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## ORIGINAL ARTICLE

# Analysis of Differentially Expressed Proteins Involved in miR-449a for Hepatocellular Carcinoma by iTRAQ Proteomics

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### SUMMARY

***Background:*** **To investigate the relationship between down-regulation of miR-449a and prognosis of hepatocellular carcinoma (HCC) and to elucidate the potential target proteins of miR-449a.**
***Material and Methods:*** **The expression of miR-449a in 142 HCC tissues was detected by RT-PCR. The correlation between down-regulation of miR-449a and prognosis of HCC was statistically analyzed during clinical follow-up. The Bel-7042 HCC cell line in miR-449a-mimic and miR-449a-inhihbitor model was used, and the potential target protein of miR-449a was screened by isobaric tags for relative and absolute quantitation (iTRAQ) technology.**
***Results:*** **miR-449a was significantly down-regulated in HCC tissues, which was significantly associated with postoperative metastasis ($p < 0.0001$) and recurrence ($p < 0.0001$). The median overall survival time in the low-expression group of miR-449a was significantly lower than that of the high-expression group (19 months vs. 37 months, $p = 0.001$). In addition, the tumor-free survival time of the low-expression group was significantly lower than that of the high-expression group (14 months vs. 24 months, $p = 0.001$). iTRAQ analysis screened out 137 differential proteins, among which 88 were up-regulated and 49 were down-regulated. GO clustering, KEGG pathway, and STRING analysis were performed, suggesting that these differential proteins have complicated functions, such as ATP binding, metal ion binding, RNA binding, human papillomavirus infection, and Epstein-Barr virus infection.**
***Conclusions:*** **miR-449a was negatively correlated with HCC prognosis. The differential proteins screened by iTRAQ can provide the basis for studying the target proteins regulated by miR-449a and understanding the pathogenesis of HCC.**
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### KEY WORDS

miR-449a, hepatocellular carcinoma, iTRAQ

### INTRODUCTION

Primary liver cancer, a highly malignant tumor, is dominated by hepatocellular carcinoma (HCC), which is considered as the second most common killer among cancers in China. HBV infection is the most important risk factor for HCC, which accounts for more than 50%. HCC is characterized by high recurrence and metastasis and relatively low long-term survival rate [1]. Moreover, the pathogenesis is not yet thoroughly understood.

In recent years, increasing evidence has demonstrated that miRNA (miR) plays an important role in HCC. miR-122 with the highest expression in hepatocytes can affect HCC metastasis by regulating ADAM17 and RhoA pathways [2,3]. miR-199a/b-3p can inhibit tumor progression by suppressing the activation of the PAK4/ Raf/MEK/ERK pathway, and its decreased expression is closely related to the poor prognosis of HCC [4]. Cheng J et al. reported that miR-34a can influence the development of HCC by regulating p53 and cell cycle [5]; miR-145 targets IRS1 and regulates its downstream Akt/FOXO1 pathway, thus inhibiting HCC cell growth [6]. The analysis of 38 HCC tissue samples showed that miR-449a exhibited a low expression in HCC tissues, and miR-449a can influence the proliferation, metastasis, and cell cycle of HCC through its target gene Met. However, it is still unclear whether decreased miR-449a is closely related to HCC recurrence, metastasis, and survival time [7]. In addition, it has been reported that HDAC-1 [8], CDK6 [9], and E2F3 [10] are direct target proteins of miR-449a, whereas other target proteins regulated by miR-449a still need to be investigated.

In this study, HCC case number was increased to 142 for investigating the relationship between abnormal expression of miR-449a, the pathological characteristics, and survival time of HCC patients. Furthermore, in the Bel-7042 cell line model, iTRAQ technique was used to search for potential target proteins regulated by miR-449a.

