Development and validation of genomic and epigenomic signatures associated with tumor immune microenvironment in hepatoblastoma

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Abstract

Background: This study aimed to probe and verify aberrantly methylated and expressed genes in hepatoblastoma and to analyze their interactions with tumor immune microenvironment.

Methods: Aberrantly methylated and expressed genes were obtained by comprehensively analyzing gene expression and DNA methylation profiles from GSE81928, GSE75271 and GSE78732 datasets. Their biological functions were predicted by the STRING and Metascape databases. CIBERSORT was utilized for inferring the compositions of tumor-infiltrating immune cells (TIICs) in each sample. Correlation between hub genes and immune cells was then analyzed. Hub genes were validated in hepatoblastoma tissues via western blot or immunohistochemistry. After transfection with sh-NOTUM, migration and invasion of HuH-6 and HepG2 cells were investigated. The nude mouse tumorigenesis model was constructed.

Results: Totally, 83 aberrantly methylated and expressed genes were determined in hepatoblastoma, which were mainly involved in metabolic and cancer-related pathways. Moreover, their expression was liver-specific. 13 hub genes were screened, which were closely related to immune cells in hepatoblastoma tissues. Among them, it was confirmed that AXIN2, LAMB1 and NOTUM were up-regulated and SERPINC1 was down-regulated in hepatoblastoma than normal tissues. NOTUM knockdown distinctly weakened migration and invasion of HuH-6 and HepG2 cells and tumor growth in vivo.

Conclusions: This study identified aberrantly methylated and expressed signatures that were in relation to immune microenvironment in hepatoblastoma. Targeting NOTUM hub gene could suppress migration and invasion of hepatoblastoma cells. Thus, these aberrantly methylated and expressed genes might act as therapeutic agents in hepatoblastoma therapy.

Keywords: Hepatoblastoma, Methylation, Tumor immune microenvironment, NOTUM, Migration, Invasion
Background
Hepatoblastoma represents a predominant pediatric liver cancer [1]. This malignancy mainly occurs in the first three years after birth [2]. Combination of surgery and chemotherapy is a typical treatment strategy. Despite overall survival rate up to 80%, patients in advanced stages are usually refractory to typical therapy [3]. Moreover, some subjects present poor prognostic factors such as non-chemotherapy resistance and metastases, thereby reducing survival time [4]. Hence, it is of significance for revealing the mechanisms of hepatoblastoma and probing novel therapeutic targets for hepatoblastoma subjects.

Genetic and epigenetic changes contribute to hepatoblastoma heterogeneity. Epigenetic changes including CpG DNA methylation possess regulatory roles on gene expression, which are involved in the etiology and pathogenesis of hepatoblastoma [5]. Methylation primarily occurs at cytosine-C5 under the background of CpG dinucleotides. Methylation status of tumor suppressor genes or oncogenes may be predictive of patients’ outcomes [6]. Reversing DNA methylation has been a promising cancer therapy strategy [7]. Here, this study screened aberrantly methylated and expressed signatures with immune microenvironment of hepatoblastoma. In vitro, targeting NOTUM hub gene distinctly restrained migratory and invasive behaviors of hepatoblastoma cells. In vivo, targeting NOTUM inhibited tumor growth. Our findings demonstrated the potential of these aberrantly methylated and expressed genes as therapeutic agents against hepatoblastoma.

Materials and methods

Hepatoblastoma dataset
GSE81928 [8], GSE75271 [9] and GSE78732 datasets were retrieved from the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) database. Among them, GSE81928 dataset was composed of hepatic RNA-seq profiling of normal (n = 3), background (n = 6), and hepatoblastoma (n = 23) tissue specimens on the Illumina HiSeq2500 platform. GSE75271 dataset contained microarray expression profiles of hepatoblastoma (n = 50) and normal liver (n = 5) tissues on the GPL570 platform. GSE78732 dataset included hepatic methylation profiling of hepatoblastoma (n = 7) and normal differentiated liver (n = 7) samples on the GPL13534 platform.

