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Original Article

Expressions of miR-23a and glucose transporter 1 in primary hepatocellular carcinoma tissues and their clinical significance

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Abstract: Objective: To investigate the expressions of miR-23a and glucose transporter 1 (Glut-1) in primary hepatocellular carcinoma (HCC) tissues and the clinical significance. Methods: A prospective study was conducted in HCC tissues and para-carcinoma tissues from 278 patients. The relative expressions of miR-23a and Glut-1 mRNA, and the ratio of Glut-1 positive cells in HCC tissues were measured. After inhibition of miR-23a in HCC cells, cell migration and Glut-1 expression were observed. The HCC cells were divided into 3 groups according to transfection or inhibition conditions in the cells: negative control group (blank group), transfection control group (NC group), and miR-23a inhibition group (Hep3B miR-23a inhibiting group). Results: We show that larger tumor size, higher alpha fetoprotein (AFP), higher T, N, and M stages, distant metastasis or lymphatic metastasis and lower degree of differentiation were followed by higher relative expression of Glut-1 mRNA and ratio of Glut-1 positive cells, and higher relative expression of miR-23a (all $P<0.05$). The median survival of Glut-1 negative patients (29 months, 95% CI=27.659-30.341) was significantly higher than that of Glut-1 positive patients (25 months, 95% CI=24.133-25.867; $X^2=35.298$, $P<0.001$). The relative expression of miR-23a in the Glut-1 positive group was higher than that in the Glut-1 negative group ($P<0.05$). There was no difference in the number of invasive cells between the blank and NC groups ($P>0.05$), while after the inhibition of miR-23a, the number of invasive cells and the expression of Glut-1 protein were decreased significantly (both $P<0.001$). Conclusion: miR-23a and Glut-1 play an important role in the development of liver cancer. High expression of miR-23a and high expression of Glut-1 suggests poor prognosis. Inhibition of miR-23a expression can inhibit the migration of HCC cells and decrease Glut-1 expression, indicating that miR-23a may be a potential biomarker for the assessment of malignancy and prognosis of liver cancer.

Keywords: miR-23a, glucose transporter 1, primary hepatocellular carcinoma, clinical significance, prognosis

Introduction

Hepatocellular carcinoma (HCC) is a common type of tumor, ranking fifth in tumor morbidity and second in tumor mortality [1]. There are more than 700,000 newly diagnosed patients and about 0.5-1 million new deaths, every year in the world, and more than half of these deaths are in China [2]. In the past two decades, the annual morbidity and mortality of HCC have increased significantly, and studies have found that HCC is highly prevalent in Asia and Africa [3]. The etiology of HCC is closely related to environmental and genetic factors [4]. At present, there are many studies on liver cancer biomarkers, among which miRNA is the most widely studied. It has been found in studies of miRNA that inhibition of miRNA can regulate the differentiation, proliferation and apoptosis of cells. mRNA plays an extremely important role in the occurrence and development of cancer and more than 50% of human malignant tumors are related to miRNA genes [5, 6].

miR-23a, a member of the miRNA gene family, it is abnormally expressed in a variety of malignant tumors [7, 8]. Studies of liver cancer have found that the abnormal expression of miR-23a is related to p53 functional status and miR-23a expression is dependent on p53 [9]. Another

study has found that p53 signaling pathway can inhibit glucose uptake by inhibiting the expression of glucose transporter 1 (Glut-1) [10]. Glut-1 is an important member of the glucose transporter protein and has low expression in normal epithelial cells and para-carcinoma epithelial cells [11, 12]. Studies have shown that Glut-1 is a key factor limiting the speed of glucose transport in tumor cells and that it is overexpressed in various tumors [13-16]. Previous research has indicated that tumor cells can increase glucose uptake under the mediation of Glut [17] and Glut plays an important role in tumor formation and metastasis [18, 19]. Glut-1 is very relevant in the occurrence and development of tumors and the growth and proliferation of tumors depend on the survival response of glycolysis [20, 21]. Glut-1 provides a large amount of energy for tumor growth and development, so it is overexpressed in cancer tissues, and it also plays a key role in tumor invasion and metastasis [19, 22]. Therefore, Glut-1 has become an important target for tumor intervention. Previous work has confirmed that inhibition of Glut-1 expression in osteosarcoma cells can make tumor cells reduce glucose uptake. The reduction of glucose supply prevents tumor cells from obtaining enough energy so they stay in low oxygen consumption stage or directly undergo programmed cell death, and tumor invasion and metastasis are reduced at the same time [23, 24].

