Proteome Expression Profile for Red Blood Cells Enables Diagnostics for Hepatocellular Carcinoma

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Research

Keywords: Hepatocellular carcinoma, Liver cirrhosis, Red blood cells, Proteomics, Biomarker, Erythropoiesis

Posted Date: November 10th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-989816/v1>

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Abstract

Background

Early diagnosis of hepatocellular carcinoma (HCC) has not been clinically resolved, which has been causing more death in patients with HCC. HCC is also a systemic disease related to disorders of blood homeostasis, and the association of red blood cells (RBCs) and HCC tumorigenesis is still elusive. This study explored the protein characteristics of RBCs at the progressive pathological stages comparing with healthy individuals, including liver cirrhosis (LC) and established HCC, to fully understand the tumorigenesis of HCC from a different view and identify potentially novel diagnostic biomarkers for HCC in RBCs.

Methods

Data independent acquisition (DIA) proteomic analyses were performed with 72 clinical RBCs samples from a cohort of subjects including HCC, LC and healthy controls. Bioinformatics analysis was conducted for significantly differentially expressed proteins (DEPs) through the whole process of tumorigenesis to characterize the clinical relevance of RBCs and tumorigenesis in HCC. The highly potential tumorigenesis-associated molecular biomarkers were evaluated with clinical samples by parallel reaction monitoring (PRM) technology.

Results

We observed that red blood cells number dynamically changes during the tumorigenesis of HCC, and LC is a developmental stage more closely approaching HCC based on the protein expression profiles in RBCs. The expression of hemoglobin (HbA, HbF) in erythroid cells also dynamically alters during the whole process of HCC tumorigenesis, suggesting immature erythroid cells exist in peripheral blood of HCC patients and erythropoiesis in the patients starts to be influenced with the occurrence of LC. We observed that the autophagy pathway is disturbed in RBCs with the onset of LC and maintained during the tumorigenesis of HCC. Oxytocin pathway and GnRH pathway are disturbed and first identified during the development of LC into HCC. SMIM1, ANXA7, HBA1 and HBE1 that are significantly altered during tumorigenesis were verified as promising biomarkers for HCC early diagnosis.

Conclusions

This study underlined the clinical relevance of the proteins in RBCs and the tumorigenesis of HCC, and provided the potential biomarkers for early diagnosis in HCC from a new perspective. Our results provided a novel strategy with RBCs for HCC early diagnosis, which will improve the translational research and application in diagnosis of HCC.

Introduction

Liver cancer is the third leading cause of cancer-related death in the world, of which HCC is the the most common primary liver cancer in adults. HCC is still difficult to cure and easy to misdiagnosis at early stage. AFP is the most widely used biomarkers in clinical diagnosis of HCC with a sensitivity of 39% ~ 65% [1], however, most HCC patients have reached the middle or late stage, and only less than 20% of them have the opportunity of surgical resection [2, 3]. Novel strategy for early diagnosis of HCC are necessary to improve the clinical treatment for HCC patients.

LC is a common overdevelopmental stage from hepatitis B to HCC. Hepatitis B patients develop into LC in 5~10 years without reasonable antiviral treatment and with unhealthy lifestyle on the premise of abnormal liver function. Clinically, around 10% of LC patients further develop into HCC in 5 years. LC is a crucial stage for HCC early diagnosis.

Liver is the main organ of fetal erythropoiesis, and the site for erythropoiesis in adults with some disorders [4]. A body of lines indicated that erythropoiesis-associated disorders occurred in HCC patients. Erythrocytosis shows an increase in the concentration of RBCs and hemoglobin in the blood of HCC patients, in which HCC cells are responsible for the production of erythropoietin for erythrocytosis [5]. Increased RBCs distribution width that is a main feature of RBCs was also clinically observed in liver disease patients [6]. Moreover, it was reported that erythroblast-like Ter-Cells are observed in enlarged-spleen HCC patients, and the elevated artemin in serum secreted from these cells highly correlates with HCC progression [7]. As a systemic disease related to disorders of blood homeostasis, cancer causes detectable changes in gene expression in blood cells and plasma [8–10]. Cancer-educated platelets, erythrocytes or leukocytes in blood have been previously reported to have potential applications for cancer diagnostics [11–13]. Therefore, we speculate that the progress of HCC tumorigenesis could be associated with molecular characteristics of RBCs in patients.