Data preprocessing
The original microarray profiles of GSE75271 dataset were read using “affy” package [10]. The raw data were standardized via robust multichip averaging (RMA) method. The, the standardized expression data were re-annotated according to the annotation file of the microarrays. Probes that did not correspond to or correspond to multiple gene symbols were deleted. For one gene symbol corresponding to multiple probes, the maximum value was chosen as the expression value of this gene. The raw data from GSE81928 dataset were dimensionally tested. The matrix was standardized by log2 (TPM + 1) to eliminate the data dimension. Principal component analysis (PCA) was presented to remove outliers. Using “ChAMP” package, the raw data of GSE78732 dataset were read [11]. After quality control, filtering and clustering, outlier samples were deleted. Then, methylation β values were normalized with the BMIQ method.

Screening for differentially expressed genes
Differential expression analyses were presented based on the GSE75271 and GSE81928 datasets. Differentially expressed genes between hepatoblastoma and normal hepatic tissues were screened by "limma" package [12]. The screening threshold was p-value < 0.05 and |log2fold change (FC)| > 1.5. Then, common up- and down-regulated genes were intersected between the two datasets.

Screening for differentially methylated genes
Differential methylation sites between hepatoblastoma and normal differentiated liver samples were analyzed via “ChAMP” package. The screening threshold was |log2FC| > 0.25 and adjusted p-value < 0.01. Using “ChAMP” package, genes corresponding to the methylated sites were annotated. The genes with differentially methylated sites were set as differentially methylated genes.

Differentially methylated and expressed genes
The jvenn tool (http://jvenn.toulouse.inra.fr/app/index.html) was utilized to comprehensively analyze the differentially expressed genes and differentially methylated genes [13]. The genes obtained by the intersection were regarded as differentially methylated and expressed genes.

Functional enrichment analysis
Differentially methylated and expressed genes were uploaded to the STRING database (version: 11.0; http://string-db.org/) [14] and Metascape database (http://metascape.org/) [15] for functional enrichment analysis. Then, this study combined the enrichment results from the two databases.

Protein-protein interaction (PPI)
PPI analysis was performed using the STRING database. The parameters were set according to the default. PPI
analysis results were visualized using the Cytoscape (https://cytoscape.org/) [16]. The MCODE plug-in was used to mine key sub-networks from the PPI network as follows: degree cutoff = 2, node score cutoff = 0.2, max depth = 100 and k-score = 2. By combining PPI and key sub-networks, hub genes were determined based on degree > 5 in the PPI network and genes in the key sub-networks. The list of hub genes was uploaded to the GENEMANIA (http://genemania.org) database [17]. Functionally similar genes of hub genes were analyzed and gene functions were then predicted.

CIBERSORT
CIBERSORT (http://cibersort.stanford.edu/) tool was used for inferring the compositions of 22 kinds of tumor-infiltrating immune cells (TIICs) in normal and hepatoblastoma samples through deconvolution algorithm [18]. The LM22 matrix was used for 1000 calculations. Monte Carlo sampling was used to calculate the p-value for the deconvolution of each sample. The differences in immune cell compositions between normal and hepatoblastoma specimens were evaluated via student’s t test. The correlation between immune cells was analyzed via Spearson correlation analysis. Furthermore, Spearson correlation between hub genes and immune cells was analyzed.

Patients and specimens
We collected 20 paraffin section specimens of hepatoblastoma in the Hunan Children’s Hospital between 2019 and 2020. Adjacent normal tissues were used as controls. Additional file 1 listed detailed clinical information for each patient. All of them did not receive any anti-cancer treatment before surgery, such as oral chemotherapy, radiotherapy, or immunotherapy. All subjects were confirmed by pathological examination. This study was approved by the Ethics committee of Hunan Children’s Hospital (HCHLL-2021-09). The research has been carried out in accordance with the World Medical Association Declaration of Helsinki, and obtained the written informed consent from their parent and/or legal guardian.