## MATERIALS AND METHODS

### Collection of HCC clinical data and HCC tissues

One hundred forty-two HCC patients who were accepted at the Department of Hepatobiliary Surgery in our hospital from May 2014 to January 2019, were involved in this study. The resected HCC tissue samples were collected as well as the neighboring non-cancerous liver tissue specimens more than 1 cm around the tumor (no tumor cells were confirmed by pathology). All the samples were immediately placed in liquid nitrogen and transferred to -80°C within 2 hours for preservation. Patient information was as follows. The patients included 101 males and 41 females. The age ranged from 19 to 75 years old, with the median of 47 years, including 79 cases $< 60$ years and 63 cases $\geq 60$ years old. There were 104 patients diagnosed with complicated HBV and 38 patients without HBV. There were 88 cases with AFP $< 400$ μg/L and 54 cases with AFP $\geq 400$ μg/L. There were 115 cases with cirrhosis and 27 cases without cirrhosis. There were 113 patients with 1 tumor and 29 patients with tumor number $\geq 2$. There were 20 cases with tumor diameter $\leq 3$ cm and 122 cases with tumor diameter $> 3$ cm. There were 82 cases with tumor capsule and 60 cases without tumor capsule. Edmonson-Steiner classification: 74 cases of level I - II and 68 cases of level III - IV. The study was approved by the Ethical Review Committee of Shenzhen University.

### Pathological characteristics of HCC

The diameter of the HCC tumor was measured, and the number of nodules and capsule was recorded. HCC was confirmed by HE staining, and the degree of differentiation for HCC was graded according to Edmonson-Steiner classification. Level I - II cancer cells exhibited similar morphology with normal liver cells, and showed higher differentiation; level III - IV cancer cells had large and hyperchromatic nuclei, less cytoplasm, and lower differentiation. HCC metastasis was defined in patients with preoperative tumor thrombi (e.g., portal vein, hepatic vein, and cholangiocarcinoma thrombi), lymph node metastasis, and extrahepatic metastasis.

### Follow-up

Survival time and tumor-free survival time were calculated from the day of surgery. Follow-up files were established through outpatient visits, letter visits, and telephone calls. Color Doppler ultrasound, CT, MRI, serum AFP measurement, angiography or surgery was adopted to determine whether there was recurrence or metastasis. Follow-up was conducted until December 31, 2019. Follow-up was terminated upon patient's death or recurrence and metastasis of HCC. If no death, recurrence, metastasis or death from other causes was observed by the deadline, the data were deleted. The follow-up time ranged from 8 months to 68 months, and the median follow-up time was 10 months.

### iTRAQ screening

According to the literature reports [7], Bel-7042 cells were transfected with miR-449a-mimic and miR-449a inhibitor by Lipofectamine 2000. After confirming the transfection and establishing the cell proliferation model, cancer cells were collected 72 hours later. Based on the iTRAQ enzymolysis labeling procedure [11], 100 μg of protein was absorbed for enzymolysis by FASP method for each sample, and the peptides were labeled by iTRAQ Reagent Multiplex Buffer Kit (AB Sciex, USA). The miR-449a-mimic and miR-449a inhibitor groups were labeled with 115 and 117 reagents, and with 114 and 116 reagents, respectively. After terminating the labeling, fractionation of 15 components was performed by HPLC. Then the Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was used to collect the data. In combination with the human database (IPI. HUMAN. v 3.87.), protein identification and inter-group comparison were conducted by Mascot 2.2.2 search engine (Matrix Science, UK).

### Bioinformatics analysis

In order to clarify the functional significance of differentially expressed proteins, biological information analysis was carried out. GO analysis (http://www.geneontology.org) was employed to categorize proteins into families and subfamilies with shared functions, which includes three main modules: biological process, cellular component, and molecular function. Pathway analy-

sis using KEGG (http://www.genome.jp/kegg/pathway.html) was used to take advantage of the current knowledge of biochemical pathways and protein-protein interaction networks using STRING database (http://www.string-db.org) which is a database of known and predicted protein interactions, including direct (physical) and indirect (functional) associations.

**Statistical analysis**

SPSS 20.0 was used for statistical analysis. The data were expressed as mean ± standard deviation and independent sample *t*-test was used. Wilcoxon rank test was used to test the differences between the paired sample groups. Chi-square test was applied to analyze the correlation between miR-449a and the clinical pathological characteristics of HCC. Overall survival rate and tumor-free survival rate of HCC patients were calculated by Kaplan-Meier method, and the overall survival time and tumor-free survival time were compared by log-rank method. $p < 0.05$ was considered statistically significant.

