European Review for Medical and Pharmacological Sciences 2016; 20: 4898-4904

Long non-coding RNA MVIH acts as a prognostic marker in glioma and its role in cell migration and invasion

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Abstract. – OBJECTIVE: High expression levels of lncRNA associated with microvascular invasion in HCC (lncRNA MVIH) were found to correlate with several solid tumors. However, little is known concerning the function of MVIH in glioma. The purpose of our study is to explore the role of lncRNA MVIH in clinical glioma samples and cell lines.

PATIENTS AND METHODS: The expression levels of MVIH were analyzed in glioma surgical resection tissues and cells by RT-PCR. Additionally, the associations of MVIH expression with clinicopathological features were analyzed. Survival and Cox proportional-hazards regression analyses were performed to determine the correlation between MVIH expression levels and prognosis in the patients. The cell proliferation, migration ability, invasion ability were measured successively by CKK-8 assay, transwell and wound healing assay.

RESULTS: We found that MVIH was significantly upregulated in glioma cell lines and tissues. Furthermore, MVIH expression was positively correlated with KPS and WHO grade. Patients with MVIH high expression tumors had a worse overall survival compared to patients with MVIH high expression tumors. Moreover, univariate and multivariate Cox regression analysis confirmed that MVIH was an independent risk factor for glioma. Finally, *in vitro*, we showed that up-regulation of MVIH expression promoted human glioma cells proliferation, invasion and migration, while down-regulation of MVIH expression suppressed human glioma cells proliferation, invasion and migration.

CONCLUSIONS: Our findings indicated that MVIH expression may serve not only as a prognostic marker but also as a potential therapeutic target in glioma.

Key Words:
lncRNAs, MVIH, Metastasis, Prognosis, Cell growth.

Introduction

Gliomas are the most common primary brain tumors in adults with a histological grade that ranges from low (WHO I, II) to high-grade (WHO III, IV)[1]. Although the microsurgical therapy, radiotherapy and chemotherapy and other treatments have been applied in the therapy of glioma, the average survival of malignant glioma patients has been improved only slightly in the past decades[2]. The major reasons that making the gliomas be incurable are the tendency of glioblastoma cells to infiltrate into surrounding brain tissue[3,4]. Therefore, it is valuable to search for valuable factors for early diagnosis for patients with a high risk of metastasis, prognosis prediction, and novel therapeutic strategies.

LncRNAs (>200 nt in length) are a class of non-coding RNAs that lack protein-coding capacity[5]. Recent research[6-8] has shown that lncRNAs participate in a large number of cellular processes, such as cell proliferation, differentiation, apoptosis, and cell cycle progression. The relatively new field of lncRNA research has focused on their value in the prognosis and treatment of cancer. Specifically, H19 is reported to contribute to gastric cancer[9], PCAT-1 related to esophageal squamous carcinoma[10], and ANRIL led to hepatocellular carcinoma[11]. However, the clinical prognosis significance and potential functions of MVIH have not been reported.

lncRNA associated with microvascular invasion in HCC (lncRNA MVIH) was a newly found lncRNA[12]. Abnormal expression of MVIH has been found in breast cancer[13], lung cancer[14] and hepatocellular carcinoma[15]. In the present study, we evaluated the expression of MVIH in tumor tissues and cells. Next, we explored the prognostic value of MVIH in glioma. Finally, based on *in vitro* assays, we explored the role of MVIH in the progression of glioma cells.

Patients and Methods

Patients and Specimens

The present study was approved by the Ethics Committee of Weifang People's Hospital, and

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each patient had written informed consent. The surgical specimens of 127 gliomas were collected in Weifang People's Hospital. The samples were obtained at the time of surgery and were immediately snap-frozen in liquid until use. None of the patients had a history of other tumor or received preoperative treatment. Histologic identification was confirmed by an experienced pathologist. The clinical characteristic of 127 patients with glioma was summarized in Table I.

Cell Lines and Cell Culture

The human U251, U87, U118 and LN18 cell lines were purchased from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Hongqiao, Shanghai, China). Primary normal human astrocytes (NHA) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and grown in humidified 5% CO_2 at 37°C.

Real-Time PCR

Total RNA was extracted from tissue samples with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The first strand of complementary DNA (cDNA) was synthesized with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). Quantitative real-time PCR (RT-PCR) was performed using an ABI Prism 7900 detection system (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. The specific primers used are as follows:

MVIH sense, 5′-AATTTTGCACATCTGAACAGCC-3′ and

reverse, 5′-TTCAAAATCCCACTACGCCCA-3′ GAPDH

sense, 5′-GTCAACGGATTTGGTCTGTATT-3′ and reverse,

5′-AGTCTTCTGGGTGGCAGTGAT-3′. GAPDH was used as an internal control. The RNA expression levels were normalized to GAPDH by using the $2^{-\Delta\Delta CT}$ methods.

