 doi:10.20944/preprints202102.0352.v1

Article

Serum cytokine analysis in patients with hepatocellular carcinoma

Running title: Cytokines in hepatocellular carcinoma

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Abstract: Background: Hepatocellular carcinoma (HCC) is one of most common cancers with a high mortality rate. HBV/HCV infection is an important risk factor to trigger HCC. Therefore, developing serum biomarkers for early diagnosis is crucial to prolong survival in HCC patients. Methods: An antibody array technology was utilized to detect serum from 20 HBV-related HCC patients, 20 chronic hepatitis B patients and 20 normal population, whose results were further validated by ELISA. Results: Both antibody array and ELISA showed that ten growth factors (SCF R, GDF-15, HGF, FGF-4, IGFBP-1, PIGF, GH, GDNF, BDNF and IGF-1) were significantly differential in HCC patients when compared to the non-HCC population. Among these growth factors, the levels of SCF R, GDF-15, HGF, GH and IGF-1 showed significant correlation with hepatitis B and its severity, indicating that these growth factors may promote HCC progression by an HBV-specific mechanism. A therapy targeting these growth factors in hepatitis B patients may help to prevent the development of HCC. FGF4 and GH were found, for the first time, to be upregulated in HCC, suggesting that these two growth factors may serve as novel serum biomarkers for the early diagnosis of HCC. Conclusion: The combined detection of all the differential growth factors may improve the diagnostic accuracy of HCC.

Key words: hepatocellular carcinoma; hepatitis B; growth factors; biomarkers; antibody array.

1. Introduction

Hepatocellular carcinoma (HCC), as one of the most common malignant tumors, involves in the third leading cause of cancer-related death [1]. It was estimated one-half of HCC cases and HCC-related deaths occur in China [2]. Infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), cirrhosis, metabolic diseases, toxins, excessive drinking and smoking are considered risk factors for HCC. As many as 80% of HCC cases in China are caused by HBV [3]. Although hepatic resection is one of the most effective therapy for HCC, the relapse free survival (RFS) rate after operation remains low due to high recurrence rate. What are worse, early HCC

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patients often present with non-typical symptoms, and most are identified only after their HCC is in the advanced state, which results in a low 5-year survival rate. Therefore, elucidating the HBV-specific molecular mechanism which induces HCC and developing serum biomarkers for the early diagnosis of HCC is crucial to prevent HBV-related HCC and to increase survival rate in HCC patients.

With the capacity of simultaneously detecting multiple proteins, antibody arrays are becoming more popular for biomarker screening in cancer research. The application of multiple protein expression profiling can produce huge information and provide deeper insights into the tumor process. However, the application of antibody microarrays in the searchof serum biomarkers for diagnosis and prognosis of HCC are sparse. In this study, we adopted this innovative technology with the aim of identifying novel cytokine biomarkers

2. Materials and Methods

Patients

Chinese patients, histologically confirmed as having HCC, who were hospitalized in the Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital, Zhengzhou University between 2018 and 2019 were recruited for the present study. Blood samples were collected prior to surgical resection, then serum was prepared by centrifugation and stored at -80 ºC. Patients with continuous or recurring chronic hepatitis (CHB) who were seropositive for the hepatitis B surface antigen (HBsAg), and healthy subjects were recruited as control subjects. None of these subjects had undergone chemotherapy or radiotherapy. All subjects signed informed consent forms prior to their participation in this study. This study was approved by the Ethics Committee of The First Affiliated Hospital, Zhengzhou University.

Antibody array experiment

In the present study, the Human Growth Factor Antibody Array G Series (Raybiotech, Inc., Norcross, GA, USA) was used for semiquantitative detection of 40 growth factors via a sandwich assay. 20 HCC patients, 20 CHB patients and 20 healthy subjects were assayed using this detection technology. Clinical data for these subjects is shown in Table 1. Briefly, sera were added into the glass arrays preprinted with 40 primary antibodies and incubated overnight at 4 ℃. Next day, the glass arrays were washed and were incubated with a biotin-conjugated anti-growth factor mix for 2 h at room temperature. After washing again, Cy3-conjugated streptavidin was added into array pools for a further 2 h incubation. Finally, after scanning slides with an InnoScan 300 Microarray Scanner (Innopsys, France), the signal values were read and normalized with analysis tool of Human Growth Factor Antibody Array G series by internal positive included in the array.

