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Transcriptomic and Proteomic Investigation of HSP90A as a Potential Biomarker for HCC

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Background: Hepatocellular carcinoma (HCC) is the third most frequent cause of cancer-related death in adults. Despite recent advances in the clinical technologies, the screening and diagnostic efficacy for HCC remains poor. Discovering novel and reliable HCC biomarkers is urgently needed.

Material/Methods: We performed a transcriptome-proteome integrated assay to track the possible HCC biomarkers from the process of HCC-derived gene expression in malignant cells to its protein product released into serum.

Results: Our screening results demonstrated that heat shock protein 90A (HSP90A), which participates in the PI3K-Akt signaling pathway and many other cancer-related pathways, warrants further investigation. The expression of HSP90A was increased in the HCC cells, serum, and tissues. Immunohistochemistry analysis on 76 clinical tissue samples also suggested the relevance between HSP90A expression and HCC metastatic behavior.

Conclusions: These findings suggest a role for HSP90A in HCC pathogenesis and the potential use of HSP90A for the screening and diagnosis of this malignancy.

MeSH Keywords: **Biological Markers • Carcinoma, Hepatocellular • HSP90 Heat-Shock Proteins • Proteome • Transcriptome**

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Background

Globally, more than 50% of HCC-related deaths occurred in China [1], with high mortality. Primary HCC usually does not show any symptoms at the early stage. By the time clinical manifestations appear, most patients have entered the terminal stage with fast and aggressive tumor progression; therefore, HCC screening and diagnosis is of utmost importance. The currently used HCC biomarker, alpha fetoprotein (AFP), has been widely used for 40 years. However, the diagnostic value of serum AFP alone may be approximated 40% for patients with early-stage HCC, and for 15–30% of HCC patients, AFP level may remain negative (<25 ng/ml) [2–5]. It is now well accepted that a single biomarker will not suffice, and that a panel of serum biomarkers is necessary to aid in diagnosis. Discovering novel and reliable HCC biomarkers has become an urgent task.

Recent remarkable progress in proteomic methods, including improved detection limits and sensitivity, has great potential for identifying novel biomarker patterns from the cancer proteome for HCC clinical application [6,7]. The cancer proteome, as the end-point of pathological processes that underlie cancer development and progression, could be an important source for the discovery of new biomarkers and molecular targets [8]. However, although many candidates have been discovered in HCC using various proteomic technologies, few of them have been confirmed as specific and sensitive biomarkers. The complexity and heterogeneity of HCC present obstacles to the discovery of specific biomarkers through direct serum proteomic methods.

The emerging integrated "omics" strategy brings hope of bridging the gap between malignant cells and peripheral fluids. Integrated transcriptomic and proteomic technologies have already been applied in cancer biomarker discovery studies [9–13]. This tactics helps to reveal the bioinformation flow between the genetic codes and the functional molecules, and dictates a reliable mapping from 2 high-throughput platforms. Nevertheless, the HCC-specific signatures, which can often be regulated by the cancerous cells and response to the disease state, have not been documented previously in HCC serum.

In the present study, in order to discover the specific serum biomarker for HCC, we performed RNA sequencing (RNA-seq) to explore HCC-derived gene expression, and further explored the expressed product in HCC serum. This integrated transcriptomic and proteomic investigation enabled us to find a higher HSP90A level in HCC and provide a new method for HCC screening and diagnosis.

Material and Methods

Cell lines

The HCC cell line SMMC7721 and the normal liver cell line L02 were bought from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Both of these cell lines were cultured in RPM11640 (HyClone, MA, USA) supplemented with 10% fetal bovine serum (HyClone, MA, USA), and 100 mg/ml penicillin and streptomycin, respectively (Invitrogen, NY, USA). Cells were maintained in a humidified atmosphere containing 5% CO_2 – 95% air at 37°C.

Patients

Access to human samples of HCC and paired controls complied with the laws of China and the guidelines of the local Ethics Committee. Blood samples from HCC patients and normal age- and sex-matched controls were obtained from the Department of Hepatobiliary Surgery, First Affiliated Hospital of Guangxi Medical University, Nanning, China). The collected blood samples were centrifuged for 10 min at 4000 g within 30–120 min after being collected, and then the isolated serum samples were stored in frozen plastic vials at –80°C before analysis. Seventy-six pairs of formalin-fixed and paraffin-embedded HCC and adjacent para-tumor tissues were obtained from the Affiliated Tumor Hospital of Guangxi Medical University (Nanning, China).

Transcriptome sequencing

For whole transcriptome sequencing, RNA from 1×10^7 L02 and SMMC7721 cells were extracted using Trizol (Invitrogen, MA, USA) and then quantified by NanoDrop 2000 (Thermo-Fisher Scientific, MA, USA). The whole transcriptome RNA-seq was performed using the Ion Total RNA-Seq Kit, Ion PI™ Chip kit, Ion PI™ Template OT2 200 Kit, and Ion PI™ Sequencing 200 Kit based on the Life Technologies Corporation's guide. In brief, mRNA was purified using oligo-dT beads from 100 µg of total RNAs for each sample and then fragmented. The cleaved RNA fragments were reverse-transcribed into first-strand cDNA, followed by second-strand cDNA synthesis. Then a single 'A' base was added to cDNA fragments at the 3' end. The cDNAs were ligated to adapters, enriched by polymerase chain reaction (PCR) to generate the final cDNA library. After amplifying the sequencing template, RNA-seq was performed using the Ion proton system (Life Technologies Corporation, MA, USA) with the standard protocol.