Western blot
RIPA lysate (Beyotime, Beijing, China) was utilized to extract total protein from tissue or cell samples, followed by BCA protein quantification. 30 μg protein was taken for SDS-PAGE gel electrophoresis and transferred to membrane. Membranes were sealed with 5% skimmed milk powder solution at room temperature for 2 h. They were then incubated with monoclonal antibodies against AXIN2 (1/1000; ab109307, abcam, USA), LAMB1 (1/1000; ab256380), NOTUM (1/1000; ab106448), SERPINC1 (1/1000; ab124808), E-cadherin (1/1000; ab238099), Vimentin (1/1000; ab137321), Snail (1/1000; ab78105) and β-actin (1/5000; ab179467) at 4°C overnight. On the next day, membranes were incubated by HRP-labeled IgG secondary antibody (1/1000; ab7090) at room temperature for 2 h. ECL chemiluminescence detection was presented. The expression of target proteins was quantified.

Immunohistochemistry
Paraffin-embedded sections were deparaffinized with xylene and hydrated with gradient ethanol. Then the sections were incubated in 3% methanol-H2O2 and transferred to 0.01 mol/L sodium citrate solution for antigen retrieval. The sections were blocked by 5% skimmed milk powder solution at room temperature and then incubated with anti-NOTUM (1/100; ab106448) antibodies and secondary antibodies (1/1000; ab7090), respectively. DAB was used to develop color and neutral gum was utilized for mounting the film.

Cell culture and transfection
Human HuH-6 and HepG2 hepatoblastoma cells (ATCC, USA) were cultured in DMEM medium (Gibco, USA) containing 10% fetal bovine serum (FBS). The cells were cultivated in a 37°C, 5% CO2 incubator. Cells in the logarithmic growth phase were inoculated on a 6-well plate (1 × 10^6/well). After incubating for 12 h, Lipofectamine 3000 (BOSTER, Wuhan, China) was utilized for transfection. According to the Lipofectamine 3000 instructions, the experiment was divided into negative control (NC) and sh-NOTUM groups. After transfection, cells were cultured for 6 h, and then replaced with DMEM medium. 48 h after transfection, transfected cells were collected. Western blot was used to detect the expression of NOTUM protein.

In vivo tumorigenicity
Totally, 10 male 5-week-old nude mice (18-22 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). This experiment was approved by the Animal Care Committee of Hunan Children’s Hospital (HCHLL-2021-09). HuH-6 and HepG2 (1 × 10^7) stably transfected with NC or sh-NOTUM were separately subcutaneously injected into the left gluteal region of nude mice (n = 5). The tumor volume was measured every 4 days, and tumor size was calculated based on the following formula: volume (mm^3) = width^2 (mm^2) × length (mm)/2. All nude mice were euthanized after 21 days, and tumors were weighted following ethical dissection and photography.

Wound healing
HuH-6 and HepG2 cells were inoculated in a 6-well class. Cells were transfected with shRNA overnight. A 10 μl pipette tip was used to make a uniform scratch on
the cell culture plate. Exfoliated cells were washed away by PBS. After culturing the cells in the incubator for 0 h, 24 h and 48 h, 3 fields of view were randomly selected to calculate the scratch width.

Transwell for invasion
The matrigel was diluted with pre-chilled serum-free DMEM and spread in the upper chamber of transwell (Corning, USA) overnight at 37 °C. 200 μL transfected cells were added to the upper chamber (2 × 10^5 cells / L). 500 μL DMEM medium containing 10% FBS was added to the lower chamber. Cells were incubated for 48 h. Then, cells were fixed in pre-cooled formaldehyde for 20 min and were stained with crystal violet for 30 min in the dark. The cells in the upper chamber were gently wiped with a cotton swab. The number of invaded cells was counted under a microscope.

Statistical analysis
R packages and SPSS 24.0 were used for statistical analysis. Measurement data were expressed as mean ± standard deviation. Comparisons between groups were presented through student’s t-test or one-way analysis of variance. Correlation analysis was performed by Spearson test. P-value< 0.05 was indicative of statistical significance.