The rapidly expanding field of searching for HCC biomarkers provide a fast growing list of biomarker candidates, including miRNAs in plasma [14], epigenetic 5hmC in peripheral blood [15], and proteins in serum [16]. However, the molecular characteristics of RBCs have been rarely explored to identify the biomarkers for HCC early diagnosis.

In this study, we revealed the association of RBCs with the progress of HCC tumorigenesis by comprehensively exploring the protein profiles of RBCs in a cohort of subjects including LC, HCC and healthy individuals. We characterized the molecular alterations in RBCs that are dynamically changed and closely associated with the progress of HCC tumorigenesis, including erythroid-specific globins, the unique proteins and signaling pathways. Novel biomarkers, including SMIM1 and ANXA7, for HCC early diagnosis in RBCs were discovered. Our study is the first to establish the link between the whole process of HCC tumorigenesis and the proteins in RBCs that provides a novel strategy for HCC early diagnosis. Our study will improve the translational research and application in diagnosis of HCC.

Material And Methods

Patients

To study whether the protein molecular characteristics is associated with the progress of tumorigenesis in HCC, 30 HCC patients with HBV infection, 17 LC patients without HCC, and 25 healthy individuals were enrolled in this study. All patients were confirmed by pathological examination. The age for the cohort ranges from 40 to 60 years old. Clinical data for each subject with regard to AFP, ALT, AST, hemoglobin and blood cells was collected from the medical records. We performed the statistical analysis for these clinical data across the cohort of the subjects. The written informed consent was obtained from all subjects.

Isolation of red blood cells

To comprehensively investigate the RBCs protein alterations during tumorigenesis of HCC by data independent acquisition (DIA) mass spectrometry, 2 mL of peripheral blood for each subject was collected in EDTA anticoagulant tube. The blood sample was centrifuged at 3,000 rpm for 5 min, and the white membrane layer in the centrifuge tube was discarded. Then, 5 mL of normal saline was added to mix and wash the cells. After 3 rounds of centrifugation at 3,000 rpm for 5 min, RBCs were collected and frozen at -80°C for later use.

Protein digestion

A total of 72 individual RBCs samples were analyzed by DIA mass spectrometry. 1 µL RBCs sample for each subject was diluted with 50 mM NH_4HCO_3 , and the proteins were reduced by 10 mM DTT at 56°C and alkylated by 50 mM iodoacetamide in darkness. Protein digestion was performed by FASP (filtered-aided sample preparation) method for mass spectrometry. The concentration was measured by nanodrop one (Thermo Fisher).

LC MS/MS analysis

Spectral library was generated by DDA (Data Dependent Acquisition) method as previously reported [17]. 10 µg peptides from each sample was pooled together and separated into 10 components by high pH reversed-phase chromatography. 2 µg peptides per components were separated by a C18 analysis column (150 µm X 15 cm, 1.9 µm) with Easy NanoLC 1200 system (Thermo Fisher) at a 75 min gradient and analyzed by Q Exactive HF mass spectrometry (Thermo Fisher). The gradient with a flow rate of 600 nL/min was set as follows: 10-14 % solvent B for 12 min, 14-26 % solvent B for 45 min, 26-42 % solvent B for 10 min. For DDA analysis, the electrospray voltage was set at 2.1 kV. Full MS1 scan ranged from 300 to 1400 m/z at 60 k resolution. 20 MS/MS spectrums were scanned per cycle at a 15000 resolution. MS1 and MS2 AGC target were set as 3×10^6 and 5×10^4 corresponding to maximum inject time 80 ms and 40 ms respectively. Isolation window was set as 1.6 Th Dynamic exclusion was set as 18 s. For DIA analysis, The MS1 ranged from 350 to 1400 m/z at 60 k resolution. A total of 30 windows covering from 400 to 1250 m/z were used for DIA with 30 k resolution and 3×10^6 AGC.

Bioinformatics analysis

Hierarchical clustering was performed using in-house R-scripts. The Wilcoxon signed-rank test was used to identify differentially expressed proteins (DEPs). Proteins with log₂-fold changes > 0.58 or < -0.58 and with P-values < 0.05 were identified as DEPs. Functional enrichment was performed based on gene ontology (GO), Kyoto Encyclopaedia of Gene and Genomes (KEGG), and STRING databases. An adjusted P-value threshold cut off was set at 0.05. Diagrams are shown with significance (-log₂ transformed) and protein number identified in the relevant protein sets.