Serum proteomics analysis

Blood samples were pooled and divided into 2 groups (HCC and normal control). The 2 groups of the pooled samples were

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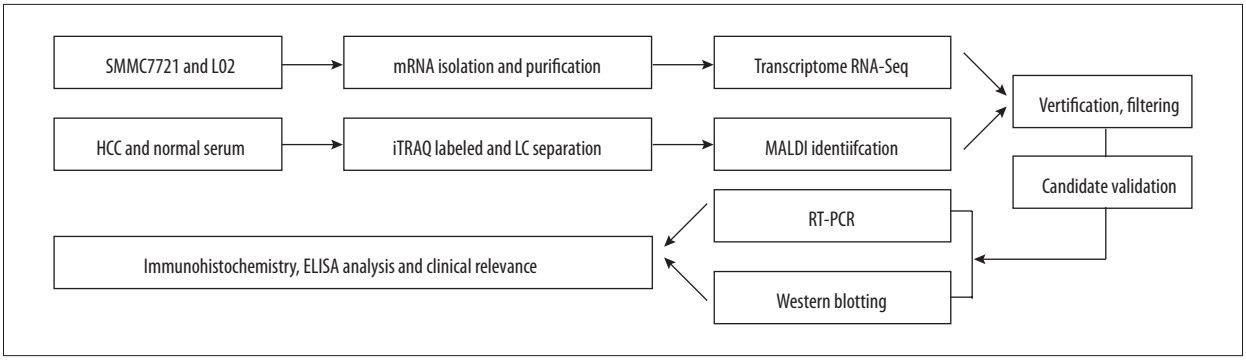

Figure 1. Study design. Candidate serum biomarker HSP90A was screened using transcriptome-proteome integrated assay. The expression of HSP90A was validated through RT-PCR and Western blotting. Immunohistochemistry was used to detect the correlation between HSP90A expression and HCC clinical significance.

used to eliminate high-abundance proteins. According to the manufacturer's instructions, the 14 highest-abundance proteins were extracted from the serum using a removal system (MARS Human 14) affinity column (Agilent Technologies, CA, USA). Total protein concentration of each group was determined by bicinchoninic acid (BCA) assay. Normal and HCC samples were reduced, alkylated, digested, and labeled with iTRAQ tag 113 and 116, respectively. The labeled peptides of 113 and 116 were pooled, then desalted and fractionated by using nano-HPLC. Twenty fractions were collected and each was spotted automatically onto the matrix-assisted laser desorption/ionization (MALDI) plates and analyzed by use of a 5800 MALDI-TOF/TOF Analyser (Applied Biosystems, MA, USA). Protein identification and quantification were performed with ProteinPilot 4.0 software (version 4.0; Applied Biosystems, MA, USA), with a novel Paragon database. The search was carried out through the SWISS-PROT database. The protein was identified through at least 2 peptides with 95% confidence. Proteins were considered up- or down-regulated when their ratios were >1.2 or <0.8, respectively.

Real-time PCR (RT-PCR)

Total RNA from cells was isolated in 2 steps using Trizol reagent (Invitrogen, MA, USA) followed by RNeasy (Qiagen, Valencia, CA, USA) purification, then subjected to reverse transcription. HSP90A was amplified with primers (5'-CCTTCTATTTGTCCCACG-3',5'-AGATCCTCCGAGTCTACCAC-3') and conditions (40 cycles, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min). GADPH was used as an internal control, ensuring cDNA quality and loading accuracy. All samples were run in duplicate on a 7700 Real-Time PCR System (Applied Biosystems, MA, USA) and the data were analyzed using Sequence Detector v1.9 software.

Western blotting

Serum proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and shifted to a 0.45-nm polyvinylidene fluoride (PVDF) membrane. The membrane was blocked at room temperature for 1–2 h with blocking reagent and then was incubated with primary antibody at a concentration of 1: 350 (Santa Cruz, CA, USA), overnight at 4°C, followed by IRDye-labeled secondary antibody at a concentration of 1:10000 (LI-COR, MA, USA) for 1 h at room temperature. The Odyssey Infrared Imaging System (LI-COR Biosciences GmbH, CA, USA) was used to obtain the image and analyze the target protein expression, which was calculated as the ratio of the intensity of target protein to that of protein standard stripe (90 KD).

ELISA

The serum HSP90A concentration was determined using a human HSP90A ELISA Kit (eBioscience, CA, USA) according to the manufacturer's instructions.

Immunohistochemistry

Seventy-six formalin-fixed and paraffin-embedded HCC and paired adjacent tissues were used in the analysis. Briefly, the paraffin sections were first hydrated. After microwave antigen retrieval, 3% H_2O_2 was used to incubate the slides to block the endogenous peroxidase activity. The non-specific binding sites were blocked with 10% goat serum. The sections were incubated with primary anti-HSP90A antibody (Abcam, CA, USA) and then secondary antibody, according to the antibody manufacturer's instructions. After incubation, the sections were counterstained with hematoxylin. Negative control slides were included in all assays.