## RESULTS

**miR-449a was significantly correlated to the survival time of HCC patients**

The expression level of miR-449a in 142 HCC tissues was significantly lower than that in paracellular tissues (ratio 1:3.8, $p < 0.001$, Figure 1A). According to the median RT-PCR results of the HCC tissues, 142 cases with HCC were divided into miR-449 low-expression group ($n = 95$) and high expression group ($n = 47$). The comparison analysis showed that the median overall survival time of miR-449 low expression group was significantly lower than that of the high-expression group (19 months vs. 37 months), and the overall survival rate was also significantly lower than that high expression group ($p = 0.001$, Figure 1B). In addition, the tumor-free survival time of the low-expression group was also significantly lower than that of the high-expression group (14 months vs. 24 months), and the tumor-free survival rate was significantly lower than that of the high-expression group ($p = 0.001$, Figure 1C).

**miR-449a is significantly correlated to HCC metastasis and recurrence**

Table 1 shows the relationship between miR-449 expression level and clinical pathologic characteristics. miR-449 expression level was significantly correlated with HCC tumor diameter ($p = 0.0002$), postoperative metastasis ($p < 0.0001$), and recurrence ($p < 0.0001$), but exhibited no significant correlation with the patient's gender, age, complicated HBV, cirrhosis, AFP levels, tumor number, tumor capsule, and pathological Edmonson-Steiner classification ($p > 0.05$).

**Difference analysis by iTRAQ**

It was observed that miR-449a was down-regulated in a series of HCC cell lines, of which Bel-7042 was the most significantly down-regulated ($p < 0.001$, Figure 2A). The Bel-7042 cell lines were selected for iTRAQ analysis. First, the transfection of the mimic group and the inhibitor group of miR-449a was verified (ratio = 78:1, $p < 0.0001$), and the Bel-7042 cell proliferation was negatively correlated with miR-449a down-regulation (ratio = 1:2.5, $p < 0.01$, Figure 2B). A total of 3,159 proteins were identified by iTRAQ ($p < 0.05$ and FDR < 1%), among which 137 were differentially expressed. Compared with the miR-449a inhibitor group, there were 88 differentially expressed proteins greater than 1.5 times and 49 down-regulated differentially expressed proteins in the miR-449a-mimic group, including the reported direct target proteins of miR-449a, such as HDAC1, Met, and CDK6, which confirmed the reliability of the iTRAQ results (Table S1).

**Bioinformatics analysis**

According to the GO classification system, differentially expressed proteins were distributed into categories based on molecular function, cellular components, and biological processes. For molecular functions, 11% of proteins were related to response to ATP binding, followed by metal ion binding (10%), RNA binding (7%) and enzyme binding (7%). For cellular components, 35% were related to nucleus and 35% to cytoplasm, followed by cytosol (34%) and extracellular exosome (24%). Type I interferon signaling pathway represented 7% of the biological processes, followed by transcription (6%), DNA-template (6%), negative regulation of cell proliferation (6%) (Figure 3). Then, the 137 differential expression proteins were further investigated using the KEGG database, and they were found to be enriched in human papillomavirus infection (8%), Epstein-Barr virus infection (7%), endocytosis (6%), cellular senescence (5%), and Kaposi sarcoma-associated herpes virus infection (5%, Figure 4). Finally, in order to better understand the pathogenic mechanisms, the protein interaction network for the identified variable proteins was constructed by STRING. From the network diagram (Figure 5), many proteins were at the core of the "traffic link," such as HDAC1, Met, IRPL-27, SQSTM1, and so on, which suggest that they may play an important role in the development of HCC.

## DISCUSSION

HCC is a highly malignant tumor, with about 365,000 new cases annually, ranking fourth in incidence of malignant tumors in China. The annual death cases are about 319,000, ranking second in mortality of tumors. The 5-year survival rate of HCC is only 30% ~ 40%. The recurrence and metastasis rate can reach 50% 2 years after surgery, and the 5-year recurrence and metastasis rate can be as high as 70% ~ 80% [12]. There-

