Integrated analysis of the rhesus monkey liver transcriptome during development and human primary HCC AFP-related gene expression

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INTRODUCTION

Liver cancer is one of the most common malignant tumors.1 Hepatocellular carcinoma (HCC) accounts for 70% of liver cancer cases.2,3 In Asia, the incidence of liver cancer is particularly high due to exposure to hepatitis B, hepatitis C, and aflatoxin.4,5 In China, the incidence and mortality of liver cancer rank among the top of all malignant tumors, and it is a health problem that cannot be ignored.6 At present, the outcomes of HCC treatment are unsatisfactory.

Alpha-fetoprotein (AFP) is expressed and synthesized sequentially in cells of the yolk sac, fetal liver, and gastrointestinal tract.7–9 AFP mainly performs the following functions during embryonic development: it interacts with estrogen to protect the fetus from circulating maternal estrogens and prevents the degradation of hormone molecules;10 exerts an immunosuppressive function to protect the fetus from the maternal immune system;11–13 and stimulates the proliferation and differentiation of the fetus.14 Since mature hepatocytes lose the ability to synthesize AFP, AFP is expressed at low levels in normal human blood.15 When malignant transformation occurs, HCC cells can regain the ability to synthesize AFP.16,17 In addition to liver cancer, malignant tumors of the stomach, pancreas, and reproductive system usually exhibit a small increase in AFP levels.18,19 AFP plays an important role in the development and progression of HCC; it not only promotes cancer cell proliferation and angiogenesis but also plays a role in inhibiting the apoptosis of cancer cells.20–22 AFP influences the antitumor effects of three important immune cell types: dendritic cells, natural killer cells, and T lymphocytes.23–25 Patients with AFPhigh HCC have significantly different protein expression profiles than patients with AFPlow HCC.26–28 Further research is needed to identify additional molecular signatures that promote the development of AFPhigh HCC.

Oncogenesis is considered similar to the early embryonic development process in a variety of ways.29–31 In this study, using data from the embryonic liver tissues of rhesus monkeys, we identified...
that the expression patterns of AFPhigh and AFPlow HCC tissues were similar to those of liver tissues during embryonic development. The expression patterns of AFPhigh HCC tissues were more similar to early embryonic liver tissues than AFPlow tissues, revealing the differences between the two types of liver cancer from the perspective of embryonic development. The gene expression profiles of AFPhigh and AFPlow HCC were investigated to explore the molecular differences between the two groups. A clear distinction of the gene expression profiles between AFPhigh and AFPlow HCC was identified, thus providing molecular evidence for the clinical relevance of the AFP level. Our study will enable us to better understand cancer pathogenesis and develop novel treatment approaches for this deadly disease.

RESULTS

Prognostic differences and clinical characteristics of patients with AFPhigh and AFPlow HCC

We conducted survival analysis to investigate the relationship between the AFP expression level and prognosis of patients with HCC. Relevant prognostic information was available for 32 (84.2%) patients with HCC enrolled in this study, including recurrence, metastasis, and survival status. The Kaplan-Meier analysis showed significant differences in prognosis between patients with AFPhigh and AFPlow HCC. A significantly longer disease-free survival (DFS) time was observed in patients with AFPlow HCC than in patients with AFPhigh HCC (Figure 1A). While the overall survival (OS) time of AFPlow patients was also longer, the difference was not statistically significant due to the small sample size (Figure 1B).