Table 1. Clinical data of subjects for antibody array

	HCC	HCB	Control	p value
n	20	20	20	
Age (mean±SD), year	48.8±2.5	48.9±2.5	48.2±1.6	0.564*
Sex (Male/Female)	10/10	10/10	10/10	
HBsAg (positive, %)	100%	100%	0%	
Serum AFP, ng/ml	363.4±72.2	18.6±4.6	12.6±3.4	<0.01*
Stage	I			

* HCC vs HCB vs Control by one-way ANOVA

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ELISA

ELISA was performed in order to validate the results of the antibody array, using ELISA kits according to the manufacturer's (Raybiotech, Inc.) instructions. ELISA was performed using a larger sample size, including 50 HCC patients, 50 CHB patients and 50 healthy subjects (Table 2). Briefly, serum samples were diluted at different dilution ratio specific to different protein and were added into the plate wells to incubate with capture antibody overnight at 4 ºC. After washing, biotin-conjugated antibody was added into plate wells bind the corresponding proteins. After washing, HRP-conjugated streptavidin was added to combine with biotin-conjugated antibody. TMB was added as the chromogenic substrate. Finally, the catalytic reaction was stopped by sulfuric acid and the optical density was measured on an ELx800NBmicroplate reader (BioTek, Inc., Winooski, VT, USA).

Table 2. Clinical data of subjects for ELISA

	HCC	HCB	Control	p value
n	50	50	50	
Age (mean±SD), year	50.5±3.1	50.3±3.1	50.7±3.7	0.815*
Sex (Male/Female)	25/25	25/25	25/25	
HBsAg (positive, %)	100%	100%	0%	
Serum AFP, ng/ml	341.5±66.9	17.9±3.5	14.6±3.1	<0.01*
Stage	I			

* HCC vs HCB vs Control by one-way ANOV

Bioinformatics Analysis

The differential proteins were selected for GO and KEGG pathway analysis, and protein-protein internetwork (PPI) analysis using the STRING database (https://string-db.org/). The corrected P-value (<0.05) was used to evaluate the significance of GO and KEGG terms in differentially expressed growth factors. In the PPI, proteins were represented with nodes, while the interactions derived from experimental repositories between any two proteins therein was represented using an edge.

Statistical analysis

Statistical analysis was performed using the Bonferroni post hoc test of one-way ANOVA using SPSS v20 (IBM Corp., Armonk, NY, USA) software. Differences between groups are considered statistically significant when P values are < 0.05. Fold change (FC) was calculated to indicate the expression tendency of cytokines between groups.

3. Result

Differential proteins analysis

The Bonferroni post hoc test of one-way ANOVA was used to analyze any two groups. Ten growth factors were selected based on their significance score (P<0.05) and signal value (>300) which was considered positive expression for the purpose of reducing false positive results. As shown in Table 3 and Figure 1, the results showed that SCF R, GDF-15, HGF, GH and IGF-1 were significantly differentially expressed between any two groups. In these growth factors, the levels of SCF R, GDF-15, HGF and GH were obviously increased in the HCC and HCB groups, compared with those in healthy group, and were obviously increased the HCC group compared with those in the HCB group. Meanwhile, IGF-1 level was significantly decreased in the HCC

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and HCB groups, compared levels in the healthy group, and was dramatically decreased in the HCC group compared the HCB group. In addition, the levels of FGF-4, IGFBP-1, PIGF, GDNF and BDNF were elevated in the HCC group compared with those in the non-HCC groups. The most representative profiles among the corresponding groups were chosen to indicate the differences between the three groups. As we know, the fluorescent intensity of proteins in arrays is proportional to their levels in the samples and each protein was measured in quadruplicate. A shown in Figure 2, the fluorescent signals of these differentially expressed growth factors were clearly diverse among the three groups, further supporting that these growth factors were differentially expressed in HCC patients.

Table 3. Antibody array data for the differentially expressed proteins

		Mean ± SD			p value		
	Protein ID	Control	HCB	HCC	HCC vs HCB	HCC vs Control	HCB vs Control
SCF R	P10721	16846.9±4793.2	33598.7±11047.3	62730.5±33562.6	<0.001	<0.001	0.038
GDF-15	Q99988	26742.5±11288.5	45006.2±12386.6	96828.3±30978.5	<0.001	<0.001	0.019
HGF	P14210	2078.7±370.8	4052.2±512.1	9574.4±4284.6	<0.001	<0.001	0.046
GH	P01241	1147.4±584.6	3159.9±440.3	7186.6±417.8	<0.001	<0.001	<0.001
IGF-1	P05019	7073.2±2208.2	3770.6±772.1	1724.8±765.7	<0.001	<0.001	<0.001
FGF-4	P08620	1164.8±392.2	963.9±68.9	4626.7±1741.2	<0.001	<0.001	1.000
IGFBP-1	P08833	25138.6±10889.8	20906.8±4628.9	60456.1±20291.0	<0.001	<0.001	0.984
PIGF	P49763	1443.1±740.9	1440.0±302.3	4933.4±6116.5	0.009	0.009	1.000
GDNF	P39905	1209.4±759.3	1056.5±200.6	3457.2±2432.1	<0.001	<0.001	1.000
BDNF	P23560	28686.2±15927.3	26417.2±10218.3	78147.9±17979.9	<0.001	<0.001	1.000