Plasmid Vector Construction and Transfection

The plasmid constructs, pcDNA-MVIH and the empty vector pcDNA (used as negative control) were obtained from Genepharma (Pudong, Shanghai, China). Non-specific siRNA (si-NC) and si-MVIH were purchased from Invitrogen (Carlsbad, CA, USA). Plasmid transfections were performed using Lipofectamine 2000.

CCK-8 Assay

The in vitro cell proliferation was measured using the CCK-8 method. Briefly, U87 cells were plated at 5.0×103 cells per well in 96-well plates and incubated overnight in Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS). After transfection, 10 µl CCK-8 liquid was added to the test well and incubated for 3 h. cellular viability was determined by measuring the absorbance of the converted dye at 450 nm. Each data point was determined three times before analysis.

Trans-well Invasion Assay

Cell invasion was examined using the 24-well transwell chambers with a layer of matrigel (Chemicon, Carlsbad, CA, USA). Briefly, U87 cells were transfected with pcDNA-MVIH or si-MVIH, or their respectively negative controls and then incubated for 24 h at 37°C in DMEM. RPMI-

Table I. Associations between MVIH expressions with the clinicopathological characteristics of glioma patients.

		MVIH expression		
Variable	**Number**	**Low**	**High**	**p**
Age (years)				NS
<50	48	21	27	
≥50	79	44	35	
Gender				NS
Male	53	30	23	
Female	74	35	39	
Tumor size (cm)				NS
<5	86	47	39	
≥5	41	18	23	
Tumor location				NS
Supratentorial	95	50	45	
Subtentorial	32	15	17	
KPS				0.001
<80	59	21	38	
≥80	68	44	24	
WHO grade				0.015
I-II	57	36	21	
III-IV	70	29	41	
Tumor recurrence				NS
Absent	87	47	40	
Present	40	18	22	
Surgery				NS
GTR	66	35	31	
PR	61	30	31	

Abbreviation: NS, difference between groups was not statistically significant.

1640 medium containing 20% FBS was added to the lower chamber. Following a 24-h incubation, the non-filtered cells were gently removed with a cotton swab. After 48 h, the cells that had invaded through the membrane were fixed with 20% methanol and stained with 0.1% crystal violet for 30 min, imaged, and counted using a microscope.

Wound Healing Assay

To examine the migration ability of cells in vitro, a wound-healing assay was performed. The artificial wounds were produced on the confluent cell monolayer with FBS-free, using a 200 µl pipette tip at 24 h post pcDNA-MVIH or si-MVIH, or their respectively negative controls transfection. Migration of cells into the wound was observed and photographed under an inverted microscope at indicated time (0 h and 24 h).

Statistical Analysis

All data are showed as mean ± standard deviation (SD), and all experiments were repeated at least three times independently. The difference among the groups in the proliferation, invasion and migration assay was estimated by Student's t-test or one-way ANOVA. Multiple comparisons between the groups were performed using S-N-K method. The correlation analysis between MVIH level and clinical stages was evaluated using the X^2-test. Survival curves were plotted using the Kaplan-Meier method and differences in survival rates were analyzed using the log-rank test. The multivariate analyses were evaluated with Cox proportional hazards models. Differences were considered statistically significant when $p < 0.05$. SPSS software for Windows (version 13.0; SPSS Inc., Chicago, IL, USA) was used to perform statistical analyses in this study.

Results

MVIH is Significantly Upregulated in Glioma Tissues and Cell Lines

To explore the role of MVIH in the development of glioma, we measured the levels of MVIH expression in glioma and normal tissues by qRT-PCR. As shown in Figure 1A, MVIH was markedly upnregulated in glioma samples when compared with that in the adjacent normal tissues ($p < 0.01$). We also measured MVIH levels in glioma cell lines (U251, U87, U118 and LN18) and primary normal human astrocytes (NHA). As shown in Figure 1B, the results showed that MVIH expression was upregulated in U251, U87, U118 and LN18 cells (all $p < 0.01$). Those results showed that deregulated expression of MVIH may play a role in the development of glioma.