We hypothesized that the abnormal expression of miR-23a in HCC tissues might affect the expression of Glut-1, thereby affecting tumor invasion and metastasis. The expression of miR-23a and Glut-1 in HCC tissues and their clinical pathology and prognosis, and the relationship between miR-23a and Glut-1 were explored in this study.

Materials and methods

General information

A prospective study was conducted in hepatocellular carcinoma (HCC) tissues and para-carcinoma tissues from 278 patients admitted in the Department of Oncology in the Affiliated Tumor Hospital of Guangxi Medical University from March 2014 to July 2015. These patients included 227 males and 51 females, aged from 25 to 71 years old, with an average of 49.1± 10.0 years old. The study was approved by the Ethics Committee of Affiliated Tumor Hospital of Guangxi Medical University and all patients signed an informed consent form.

Inclusion criteria were: 1) Diagnosis criteria of liver cancer and TNM staging referred to *The standard for diagnosis and treatment of primary liver cancer (2017 edition)* [25]; 2) Patients aged between 18-75 years old; 3) All patients underwent liver cancer surgery in the Affiliated Tumor Hospital of Guangxi Medical University to obtain HCC tissues and para-carcinoma tissues, and the obtained tissue specimens were stored at -80°C. Patients with incomplete clinical data, serious heart, liver, kidney and other diseases, mental illness or cerebrovascular disease, or other cancers were excluded in this study.

Methods

Qualitative determination of Glut-1 in HCC tissues and para-carcinoma tissues: The HCC tissues and para-carcinoma tissues were fixed in formaldehyde solution. The samples were dehydrated and transparentized using ethanol and xylene, and then embedded in paraffin. The specimens were cut into ~ 2 to 3 μm slices. In this study, mouse anti-human Glut-1 polyclonal antibody (Beijing Zhongshan Goldenbridge Biotechnology Co. Ltd., China) and kit (Guangzhou Dingguo Bio-technology Co., Ltd., China) were used to determine the expression of Glut-1 protein by immunohistochemistry SP method. Details are as follows. After routine sectioning of the tissues, the samples underwent thermal antigen retrieval under high temperature and pressure. To the samples we added goat serum, primary antibody (1:200) and secondary antibody (1:1,500), and then were stained with DAB solution (Guangzhou Dingguo Bio-technology Co., Ltd., China) after 12 hours. The samples were washed with water, dehydrated in gradient alcohol and then fixed in xylene (Guangzhou Dingguo Biotechnology Co., Ltd., China). Qualitative determination of Glut-1 is defined as the product of the percentage of positive cells and staining degree observed under random 5 high power microscope viewing regions (Shanghai Mubo Biotechnology Co., Ltd., China). The percentage of positive cells: ≤1%, 0 score; 2-10%, 1 score; 11-50%, 2 scores; 51-80%, 3 scores; 81-100%, 4 scores. Staining degree: negative, 0 score; weak posi-

tive, 1 score; positive, 2 scores; strong positive, 3 scores. Product score: 0, negative Glut-1; 1-4, weak positive Glut-1; 5-7, positive Glut-1; 8-12, strong positive Glut-1. Glut-1 positive rate = Number of cases of (weak positive + positive + strong positive)/total number of cases [26].

Determination of relative expressions of miR-23a and Glut-1 mRNA in HCC tissues and para-carcinoma tissues: The specimens confirmed by pathological sections were confirmed to be 2-3 mm^2 HCC tissues and para-carcinoma tissues from the -80°C freezer. The Trizol kit was purchased form Molecular Research Center, Inc., USA, and the upstream and downstream primers were designed and supplied by Guangzhou Ruibo Biotechnology, China [27]. miRNA was reversely transcribed into cDNA using reverse transcription kit (Fernentas, Canada) and DNA amplification was performed according to this template. Then the expressions of miRNA-23a and Glut-1 mRNA in the samples were determined by fluorescent probe. The upstream and downstream primer sequences of miRNA-23a were 5'-ATCACATTGCCAGGGATTTCC-3' and 5'-CAGTGCGTGTCGTGGAGT-3', and those of Glut-1 mRNA were 5'-AACTCTTCAGCCAGGGTCCAC-3' and 5'-CACAGTGAAGATGATGAAGAC-3'. The reaction mixture (25 μL) consisted of: (2×) 12.5 μL SYBR premix, 0.5 μL each of the forward and reverse primers, 2.0 μL cDNA, and 9.5 μL ddH_2O. The reaction conditions were: 94°C for 4 min, 95°C for 40 s, 60°C for 30 s, 72°C for 30 s, and after 35 cycles, 72°C for 1 min. The products of PCR amplification were detected by quantitative fluorescence. The relative expression of each gene was calculated by the $2^{-\Delta\Delta Ct}$ method with U6 snRNA as an internal reference.