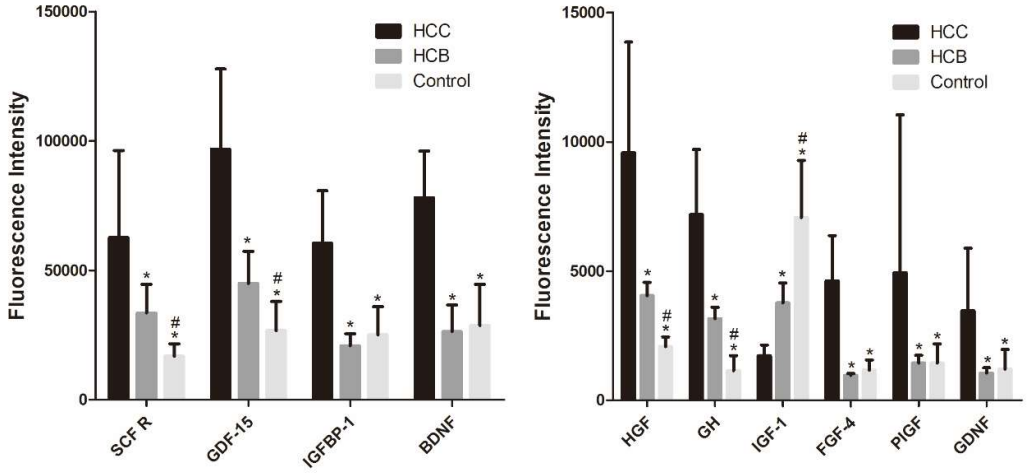

Figure 1. Histogram of antibody array data. The fluorescence values of differentially expressed proteins representing their expression levels are shown by histogram. * $p<0.05$ versus HCC group, # $p<0.05$ versus HCB group.

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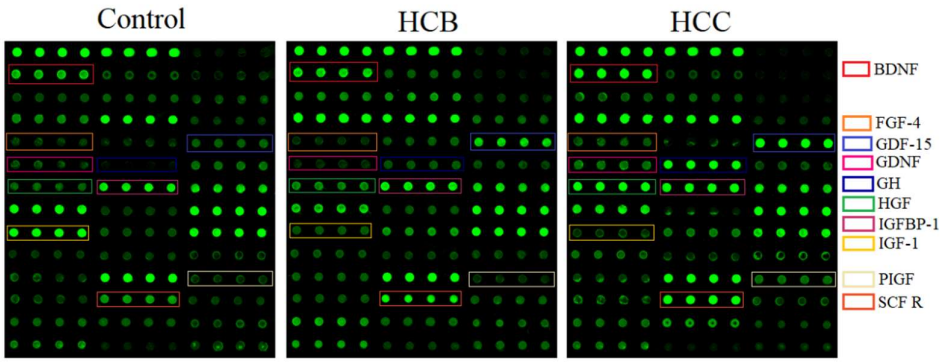
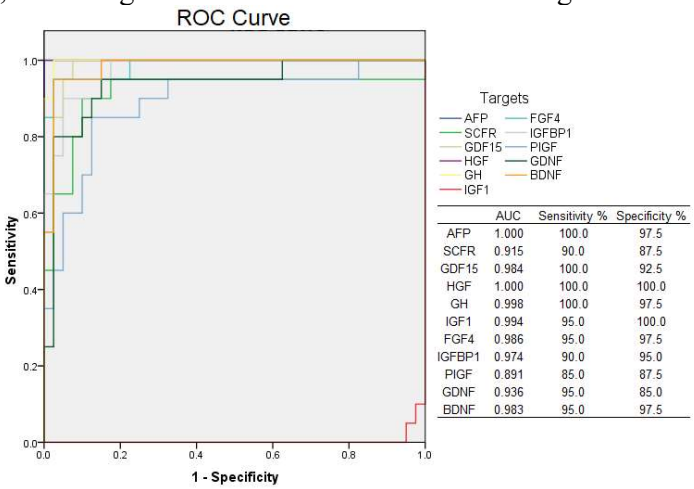


Figure 2. Antibody array profiles. The locations of the differential proteins are noted in colored boxes. Each antibody was printed in four duplicates. The fluorescence intensities are proportional to their expression levels.