Flow cytometry

To analyze the alteration in hemoglobin expression during the progress of HCC tumorigenesis, 2 mL peripheral blood from another batch of subjects including 8 HCC, 9 LC and 5 healthy individuals were conducted for this analysis. The collected sample was fixed in 0.05% glutaraldehyde solution in 1 × PBS for 10 min, permeabilized for 5 min in 0.01% Triton X-100 in 1 × PBS/1% FBS for 5min, and stained with PE-conjugated fetal hemoglobin antibody (Miltenyi Technology) and AF647-conjugated hemoglobin β antibody (Santa Cruz Biotechnology) for 15min in the dark. After incubation, the cells were washed twice and resuspended in 200 μL 1 × PBS buffer to prepare the cell suspension. A BD FACSAria II instrument was used for flow cytometric analysis, and the data analysis was performed with Flowjo software (Version 7.6, Three Star).

PRM validation

The validation of proteins was carried out by parallel reaction monitoring (PRM) technology [18]. Briefly, the analyses of PRM were performed on 40 min gradient LC with a flow rate of 600 nL/min: 8-13 % solvent B for 2 min, 13-35 % solvent B for 24 min, 35-45 % solvent B for 4 min. The mass spectrometer were acquired using the the following parameter: PRM scans were performed at a resolution of 30,000 at 200 m/z, individual isolated window of 1.6 Th, retention time window was set to ± 4 min and maximum injection time of 40 ms. A normalized collision energy of 27 in an HCD collision cell was employed for fragmentation. All PRM data analysis and data integration was performed with the software of Skyline software (3.5.0).

Immunohistochemistry

Paraffin-embedded tissue sections of HCC and matched control were analyzed by immunostaining. SMIM1 antibody (Cusabio Biotech) was used at the dilution of 1:100. Digital imaging was performed using the software LAS V4.5 (Leica DM 2000). Pictures were acquired using HistoFAXS system and the staining was visualized using K-viewer software.

Result

Clinical characteristics of RBCs associates with the progression of HCC tumorigenesis

To explore the relationship between the tumorigenesis of HCC and RBCs in patients, we collected the clinicopathologic characteristics of LC (N=17), HCC (N=30) and healthy controls (HC, N=25) (Table S1). The tested ALT, AST and AFP level in peripheral blood are extremely high in HCC comparing to LC and HC, indicating the functional damages in liver with the onset of HCC. The AFP level in HCC fluctuates extremely largely (Fig. 1A), and over 50% of HCC patients are included in the normal range, which could result in the missed detection of HCC cases. The AFP level in LC is similar to HC, indicating that AFP could not be used as a biomarker for early diagnosis of HCC. The level of hemoglobin (Fig. 1B) and RBCs (Fig. 1C) in peripheral blood are significantly lower in LC than HC, suggesting that RBCs start to be influenced with the occurrence of LC. Interestingly, this disturbance is gradually recovering in established HCC, approaching to HC, indicating the association of molecular characteristics in RBCs with the process of HCC tumorigenesis. Tumor-educated blood platelets were previously characterized to distinguish six types of cancers from healthy controls using mRNA profiling besides HCC [13]. We first observed that platelets are also associated with HCC tumorigenesis, since the number of platelets in peripheral blood changes with the similar trend as RBCs (Fig.1D). Leukocyte changes slightly during the tumorigenesis of HCC. Taken together, our results demonstrate that the molecular characteristics of RBCs during tumorigenesis could reflect the progression of HCC and provide a novel strategy for early diagnosis.

LC is a crucial stage developing into HCC

RBCs could clinically indicate the progression of HCC, however, the molecular characteristics in RBCs through the whole process of HCC tumorigenesis has been largely unknown. Here we utilized DIA mass spectrometry to comprehensively analyze the protein profiles in RBCs from the cohort including HCC (N=30), LC patients (N=17) and HC (N=25) (Table 1). 659 proteins were identified for characterization (Table S2), of which most proteins are present in vesicles and cytosol of RBCs (Fig. 2A), and fulfill a variety of cellular responses and metabolism related functions (Fig 2. B). The hierarchical clustering analysis showed that protein expression profiles of RBCs in LC and HCC patients are quite different from HC, and LC is closer to HCC (Fig. 2C), which was also revealed by PCA analysis (Fig. 2D). In terms of proteome in RBCs, we demonstrated that the pathology of LC is a developmental stage towards HCC, and LC is a crucial stage developing into HCC. The dynamic changes of proteins in LC could facilitate the early diagnosis of HCC.