Statistical analysis

Statistical analyses were performed using SPSS statistics software (SPSS, Chicago, IL). P value <0.05 was considered statistically significant.

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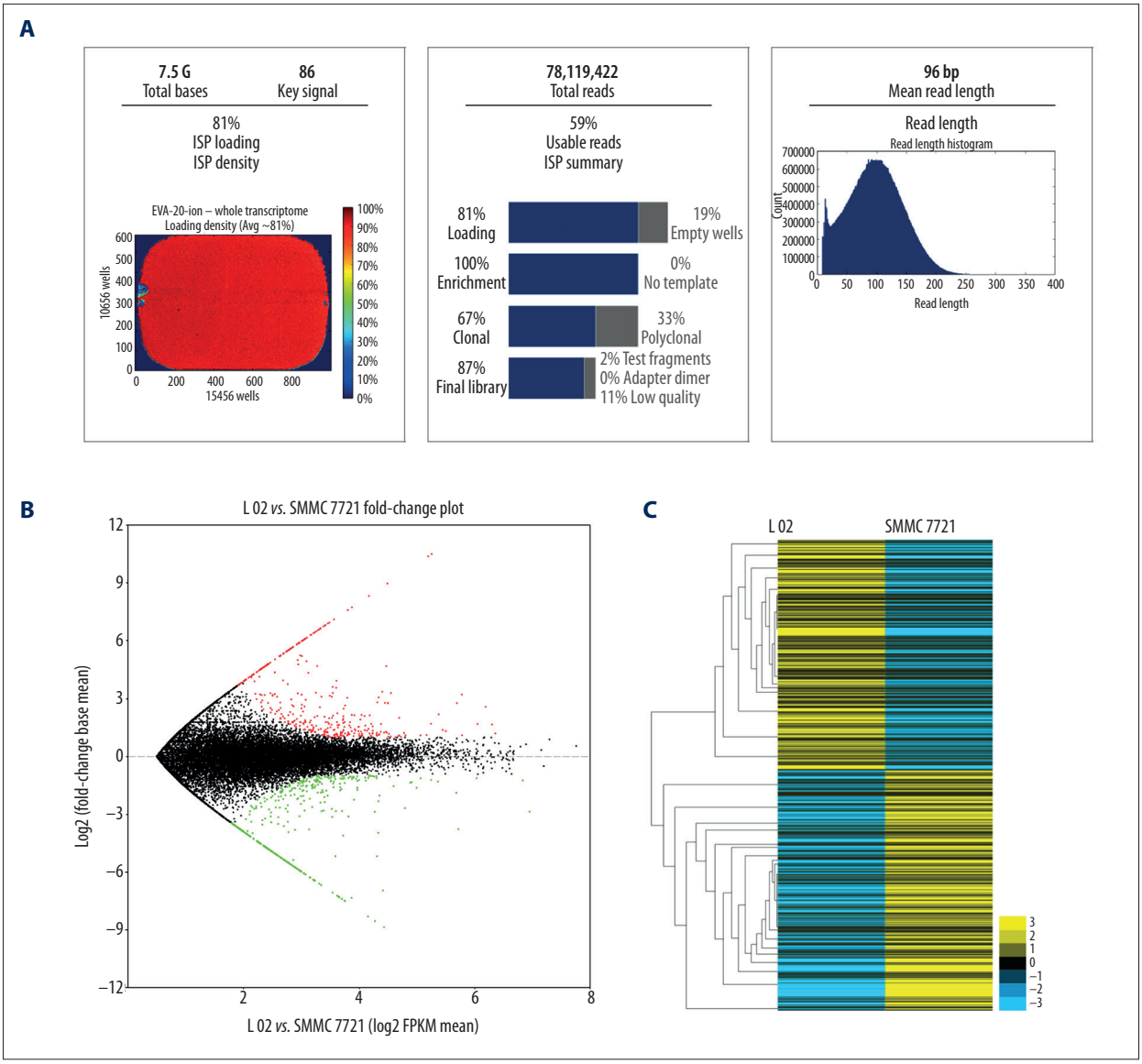

Figure 2. Raw data of RNA-seq. (**A**) Description for all data. (**B**) Log2 ratio plot for all the genes. The red and green dots indicated that up- and down-regulated DEGs were significant at P values less than 0.05. (**C**) Hierarchical clustering of DEGs.

Results

Study design

As shown in Figure 1, the study consisted of the following parts: (1) transcriptomic RNA-seq to discover HCC-derived gene expression; (2) iTRAQ-MALDI-TOF-MS/MS analysis to screen expression product in serum; (3) a validation step to confirm the expression level of the candidate through RT-PCR and Western blotting; and (4) clinical analysis to identify and verify the correlation between HSP90A and HCC clinical characters so as to evaluate its role in HCC clinical application.