According to the clinical information of patients with HCC, the status of cirrhotic and hepatic capsule invasion were significantly different between the AFPhigh and AFPlow groups (Table S1, \( p < 0.05 \)). Cirrhosis is defined as the histological development of regenerative nodules surrounded by fibrous bands in response to chronic liver injury and is a stage often experienced during the progression of normal liver tissue to liver cancer.\(^3\) Serum AFP levels were evaluated in patients with liver cirrhosis.\(^1\) In cirrhotic livers where hepatocyte proliferation is compromised, liver progenitor cells (LPCs) are activated and then differentiate into hepatocytes and cholangiocytes, leading to the generation of regenerative nodules and functional restoration. AFP is secreted by LPCs in the liver cells of patients with cirrhosis.\(^3\) Regeneration after an acute exacerbation of cirrhosis might have been closely related to the dramatic increase in AFP levels. AFP has been reported to mediate the invasion and metastasis of HCC in vivo and in vitro by upregulating the expression of metastasis-related proteins, including epithelial cell adhesion molecule (EpCAM), keratin 19 (KRT19), matrix metallopeptidases (MMPs), and C-X-C motif chemokine receptor 4 (CXCR4), or activating the phosphoinositide 3-kinases (PI3K)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) signaling pathway,\(^25,26\) which might be the underlying mechanism related to the increase in AFP levels and hepatic capsule invasion.

AFPhigh and AFPlow HCC samples had distinct gene expression profiles

We conducted a t-distributed stochastic neighbor embedding (t-SNE) analysis to verify differences in the gene expression status between AFPhigh and AFPlow HCC and found a high degree of discrimination between the two groups of patients (Figure 2A). We performed a differential expression analysis of the two sets of samples and identified 744 differentially expressed genes, including 382 upregulated and 362 downregulated genes in AFPhigh HCC versus AFPlow HCC (Figure 2B).

We performed a Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis on differentially expressed genes to explore the differences in the biological functions of AFPhigh and AFPlow HCC and revealed that the upregulated genes were mainly related to the cell cycle (Figure 2C), while the downregulated genes were closely related to metabolic processes (Figure 2D). Gene set enrichment analysis (GSEA) of the KEGG pathway-related gene set showed that some pathways related to liver function, such as drug metabolism, three major metabolic pathways, and complement and coagulation-related pathways, were more active in AFPlow HCC tissues than in AFPhigh tissues (Figures 2E–2G).

We examined expression levels of representative genes involved in the cell cycle (CDK1, CDC25C, CCNB1, and CCNB2) and metabolic processes (CYP2A13, UGT2B15, and HSD11B1) using real-time PCR in an independent cohort of tissues from patients with HCC (\( n = 55 \), 16 AFPhigh, 39 AFPlow) to confirm the expression levels of representative genes involved in identified differentially altered pathways between AFPhigh and AFPlow HCC tissues from the RNA microarray. The results confirmed that genes involved in the cell cycle process were expressed at high levels in the AFPhigh group and that genes involved in metabolic processes were expressed at high levels in the AFPlow group (Figure 3A). We downloaded primary cancer transcriptome data from the Liver Hepatocellular Carcinoma Project of The Cancer Genome Atlas (TCGA-LIHC). The expression levels of the aforementioned genes were also verified in the cohort TCGA-LIHC and showed the same expression
patterns as the real-time PCR cohort (Figure 3B). Similar to previously reported results, our real-time PCR results and microarray results both showed that patients with high serum AFP levels exhibited a similar increase in AFP expression in HCC tissues (Figure 3A; Table S2).

Figure 2. Characteristics of gene expression in patients with AFP<sup>high</sup> and AFP<sup>low</sup> HCC
(A) The t-SNE analysis shows a significant distinction between patients with high and low AFP levels. (B) Volcano plot of the differentially expressed genes between the two groups of patients. (C and D) KEGG pathway analysis of upregulated and downregulated genes in AFP<sup>high</sup> HCC. (E–G) KEGG pathway-related gene set identified by the GSEA algorithm in AFP<sup>low</sup> HCC samples.

**AFP<sup>high</sup> and AFP<sup>low</sup> HCC tissues had different immune statuses**
The antitumor immune response plays a major role in the clinical outcomes of patients with cancer. Previous studies have reported that AFP inhibits the maturation of important immune cells,
including dendritic cells and natural killer cells, to promote tumor growth. Therefore, in the present study, the single-sample GSEA (ssGSEA) algorithm was used to score the immune cell-specific genes in each HCC tissue. According to the results of the t test, the scores for dendritic cells (Figure 4A), neutrophils (Figure 4B), and natural killer cells (Figure 4C) were significantly different in patients with AFPhigh and AFPlow HCC. The scores of the AFPlow samples were higher than those of the AFPhigh group, suggesting that the immune response in patients with AFPlow HCC may be more active than that in AFPhigh patients, and these patients also have more effective antitumor immunity.