Erythroid-specific proteins could indicate the pathological process of HCC

The clinical characteristics of RBCs and hemoglobin in peripheral blood from the cohort fluctuates through the tumorigenesis of HCC. We next wonder the relationship between the expression profiles of erythroid-specific proteins and the progression of HCC, which might suggest the biomarkers for early diagnosis of HCC. Hemoglobin is the most abundant protein in RBCs, combines and transports oxygen to organs in the body through its tetrameric structure. Two alpha chains together with two gamma chains constitute fetal hemoglobin (HbF: $\alpha_2\gamma_2$) which is normally replaced by adult hemoglobin (HbA: $\alpha_2\beta_2$) at

birth. We observed that the components of hemoglobin, including HBA1, HBE1, HBG2 and HBB, showed differentiated expression during the tumorigenesis of HCC (Fig 3. A-D). Specifically, HBA1 expression increases significantly at the stage of LC, while HBB expression decreases at the same stage. HBG2, that is predominant fetal globin at birth, remains similar expression level as HC, but increases the expression at HCC. As an embryonic globin, HBE1 expression increases at both the stage of LC and HCC.

PRM is a targeted proteomics technology based on high-resolution, high-precision mass spectrometry, which can selectively detect target proteins and target peptides to achieve absolute quantification of the target protein/peptide. In this study, we verified the expression of differentially expressed globins in another cohort of LC, HCC and healthy controls with RPM technology (Table S3-4), and observed that the expression pattern of HBA1 and HBE1 is similar with the expression pattern in proteomic data (Fig. E, F).

Consistently, we observed decreased expression of GYPA, a specific marker of mature erythrocytes, in RBCs of HCC (Fig. 3G), and the nucleated erythroid cells were observed in the peripheral blood of HCC patients (Fig. 3H). These results indicate that the immature erythroid cells could exist in the peripheral blood of HCC. To test this hypothesis, we counted the erythroid cells respectively expressing HbF and HbA in peripheral blood from another cohort of HCC, LC and HC by flow cytometry analysis. Interestingly, we observed that the number of erythroid cells expressing HbF is significantly higher in LC patients than HC (Fig 3. I), indicating that the production of erythroid cells is initially affected at the stage of LC, and the immature erythroid cells could be released into peripheral blood at this stage. Consistently, we observed the lowest number of erythroid cells expressing HbA consisting of two alpha chains together with two beta chains at the stage of LC comparing with HC and HCC (Fig. 3J), even though no statistically significant difference was observed among them. Together, our results demonstrated that the expression changes of erythroid-specific proteins in the process of HCC tumorigenesis could indicate the pathological process. The increase of HbF and the decrease of HbA in LC could be used for early diagnosis for HCC.

Impairments in RBCs on the onset of liver cirrhosis

We next explored the disturbance to RBCs with the occurrence of LC, which might be a sign of the development into HCC. A total of 157 DEPs (79 up-regulated, 78 down-regulated) were identified in RBCs with the onset of LC compared with HC (Table S5, Fig4. A, B). The altered proteins are much more than the process from LC to HCC (57 DEPs), indicating that the occurrence of LC leads to more drastic changes in RBCs during the tumorigenesis of HCC.

Autophagy is a major player in LC and considered as an anti-fibrosis pathway, because it provides survival signals for hepatocytes and acts as the gate keeper of HCC [19]. In this study, we also observed the significantly disturbed autophagy pathway with the onset of LC in RBCs. The impairment of m-TOR and tight junction pathway are firstly characterized in LC (Fig4. C).

Interestingly, we observed two proteins, SMIM1 and ANXA7, that were associated with the cellular characteristics of red blood cells [20-22] and the tumorigenesis of hematocellular carcinoma and functions of erythroid cells, respectively [23-26]. Since these two proteins are significantly changed proteins in RBCs on the occurrence of LC (Fig. 4A-B), we speculate that they could be potential biomarkers for early diagnosis of HCC. These two proteins were further verified with clinical samples by RPM technology in this study. In summary, these identified disturbed proteins or pathways in RBCs with the occurrence of LC could facilitate the early diagnosis of HCC.