Effects of the inhibition of miR-23a on the migration of HCC and the expression of Glut-1 protein

Cell culture and transfection: Hepatoma cell line Hep3B (Shanghai Cell Bank of Chinese Academy of Sciences, China) was cultured in the medium containing 10% fetal bovine serum, 100 IU/mL penicillin and 100 μg/mL streptomycin (all purchased from Guangzhou Dingguo Bio-technology Co., Ltd., China). Cell transfection was performed according to the instruction of Lipofectamine™ 2000 reagent (Guangzhou Dingguo Bio-technology Co., Ltd., China). The cells were inoculated in 6-well plates and cultured until the cells reached 80% confluency. Twenty-four hours before transfection, normal serum was replaced with DMEM medium containing 100 mL/L fetal bovine serum without double antibody. Then 100 nmol miR-23a mimics (miR-23a) and 100 nmol negative control (miR-control) were transfected into Hep3B cells, and the transfection reagent was Lipofectamine™ 2000. The cells were divided into 3 groups: negative control group (blank group), transfection control group (NC group), and miR-23a inhibition group (Hep3B miR-23a inhibiting group).

Cell invasion: The cells of three groups were prepared into cell suspensions at a concentration of 1×10^6/mL in serum-free medium. The suspensions were placed in the upper chamber of Transwell and 20% fetal bovine serum was added to the lower chamber as a medium. The suspensions were cultured for 24 h at 37°C. Then the cells in the suspension were stained with 5% crystal violet, and the number of cells was observed under a microscope.

Inhibition of miR-23a expression of Glut-1 protein: Western blot was performed in this study. The cells were washed three times with PBS, and were added to lysis buffer (Beijing Zhongshan Goldenbridge Biotechnology Co. Ltd., China) containing protease inhibitor for 5 min at 100°C. Then the total protein was extracted. The protein samples were fractionated by SDS-PAGE and transferred on PVDF membrane (Beijing Zhongshan Goldenbridge Biotechnology Co. Ltd., China). The membrane was blocked with 5% BSA for 1 h, and then incubated with primary antibody overnight at 4°C. The primary antibody was mouse anti-human Glut-1 polyclonal antibody (1:200). Then the membrane was incubated with peroxidase-labeled secondary antibody (1:1,500; Beijing Zhongshan Goldenbridge Biotechnology Co. Ltd., China) at room temperature. The membrane was placed in the MultiImager (Beijing Zhongshan Goldenbridge Biotechnology Co. Ltd., China) for exposure and photographing. Then the gray-scale values were calculated.

The illuminating liquid gel (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) was subjected to exposure imaging and the relative expression amount of the gray value was calculated.

Table 1. Comparison of general information and baseline information

Item	Number of cases (n, %)
Age (year old)	
≥60	49 (17.63)
<60	229 (82.37)
Gender	
Male	227 (81.65)
Female	51 (18.35)
Tumor size	
≤2.0 cm	26 (9.36)
2.1-4.9 cm	123 (44.24)
≥5.0 cm	129 (46.40)
AFP level	
<40	70 (25.18)
40-399	106 (38.13)
≥400	102 (36.69)
T staging	
T1	85 (30.58)
T2	50 (17.99)
T3	117 (42.09)
T4	26 (9.35)
N staging	
N0	258 (92.81)
N1	20 (7.19)
M staging	
M0	244 (87.77)
M1	34 (12.23)
Degree of tumor differentiation	
Low	74 (26.62)
Middle	160 (57.55)
High	44 (15.83)

Note: AFP, alpha fetoprotein.