Construction of the landscape of embryonic liver development in rhesus monkeys
Since AFP is regarded as a protein related to embryonic development, we conducted comparative analysis of gene expression data for rhesus monkey embryonic development and HCC to reveal the differences between the two groups of samples. Principal component analysis (PCA) was applied to evaluate 10,632 individual homologous monkey genes based on transcripts per million (TPM) values to construct a developmental gene landscape (Figure 5A). The HCC expression profile data were projected onto liver development patterns. The samples from the four phases of development (Figure 5B) were arranged in sequence, and the distribution was consistent with the developmental time series (especially on PC1).

Genes were divided into 9 groups according to their positions on the developmental landscape (Figure 5C). Line graphs of the mean expression in each group at each time point of liver development were generated. The black line represents the average expression level of each group of genes. The expression levels of groups 1 and 2 gradually decreased during the development process, but the levels of groups 5 and 6 gradually increased (Figure 5D).

The similarities and differences between AFPhigh and AFPlow HCC in the liver developmental landscape
PC1 was used as a measure of developmental time to calculate the average distance between AFPhigh and AFPlow HCC samples and samples from each developmental phase (Figures 6A and 6B). The liver cancer samples were distributed in a certain area between T1 (early development of the fetal liver, gestational age less than or equal to 100 days) and T2 (late development of the fetal liver, close to full-term pregnancy, gestational age greater than 155 days). AFPlow HCC tissue was most similar to the end-stage (T2) developing liver tissue in terms of the mRNA expression pattern. In contrast, AFPhigh HCC tissue was most similar to early stage developing liver tissue (T1); furthermore, the latter tissue type was more similar to the two points after birth than the former tissue type. Overall, the AFPhigh HCC tissue was more similar to early stage developing liver tissue (T1); furthermore, the latter tissue type was more similar to the two points after birth than the former tissue type. Overall, the AFPhigh and AFPlow HCC mRNA expression profiles were similar to the liver embryonic development expression profiles; however, AFPhigh tissues were more similar to early embryonic liver tissues than AFPlow HCC tissues.

The differentially expressed genes between AFPhigh and AFPlow HCC were projected onto the gene development landscape (Figures 6C and
Genes that were expressed at high levels in \( \text{AFP}^{\text{high}} \) HCC were concentrated in the left pole of the graph (groups 1 and 2, chi-square test, \( p < 0.0001 \)), while the genes expressed at high levels in \( \text{AFP}^{\text{low}} \) HCC were concentrated in the right pole (groups 5 and 6, chi-square test, \( p < 0.0001 \)).

**DISCUSSION**

Liver cancer is one of the leading causes of cancer-related death, and HCC comprises more than 70% of all liver cancers.\(^4,5\) AFP is an important protein related to embryonic development and a clinically relevant biomarker for the diagnosis of primary liver cancer.\(^39,40\) In Asian regions, approximately 90% of patients with HCC present elevated AFP expression levels, and patients with \( \text{AFP}^{\text{high}} \) and \( \text{AFP}^{\text{low}} \) HCC generally exhibit significant differences in clinicopathological features and prognosis.\(^41,42\) Therefore, we must explore differences in the molecular mechanism of tumorigenesis between \( \text{AFP}^{\text{high}} \) and \( \text{AFP}^{\text{low}} \) HCC. In the 1980s, Pierce et al. proposed the theory of “cancer, a developmental biology problem,” and many studies have linked tumorigenesis to embryonic development.\(^43,44,45\) Similarities between liver cancer development and development have already been observed in previous studies, and the transcriptional reprogramming induced in HCC mimics that of developing liver cells.\(^46,47\) Previous studies have identified multiple gene expression profiles of HCC, and variations in gene expression profiles are closely related to the initiation and maintenance of malignant phenotypes.\(^58,59\) However, few studies have compared \( \text{AFP}^{\text{high}} \) and \( \text{AFP}^{\text{low}} \) HCC. This study mainly aimed to identify differences in the molecular mechanism between \( \text{AFP}^{\text{high}} \) and \( \text{AFP}^{\text{low}} \) HCC by comparing the microarray data for AFP-stratified HCC tissues to the landscape of embryonic liver development.