RBCs molecular characteristics changes from LC to HCC during tumorigenesis

Liver cirrhosis is a developmental stage that develops into HCC, during which the maturation of RBCs in peripheral blood are influenced. We next explored what happened to RBCs during LC-HCC transition by analyzing the differentially expressed proteins (DEPs) between LC and HCC patients. We identified 57 DEPs (26 up-regulated, 31 down-regulated) in HCC compared to LC (Table S6, Fig. 5A), and these disturbed DEGs were also enriched in autophagy pathway (Fig. 5B) that is necessary for the suppression of spontaneous tumorigenesis through a cell-intrinsic mechanism, and the impairment of autophagy initiates spontaneous liver tumorigenesis in aged mice [27, 28]. This finding suggested the disruption of the gate keeper of HCC during this process. The estrogen pathway (Fig. 5B), another HCC associated pathway, was also disturbed during this process [29]. However, the disturbed oxytocin and GnRH pathways were first identified during this process.

During this transition, we observed SMIM1 is down-regulated with the onset of LC and gradually increases towards normal level at HCC (Fig. C), and ANXA7 gradually increases during the whole process of HCC tumorigenesis (Fig. 5D). PRM assay confirmed the dynamic changes of SMIM1 and ANXA7 in RBCs during the whole process of HCC tumorigenesis with clinical samples (Fig. 4E, F; Table S3-4).

SMIM1 dramatically decreases in LC and gradually increases in HCC, while ANXA7 continuously increases from HC to HCC, both of which could indicate the tumorigenesis of HCC. The dramatic decrease of SMIM1 expression in RBCs from LC patients could act as early diagnosis biomarker for HCC. Moreover, we selected SMIM1 to be tested with HCC tissue. The current result showed that it is highly expressed in HCC tissue but not in precancerous lesions (Fig. 5G), suggesting it is probably associated with the production of erythroid cells or progression of HCC. The underlying mechanism needs to be further explored in the future.

A few pathways involving erythropoiesis are affected between HCC and HC

By comparing with HC, we next wonder the alterations in RBCs that could be caused by HCC tumorigenesis. The DEGs between HC and HCC can clearly distinguish these two stages (Fig. 6A). The disturbance of oxygen transport, folate metabolic pathway, HIF-1 pathway and glycolysis pathway in

RBCs of HCC patients that are closely related to erythroid differentiation [30-33], suggesting erythropoiesis is abnormal in established HCC (Fig. 6B, Table S7). Interestingly, the disturbed mTOR pathway, that is first identified in LC in this study, was also identified in established HCC (Fig. 6B). A few cancer-related pathways are also altered in HCC, including autophagy, cell death, protein degradation, proteolysis, response to tumor necrosis, and a variety of metabolic pathways that are enriched by the down-regulated proteins (Fig. 6C), demonstrating that cancer-associated dysfunctions can also be revealed in RBCs in established HCC.

Discussion

HCC is rarely detected early and usually fatal within a few months of diagnosis. Early diagnosis of HCC is currently the most important step in HCC management. As a non-invasive screening of tumors, blood testing, has been paid more and more attention. In addition to circulating tumor cells in peripheral blood, some factors released by tumor cells were found in plasma, and a large number of tumor biomarkers were characterized including CA125 and AFP, of which CA125 is also a sensitive factor in liver cirrhosis [34, 35]. The transcriptional information carried by platelets in peripheral blood can be utilized to judge the occurrence and type of tumors [13]. Red blood cells have a life span of 120 days, and could change during the circulation of peripheral blood experiencing tumor microenvironment. We hypothesize that RBCs may enable clinical advances in blood-based “liquid biopsies”, and RBCs could be also used as markers for cancer diagnosis even at earlier stage.

Hepatitis B patients can gradually develop from cirrhosis to liver cancer, therefore it is indispensable to assess the transformation of cirrhosis into HCC for early diagnosis. We found the blood routine index of liver cirrhosis patients appears to be abnormal, and the protein expression profile in RBCs was significantly different from that of healthy individuals. The number of platelets and RBCs in LC patients is less than that in patients with HCC and healthy individuals. We speculate some disturbed proteins could be used as a biomarker for the progression of LC to HCC. SMIM1, a small and conserved membrane protein, is an antigen for the Vel blood group and participates in red blood cell formation [20, 21]. SMIM1 decreases significantly in LC stage, but increases 18 folds in HCC, returning to near normal level (Fig. 5C, E). SMIM1 is not an indicator to screen HCC in healthy individuals, but it could indicate the development of liver cirrhosis to HCC. The dramatic decrease of SMIM1 in LC could be used as a sign for the early diagnosis of HCC. We suggest the combined detection of SMIM1, AFP and CA125 could improve the efficiency of early diagnosis of HCC. ANXA7, a Ca^{2+} -binding protein that is involved in membrane organization and dynamics, plays a role in promoting the proliferation of liver cancer, and loss of function slows down the proliferation of liver cancer [36]. We found ANXA7 could reflect the development of HCC, since its expression gradually increases during tumorigenesis. ANXA7 could serve as a potential biomarker for early diagnosis in HCC.