Diagnostic values of differential growth factors

To evaluate the accuracy of differential growth factors in predicting HBV-related HCC, receiver operating characteristic (ROC) were performed. As shown in Figure 3, all differential growth factors showed high sensitivity, specificity, and area under curves (AUC), indicating their valuable functions for the diagnosis of HCC.



	AUC	Sensitivity %	Specificity %
AFP	1.000	100.0	97.5
SCFR	0.915	90.0	87.5
GDF15	0.984	100.0	92.5
HGF	1.000	100.0	100.0
GH	0.998	100.0	97.5
IGF1	0.994	95.0	100.0
FGF4	0.986	95.0	97.5
IGFBP1	0.974	90.0	95.0
PIGF	0.891	85.0	87.5
GDNF	0.936	95.0	85.0
BDNF	0.983	95.0	97.5

Figure 3. ROC analysis of differential proteins. The array data of the differential proteins was used to perform ROC analysis to determine HCC cases.

Correlation of differential growth factors with HBeAg status and AFP

To indicate the correlation of differential expression of growth factors with HBeAg status and serum AFP, linear regression analysis and statistical analysis were performed. As a result, among the ten differentially expressed growth factors, the levels of SCF R, GDF-15, HGF and GH were significantly positively correlated with AFP level in HCC patients, while IGF-1 level showed significant negative correlation with AFP level (Figure 4). When CHB patients and HBV-related HCC patients were divided into HBeAg-negative and HBeAg-positive groups, these five proteins also showed significantly differential expression between the HBeAg-negative and HBeAg-positive groups (Figure 5).

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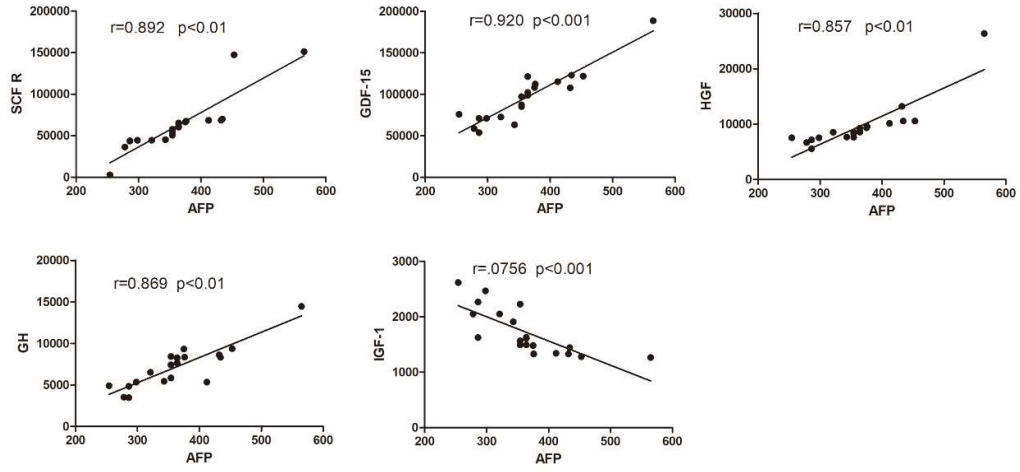

Figure 4. Correlation analysis between differential proteins with AFP. The correlation between differential proteins with AFP was performed to evaluate their diagnostic value in HCC.