Raw data of RNA-seq

On completion, a 78.1 million 96bp long sequencing read was generated and it corresponded to 7.5 G raw sequence data (Figure 2A). The normalized gene expression was measured as fragments per kilobase of exon per million fragments mapped (FPKM) (Figure 2B). The result of clustering analysis indicated that the transcriptome of hepatoma cells was distinct from that of normal liver cells (Figure 2C). To estimate the gene expression level, we conducted enrichment analysis to determine the gene expression and to identify significantly dysregulated genes. A threshold of 2-fold up-regulation (log 2 ratio >1) or down-regulation (log 2 ratio <1) of gene expression was used. The results showed 611 differentially expressed genes

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Table 1. HCC-derived transcription factors and the downstream regulated genes.

Gene ID	Genesymbol	Regulation	Q Value	Inner link
ENSG00000102878	HSF4B	Up	0.004	Possitive transcription factor for HSP90A,CRYAB expression
ENSG00000197825	NF-kappa-B	Up	0.000	Possitive transcription factor for EGR1, SAA1 expression
ENSG00000080824	HSP90A	Up	0.000	Downstream gene of HSF4B transcription factor
ENSG00000109846	CRYAB	Up	0.000	Downstream gene of HSF4B transcription factor
ENSG00000120738	EGR1	Up	0.000	Downstream gene of NF-kappa-B transcription factor
ENSG00000173432	SAA1	Up	0.000	Downstream gene of NF-kappa-B transcription factor

Table 2. The differentially expressed proteins in HCC serum.

Number	Accession	Symbol	Unused	% Cov (95)	Peptides	Regulation	P value
1	P01009	SERPINA1	48.3	42.3	26	Up	0.0000
2	P0C0L5	C4B	139.2	37.3	97	Up	0.0001
3	P04114	APOB	246.3	25.2	139	Up	0.0015
4	P01023	A2M	100.7	33.5	65	Up	0.0066
5	P10643	C7	19.00	15.0	10	Up	0.0085
6	Q06033	ITIH3	17.3	11.0	10	Up	0.0108
7	P00738	HP	42.8	40.6	33	Up	0.0123
8	P02741	CRP	7.7	17.0	4	Up	0.0123
9	P00751	CFB	49.5	24.1	33	Up	0.0147
10	P0C0S5	H2AFZ	2.0	7.0	1	Up	0.0155
11	p02748	C9	20.7	14.9	13	Up	0.0158
12	P11226	MBL2	2.0	3.2	1	Up	0.0167
13	P08637	FCGR3A	2.1	4.3	1	Up	0.0172
14	P07900	HSP90A	12.5	7.0	11	Up	0.0187
15	P01011	SERPINA3	40.9	34.8	37	Up	0.0190
16	P02652	APOA2	70.7	47.5	61	Up	0.0279
17	P62805	HIST1H4A	4.0	17.5	2	Up	0.0299
18	P06396	GSN	23.7	14.8	12	Down	0.0000
19	P05155	SERPING1	27.3	25.6	15	Down	0.0001
20	P06727	APOA4	63.5	62.4	41	Down	0.0008
21	P01871	IGHM	27.5	29.0	16	Down	0.0081
22	P43652	AFM	27.8	17.5	17	Down	0.0090
23	P02647	APOA1	45.9	69.3	32	Down	0.0094
24	P01008	SERPINC1	42.5	34.1	27	Down	0.0118
25	P02743	APCS	12.8	24.2	7	Down	0.0159
26	P09871	C1S	12.8	7.6	6	Down	0.0173
27	P19827	ITIH1	33.7	16.9	22	Down	0.0184
28	P63261	ACTG1	7.8	9.6	4	Down	0.0269
29	P02765	AHSG	28.2	35.2	20	Down	0.0317
30	Q6EMK4	VASN	2.1	2.1	1	Down	0.0423
31	Q6UXB8	PI16	2.0	2.2	1	Down	0.0432