Statistical analysis

SPSS 17.0 statistical software was used to analyze the data. Continuous variables were expressed as the mean ± standard deviation. Data following a normal distribution and homogeneity of variance were compared by t-test and expressed by t, conversely by rank sum test and Z. Comparison of data between multiple groups was made using one-way analysis of variance (ANOVA) and pairwise comparison between two groups was performed using Turkey method. Count data were expressed as percent (%) and were analyzed by Pearson chi-square test. Survival analysis was performed by Kaplan-Meier method and log-rank test, and was expressed as X^2. $P<0.05$ is considered statistically significant.

Results

General information and baseline information

A total of 278 patients with HCC were included in this study and the details are shown in **Table 1**.

Expression of Glut-1 protein in HCC

It was observed under a microscope that, ≤1% positive Glut-1 cells and negative staining were seen in the HCC tissues of Glut-1 negative patients and the opposite results were seen in that of Glut-1 positive patients. See **Figure 1**.

Comparisons of the expressions of miR-23a and Glut-1 in the general and baseline information

In HCC, the relative expression of miR-23a and Glut-1 miRNA, and the positive rate of Glut-1 expression showed no difference in regard to age and gender (all $P>0.05$). Larger tumor size, higher alpha fetoprotein (AFP), higher T, M and N stages, and lower degree of differentiation were followed by higher relative expression of Glut-1 mRNA and ratio of Glut-1 positive cells, and higher relative expression of miR-23a in HCC tissue (all $P<0.05$). See **Table 2**.

Comparison of median survival between Glut-1 positive and negative patients

The median survival of Glut-1 negative patients (29 months, 95% CI=27.659-30.341) was significantly higher than that of Glut-1 positive patients (25 months, 95% CI=24.133-25.867; $X^2=35.298$, $P<0.001$). See **Figure 2**.

Comparison of cell invasion and the effects of inhibition of miR-23a on Glut-1 protein

There was no difference in the number of invasive cells and the expression of Glut-1 protein between the Blank group and the NC group (both $P>0.05$), while after the inhibition of miR-23a, those were decreased significantly (both $P<0.001$). See **Figures 3**-**5**.

Inhibition of miR-23a on the expression of Glut-1 protein

The experimental study found that there was no difference in the expression of Glut-1 between

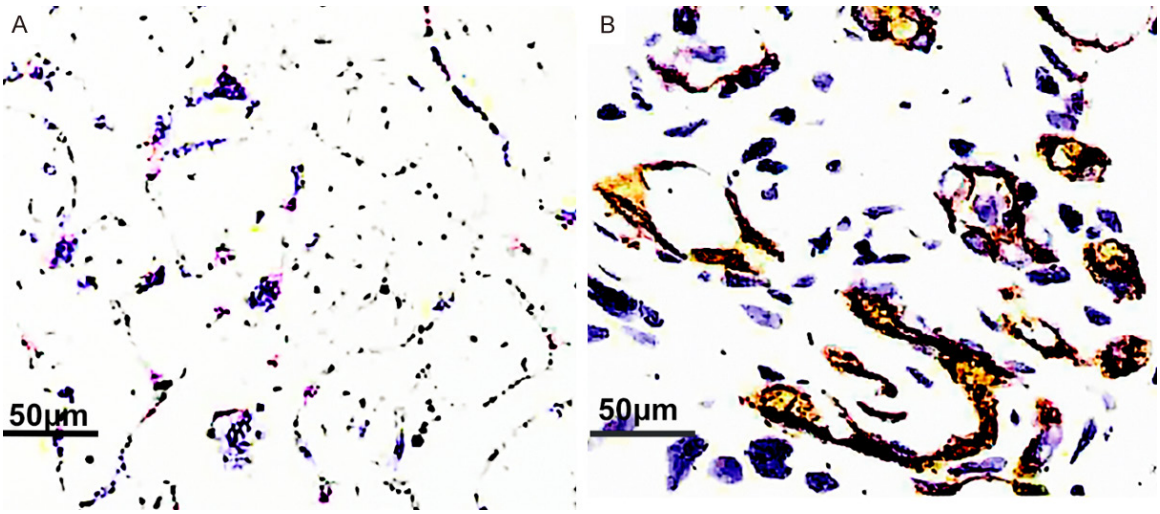

Figure 1. The expression of Glut-1 protein in hepatocellular carcinoma. A. Glut-1 negative expression; B. Glut-1 positive expression.