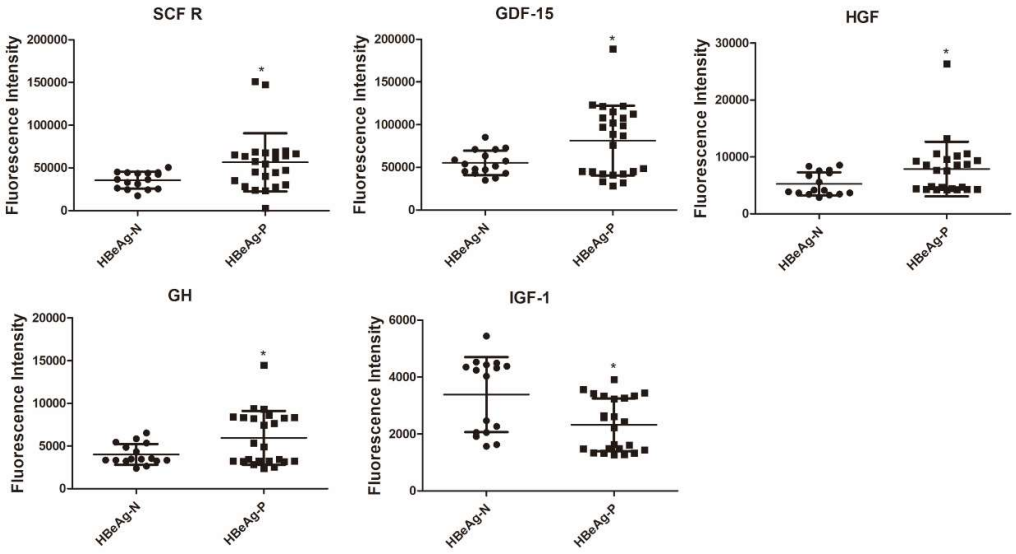

Figure 5. Correlation analysis between differential proteins and HBeAg status. HCB and HCC patients were divided into HBeAg-negative and HBeAg-positive groups, and five differential proteins showed significantly differential between the HBeAg-negative group and the HBeAg-positive group.

Bioinformatics Analysis of differential cytokines

To clarify the potential function of the differentially expressed growth factors on the regulation of the pathology of HCC, GO enrichment and KEGG pathway enrichment analyses and PPI analysis were performed. The GO terms included biological process, molecular function, and cellular component, and the two most enriched terms and two most enriched KEGG pathways are shown in Table 4. Furthermore, the ten differentially expressed growth factors were mapped into the PPI network to identify the more important factors. As shown in Figure 6, HGF, SCF R, FGF4 and IGF1 are in the central position of this PPI network, suggesting these factors may be the ones most involved in the pathological processes of HCC.

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Table 4 The most enriched GO terms and KEGG pathways

term ID	term description	p value	matching proteins in your network
GO:0007169	transmembrane receptor protein tyrosine kinase signaling pathway	<0.01	BDNF, FGF4, GDF15, GH, HGF, IGF1, IGFBP1, SCFR, PGF
GO:0010469	regulation of signaling receptor activity	<0.01	BDNF, FGF4, GDF15, GDNF, GH, HGF, IGF1, PGF
GO:0008083	growth factor activity	<0.01	BDNF, FGF4, GDF15, GDNF, GH, HGF, IGF1, PGF
GO:0005102	signaling receptor binding	<0.01	BDNF, FGF4, GDF15, GDNF, GH, HGF, IGF1, IGFBP1, PGF
GO:0005576	extracellular region	<0.01	BDNF, FGF4, GDF15, GDNF, GH, HGF, IGF1, IGFBP1, SCFR, PGF
GO:0005615	extracellular space	<0.01	GDF15, GH, HGF, IGF1, IGFBP1, SCFR, PGF
hsa04151	PI3K-Akt signaling pathway	<0.01	BDNF, FGF4, GH, HGF, IGF1, SCFR, PGF
hsa04014	Ras signaling pathway	<0.01	BDNF, FGF4, HGF, IGF1, SCFR, PGF

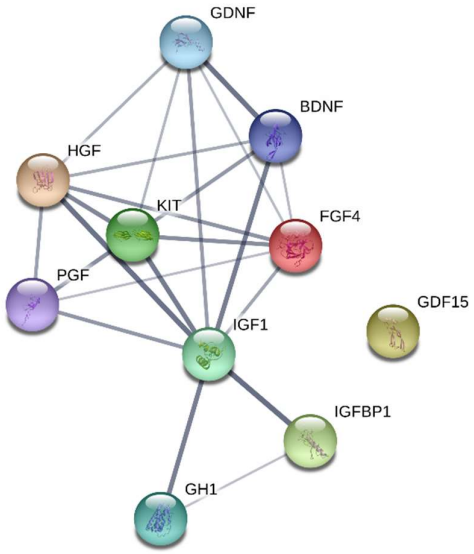

Figure 6. PPI analysis. The correlation of the differential proteins was evaluated by PPI analysis. The line linking two proteins identifies a correlation in the biological function between these two proteins and the line thickness is positively related to the strength of data support for the correlation.

ELISA results

To further validate the reliability of microarray results, ELISA was performed. The results of these validation experiments are identical to those of the microarray experiment (Figure 7).