Association between MVIH Upregulation and Clinicopathological Parameters of Patients with Glioma

To investigate the associations of MVIH expression with various clinicopathological parameters of glioma, the patients with glioma were classified into two groups based on the median expression levels. We found that found that high expression of MVIH was significantly correlated

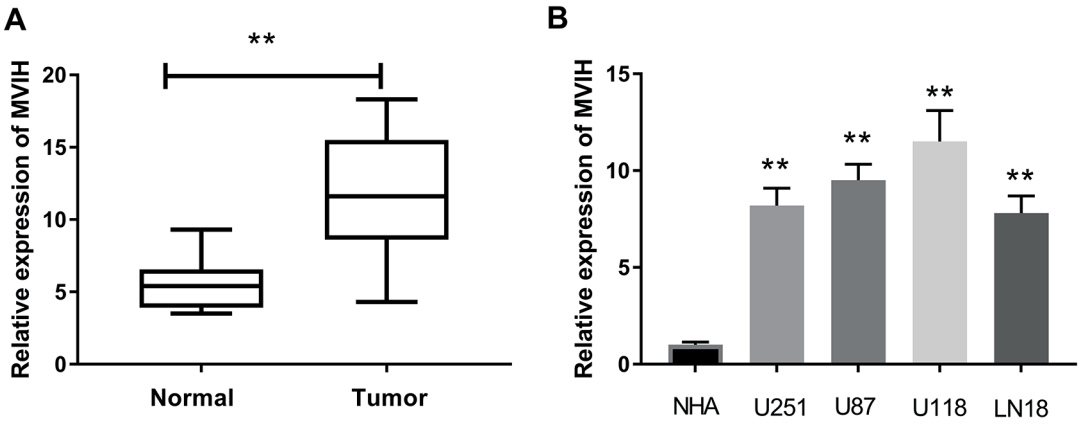

Figure 1. Comparison of MVIH expression levels between normal cells and glioma cancerous tissues and cell lines. ***A,*** Relative levels of MVIH in surgical specimens of glioma and matched adjacent normal tissues were quantified by qRT-PCR. ***B,*** qRT-PCR analysis of MVIH expression in glioma cell lines. $^{*}p < 0.05$. $^{**}p < 0.01$

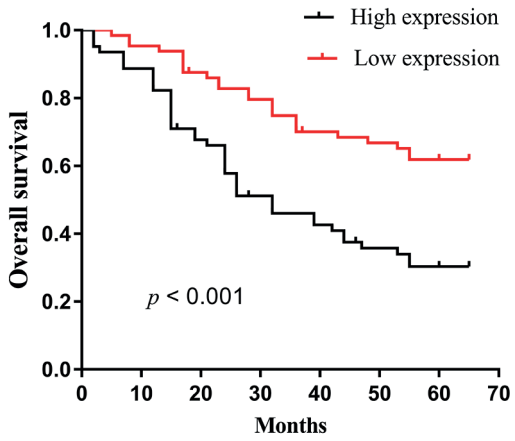

Figure 2. The 5-year overall survival rate of glioma patients with high MVIH was significantly lower than that of those patients with low MVIH ($p < 0.001$). Corresponding p values analyzed by log-rank tests are indicated.

with KPS ($p = 0.001$), and advanced WHO grade ($p = 0.015$). However, There was no significant association between MVIH expression and other clinicopathologic characteristics, including age, gender, tumor size, tumor location, Tumor recurrence and surgery (all $p > 0.05$).

MVIH Expression and Patient's Survival

To further evaluate the prognostic value of MVIH expression in patients with glioma, Kaplan-Meier survival analysis was applied to compare overall survival (OS) according to MVIH expression. We found that glioma patients with high MVIH expression had a worse prognosis than those patients with low MVIH expression (Figure 3). To identify the prognostic significance of clinicopathological factors for overall survival, univariate Cox analysis was conducted. KPS ($p = 0.001$), WHO grade ($p = 0.001$) and MVIH expression ($p = 0.001$) were risk factors that were correlated with the overall survival of glioma patients (Table II). The further multivariate analysis confirmed that high MVIH expression was a significant independent predictor of poor survival in glioma (HR = 3.69, 95% CI: 1.89-7.73, $p = 0.001$).