In addition, distinct gene expression profiles between the two groups were revealed by a bioinformatics analysis.

In the current study, we finally identified 744 differentially expressed genes, including 382 upregulated and 362 downregulated genes, in \( \text{AFP}^{\text{high}} \) tissues compared with \( \text{AFP}^{\text{low}} \) tissues. The gene ontology (GO) enrichment analysis revealed that the upregulated genes were mainly related to the cell cycle, while the downregulated genes had pathways in HCC tissues with low levels of AFP. \( \text{AFP}^{\text{low}} \) HCC displayed a stronger immune response according to the scoring of immune-specific genes. Based on these results, \( \text{AFP}^{\text{high}} \) and \( \text{AFP}^{\text{low}} \) HCC have different molecular characteristics; furthermore, the immune responses of \( \text{AFP}^{\text{high}} \) tissues were weaker than those of \( \text{AFP}^{\text{low}} \) tissues, suggesting that \( \text{AFP}^{\text{high}} \) tissues are more malignant.

Many studies have shown several similarities between embryonic development and cancer.\(^50\) AFP has been shown to have similar biological functions in embryonic development and tumorigenesis. The immunosuppressive effect of AFP on embryonic development protects the embryo from attack by the maternal immune system and allows malignant cells in tumors to escape the effector cells of the immune system.\(^51,52\) The physiological function of AFP to promote proliferation could ensure the growth and development of the fetus, and it would cause the proliferation of malignant tumors.\(^53\) Therefore, an integrated study on the rhesus monkey liver transcriptome during development and human primary HCC AFP-related gene expression profiles was necessary.

This study combined rhesus embryonic liver development data with microarray data to explore the differences among \( \text{AFP}^{\text{high}} \) and \( \text{AFP}^{\text{low}} \) HCC tissues at the embryonic developmental level. \( \text{AFP}^{\text{high}} \) and \( \text{AFP}^{\text{low}} \) HCC mRNA expression profiles showed similar expression patterns as the developing embryonic liver, and \( \text{AFP}^{\text{high}} \) tissues were more similar to early embryonic liver tissue than \( \text{AFP}^{\text{low}} \) tissues. PCA was applied to assess 10,632 individual monkey homologs based on TPM values to construct a landscape of the development-related genes and to verify this result. The genes were divided into 9 groups according to the position of the gene in the development landscape. The differentially expressed genes between \( \text{AFP}^{\text{high}} \) and \( \text{AFP}^{\text{low}} \) HCC were projected onto gene expression in the developmental landscape. The upregulated genes in \( \text{AFP}^{\text{high}} \) HCC were concentrated in the left pole of the graph (groups 1 and 2, chi-square test, \( p < 0.0001 \)), but the upregulated genes in \( \text{AFP}^{\text{low}} \) HCC were concentrated in the right pole (groups 5 and 6, chi-square test, \( p < 0.0001 \)).
result confirms the conclusion stated above that the AFPhigh tissues are more similar to the earlier embryonic liver tissue.