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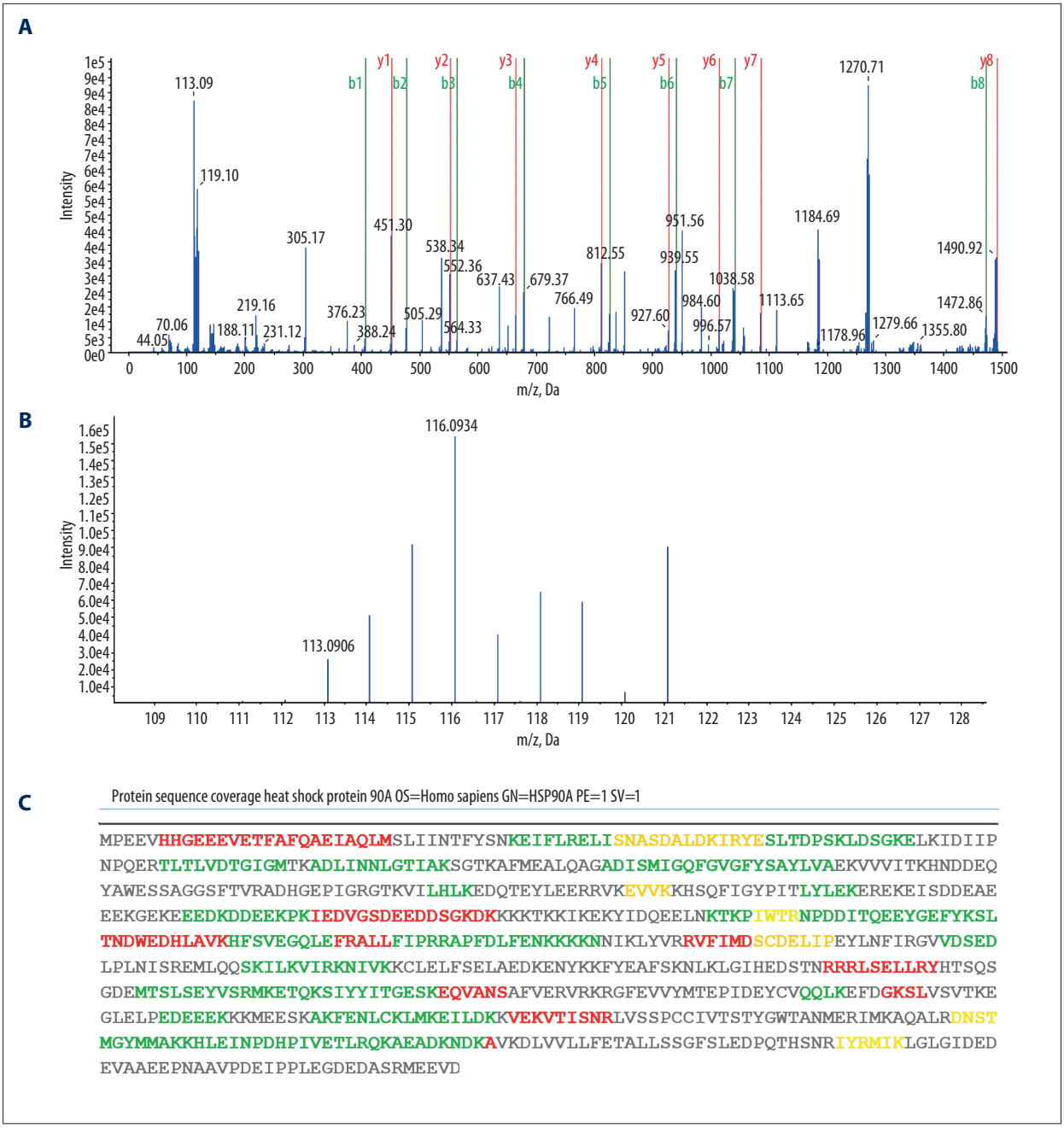

Figure 3. HSP90A protein sequence. (**A**) MS/MS spectrum of the HCC-derived HSP90A peptides. (**B**) The relative level of TALDO was significantly up-regulated during HCC progression. (**C**) Aligned TALDO sequences.

(DEGs) were detected, including 368 up-regulated and 243 down-regulated DEGs.

Identification of HCC-derived genes

Although we identified approximate numbers of up- and down-regulated DEGs in the cellular HCC transcriptome analysis, we still noticed that 2 transcription factors – heat shock transcription factor 4B (HSF4B) and NF-kappa-B – were up-regulated, with a consistently increased tendency of their downstream genes (Table 1). We deduced that the transcription factor binds to specific DNA sequences, thereby controlling the flow of genetic information from DNA to mRNA, then, mRNA conveyed genetic information from DNA to the protein products of gene expression, and finally the HCC-derived products were secreted from the cells and flowed into the serum. Thus, the downstream genes of HSF4B and NF-kappa-B – HSP90A, bovine alpha-crystallin B chain (CRYAB), early growth response protein

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Table 3. KEGG pathway of HSP90A.

Pathway ID	Definition	Relationship with cell transformation
hsa04141	Protein processing in endoplasmic reticulum	Cell apoptosis
hsa04151	PI3K-Akt signaling pathway	Cell apoptosis and cell cycle
hsa04612	Antigen processing and presentation	Organismal systems; immune system
hsa04621	NOD-like receptor signaling pathway	Immune response; cell death
hsa04915	Estrogen signaling pathway	Cell cycle; cell apoptosis and cell adhesion
hsa05200	Pathways in cancer	Tissue invasion and metastasis; apoptosis; proliferation; cell damage
hsa05215	Prostate cancer pathway	Prostate cancer

1 (EGR1), and serum amyloid A 1 (SAA1) – were considered as the significant HCC-derived products that should be focused on in the serum proteomic analysis.

Raw data of iTRAQ-MALDI-TOF-MS/MS analysis

MS spectra were acquired from 900 to 4000 m/z, for a total of 1500 laser shots. Laser intensity remained fixed throughout the analyses. MS/MS analysis was performed using collision energy of 2kV with air at 2×10^{-6} Torr as the collision gas. Metastable ions were suppressed, for a total of 1500 laser shots. Twenty of the most intense ion signals, characterized by an S/N >45, were automatically selected as precursors for MS/MS acquisition. Peptides matched the mass spectra identification conditions, identified with software on the 5800 MALDI-TOF/TOF Analyzer (P<0.05, proteins were classified as up- or down-regulated when their ratios 113/116 were >1.2 or <0.8, respectively). After filtering, a total of 31 proteins in HCC serum were screened through the iTRAQ technology platform. The expression levels of 17 proteins were up-regulated in HCC samples while the other 9 proteins were down-regulated (Table 2).