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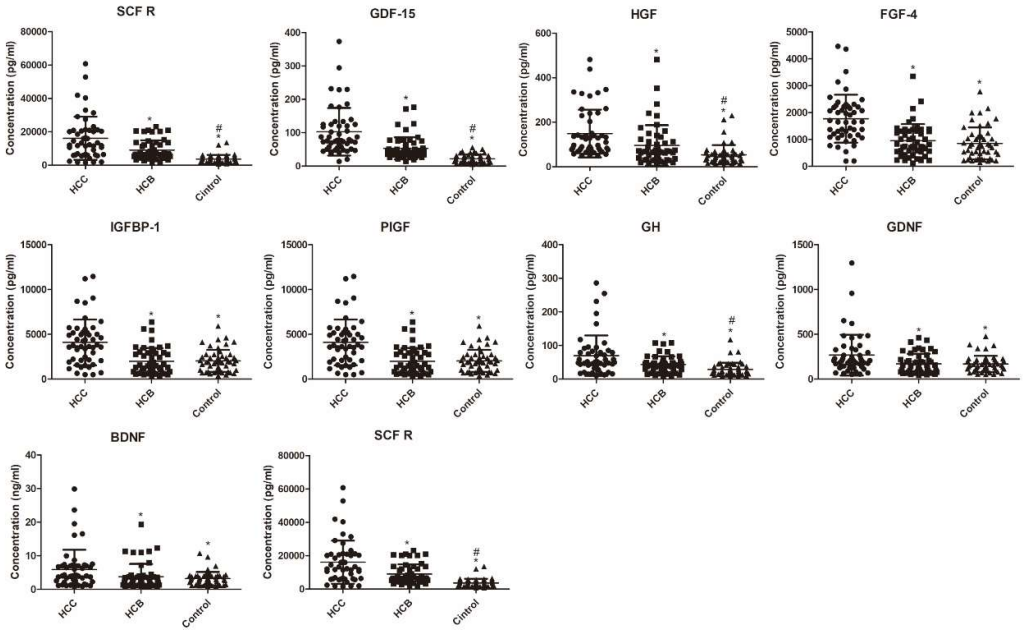

Figure 7. The ELISA results. The ELISA data are shown by the scatter plot with median values and validates the differences of the ten proteins among the three groups. * p<0.05 versus HCC group, # p<0.05 versus HCB group.

4. Discussion

In previous studies, the search for HCC biomarkers focused on gene profiles [4-6]. However, altered gene expression patterns do not directly translate into the pathogenesis of diseases, but the proteins they code are directly involved. Increasingly, protein biomarkers have been used for the diagnosis and prognosis of diseases, including cancer detection at sufficiently low risk to safely omit systemic treatment. For example, alpha-fetoprotein [AFP] is the most widely-used serum biomarker for HCC diagnosis [7]. However, AFP is not specific for HCC diagnosis. Increased serum AFP levels also occur in patients with chronic liver disease [8], and it is hard to distinguish between HCC patients and patients with chronic liver diseases by AFP level alone. Furthermore, it has been recognized that HBV/HCV infection contributing to CHB is an important risk factor for HCC. Therefore, identifying serum biomarkers can improve the diagnosis of HCC and even prevent the development of HBV-related HCC when detected early enough. In addition, HCC is a hyper-vascular tumor, and angiogenesis is essential for tumor growth in HCC [9]. Thus, growth factors play an important role in tumor angiogenesis of HCC [10]. Therefore, in the present study, an antibody array detecting multiple growth factors was utilized.

As a result, the levels of ten growth factors were significantly altered in HCC patients, compared with those in HCB patients and healthy subjects. Nine growth factors were up-regulated (SCF R, GDF-15, HGF, FGF-4, IGFBP-1, PIGF, GH, GDNF and BDNF) and IGF-1 was found to be down-regulated. Furthermore, among these differentially expressed growth factors, SCF R, GDF-15, HGF and GH were increased in the HCB group, compared with the healthy group, and increased further in the HCC group. Meanwhile, IGF-1 showed decreased expression in HCB patients and further down-regulation in HCC patients. These proteins showed significant correlation with HBeAg status and AFP, suggesting that these growth factors might be involved in HBV-specific mechanisms contributing to the development of HCC, and have high

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value for the diagnosis of HCC. In addition, ELISA experiments using fresh samples showed the results identical to those from the antibody microarray. These results demonstrate that these ten growth factors play important roles in the specific pathophysiological processes of HCC and may serve as serum biomarkers for the diagnosis of HCC.