Results
Identification of differentially expressed genes in hepatoblastoma
To obtain hepatoblastoma-related genes, this study retrieved GSE75271 and GSE81928 datasets. Firstly, we normalized microarray profiles of GSE75271 dataset by RMA method (Fig. 1A). Figure 1B showed the results of dimension analysis. By PCA, we removed three hepatoblastoma samples “GSM1948566”, “GSM1948577”, “GSM1948562” that were close to normal liver tissues. Figure 1C displayed the PCA results of GSE75271 expression matrix after removing outliers. Totally, 1364 differentially expressed genes were screened for hepatoblastoma than normal samples, including 756 up- and 608 down-regulated genes (Fig. 1D). Their expression in each sample was visualized into a heat map (Fig. 1E).

For RNA-seq data of GSE81928 dataset, we standardized the expression matrix by log2 (TPM + 1) in Fig. 2A, B. Figure 2C depicted the dimension analysis results following normalization. Background profiles were then removed (Fig. 2D). According to differential expression analysis, 528 up- and 274 down-regulated genes were screened for hepatoblastoma (Fig. 2E). These genes may distinguish hepatoblastoma from normal samples (Fig. 2F).

After intersection of differentially expressed genes between GSE75271 and GSE81928 datasets, 134 up-regulated genes were identified for hepatoblastoma (Fig. 3A). Meanwhile, 191 down-regulated genes were intersected for hepatoblastoma (Fig. 3B). These common genes were further analyzed.

Identification of differentially methylated and expressed genes in hepatoblastoma
Methylation profiles were obtained from GSE78732 dataset. Firstly, all samples were clustered before normalization. An outlier sample “GSM2074844” was removed (Fig. 4A). Afterwards, we analyzed differentially methylated sites between hepatoblastoma and normal samples. As a result, 1125 hypermethylated and 2337 hypomethylated sites were screened in hepatoblastoma (Fig. 4B). There was a distinct difference in methylation between hepatoblastoma and normal specimens (Fig. 4C). Here, genes corresponding to differentially methylated sites were considered as differentially methylated genes. Then, we identified 83 differentially methylated and expressed genes in hepatoblastoma (Fig. 4D), as follows: TNFRSF19, SP5, NOTUM, NKD1, BMP4, ODAI, ROBO1, BAMBI, AXIN2, TRH, TRIB2, ASPSCR1, MSX1, HOXA3, ARHGEF3, PTK7, LAMB1, TXB3, C6orf48, IGDC3, NT5DC2, GSTP1, RERE, COL4A1, LZTS2, RPS12, NPM1, PLCG1, CYP2C8, SLC22A1, HAL, SLC10A1, XDH, HAO2, C3P1, TDO2, PGLYRP2, CYP1A2, GLS2, TAC3, HABP2, ADR1A1, HP, BP, CD3, FMO3, LBP, THRSP, CDA, ALDOB, HFE2, SRD5A2, GOT1, NAMPT, MAT1A, CLU, LAT, CAD1, TRPM8, GREM2, ATF3, AKR7A3, CBS, G0S2, MT1G, CSRNP1, ACSM5, DPYS, FND3, IGFALS, DAO, STEAP3, DUSP1, MARCO, APBB1IP, SLC4A3, SERPINC1, NRG1, AGXT2, ACSL1, ABCB11, UGP2 and MGST1.