Table 2. Comparisons of the expressions of miRNA-23a and Glut-1 in the general and baseline information

Item	Relative expression of mirna-23a	Number of Glut-1 negative cases	Number of Glut-1 positive cases	Relative expression of Glut-1 miRNA
Age (year)				
≥60 (n=49)	3.42±0.34	9 (18.37)	40 (81.63)	1.93±0.16
<60 (n=229)	3.41±0.64	34 (14.85)	195 (85.15)	1.93±0.21
F/t	0.123	0.383		0.099
P	0.876	0.563		0.921
Gender				
Male (n=227)	3.41±0.64	32 (14.10)	195 (85.90)	1.93±0.20
Female (n=51)	3.40±0.68	11 (21.57)	40 (78.43)	1.93±0.18
F/t	0.283	1.778		0.087
P	0.804	0.182		0.931
Tumor size				
≤2.0 cm (n=26)	3.25±0.40	13 (50.00)	13 (50.00)	1.74±0.09
2.1-4.9 cm (n=123)	3.66±0.22^{aa}	22 (17.89)	101 (82.11)^{aaa}	1.86±0.13^{aa}
≥5.0 cm (n=129)	3.76±0.36^{aaa,bbb}	8 (6.20)	121 (93.80)^{aaa,bbb}	2.03±0.22^{aaa,bbb}
F/t	63.053	578.178		43.030
P	<0.001	<0.001		<0.001
AFP level				
<40 (n=70)	3.22±0.40	21 (30.00)	49 (70.00)	1.81±0.12
40-399 (n=106)	3.62±0.26^{cc}	17 (16.04)	89 (83.96)^{c}	1.86±0.13^{c}
≥400 (n=102)	3.71±0.40^{ccc,ddd}	5 (4.90)	97 (95.10)^{ccc,d}	2.08±0.20^{ccc,ddd}
F/t	65.232	612.234		43.030
P	<0.001	<0.001		<0.001
T staging				
T1 (n=85)	3.08±0.46	21 (24.71)	64 (75.29)	1.79±0.11
T2 (n=50)	3.19±0.54	10 (20.00)	40 (80.00)	1.88±0.12^{eee}
T3 (n=117)	3.27±0.69	12 (10.26)	105 (89.74)^{ee}	1.98±0.16^{eee,fff}
T4 (n=26)	4.02±0.41^{eee,fff,ggg}	0 (0.00)	26 (100.00)^{eee,f}	2.20±2.00^{eee,fff,ggg}
F/t	6.055	13.337		26.365

P	0.001	0.004		<0.001
N staging				
N0 (n=258)	3.23±0.44	41 (15.89)	217 (84.11)	1.90±0.17
N1 (n=20)	3.44±0.58	2 (10.00)	18 (90.00)	2.35±0.18
F/t	2.662	2.231		10.285
P	0.009	0.067		<0.001
M staging				
M0 (n=244)	3.13±0.32	41 (16.80)	203 (83.20)	1.90±0.17
M1 (n=34)	3.44±0.56	2 (5.88)	32 (94.12)	2.22±0.21
F/t	4.661	3.168		9.650
P	0.001	0.075		<0.001
Degree of tumor differentiation				
Low (n=74)	3.25±0.38	6 (8.11)	68 (91.89)	2.14±0.19
Middle (n=160)	3.61±0.30^{hhh}	21 (13.12)	139 (86.88)	1.89±0.14^{hhh}
High (n=44)	3.73±0.42^{hhh,iii}	16(36.36)	28(63.64)^{hhh,iii}	1.73±0.08^{hhh,iii}
F/t	72.382	20.216		43.030
P	<0.001	<0.001		<0.001

Note: AFP, alpha fetoprotein. Compared with tumor size ≤2 cm, ^{aa}P<0.01, ^{aaa}P<0.001; compared with tumor size 2.1-4.9 cm, ^{bbb}P<0.001; compared with AFP<40, ^{c}P<0.05, ^{cc}P<0.05, ^{ccc}P<0.001; compared with AFP between 40 to 399, ^{d}P<0.05, ^{ddd}P<0.001; compared with T1, ^{ee}P<0.01, ^{eee}P<0.001; compared with T2, ^{f}P<0.05, ^{fff}P<0.001; compared with T3, ^{ggg}P<0.001; compared with low tumor differentiation, ^{hhh}P<0.001; compared with middle tumor differentiation, ^{iii}P<0.001.