To further reveal the detailed mechanism of these factors in the pathophysiological processes of HCC, GO enrichment and KEGG pathway enrichment analysis were performed. The analysis results revealed that the two most enriched GO terms included 'transmembrane receptor protein tyrosine kinase signaling pathway' and 'signal transduction' for biological process, 'growth factor activity' and 'signaling receptor binding' for molecular function, and 'extracellular region' and 'extracellular space' for cellular component. The two most enriched pathway terms were 'PI3K-Akt signaling pathway' and 'Ras signaling pathway'. Growth factor signal transduction from the cell surface is activated to regulate cellular functions through binding with transmembrane receptors which act as tyrosine kinases. Growth factors and their receptors are frequently overexpressed in cancer cells and are involved in cell functions such as differentiation, growth, angiogenesis, and inhibition of cell death and apoptosis [11, 12]. Taken together, these suggest that the differentially expressed growth factors identified in this study participate in HCC pathophysiological processes mainly through the identified GO functions. More importantly, the KEGG pathway enrichment analysis showed that the most enriched pathway terms were 'PI3K-Akt signaling pathway' and 'Ras signaling pathway'. Activation of PI3K-Akt pathway plays a pivotal role in essential cellular functions including proliferation, migration and differentiation that contribute to the development of human cancers, and activation of PI3K-Akt signaling is known to be aroused by various growth factors and cytokines, such as HGF, IGF-1 and PIGF [13-17]. Numerous studies have shown that activation of the PI3K-Akt signaling pathway plays an essential role in the development and progression of HCC by regulating cell proliferation, invasiveness, angiogenesis, and metastasis [18-21]. The Ras pathway is also a dominant signaling network responsible of cell proliferation and survival, which is activated by growth factors such as HGF, IGF-1 and GDNF [22-25]. Recently, it has become evident that the Ras signaling pathway is important in the occurrence and development of HCC [26, 27]. Therefore, these differentially expressed growth factors may participate in the pathophysiological processes of HCC via the PI3K-Akt signaling pathway and Ras signaling pathway.

Stem cell factor receptor (SCF R c-kit, CD117) is a transmembrane tyrosine kinase involved in the early growth and development of hepatic progenitor cells. Previous researches revealed that the expression of SCF R was associated with hepatitis B virus-induced chronic hepatitis, cirrhosis, and hepatocellular carcinoma [28-30]. Growth differentiation factor 15 (GDF15) is a novel TGF-beta superfamily cytokine and macrophage inhibitory cytokine-1. Clinical evidences have shown that GDF15 levels are significantly related to disease severity and poor prognoses in liver diseases [31-33], and serum GDF15 levels are significantly increased in chronic hepatitis C patients and HCC patients [34-36]. Youhui et al. [36] found that GDF15 might promote HCV progress by altering the signaling and growth of host cells. Hepatocyte growth factor [HGF] is a growth factor responsible for liver regeneration, embryogenesis, and wound healing [37-39]. Furthermore, HGF plays essentially functions in tumor invasion, metastasis, and angiogenesis through the HGF/Met axis pathway, including HCC progression [40-42]. Upregulated serum HGF levels are significantly correlated with fibrosis score and hepatic activity index in patients with

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chronic hepatitis B, and poor prognoses in HBV-positive HCC patients [43, 44]. Consistently, the present study showed SCF R, GDF-15 and HGF were upregulated in HCB patients and further increased in HBV-induced HCC. Growth hormone (GH) is an important factor to regulate body energy homeostasis and liver physiology [45, 46]. It has been observed as having a protective role for GH-STAT5 signaling in HCC development, and GH overexpression reduces life expectancy [47]. Previous research has indicated that GH levels in chronic severe hepatitis patients were high [48], and the present study showed that GH was upregulated in HCB patients and increased further in HBV-induced HCC for the first time. Insulin-like growth factor-1 (IGF1), produced by liver tissue, is involved in cellular proliferation, differentiation and apoptosis, and the pathogenesis of malignancies [49-51]. Decreased serum IGF-1 has been shown to be closely related to liver reserve function, severe viral hepatitis, and poorer overall survival of HCC patients, and IGF-1 is considered to be a important factor for predicting the risk of developing C virus-related cirrhosis [52-55]. In the present study, IGFI level was also found to be decreased in hepatitis B patients and downregulated further in HBV-induced HCC. Furthermore, the GH-IGF axis has an important function in liver diseases [56, 57]. Our results indicated that elevated serum SCF R, GDF-15, HGF and GH, and decreased IGF1 were associated with hepatitis B, HBV infection severity and HBV-related HCC, suggesting their abnormal levels may be involved in an HBV-specific mechanism for the development of HCC. A therapy targeting SCF R, GDF-15, HGF and the GH-IGF axis in CHB patients may be useful in preventing HCC progression.