Differentially methylated and expressed genes are closely related to hepatoblastoma
To investigate the biological functions of differentially methylated and expressed genes, we presented functional enrichment analysis by the STRING database. Our data showed that these genes were mainly enriched in metabolic or catabolic processes such as carboxylic acid metabolic process, alpha-amino acid catabolic process, small molecule catabolic process, small molecule metabolism, carboxylic acid catabolic process, small molecule metabolic process, carboxylic acid catabolic process, small molecule biosynthetic process, xenobiotic metabolic process, cellular amino acid metabolic process, catabolic process, and organic substance catabolic process (Fig. 5A). Cellular components enriched by these genes were then analyzed. The results showed that only peroxisome and peroxisomal membrane were enriched. Furthermore, they had various molecular functions such as cofactor binding, oxidoreductase activity, protein binding, identical protein binding, oxidoreductase activity, acting on CH or CH2 groups, coenzyme binding,
Fig. 1 Normalization, filtration, and differential expression analysis for microarray profiles of GSE75271 dataset. (A) Dimensional distribution of expression matrix data after normalization. (B) Scree plot for dimensional distributions following normalization. (C) PCA for the classifications of normal and hepatoblastoma samples. (D) Volcano plot for up- and down-regulated genes between hepatoblastoma and normal samples. (E) Heat map for the expression of these genes in hepatoblastoma and normal samples.
Fig. 2 Normalization, filtration, and differential expression analysis for RNA-seq data of GSE81928 dataset. (A, B) Dimensional distribution of expression matrix data before and after standardization. (C) Scree plot for dimensional distributions following standardization. (D) PCA for the classifications of background, normal and hepatoblastoma samples. (E, F) Volcano and heat maps for up- and down-regulated genes between hepatoblastoma and normal samples.
binding, pattern recognition receptor activity, pyridoxal phosphate binding and transaminase activity, indicating that they were mainly involved in catabolic processes. KEGG pathway enrichment analysis also confirmed that these genes primarily participated in metabolic pathways (such as drug metabolism - cytochrome P450 and other enzymes, cysteine and methionine metabolism, phenylalanine metabolism, metabolism of xenobiotics by cytochrome P450, biosynthesis of amino acids, caffeine metabolism, phenylalanine, tyrosine and tryptophan biosynthesis, peroxisome, chemical carcinogenesis, alanine, aspartate and glutamate metabolism, tyrosine metabolism, ubiquinone and other terpenoid-quinone biosynthesis, carbon metabolism, pentose phosphate pathway, linoleic acid metabolism, arginine biosynthesis and bile secretion) and cancer-related pathways (such as Wnt signaling pathway, hepatocellular carcinoma, pathways in cancer, NF-κB signaling pathway) in Fig. 5B.

Through the Metascape database, we performed DisGeNET annotation analysis on 83 methylated and expressed genes. We found that these genes were closely related to liver diseases, such as drug-induced liver disease, chemical and drug-induced liver injury, hepatitis, toxic, chemically-induced liver toxicity, drug-induced acute liver injury, hepatitis, drug-induced, fatty liver disease, cholestasis, diabetes mellitus, experimental malnutrition, juvenile arthritis, congenital defects, endotoxemia, thrombosis of cerebral veins, prostatic hypertrophy, homocystinuria, and hypertryrosinemia (Fig. 5C). PaGenBase annotation analysis results revealed that these genes were liver tissue-specific and liver cell-specific (Fig. 5D). Figure 5E indicated the complex interactions between pathways enriched by these genes.

**Construction of a PPI network and subnetworks in hepatoblastoma**

This study explored the interactions between proteins from differentially methylated and expressed genes in depth. A PPI network was established, composed of 60 nodes (Fig. 6A). Among all nodes, 16 genes were up-regulated, while 44 genes were down-regulated in hepatoblastoma. BMP4 (degree = 10) had the highest degree in the network, which had the closest connection with other nodes, indicating that their status in the network was the most important. Using the MCODE plugin, we established two subnetworks from the PPI network. In Fig. 6B, there were CYP1A2, GSTP1, CYP2C8, ABCB11 and MGST1 in the cluster 1. Furthermore, there were BMP4, SERPINC1, LAMB1 and NOTUM in the cluster 2 (Fig. 6C). In this study, 13 hub genes were determined by integration of genes with degree > 5 in the PPI network and genes in the two sub-networks, including
AGXT2, HPD, TAT, HAO2, GSTP1, ABCB11, MGST1, LAMB1, CYP1A2, CYP2C8, BMP4, SERPINC1 and NOTUM.