Effect of MVIH on Proliferation, Invasion and Migration of Glioma Cells

To investigate the functional effects of MVIH in glioma cells, we selected U87 cells for MVIH overexpression and knockdown. pcDNA-MVIH or si-MVIH, or their relative negative controls were transfected into U87 cells. As shown in Figure 3A, pcDNA-MVIH transfection in U87 cells significantly increased the expression levels of MVIH when compared to pcDNA transfection ($p < 0.001$). While MVIH expression was decreased in U87 cells with si-MVIH transfection compared to the scrambled siRNA. Next, we performed CKK-8 and found that U87 cells transfected with pcDNA-MVIH showed significant cell proliferation promotion. On the contrary, U87 cells transfected with MVIH siRNA showed significant cell proliferation inhibition (Figure 3B). Furthermore, we examined the migration and invasion ability by wound healing assay and transwell assay and we found that MVIH overexpression significantly increased the migration and invasion of U87 cells ($p < 0.01$) (Figure 3C and 3D). While MVIH knockdown significantly reduced the migration and invasion of U87 cells ($p < 0.01$) (Figure 3C and 3D). Collectively, our results suggest that MVIH increased glioma cell tumorigenicity *in vitro*.

Table II. Univariate and multivariate analyses of prognostic variables of overall survival in glioma patients.

		Univariate analysis		Multivariate analysis	
Parameters	**Categories**	**HR (95% CI)**	***p*-value**	**HR (95% CI)**	***p*-value**
Gender	Female vs. Male	1.38 (0.73-2.25)	NS		
Age	<50 vs. ≥50	0.76 (0.34-1.23)	NS		
Tumor size	<5 cm vs. ≥5 cm	1.66 (1.02-2.36)	NS		
Tumor location	Supratentorial vs. Subtentorial	2.63 (1.22-3.88)	NS		
KPS	<80 vs. ≥80	3.82 (1.65-7.53)	0.001	3.13 (1.19-5.99)	0.003
WHO grade	I-II vs. III-IV	4.81 (1.33-8.72)	0.001	3.71 (1.13-7.62)	0.001
Tumor recurrence	Absent vs. Present	3.22 (1.76-4.96)	NS		
Surgery	GTR vs. PR	2.33 (1.42-3.72)	NS		
MVIH expression	Low vs. high	4.22 (2.19-8.81)	0.001	3.69 (1.89-7.73)	0.001

Abbreviation: NS, difference between groups was not statistically significant.