Integrated analysis of candidate biomarker

From the clues provided by the HCC transcription regulation, we examined the expression level of HSP90A, CRYAB, EGR1, and SAA1 in proteomics data to trace the serum biomarker from hepatoma cells. Excitingly, HSP90A level was 30.479 times higher in the HCC group than in the normal control (P=0.0187, Figure 3). We further performed the gene annotation analysis of HSP90A through the GENE Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) database. The result suggested that HSP90A was involved in the biological processes of cell localization, motility, differentiation, migration, and, especially, neuron migration, and participated in the 7 KEGG pathway, including protein processing in endoplasmic reticulum, PI3K-Akt signaling pathway, antigen processing and presentation, NOD-like receptor signaling pathway, estrogen signaling pathway, pathways in cancer, and the prostate cancer pathway (Table 3). The results suggested that functional HSP90A was significantly associated with cellular transformation.

Validation and clinical analysis of HSP90A

We used RT-PCR and Western blot analysis to validate the mRNA and protein level of HSP90A. The results showed the level of HSP90A mRNA in SMMC7721 was 4.177 times higher than that of L02 (P<0.05) (Figure 4A, 4B), and the level of HSP90A protein in the HCC group was 7.259 times higher than in the normal group (P<0.05) (Figure 4C, 4D). We then detected 90 cases of HCC and control serum through ELISA, and the results suggested that the serum HSP90A level was significantly higher in the HCC subjects (273.6±20.3 μg/ml) than in the normal controls (186.2±18.3 μg/ml) (P<0.05). Finally, we used immunohistochemistry to explore the clinical significance of HSP90A expression in 76 HCC cases. The clinical characteristics of these patients are listed in Table 4. The percentage and intensity of positively staining cells varied widely among cases; tumor samples were classified as strong in 29 cases (38.2%), while adjacent samples were classified as strong in 13 cases (17.1%) (P<0.05). Statistical analysis also revealed that patients with high HSP90A expression levels (up to +++) had significantly higher metastasis rates (66.6%) than those with low expression (25.0%), P=0.01 (Figure 5).

Discussion

This study demonstrates that an integrated omics strategy could be used to trace protein expression and to discover a serum HCC biomarker. We used cell transcriptomic and serum proteomic analysis to screen HCC-derived biomarkers. A potential candidate,

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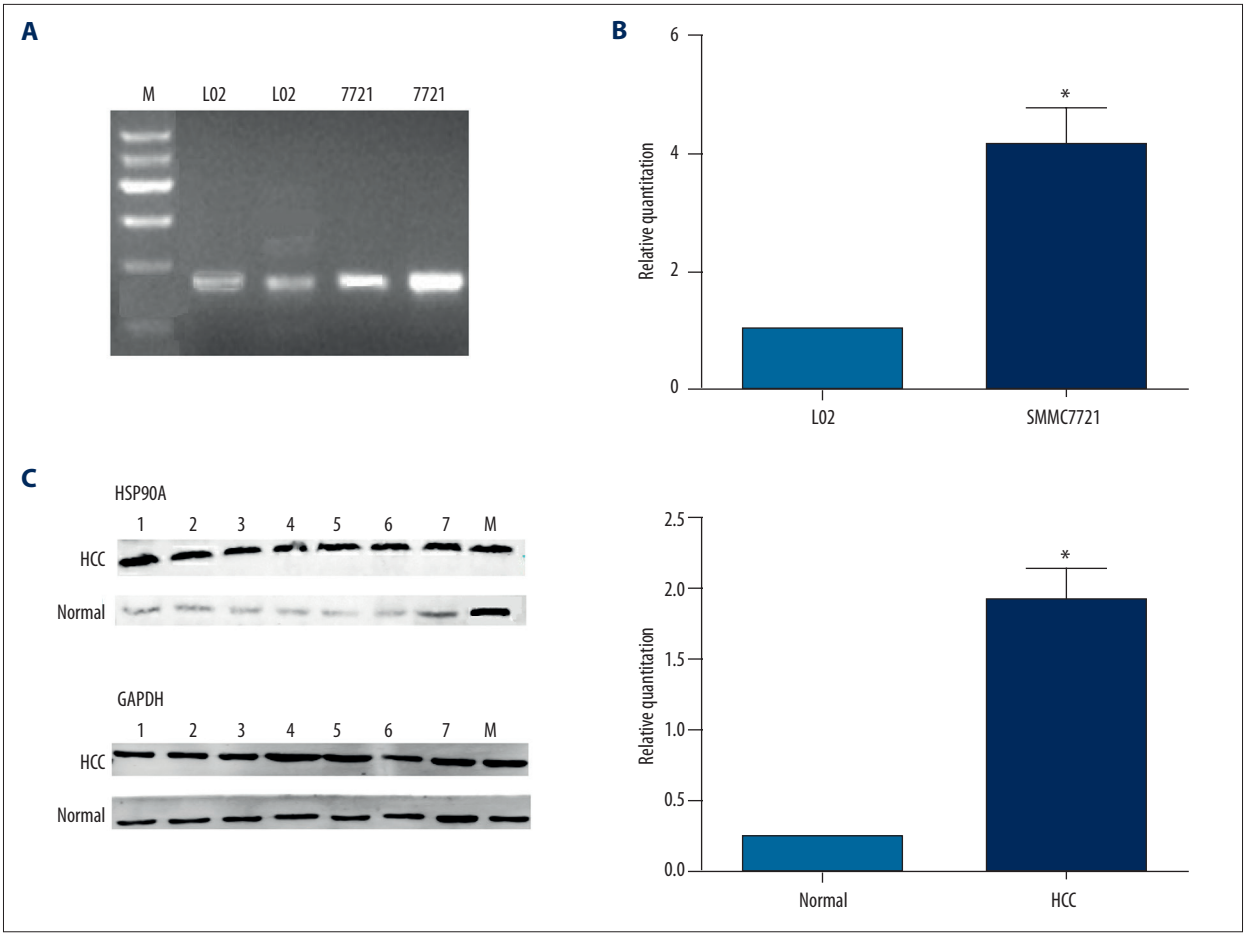