Brain-derived neurotrophic factor [BDNF] is from nerve growth factor family, and supports the survival and growth of neurons. BDNF is up-regulated in a variety of human cancers including breast [58], bladder [59], ovarian [60] and liver [61] cancers, and in viral hepatitis [62]. Although BDNF showed no significant alteration in HCB patients in the present study, BDNF was elevated in HCC patients. Therefore, determining whether or not BDNF promotes HCC development by an HBV-specific mechanism requires further research. Fibroblast growth factor 4 [FGF4] is a member of the FGF superfamily, which is involved in numerous stages of embryonic development and enhances multiple oncogenic activities, including tumor proliferation, invasion/migration, and colony initiation abilities [63, 64]. Although there are some reports on the involvement of FGF4 in the processes of cancers, including lung adenocarcinoma, colorectal cancer, breast cancer, and ovarian cancer [65-68], little is known about the involvement of FGF4 in HCC. In this study, we found, for the first time, that FGF4 was highly expressed in HCC, suggesting a role as a novel serum biomarker for HCC. Insulin-like growth factor-binding protein-1 [IGFBP-1], the most predominant IGFBP which is primarily secreted by the fetal liver, has been shown to involve in cellular proliferation, development, apoptosis, and tumor growth [69-71]. Paradoxical data have been reported on the expression of IGFBP1 in HCC [72, 73]. However, consistent with the report from Hwang et al. [74], we found elevated serum IGFBP-1 levels in HCC, implying that IGFBP1 may be potential target in the early diagnosis of HCC. Placenta growth factor (PIGF) is a dimeric glycoprotein with structural and functional similarities to VEGF [75]. PIGF serves a function in tumor angiogenesis [76-78]. Previous studies indicated that overexpression of PIGF had a close relation with early recurrence of HCC [79, 80], and our finding showed PIGF had higher serum levels in HCC, suggested that PIGF may be a prognostic indicator and a diagnostic biomarker of HCC. Glial-derived neurotrophic factor (GDNF), as a member of the transforming growth factor-beta superfamily, mediates endothelial cell network formation and protects against a

 doi:10.20944/preprints202102.0352.v1

variety of neuronal damage [81, 82]. In addition, GDNF is involved in angiogenesis [83]. Zhong et al. [84] reported that HCC cells overexpressed GDNF and proposed that GDNF might be an important factor promoting pathological neovascularization. Consistently, our study revealed GDNF was increased in the serum of HCC patients, suggesting GDNF may be also a serum biomarker for the early diagnosis of HCC. However, few researches have shown a correlation of FGF4, IGFBP-1, PIGF and GDNF with viral hepatitis, indicating that these proteins may contribute to HCC progression independent of an HBV-specific mechanism.

5. Conclusions

In conclusion, we found that SCF R, GDF-15, HGF, FGF-4, IGFBP-1, PIGF, GH, GDNF and BDNF were increased in the HCC group, compared with the non-HCC groups, while IGF-1 was decreased. Among these proteins, the levels of SCF R, GDF-15, HGF, GH and IGF-1 also showed correlation with hepatitis B and its severity, indicating that these proteins may promote HCC progression by an HBV-specific mechanism. Treatments targeting these factors in CHB patients may be useful for preventing HCC progression. FGF4 and GH were found, for the first time, to be abnormally expressed in HCC, suggesting that these two proteins may serve as novel serum biomarkers for the early diagnosis of HCC. Taken together, the combined detection of all the differentially expressed proteins may improve the diagnostic accuracy of HCC.

Declarations

Acknowledgments

Not applicable.

Funding

No funding was received for the study.

Availability of data and materials

The datasets analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

SQ, YZ, YG and XD developed the study design and experimental setup. SQ, GZ, YP, MH contributed to sample collection and processing. SQ and YZ performed antibody array and ELISA experiments and data collection and analysis. SQ and XD conducted statistical analyses. SQ, YZ, MH and XD drafted and/or revised the manuscript or revised. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Ethical Review Board of the First Affiliated Hospital, Zhengzhou University (permit no. 201801101) and written informed consent was obtained from each participant prior to their inclusion in this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that no competing interests exist.

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