In summary, this study assessed HCC expression profiles and liver development data from rhesus monkey embryos and found that AFPhigh HCC tissues were more similar to early embryonic liver tissues than AFPlow HCC tissues, although the AFPhigh and AFPlow HCC mRNA expression profiles and the embryonic liver developmental landscape exhibited similar patterns. Moreover, AFPhigh and AFPlow HCC tissues have different gene expression profiles, and the differentially expressed genes in AFPhigh tissues are mainly related to the cell cycle and metabolism. Additionally, the immune response of patients with AFPhigh HCC is weaker than that of patients with AFPlow HCC, suggesting that AFPhigh HCC may have a stronger immune escape mechanism. For the first time, this study revealed differences between AFPhigh and AFPlow HCC from the perspective of embryonic development. This study provides new insights into the molecular mechanisms of HCC development and reveals new strategies for targeted therapy.

MATERIALS AND METHODS

Patients and samples

Tissues for the mRNA microarray analysis were collected from 38 patients with HCC prior to hepatectomy at Cancer Hospital of Peking Union Medical College and Chinese Academy of Medical Science (PUMC&CAMS) from July 2009 to September 2011. According to the serum AFP levels of patients described in the inspection reports of the Department of Clinical Laboratory, PUMC&CAMS, the patients were divided into the AFPhigh group (n = 19) and AFPlow group (n = 19) with 25 ng/mL as the cutoff value. The average serum AFP level in the AFPhigh group was 4,087.2 ng/mL, and the average serum AFP level in the AFPlow group was 7.2 ng/mL. All patients were positive for hepatitis B virus (HBV) infection. The clinical characteristics of the two groups of patients are shown in Table S1.

Tissues for real-time PCR experiments were collected from 55 patients prior to hepatectomy at Cancer Hospital, PUMC&CAMS. According to the serum AFP level of patients described in the inspection reports of the Department of Clinical Laboratory, Cancer Hospital, PUMC&CAMS, the patients were divided into the AFPhigh group (n = 16) and AFPlow group (n = 39) with 25 ng/mL as the cutoff value. The average serum level AFP in the AFPhigh group was 3,445.7 ng/mL, and the average serum AFP level in the AFPlow group was 5.7 ng/mL.

All patients provided written informed consent before surgery, and the treatments were performed in accordance with present ethical principles. The inclusion criteria were as follows: patients did not receive radiotherapy, chemotherapy, interventional therapy, or radiofrequency ablation therapy before surgery; postoperative pathology confirmed the diagnosis of HCC; and the pathological margin was negative and not associated with malignant tumors of other organs.

All rhesus monkeys were raised at the Institute of Laboratory Animal Science, PUMC&CAMS. Please refer to the article “Extensive ceRNA–ceRNA interaction networks mediated by miRNAs regulate development in multiple rhesus tissues” for a more detailed description.

RNA preparation and microarray analysis

Total RNA was isolated with TRIzol reagent (Invitrogen, CA, USA) and then purified with the RNeasy kit (QIAGEN, MD, USA). The RNA was quantified with an ND-1000 UV-VIS spectrophotometer (NanoDrop Technologies, DE, USA), and its integrity was assessed.
using an Agilent 2100 Bioanalyzer (Agilent, CA, USA). RNA samples exhibiting optical density (OD) 260/280 ratios > 1.9 and RNA integrity numbers (RINs) greater than 6.5 were used in this study.

The sample labeling, hybridization, washing, and scanning steps were conducted according to the manufacturer’s specifications. Briefly, Cy3-labeled cRNA was generated from 500 ng input total RNA using Low RNA Input Linear Amplification Kit PLUS (Agilent, CA, USA) and hybridized to the Whole Human Genome Oligo Microarray (Agilent, CA, USA). After hybridization, the slides were washed and then scanned with the Agilent G2505B Microarray Scanner System. The fluorescence intensities of scanned images were extracted and preprocessed using Agilent Feature Extraction Software (v9.1). The raw data were normalized with the median scale method using the R package “limma” (https://www.r-project.org/). Probes representing the same gene were further screened, and only the probe exhibiting the highest mean intensity was retained. Consequently, an expression matrix containing 19,503 unique genes was obtained and used in the subsequent analysis. The raw and processed data are publicly available on the China National Center for Bioinformation, National Genomics Data Center (CNCB-NGDC) website (https://bigd.big.ac.cn/omix/) under accession number PRJCA003750.