In this study, we first observed the alterations of globins in RBCs during the whole process of tumorigenesis in HCC. Globins, covering a range of myoglobin, hemoglobin, cytoglobin, and neuroglobin, are present in all kingdoms of living organisms where they display a variety of functions, including O₂

sensing, transport, storage and heme-based catalysis [37]. Myoglobin and cytoglobin were reported as a tumor suppressor in breast and lung tumors. Loss of cytoglobin accelerates liver fibrosis and cancer development despite its etiology in mouse models of chronic liver injury [38]. Neuroglobin is the unique globin identified as a critical player in cancer cell adaptations and resistance to detrimental oxidative stress conditions [39]. β -globin is selectively increased in cancer cells, mediating a cytoprotective effect during blood-borne metastasis [40]. The normal adult hemoglobin is a predictive clinical indicator for liver cancer combining with combined with CA-125 [35]. In this study, we observed that the expression of hemoglobin, especially HbF, dramatically alters during tumorigenesis of HCC. The maturity of erythroid cells in HCC patients decreases. Consistently, embryonic and fetal globins are increased in established HCC, suggesting immature erythroid cells are present under HCC conditions and gamma and/or epsilon chain production could continue into adulthood. The alteration of erythroid-specific globins expression could indicate the progression of HCC and serve as biomarker for early diagnosis in HCC. This finding could be an advancement in clinical application and translational research in this field, even though the molecular mechanism underlying the association of the biosynthesis of different types of erythroid-specific globins in peripheral blood and tumorigenesis of HCC is still a mystery.

Interestingly, we have observed the preoperative anemia rate in HCC(34.45%) is lower than many cancers, such as colorectal cancer (45.62%) and uterine cancer (46.94%) (unpublished data). We speculate that the erythropoietin-driven extra production of red blood cells stimulated by the pathogenesis of HCC could partially solve the problem of oxygen-carrying of RBCs in the body, although the maturity of some erythroid cells in the peripheral blood of HCC patients is abolished.

We revealed immature red blood cells in the peripheral blood of patients with HCC. Physiologically, nucleated erythroid cells only exist in the bone marrow but not peripheral blood in adult. The presence of these nucleated red blood cells in the peripheral blood could indicate the pathological status including cancers. We speculate the possibilities that the immature erythroid cells exist in peripheral blood. Firstly, liver is an extramedullary hematopoietic organ, and CD71+CD45+ erythroid cells were characterized in situ in tumor tissue of HCC [41]. These in-situ generated immature red blood cells by tumor tissue could be released into the peripheral blood. Secondly, under pathological conditions of HCC the bone marrow could be stimulated to release the nucleated erythroid cells into peripheral blood. Peripheral red blood cells could be a good indicator of tumor occurrence and development, and serve as marker for early diagnosis of cancers including HCC.

Conclusion

Our results revealed that molecular characteristics changes of RBCs in peripheral blood is closely associated with the process of HCC tumorigenesis. The defects in RBCs initiate at the stage of liver cirrhosis, and continue with the onset of the established HCC that is represented by the presence of immature erythroid cells. We discovered several novel biomarkers including SMIM1 and ANXA7 in RBCs that could imply the early diagnosis of HCC. Our study will expand the clinical applications of “liquid biopsies” in cancer early diagnosis.

Abbreviations

HCC, hepatocellular carcinoma

LC, liver cirrhosis

HC, healthy control

RBCs, red blood cells

DIA, data independent acquisition

DEPs, differentially expressed proteins

PRM, parallel reaction monitoring

LAP3, leucine aminopeptidase 3

QDPR, quinoid dihydropteridine reductase

KEGG, Kyoto Encyclopaedia of Gene and Genomes

GO, gene ontology

Declarations

Ethics approval and consent to participate

This study was performed in accordance with the ethical standards of the institutional research committee and the latest Declaration of Helsinki and approved by ethics committee of the PLA General Hospital.