Figure 4. Validation of HSP90A expression level. (**A, B**) RT-PCR analysis for the mRNA level of HSP90A in L02 and SMMC7721. The relative level of HSP90A was calculated as the ratio of the intensity of target protein to that of GAPDH. (**C, D**) Western blot analysis for the protein level of HSP90A in normal and HCC serum. The relative level of HSP90A was calculated as the ratio of the intensity of target protein to that of protein standard stripe (90KD).

HSP90A, which was associated with cell location, motility, differentiation, migration, and neuron migration, was identified. The abnormal expression of HSP90A was validated through RT-PCR and Western blot analysis. Furthermore, we demonstrated that HSP90A seems to be correlated with HCC, especially the HCC metastasis, and that this is appears to be clinically relevant.

With the development of next-generation sequencing technologies, RNA-seq has become a powerful approach in defining the abnormal transcriptomes of specific diseases [14–16]. Taking advantage of RNA-seq, we are now able to monitor the changes in gene expression at the genome-wide level in disparate biological process. It is well known that reprogramming of the transcriptome leads to aberrant cellular behavior and thus directly contributes to cancer progression. Specific to HCC, by comparing the transcriptomes between health and HCC patients, we can develop a deeper understanding of how transcriptional changes contribute to the development of HCC [17]. In addition to transcriptome change, the technological platform integrating transcriptomic data with proteomic data guarantees more precise definition of the proteome at multiple levels [18,19], which further contributes to a deeper understanding of the dynamic and complex regulatory relationship between the transcriptome and the proteome [20–22]. Here, we used a transcriptome-proteome strategy to analyze the entire landscape, from transcription regulation to protein expression, and to avoid nonspecific readouts. We consider that integrated transcriptome-proteome analysis is an effective method for biomarker discovery.

In this study, we identified HSF4B as an activated transcription factor in an HCC cell line. We also observed up-regulated downstream gene (HSP90A) expression at the mRNA level. In proteomic analysis, a high protein level of HSP90A was also found in HCC serum. HSP90A is one of the cytosolic isoforms of HSP90. Quiescent normal cells do not secrete HSP90 [23–25] unless being triggered by environmental stress, including reactive oxygen species (ROS) [26], heat [27,28], hypoxia [23],

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Table 4. Clinical data of 76 cases of HSP90A for HCC.

Characteristics	No. of cases	HSP90A				U/c2	P-Val
		–	+	++	+++		
Age (year)							
<40	14	0	2	4	8	2.496	0.287
40–60	47	0	7	22	18		
>60	15	0	2	10	3		
AFP (ng/ml)							
<25	32	0	4	16	12	4.126	0.127
25–400	15	0	4	8	3		
>400	29	0	3	12	14		
Gender							
Male	64	0	10	30	24	–0.483	0.629
Female	12	0	1	6	5		
Tumor size (T) (cm)							
<5	18	0	3	6	9	2.34	0.31
5–10	37	0	4	24	9		
>10	21	0	4	6	11		
Metastasis							
+	24	0	4	4	16	–2.568	0.01
–	52	0	7	32	13		
Diolame complete							
+	38	0	5	20	13	–0.421	0.674
–	38	0	6	16	16		
Cirrhosis							
+	61	0	9	28	24	–0.278	0.781
–	15	0	2	8	5		
Tumor number							
One	41	0	5	21	15	–0.011	0.991
More	35	0	6	15	14		

gamma-irradiation [29], and tissue injury-released cytokines, such as transforming growth factor α (TGFα) [24]. These kinds of environmental stresses are known to be involved in the growth, invasion, and metastasis of many solid tumors. In this case, secretion of HSP90 has an important implication in cancer therapy because it plays a natural role in predicting cancer.