Functionally similar genes and biological functions for hub genes
Using the GENEMANIA database, we identified functionally similar genes for hub genes, including genes with co-expression, co-localization, and shared protein domains (Fig. 7A). Furthermore, we predicted the gene functions of these hub genes. The data showed that these hub genes were mainly enriched in various metabolic processes and physiological functions of blood (Fig. 7B). Especially, demethylation was distinctly enriched by them. These data highlighted the pivotal role of these hub genes in hepatoblastoma.

Tumor immune microenvironment in hepatoblastoma
We employed CIBERSORT to assess compositions of TIIcs in normal and hepatoblastoma specimens. Figure 8A showed the compositions of each TIIc for each hepatoblastoma sample. Observably, T cells CD4 memory resting and macrophages M2 were main immune cell types in hepatoblastoma tissues (Fig. 8B). Furthermore, we compared the differences in immune cell compositions between normal and hepatoblastoma specimens. Our data
showed that there were significantly higher compositions of B cells naïve, T cells CD8, T cells CD4 memory resting, T cells follicular helper, T cells regulatory (Tregs), T cells gamma delta, NK cells resting, NK cells activated, macrophages M0, macrophages M2, dendritic cells resting, dendritic cells activated and mast cells resting in hepatoblastoma compared to normal tissue specimens (Fig. 8C). Meanwhile, B cells memory, plasma cells, monocytes, mast cells activated, eosinophils and neutrophils exhibited distinctly lowered compositions in hepatoblastoma than normal tissues. These data were indicative that abnormal immune cells could be in relation to hepatoblastoma progression.

Interactions between immune cells in hepatoblastoma
To explore the interactions between tumor immune cells, we analyzed the correlations between immune cells in hepatoblastoma. Figure 9A, B showed the complex

**Fig. 5** Biological functions of differentially methylated and expressed genes. (A) GO enrichment results of differentially methylated and expressed genes. (B) KEGG pathways enriched by these genes. (C) DisGeNET annotation results from Metascape database. (D) PaGenBase annotation results from Metascape database. (E) Enriched pathway interaction network.
interactions between immune cells in tumor microenvironment of hepatoblastoma. Specially, B cells naïve were negatively correlated to B cells memory (cor = −0.633 and p-value = 2.171e-07; Fig. 9C). In Fig. 9D, there was a negative correlation between macrophages M1 and dendritic cells activated (cor = −0.548 and p-value = 1.509e-05). Furthermore, a negative correlation between macrophages M1 and macrophages M2 was found in hepatoblastoma (cor = −0.436 and p-value = 0.0008716; Fig. 9E). In Fig. 9F, mast cells resting had a negative association with mast cells activated (cor = −0.618 and p-value = 4.922e-07). Also, there was a negative association between NK cells resting and NK cells activated (cor = −0.612 and p-value = 6.798e-07; Fig. 9G). In Fig. 9H, plasma cells were negatively associated with mast cell resting (cor = −0.435 and p-value = 0.0009076). There was a positive correlation between plasma cells and Tregs (cor = 0.403 and p-value = 0.002263; Fig. 9I).

Hub genes are closely correlated to immune cells in hepatoblastoma

We further analyzed whether hub genes were in relation to immune cells in hepatoblastoma. The data showed that ABCB11 was negatively correlated to mast cells resting (cor = −0.41 and p-value = 0.001876; Fig. 10A) and positively associated to monocytes (cor = 0.431 and p-value = 0.001021; Fig. 10B) and plasma cells (cor = 0.414 and p-value = 0.001657; Fig. 10C). Moreover, BMP4 exhibited a positive association with mast cell resting (cor = 0.431 and p-value = 0.001017; Fig. 10D) and showed a negative correlation to plasma cells (cor = −0.482 and p-value = 0.0002354; Fig. 10E). In Fig. 10F, CYP1A2 was positively linked to plasma cells (cor = 0.433 and p-value = 0.001081). CYP2C8 (cor = 0.479 and p-value = 0.0002154) and HAO2 (cor = 0.444 and p-value = 0.0006848) both displayed positive interactions with eosinophils in Fig. 10G, H. LAMB1 possessed a positive association with macrophages M0 (cor = 0.424 and p-value = 0.001242; Fig. 10I). NOTUM was negatively correlated to B cells memory (cor = −0.487 and p-value = 0.0001653; Fig. 10J) and plasma cells (cor = −0.405 and p-value = 0.002342; Fig. 10K). SERPINC1 was negatively associated with macrophages M0 (cor = −0.524 and p-value = 4.093e-05; Fig. 10L) and was positively correlated to plasma cells (cor = 0.444 and p-value = 0.0007725; Fig. 10M). TAT exhibited a positive association with plasma cells (cor = 0.422 and p-value = 0.001473; Fig. 10N). Hence, hub genes could be closely correlated to immune cells in hepatoblastoma.