**Table 1. Correlation of miR-449a expression with patients' clinicopathologic variables in HCC.**

| Content | Variable | Cases | miR-449a | | $\chi 2$ | p-value | Correlation |
|---|---|---|---|---|---|---|---|
| | | | Low expression | High expression | | | |
| **Gender** | | | | | **3.0391** | **0.0813** | **no** |
| | **male** | **101** | **72** | **29** | | | |
| | **female** | **41** | **23** | **18** | | | |
| **Age (years)** | | | | | **0.5944** | **0.4407** | **no** |
| | **< 60** | **79** | **55** | **24** | | | |
| | **≥ 60** | **63** | **40** | **23** | | | |
| **HBV** | | | | | **3.1741** | **0.0748** | **no** |
| | **yes** | **104** | **74** | **30** | | | |
| | **no** | **38** | **21** | **17** | | | |
| **Liver cirrhosis** | | | | | **3.4101** | **0.0648** | **no** |
| | **yes** | **115** | **81** | **34** | | | |
| | **no** | **27** | **14** | **13** | | | |
| **AFP (μg/L)** | | | | | **2.2981** | **0.1295** | **no** |
| | **< 400** | **88** | **63** | **25** | | | |
| | **≥ 400** | **54** | **32** | **22** | | | |
| **Tumor multiplicity** | | | | | **2.2641** | **0.1324** | **no** |
| | **1** | **113** | **79** | **34** | | | |
| | **≥ 2** | **29** | **16** | **13** | | | |
| **Tumor size (cm)** | | | | | **14.3210** | **<u>0.0002</u>** | **significant** |
| | **≤ 3** | **20** | **6** | **14** | | | |
| | **> 3** | **122** | **89** | **33** | | | |
| **Envelope** | | | | | **3.4451** | **0.0635** | **no** |
| | **yes** | **82** | **60** | **22** | | | |
| | **no** | **60** | **11** | **5** | | | |
| **Edmonson-Steiner stage** | | | | | **0.2894** | **0.5906** | **no** |
| | **I ~ II** | **74** | **48** | **26** | | | |
| | **III ~ IV** | **68** | **47** | **21** | | | |
| **Metastasis [a]** | | | | | **62.5010** | **< <u>0.0001</u>** | **significant** |
| | **yes** | **85** | **78** | **7** | | | |
| | **no** | **48** | **12** | **36** | | | |
| **Recurrence [b]** | | | | | **26.4710** | **< <u>0.0001</u>** | **significant** |
| | **yes** | **102** | **80** | **22** | | | |
| | **no** | **29** | **8** | **21** | | | |

**Note: Pearson's Chi-square test was used to analyze the correlation of miR-449a expression with patients' clinicopathologic variables in HCC, $\chi^2$ - indicates chi-square value, p < 0.05 was considered statistically significant, HBV - hepatitis B virus, AFP - alpha-fetoprotein.**
**[a] 9 cases of lost visit.**
**[b] 9 cases of lost visit, excluding 2 patients without recurrence but with a follow-up time of less than 12 months.**

fore, HCC has heavily threatened the health of Chinese and brings about a huge economic and social burden. Currently, effective treatment for the recurrence and metastasis of advanced HCC and postoperative HCC is still absent, and the pathogenesis is still not clear.

miRNAs are a class of non-coding RNAs with a length of about 19 ~ 25 nucleotides, which are characterized by high conservation, sequence homology, timing sequence, and tissue specificity. They play a transcriptional regulatory role by binding to the 3'-UTR of its target