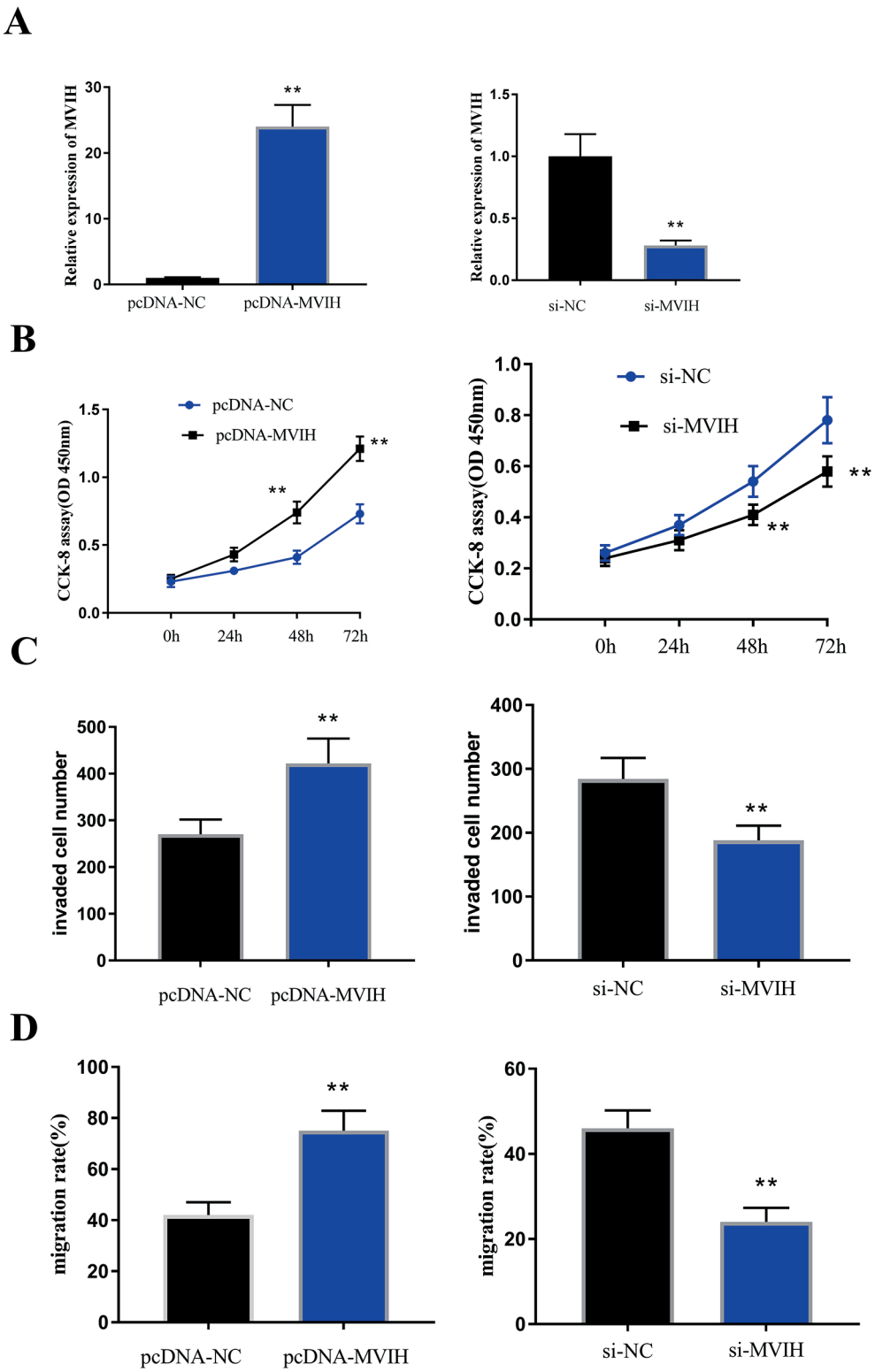

Figure 3. MVIH repressed gliomas growth, migration and invasion. A. U-87 cells were transfected with pcDNA-MVIH constructs or siNEAT, followed by qPCR detection of MVIH expression. B. Effect of MVIH on the growth of glioma cells U87 detected by the CKK-8 assay. C. The cell invasion abilities were determined in U87 cells. D. Wound-healing assay was performed to evaluate the effect of MVIH on U87 cell migration. Data are the means ± standard deviation of three independent experiments. $^{*}p < 0.05$. $^{**}p < 0.01$

Discussion

Previous studies have demonstrated that MVIH played an important role in several tumors. Shi et al[15] found that MVIH expression was significantly increased in hepatocellular carcinoma tissues and cells. Furthermore, they confirmed that MVIH promoted cell growth and inhibited cell apoptosis of HCC via inhibiting miR-199a expression in *vitro* and *in vivo*. Nie et al[14] showed that elevation of MVIH was associated with advanced progression and poor prognosis in non-small cell lung cancer. Lei et al[13] reported that upregulated MVIH expression levels could promote cell proliferation and cell cycle, and inhibited cell apoptosis. They also confirmed that increased expression of MVIH frequently indicated a poor prognosis in breast cancer. However, little is known about the possible involvement of MVIH expression with the clinicopathological characteristics and prognosis of glioma.

Non-coding RNAs (ncRNAs) were once neglected and considered non-function RNA in for a long time. However, now a growing line of evidence supports a role for lncRNAs as predictive biomarkers or tumor targets in human cancers[16]. For instance, Chen et al[17] reported that cervical cancer patients with high expression of lncRNA CCAT2 had poor overall survival and progression-free survival. Qi et al[18] found that lncRNA LOC285194 was significantly down-regulated and is associated with poor prognosis in colorectal cancer. For glioma, it has been reported that lncRNA AB073614[19], MALAT1[20] and SPRY4-IT1[21] may serve as a new prognostic biomarker and therapeutic target.

To the best of our knowledge, this is the first report of MVIH being involved in the development of glioma. In the present study, we discovered that MVIH was upregulated in glioma tissues and cells. High expression of MVIH closely correlated with KPS and WHO grade. Kaplan-Meier analysis revealed that glioma patients with high MVIH expression had poorer overall survival. Univariate and Multivariate Cox proportional hazards model analysis indicated that MVIH was independent prognostic factors for overall survival in glioma patients. To further understand the mechanism of MVIH in glioma process, *in vitro* experiments were conducted. We found that overexpression of MVIH in glioma cells promoted cell proliferation, invasion and migration, while knock-down of MVIH had the opposite effects. All our findings revealed that MVIH may function as a tumor promoter in glioma.

Conclusions

We firstly offered evidence that MVIH may be associated with the aggressive tumor behaviors. What was more, MVIH was proven to be an independent factor for predicting the prognosis of glioma, suggesting that it might have potential clinical value.

Conflicts of interest

The authors declare no conflicts of interest.

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