Validation of AXIN2, LAMB1, NOTUM and SERPINC1 proteins in hepatoblastoma

This study collected 20 pairs of paraffin section specimens from hepatoblastoma and adjacent normal tissues.
The expression of AXIN2, LAMB1, NOTUM and SERPINC1 proteins was examined by western blot. Our results demonstrated that AXIN2 ($p < 0.01$), LAMB1 ($p < 0.05$) and NOTUM ($p < 0.001$) were all distinctly up-regulated in hepatoblastoma compared to normal tissues (Fig. 11A-D). SERPINC1 protein ($p < 0.01$) exhibited lowered expression in hepatoblastoma than normal tissues (Fig. 11E). NOTUM protein was also detected in hepatoblastoma and adjacent normal tissues via immunohistochemistry. There was a higher NOTUM expression in hepatoblastoma than normal specimens ($p < 0.05$; Fig. 11F, G).

**NOTUM knockdown inhibits tumor growth in vivo**

To observe the function of NOTUM hepatoblastoma progression, NOTUM was silenced by transfection with sh-NOTUM in HuH-6 or HepG2 hepatoblastoma cells.
Western blot confirmed the decrease in NOTUM expression in HuH-6 cells \((p < 0.01; \text{Fig. 11H-J})\). This study established nude mouse tumor xenograft models injected by HuH-6 or HepG2 cells that were transfected with NC or sh-NOTUM. Tumor volume was measured every 4 days. We found that NOTUM knockdown significantly lessened the tumor volume (Fig. 11K, L). After 21 days, we measured the tumor weight. We observed that tumor weight was distinctly lowered by NOTUM knockdown (Fig. 11M-P).

**NOTUM knockdown lessens migration and invasion of hepatoblastoma cells**

Migration and invasion of HuH-6 and HepG2 cells were assessed after the deletion of NOTUM. In Fig. 12A-C, the number of invasive HuH-6 and HepG2 cells was reduced under transfection with sh-NOTUM \((p < 0.01)\). Furthermore, NOTUM knockdown distinctly suppressed the migratory ability of HuH-6 and HepG2 cells \((p < 0.01; \text{Fig. 12D-F})\). Epithelial-mesenchymal transition (EMT) is essential for cancer migration and invasion.
The expression of EMT markers (E-cadherin, Vimentin and Snail) was examined following the deletion of NOTUM. Our results showed that NOTUM knockdown significantly enhanced the expression of E-cadherin as well as lessened the expression of Vimentin and Snail in HuH-6 and HepG2 cells (Fig. 12G–J).

Discussion
At current, chemotherapy drugs like doxorubicin are applied as first-line agents for liver cancer [19]. However, they are non-selective cytotoxic molecules with prominent side effects. Sorafenib represents the only approved targeted drug for liver cancer [19]. Nevertheless, due to adverse side effects and limited therapeutic effects, it is of significance to explore novel targeted drugs beyond sorafenib. This study identified 83 abnormally expressed genes induced by DNA methylation in hepatoblastoma. It was predicted that these genes were closely related to metabolic processes and pathways as well as cancer-related pathways, highlighting their implications on hepatoblastoma progression. 13 hub genes were determined, which played pivotal roles in hepatoblastoma.
Also, these hub genes exhibited distinct correlations to immune cells, indicating that they might be related to immune response. Among them, we verified NOTUM functions in vitro and in vivo. Our data suggested that targeting NOTUM restrained tumor growth, migration and invasion of hepatoblastoma cells. Hence, these findings might provide promising therapeutic agents for hepatoblastoma.