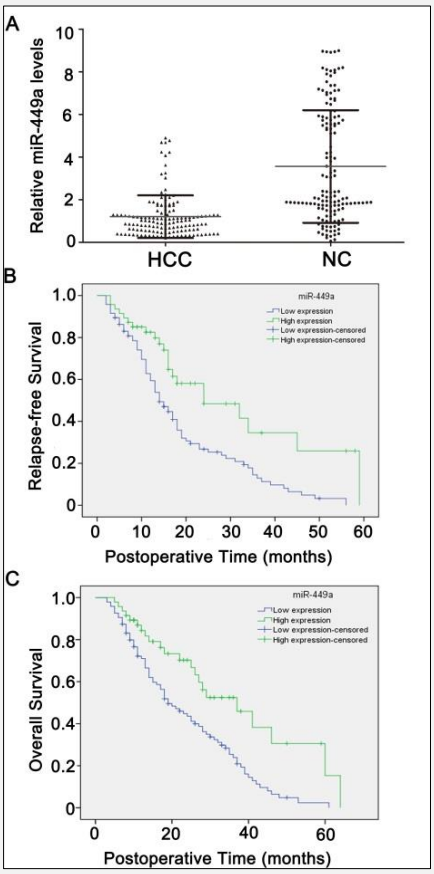


**Figure 1. The expression of miR-449a was significantly down-regulated in HCC tissues by RT-PCR compared with paracellular tissues (n = 142, A). Kaplan-Meier method was used to calculate the total survival time and tumor-free survival time of the low-expression group and the high-expression group of miR-449a, and the difference between the total survival time and tumor-free survival time was analyzed by log-rank method (n = 95 in the low-expression group, B, and n = 47 in the high-expression group of miR-449a, C).**

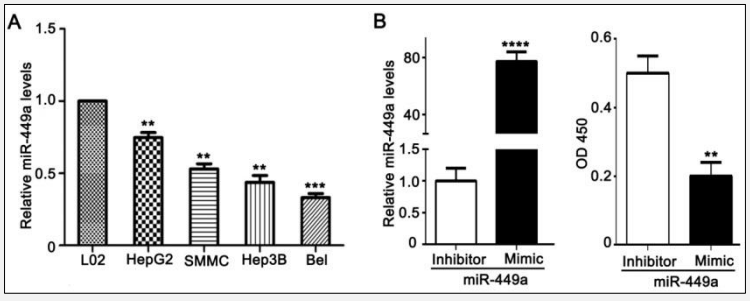


**Figure 2. miR-449a was down-regulated in HCC cell lines HepG2, SMMC, Hep3B, and Bel-704, among which the most significant down-regulation was in Bel-704 (A).**

**In both miR-449a-inhibitor and miR-449a-mimic groups, miR-449a was successfully transfected by RT-PCR. After 72 hours, OD450 of Bel-704 cells was detected to observe cell proliferation.**
**** p < 0.01, *** p < 0.001, **** p < 0.0001.**

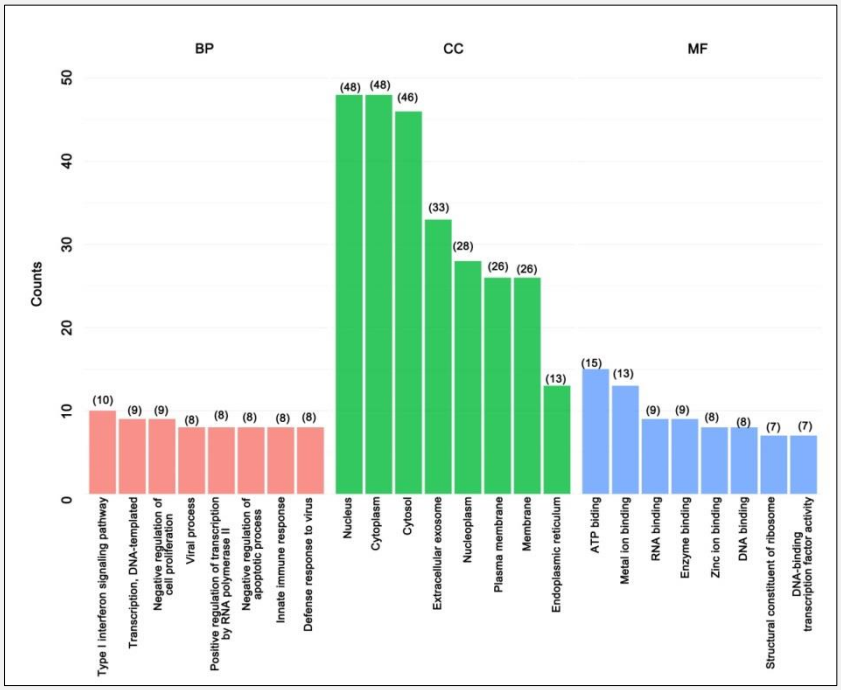


**Figure 3. GO assignment of differential expression of proteins. including molecular function (MF), cellular component (CC), and biological processes (BP).**