**Real-time PCR assays**

RNA (1 μg) was reverse transcribed in a final volume of 20 μL using random primers under the standard conditions recommended by the PrimeScript RT Reagent Kit (Takara, Kyoto, Japan). Real-time PCR analyses were performed with SYBR Premix Ex Taq (Takara, Kyoto, Japan). The following sense and antisense primers were synthesized: CDK1 (forward 5’-CTGGGGTCAGCTGCTACT-3’, reverse 5’-TCCACTTCTGGCCACACTTC-3’), CDC25C (forward 5’-TCA GAGGCCGTAACTTTGGC-3’, reverse 5’-CAGCCGGAAGACTTG AGCAGA-3’), CCNB1 (forward 5’-CGGCCCTCTACCTTTGCA-3’, reverse 5’-TGTTCCTTGACAGTCCATTCACACG-3’), CCNB2
Comparative analysis of expression data and rhesus liver development data

The liver transcriptome data of 11 rhesus monkeys covered 4 stages of monkey development (T1, early development, including 1 sample at 45 days of gestation, 1 sample at 70 days of gestation, and 1 sample at 100 days of gestation; T2, 2 samples at 160 days of gestation; T3, 3 samples from monkeys within 7 days of birth; T4, 3 samples from adult monkeys over 5 years old). For the transcriptome sequencing-PE90, the data were subjected to cutadapt and sickle analyses to remove the sequencing linker and the low-quality sequence. Macaca mulatta 8 from the NCBI and Ensembl databases was used as the reference genome, and sequence alignment was performed with hisat2. The splicing and quantification of the transcript was performed by salmon. A total of 11,168 genes were expressed in 11 samples, of which 10,632 (95.20%) were homologous to human genes and were detected by the Agilent chip used in this study. The liver development landscape was constructed by PCA of 10,632 human homologous monkey genes, depicted in TPM, from rhesus liver development samples. The HCC expression profile data were projected onto the liver development pattern, and PCI was used as a measure of developmental time to calculate the average distance between AFB<sup>high</sup> and AFB<sup>low</sup> HCC samples.

Data downloaded and statistical analysis

“GDC-Client” was implemented to download the transcriptome data from TCGA-LIHC, and log2 transformed (fragments per kilobase million [FPKM] +1) values were applied as the gene expression value. All patients (n = 371) were divided into the AFB<sup>high</sup> group (n = 186) and AFB<sup>low</sup> (n = 185) group according to AFP expression. The Wilcoxon rank-sum test was adopted to compare differences between two groups. The liver transcriptome data of 11 rhesus monkeys covered 4 stages of monkey development (T1, early development, including 1 sample at 45 days of gestation, 1 sample at 70 days of gestation, and 1 sample at 100 days of gestation; T2, 2 samples at 160 days of gestation; T3, 3 samples from monkeys within 7 days of birth; T4, 3 samples from adult monkeys over 5 years old). For the transcriptome sequencing-PE90, the data were subjected to cutadapt and sickle analyses to remove the sequencing linker and the low-quality sequence. Macaca mulatta 8 from the NCBI and Ensembl databases was used as the reference genome, and sequence alignment was performed with hisat2. The splicing and quantification of the transcript was performed by salmon. A total of 11,168 genes were expressed in 11 samples, of which 10,632 (95.20%) were homologous to human genes and were detected by the Agilent chip used in this study. The liver development landscape was constructed by PCA of 10,632 human homologous monkey genes, depicted in TPM, from rhesus liver development samples. The HCC expression profile data were projected onto the liver development pattern, and PCI was used as a measure of developmental time to calculate the average distance between AFB<sup>high</sup> and AFB<sup>low</sup> HCC samples.

Data Availability Statement

Microarray data that support the findings are publicly available on the China National Center for Bioinformation, National Genomics Data Center (CNCG-NGDC) website (https://bigd.big.ac.cn/omix/) under accession number PRJCA003750.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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