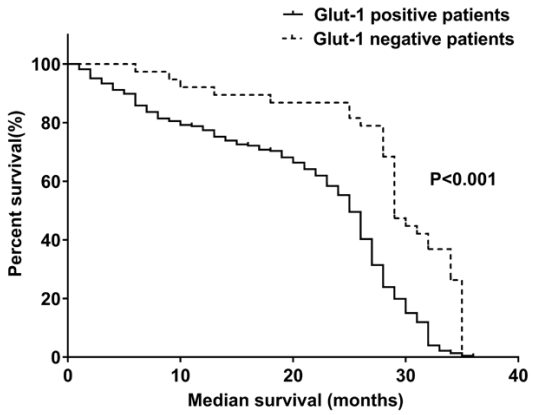

Figure 2. Comparison of median survival between Glut-1 positive and negative patients. OS, overall survival.

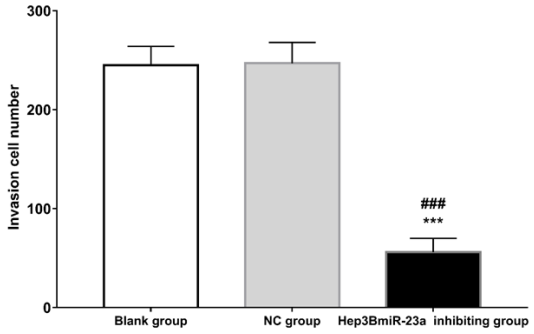

Figure 3. Detection of cell invasion. Compared with blank group, ***P<0.001; compared with NC group, ###P<0.001.

the blank and NC groups, but the expression of Glut-1 protein decreased after miR-23a inhibition, as shown in **Figure 5**.

Discussion

Studies have shown that miR-23a is closely related to the growth and metastasis of a variety of malignant tumors [8]. Research on gastric cancer has found that the inhibition of miR-23a can reduce tumor cell apoptosis and increase tumor invasion, and promote malignant metastasis of tumor cells [25]. Studies of lung cancer have indicated that the expression of miR-23a is associated with tumor cell proliferation, migration, and clinical malignancy [26]. Work on colon cancer has demonstrated that miR-23a can inhibit cell growth and metastasis in colon cancer [27]. The above studies have shown that the expression of miR-23a is downregulated in tumor tissues. This study also found that larger tumor size, higher AFP, higher T, M and N stages, and lower degree of differentiation were followed by lower relative expression of miR-23a, which is consistent with the above studies.

Research from abroad has shown that high expression of Glut-1 in oral epithelial dysplasia indicates an increased risk of oral cancer [28]. Studies also have found that there is no Glut-1 expression in normal endometrium; while Glut-

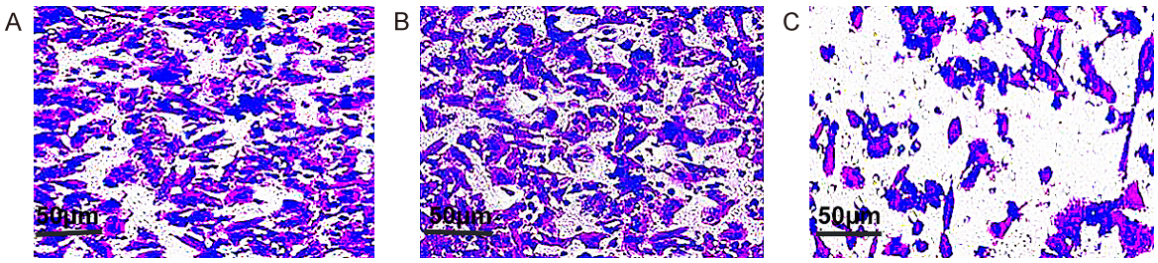

Figure 4. Comparison of cell invasion under the microscope. A: Blank group; B: NC group; C: Hep3BmiR-23a inhibiting group.