**Ordinate represents the number of proteins, abscissa represents specific enrichment pathway.**

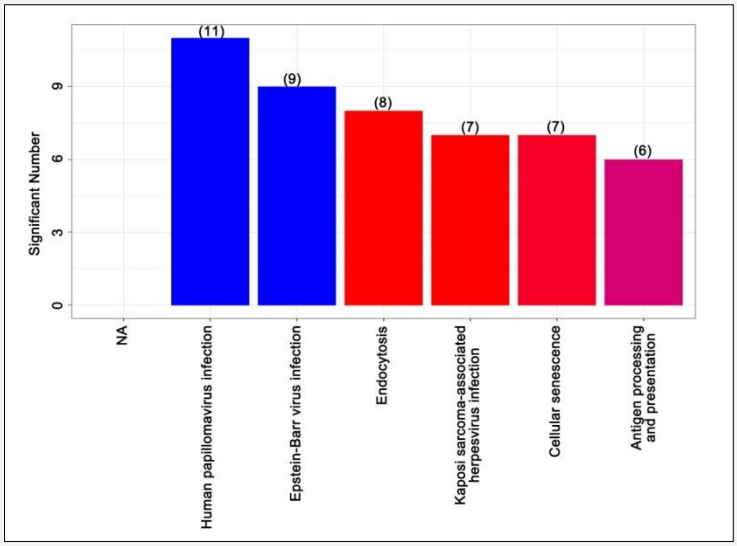


**Figure 4. KEGG enrichment analysis of differential expression of proteins, ordinate represents the number of proteins, abscissa represents specific enrichment classification.**

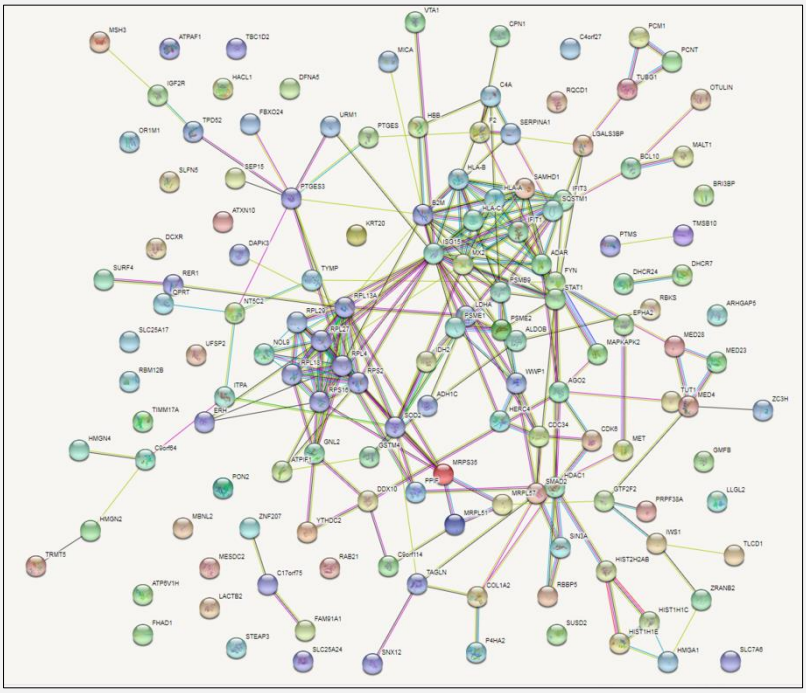

**Figure 5. Interaction network analysis of differential expression of proteins.**

**In this network, nodes are proteins, lines represent functional associations between proteins, and different line colors represent the types of evidence for the predicted functional association. A red line indicates the presence of fusion evidence; a green line indicates neighborhood evidence; a blue line indicates co-occurrence evidence; a purple line indicates experimental evidence; a yellow line indicates text mining evidence; a light blue line indicates database evidence; a black line indicates co-expression evidence.**

