosomes 1q, 2, 8q, 12, 1p, and 20,\(^{(16,32,54)}\) as well as novel focal alterations. Gains in 1q and losses in 20q were more frequently identified in the high-risk HBs, but our study was not sufficiently powered to confirm significance of this or other potential associations, highlighting the need for large collaborative studies to obtain sufficient numbers of clinically annotated specimens.

**HISTOLOGY AND BIOLOGY**

Risk classes were associated with characteristic histological types. All tumors showing fetal histology with low mitotic activity (well-differentiated fetal HB) clustered in the favorable prognostic group. These features are known to be associated with an indolent clinical course, and well-differentiated fetal HBs are treated by surgery alone according to Children’s Oncology Group protocols with nearly 100% survival.\(^{(51)}\) However, some HBs in the low-risk group displayed other epithelial histology, including three with a minor small-cell component. None of the tumors in this group showed anaplasia, significant atypia, or HCC features; and none were classified as hepatocellular neoplasm not otherwise specified following the new international consensus classification.\(^{(11)}\) Tumors in the high-risk group included embryonal and small-cell epithelial components, and only one (TLT-074) showed...
FIG. 5. Hepatic stem/precursor cell differentiation and HB hypothesis. We postulate about the cellular origin of HBs and main dysregulated pathways and genes involved in HB pathogenesis, suggesting that expression of relevant hepatic markers and signaling pathways involved in development and hepatoblast, hepatocyte, and cholangioblast differentiation could be indicative of the developmental stage of any tumor progenitors. (A) Dysregulation of developmental pathways may determine the ability of mutated liver progenitor/stem cells to differentiate and progress to more or less aggressive HBs. (B) Prognostic biomarkers may indicate the stage of liver cell differentiation at HB genesis. Activated signaling pathways and mutated genes associated with corresponding prognostic HB groups. Abbreviations: Alb, albumin; BMP, bone morphogenetic protein; CK19, cytokeratin 19; FGF, fibroblast growth factor; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog; TLCT, transitional liver cell tumor.
exclusively mitotically active fetal features. Intermediate-risk HBs demonstrated both mixed epithelial and mesenchymal or only epithelial histological patterns, including small-cell component and atypia, as well as variable genomic instability, and clinical parameters intermediate between the other two risk groups.

HBs demonstrate a diverse range of histological phenotypes and clinical behaviors that may result from the proliferation of transformed stem cells or early hepatic precursors with varying degrees of differentiation. Dysregulation of various pathways may determine the ability of mutated liver stem cells to differentiate and progress to aggressive neoplasms (Fig. 5A). Consequently, we expect that, for example, mixed epithelial and mesenchymal HBs may arise from transformed precursor cells that retained the ability to differentiate to both lineages; note that the mesenchymal component of HBs does not produce GPC3. Our hypothesis is that HB phenotypes correspond to varying degrees of differentiation and activation of oncogenic pathways (Fig. 5B). High HNF1A and NOTCH induction, representing hepatic and biliary specification, is seen in more differentiated tumors, suggesting that these better-differentiated HBs develop from cells at later stages of the lineage. On the other hand, tumors expressing stem-cell and liver-precursor markers such as LIN28B, EpCAM, and SALL4 are more aggressive and often show chemoresistance. In addition, activation of the NFE2L2 signaling pathway or TERT promoter mutations, which are present in tumors of older children and with biological features that overlap those of HCC, are associated with particularly poor prognosis.

Only three high-risk HBs showed significant atypia (TLT-070) or anaplasia (TLT-009, TLT-058). Interestingly, TLT-009 and TLT-058 were the oldest children included in our series, and both carried mutations at the TERT promoter. This is of particular interest as tumors with similar histological and clinical features were described and initially designated as transitional-cell liver tumors. Histological review of a large series of these tumors by a group of international experts indicated that these hepatocellular neoplasms, which are usually diagnosed in older children, may represent a distinct biological group of HB with HCC features that should be included under the new hepatocellular neoplasm not otherwise specified provisional category. Ultimately, we expect that, for example, mixed epithelial and mesenchymal HBs may arise from transformed precursor cells that retained the ability to differentiate to both lineages; note that the mesenchymal component of HBs does not produce GPC3. Our hypothesis is that HB phenotypes correspond to varying degrees of differentiation and activation of oncogenic pathways (Fig. 5B). High HNF1A and NOTCH induction, representing hepatic and biliary specification, is seen in more differentiated tumors, suggesting that these better-differentiated HBs develop from cells at later stages of the lineage. On the other hand, tumors expressing stem-cell and liver-precursor markers such as LIN28B, EpCAM, and SALL4 are more aggressive and often show chemoresistance. In addition, activation of the NFE2L2 signaling pathway or TERT promoter mutations, which are present in tumors of older children and with biological features that overlap those of HCC, are associated with particularly poor prognosis.

In conclusion, our analysis suggests that testing the proposed prognostic markers using a combination of targeted DNA sequencing, RNA expression profiling, and immunohistochemistry may be useful for predicting risk and response to therapy for HB patients. We propose a new HB stratification algorithm that could be used in combination with clinical parameters and histopathology. Immunohistochemical stains incorporating diagnostic antibodies for CTNNB1, AFP, GPC3, and integrase interactor 1 were previously recommended by the International Consensus HB Classification group. We have described five additional predictive biomarker candidates: HNF1A, NFE2L2, SALL4, HMGA2, and LIN28B. These, together with copy-number assessment by cytogenetic or cytogenomic analysis, should be tested within a large-scale prospective clinical study. Integration of biomarkers associated with clinical outcomes into clinical stratification algorithms may be useful for HB prognostic classification and therapeutic guidance, particularly in the case of biomarkers for which emerging therapies may be available (let-7, LIN28B, NFE2L2, SALL4). Optimally designed, prospective collaborative clinical trials that include rigorous specimen banking and molecular analysis will be needed to prospectively characterize additional HB tumor specimens and further validate the biomarkers described here.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.28888/supinfo.