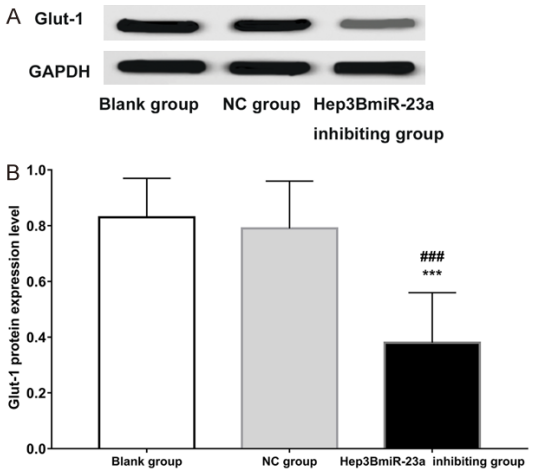

Figure 5. Comparison of Glut-1 protein expression. A: Qualitative comparison; B: Quantitative comparison. Compared with blank group, ***P<0.001; compared with NC group, ###P<0.001.

1 expression is elevated in endometrial carcinoma and atypical endometrial hyperplasia, and Glut-1 expression in the former is higher than that in the latter [29]. Studies on colorectal cancer have indicated that Glut-1 expression is associated with patient's age, tumor stage and lymphatic metastasis [30]. The above data show that Glut-1 plays an important role in the occurrence and development of tumors, because high expression of Glut-1 can provide energy for tumor growth to enhance tumor invasion and distant metastasis of surrounding tissues. This study also found that larger tumor size, higher AFP, higher T, M and N stages, and lower degree of differentiation were followed by higher relative expression of Glut-1 mRNA, indicating that high expression of Glut-a is related to the enhancement of tumor invasion, which is consistent with the above studies.

A previous study on Glut-1 in the prognosis of cancer included 269 patients with lung cancer and it reported that the positive rate of Glut-1 expression in lung squamous cell carcinoma (99%) was higher than that in lung adenocarcinoma with a statistical difference. Further multivariate regression analysis found that high expression of Glut-1 was negatively correlated with survival time in patients with lung adenocarcinoma [31]. It is also found that high expression of Glut-1 in pancreatic cancer cells is related to the decline of overall survival (OS) after pancreatic cancer resection [32]. A meta-analysis had described that high expression of Glut-1 is associated with tumor stage, degree of differentiation and tumor size, and is negatively correlated with disease-free survival and OS [33]. The aforesaid studies show that high expression of Glut-1 can enhance tumor invasion, metastasis, and rapid growth, leading to the decrease of patients' survival time. In this study, we also found that Glut-1 negative patients had higher median survival than Glut-1 positive patients with a statistical difference, suggesting that high expression of Glut-1 enhances tumor invasion and metastasis, resulting in shorter survival time in the patients. The results of this study were consistent with those of above studies.

Previous studies have shown that miR-23a can accelerate lung cancer progression by enhancing invasion and metastasis of lung cancer A549 cells [34]. Another research has found that up-regulation of miR-23a promotes proliferation of lung adenocarcinoma cells [35]. Inhibition of miR-23a expression in osteosarcoma cells, renal cell carcinoma and breast cancer cells can significantly reduce tumor invasion [36-38]. Transfected pancreatic cancer cells can inhibit miR-23a and reduce cell migration [39]. In the study on the effects of RNA on Glut-1 protein signaling pathway, a study has shown that low expression of lincRNA-p21 in the patients with liver cancer can

inhibit P53-mediated glucose metabolism. p53 signaling pathway is a negative regulator for glycolysis [40], and it can inhibit Glut-1 to limit glucose uptake [10]. p53 is an important signaling pathway for glycolysis. Previous studies on liver cancer have found that miR-23a can affect the functional status of p53 signaling pathway [9]. Therefore, we speculated that miR-23a may regulate Glut-1 and affect the migration of liver cancer cells through affecting the p53 signaling pathway. In this study, it was also confirmed that inhibition of miR-23a expression inhibited the migration of HCC cells and decreased Glut-1 expression, which is consistent with the above studies.

The shortcoming of this study is small sample size. Therefore, the sample size can be further expanded for multi-center research, and the mechanisms of miR-23a and Glut-1 in liver cancer can be further studied.

In summary, miR-23a and Glut-1 are important in the development of liver cancer. Low expression of miR-23a and high expression of Glut-1 indicate poor prognosis. Inhibition of miR-23a expression can inhibit liver cancer cell migration and reduce Glut-1 expression; therefore, miR-23a may be a potential biomarker for the malignancy and prognosis of liver cancer.

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Disclosure of conflict of interest

None.

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