Up-regulation of HSP90A was also observed during HCC metastasis in our clinical samples. Certain tumor cells, such as those that maintain a steady-state level of hypoxia-inducible factor 1 (HIF-1), acquired the ability to constitutively secrete HSP90A for invasion and metastasis. Some studies suggested that even outside the cells, HSP90A still acts as a chaperone protein by assisting proper folding and activation of other surface-bound or secreted proteins, such as human epidermal growth factor receptor 2 (HER2) tyrosine kinase receptor, matrix metalloproteinase 2 (MMP2), and extracellular matrix (ECM) proteins [30,31]. Although numerous metastasis-associated biomarkers have been discovered, most of them were found by using HCC tissue and cell lines [32,33]. Essentially, a

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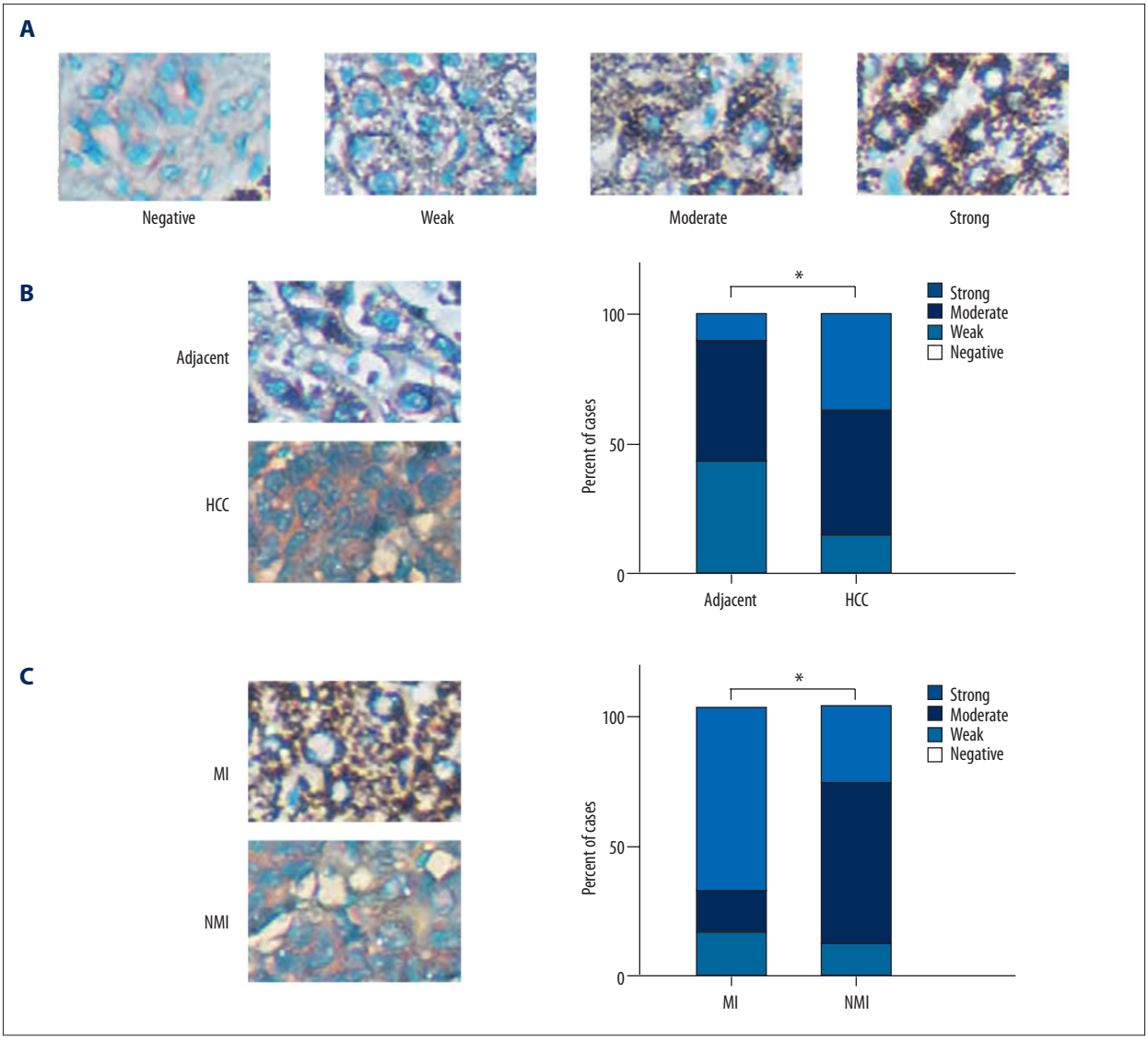

Figure 5. Immunohistochemical analysis of HSP90A in 76 tissue samples. (**A**) Expression intensity of positively staining cells with different grades. (**B, C**) HSP90A expression was significantly increased in HCC and metastatic specimens (MI), compared with adjacent and non- metastatic ones (NMI), P<0.05.

useable biomarker for HCC screening and monitoring should be detected in the serum. As a specific serum biomarker particularly useful for screening and monitoring HCC, HSP90A should be further examined through more clinical validation studies.

In conclusion, we developed a transcriptome-proteome integrated assay to screen HCC-derived serum signature. A candidate biomarker, HSP90A, might serve as a potential biomarker for HCC.

Conclusions

This study not only described the differential expression of HSP90A in HCC cells, serum, and tissues, but also focused on the origin of high-level HSP90A from initial transcriptional regulation to protein expression. These tactics effectively discarded those nonspecific serum proteins that were easily misunderstood as HCC biomarkers. The role of HSP90A in HCC metastasis also warrants further investigation.

Acknowledgements

YZ conceived of the study, and participated in its design and coordination and helped to draft the manuscript. XFD carried out the RNA-seq. NZ and HTL performed the proteomics studies. GL cultivated the cells. CPL drafted the manuscript. MH designed the study and performed the statistical analysis.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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