Here, we screened 83 abnormally expressed and methylated genes in hepatoblastoma. After prediction, they might contribute to hepatoblastoma progress. More importantly, these genes were liver-specific. The epigenetic changes of hepatoblastoma are mainly reflected in the hypermethylation of tumor suppressor genes and the hypomethylation of oncogenes. Studying the DNA methylation of hepatoblastoma can provide new molecular
Fig. 11 (See legend on next page.)
Fig. 11 Validation of expression of hub genes in hepatoblastoma and effects of NOTUM on tumor growth. (A) Western blot for (B) AXIN2, (C) LAMB1, (D) NOTUM and (E) SERPINC1 proteins in hepatoblastoma and adjacent normal tissues. (F, G) Immunohistochemistry of NOTUM protein in hepatoblastoma as well as normal tissues. Bar = 50 μm. (H, J) Western blot for NOTUM expression in HuH-6 and HepG2 cells transfected with sh-NOTUM or NC. (K, L) Tumor volume was measured every 4 days in nude mice injected with HuH-6 or HepG2 cells transfected with sh-NOTUM or NC. (M-P) Tumor weight was measured in nude mice injected with HuH-6 or HepG2 cells transfected with sh-NOTUM or NC after 21 days. *p < 0.05; **p < 0.01; ****p < 0.0001

Fig. 12 Effects of NOTUM on migration and invasion of hepatoblastoma cells. (A-C) Transwell for assessing invasion of HuH-6 or HepG2 cells transfected with sh-NOTUM or NC. (D-F) Wound healing for evaluating migration of HuH-6 or HepG2 cells with sh-NOTUM or NC transfection. (G-J) Western blot for the expression of EMT markers (E-cadherin, Vimentin and Snail) in HuH-6 or HepG2 cells with sh-NOTUM or NC transfection. *p < 0.05; **p < 0.01; ***p < 0.001
markers and offer a reliable basis for early diagnosis, selection of treatment options and prognosis evaluation. In addition, designing demethylation drugs targeting specific targets to reactivate tumor suppressor gene functions is expected to become a new treatment for hepatoblastoma. Hub genes usually play pivotal roles in the process of EMT in hepatoblastoma cells. However, more experiments should be performed for verifying NOTUM functions during hepatoblastoma progression.

Conclusion
Taken together, this study proposed aberrantly methylated and expressed signatures that were related to immune microenvironment in hepatoblastoma. Among the, NOTUM was validated in depth. Targeting NOTUM could inhibit tumor growth, migration and invasion of hepatoblastoma cells. Thus, these signatures might act as therapeutic agents against hepatoblastoma.

Abbreviations
GEO: Gene Expression Omnibus; RMA: robust multichip averaging; PCA: principal component analysis; FC: fold change; PPI: protein-protein interaction; TIICs: tumor-infiltrating immune cells; FBS: fetal bovine serum; NC: negative control; Tregs: T cells regulatory

Supplementary Information
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Additional file 1. The clinical information of 20 cases of hepatoblastoma patients

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Authors’ contributions
LY, YQ conceived and designed the study. ZYB conducted most of the experiments and data analysis, and wrote the manuscript. ZT participated in collecting data and helped to draft the manuscript. All authors reviewed and approved the manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files.

Declarations
Ethics approval and consent to participate
This study was approved by the Ethics Committee of Hunan Children’s Hospital (HCHLI-2021-09). The research has been carried out in accordance with the World Medical Association Declaration of Helsinki.

Consent for publication
In this study, paraffin section specimens were collected from children. We have obtained the written informed consent from their parent and/or legal guardian.

Competing interests
The authors declare that they have no competing interest.
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