gene, thus affecting the downstream signaling pathway [13]. It has been reported that miR-21 [14], miR-221 [15], miR-222 [16], and so on are highly expressed in HCC, while miR-122a [17], miR-139 [18], miR-150 [19], and so on are less expressed in HCC tissues. Muralkami et al. used chip technology to analyze miRNA expression patterns in 24 HCC cancer tissues, 22 adjacent HCC tissues, and 9 HBV liver tissues, demonstrating that miR-222, miR-106a, miR-92, and miR-17-5p are significantly related to the degree of HCC differentiation [20]. Our team reported that abnormal expressions of miR-34a [5], miR-145 [6], and miR-449a [7] are involved in the regulation of HCC. It was reported that down-regulation of miR-449a was observed in 38 HCC tissues, and down-regulation of miR-449a in Bel-7042 cell lines promoted the proliferation, metastasis, and invasion of HCC. In this study, the sample size of HCC was expanded to 142 cases; the down-regulation of miR-449a in HCC tissues was further confirmed. It was demonstrated that the down-regulation of miR-449a was significantly related to HCC metastasis, recurrence, and survival time. Therefore, miR-449a is expected to be a potential biomarker for predicting the prognosis of HCC and a target for the prevention and treatment of HCC recurrence and metastasis [21].

Although miR-449a is very important, previous studies have discussed how to regulate HCC with miR-449a. It was reported that miR-449a is down-regulated in lung cancer tissues and negatively correlated with the prognosis of lung cancer, which can regulate the proliferation of lung cancer cells by acting on target gene E2F3 [10]. In addition, miR-449a was demonstrated to intervene in the occurrence of prostate cancer by mediating the expression of HDAC-1. Besides, miR-449a can also directly regulate the target genes CDK6 and CDC25A to affect tumor progression [8]. Our team has previously reported that miR-449a can affect the proliferation, metastasis, and invasion of HCC by regulating the target gene Met [7].

Additionally, other downstream target proteins regulated by miR-449a need to be identified.

iTRAQ technology was used to screen the downstream proteins involved in the regulation of miR-449a. iTRAQ is currently used for the global evaluation of protein expression in a specific disease or disease stage. Compared with traditional two-dimensional electropho-

resis, iTRAQ has higher throughput, sensitivity, and repeatability, which has been widely used in screening the biomarkers and differentially expressed proteins for various diseases. This iTRAQ research method is original and can find the target gene regulated by miR-449a from the source, but it also has some limitations, for example, the differential proteins cannot be directly considered as the direct target protein regulated by miR-449a, which needs to be verified by other molecular biological techniques [22]. In this study, a total of 137 differentially expressed proteins were screened through analysis. Compared with the miR-449a inhibitor group, 88 differentially expressed proteins greater than 1.5 times were observed in the miR-449a-mimic group, as well as 49 down-regulated proteins, including reported target proteins HDAC1, Met, and CDK6. GO analysis revealed that the molecular functions mainly include ATP binding, DNA/RNA binding, and ion binding, which was assumed to be related to the ATP energy consumption required for the proliferation and metastasis of HCC and the regulatory characteristics of miR-449a [23]. In the biological processes such as transcription regulation, cell proliferation and apoptosis, and viral infection, HCC is mainly infected by HBV virus, which will influence the proliferation and apoptosis of HCC cells by affecting the transcription and translation of a class of proteins. Thus, it was proposed that miR-449a plays a certain role in this intermediate link [24, 25]. The KEGG pathway was found to be mainly related to viral infection and immune antigen body, indicating that miR-449a plays a role in some key pathways affecting the pathogenesis of HBV infection [26]. Finally, STRING analysis showed that these differentially expressed proteins have complicated functions, especially a large class of proteins in the interlaced "hub center", which may play an important role in the development and progression of HCC, such as ITIT1, PRL-18, SOD2, and B2M, which also include the reported direct target genes Met and HDAC1. In the future, to determine if they are direct target proteins of miR-449a needs further studies.

In this study, it was confirmed that the down-regulation of miR-449a was significantly related to the metastasis, recurrence, and survival time of HCC. Second, iTRAQ technique was used for the first time to screen potential target proteins of miR-449a, which will provide a basis for further elucidating the mechanism of miR-449a-induced HCC.

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**Ethics approval:**
All the experimental protocols were approved by the Animal Ethics Committee of the First Affiliated Hospital of Shenzhen University, Health Science Center (Shenzhen, China).

**Declaration of Interest:**
None of the authors have any conflict of interest.

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