Consent for publication

Not applicable

Availability of data and materials

Not applicable.

Competing interests

The authors have declared no competing interests.

Funding

This study was supported by the National Natural Science Foundation of China (Grant No. 32071117, 81870097), the National Key Research and Development Program of China (Grant Nos. 2017YFC0907400, 2018YFC0910701).

Authors' contributions

DW and ZZ conceived and supervised the study; DW and SW designed the experiments; GW and SW analyzed the data; HY, JZ, XC, YZ and FP performed the experiments; LW and XH conducted clinicopathological examination for the patients; SL, JZ, WZ and BF collected the clinical data for the cohort of this study; SW and ZZ drafted the manuscript; DW and ZZ revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable

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Figures

Figure. 1

Figure 1

Clinical characteristics of peripheral blood during tumorigenesis of HCC A-B. Changes of AFP and hemoglobin level in peripheral blood of a cohort of LC, HCC and HC during tumorigenesis of HCC. AFP was clinically detected by electrochemiluminescence immunoassay. Hemoglobin was clinically detected by cyanmethemoglobin method; C-E. Changes in the number of RBCs, platelets and leukocytes in the

peripheral blood from a cohort of LC, HCC and HC. These blood cells were clinically detected with full-automatic hematology analyzer.

Figure 2

Figure 2

Analysis of the identified proteins of RBCs in the cohort of HCC, LC and HC A. Cellular localization of the identified 659 proteins in RBCs by GO analysis; B. Functional enrichment of the identified proteins in

RBCs by GO analysis; C. Hierarchical clustering analysis of LC, HCC and HC groups based on 659 DEPs; D. PCA analysis of LC, HCC and HC groups based on DEPs.

Figure. 3

Figure 3

Analysis for the expression of erythroid-specific proteins in a cohort of HCC, LC and HC A-D. Analysis of globins expression changes in the cohort of LC (N=17), HCC (N=30) and HC (N=25). The expression was derived from DIA mass spectrometry data; E-F. The expression of HBA1 and HBE1 in RBCs during the

tumorigenesis were verified with PRM strategy with another batch of clinical samples from LC (N=9), HCC (N=11) and HC (N=10); G. Analysis of GYPA expression changes in the cohort of LC (N=17), HCC (N=30) and HC (N=25); H. Nucleated erythroid cells was observed under microscope in HCC patients; I-J. Statistics of RBCs respectively expressing HbF (I) and HbA (J) in peripheral blood samples from HCC (N=8), LC (N=9) and HC (N=8). The cell number was calculated by flow cytometry analysis.

Figure. 4

Figure 4

Alterations in RBCs with the onset of LC A. Hierarchical clustering analysis of LC and HC groups based on DEPs between them. Solid triangle indicates SMIM1 and ANXA7, respectively; B. Volcanic plot of DEPs from protein differential expression analysis between LC and HC group (P < 0.05). SMIM1 and ANXA7

were highlighted with arrow; C. Interactive networks that connects disturbed pathways and proteins existed in RBCs with the onset of LC. The networks were constructed by significantly DEPs between LC and HC group according to STRING analysis.

Figure. 5

Figure 5

Alterations in RBCs and potential biomarker identification during tumorigenesis from LC to HCC A. Hierarchical clustering analysis of HCC and LC groups with DEPs between them; B. Interactive networks

regulating the tumorigenesis from LC to HC. This networks connects the disturbed pathways and proteins existed in RBCs and was constructed by DEPs between LC and HCC group; C-D. Analysis for SMIM1 and ANXA7 expression in a cohort of LC, HCC and HC, respectively. The protein expression was calculated from mass spectrometry data and the significant difference analysis were conducted between groups; E- F, The expression trend of SMIM1 and ANXA7 during the tumorigenesis were verified with PRM strategy with another batch of clinical samples from LC (N=9), HCC (N=11) and HC (N=10); G. Immunostaining for SMIM1 in normal liver and HCC tissue.

Figure. 6

Figure 6

A few erythropoiesis-related pathways are affected in RBCs between HCC and HC A. Hierarchical clustering analysis of HCC and HC groups with DEPs between them; B. Interactive networks involving

disturbed pathways and proteins constructed with DEPs between HCC and HC groups; C. Enriched biological processes with the top highest PAS Z score; D. Volcanic plot of DEPs between HCC and HC groups ($P < 0.05$).

Supplementary Files

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