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ORIGINAL PAPER

Higher expression of cation transport regulator-like protein 1 (CHAC1) predicts of poor outcomes in uveal melanoma (UM) patients

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Abstract
Objective The purpose of our present study was to investigate the expression of cation transport regulator-like protein 1 (CHAC1) in uveal melanoma (UM) tissues and its function in UM progression.
Methods The mRNA expression of CHAC1 in UM tissues and its prognostic value were investigated based on Gene Expression Omnibus database and The Cancer Genome Atlas database. SP6.5 and M23 UM cell lines with depleted CHAC1 were constructed using small interfering RNA. The viability and migration ability of SP6.5 and M23 UM cells were determined by MTT and wound healing assays, respectively. Western blot was conducted to test the influences of CHAC1 depletion on PI3K signaling pathway.
Results Higher expression of CHAC1 was observed in the UM tissues from patients with liver metastases compared to that from patients without metastases. High CHAC1 expression was correlated with poor prognostic and was an independent predictor for UM patients. Depletion of CHAC1 remarkably inhibited the proliferation and motility of SP6.5 and M23 UM cells. Moreover, the ratios of p-AKT/AKT and p-mTOR/mTOR were reduced notably after silencing CHAC1.
Conclusions Our results suggested that CHAC1 functioned as a facilitator in UM cell proliferation and migration and possessed the potential to be a predictor as well as a therapeutic target for UM patients.

Keywords CHAC1 · Uveal melanoma · Migration · Prognosis · PI3K signaling pathway

Introduction

As a primary intraocular tumor in adults, uveal melanoma (UM) possesses the highest morbidity and mortality around the world [1–3]. At present, the treatment options available includes: proton beam or stereotactic irradiation, radioactive plaque and enucleation. In spite of good local control, more than half of patients will die from metastases, mostly liver metastases [4–6]. Identifying and studying molecules that may involve in metastasis can help us understand the molecular mechanisms behind UM metastasize. Moreover, these findings may aid in the treatment or prevention of melanoma.

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As a stress signaling pathway in the endoplasmic reticulum, the unfolded protein response (UPR) pathway is found to be activated in many types of human solid tumors, including human melanoma [7, 8]. A range of components of this pathway were up-regulated in several human cancers [9]. Hence, the members of UPR possibly be new therapeutic targets for cancer therapy [10]. Recently, cation transport regulator-like protein 1 (CHAC1) has been characterized as a novel member of UPR pathway [11] and then a pro-apoptotic factor of this pathway [12]. Previously, Stelzer et al. [13] found that CHAC1 was negatively regulated by miRNA-370 in human parthenogenetic-induced pluripotent stem cells and possibly participated in cellular differentiation. CHAC1 has been identified to be different expressed in pancreatic ductal adenocarcinoma compared with normal pancreatic ducts [14]. Furthermore, it has been demonstrated that overexpression of CHAC1 splicing variants was correlated with poor outcome in ovarian and breast cancer patients [7]. Lately, Peng-Hsu Chen et al. [12] reported that overexpression of CHAC1 enhanced glioma apoptotic death, and CHAC1-inhibited Notch3 pathway was involved in temozolomide (TMZ)-induced glioma cytotoxicity. However, whether CHAC1 is implicated in UM progression remains unknown.

Recently, The Cancer Genome Atlas (TCGA) database has paid attention to the characteristics of UM and identified that the mutation of GNAQ or GNA11 gene was occurred in almost all the patients with UM (80 patients were analyzed) [5]. In our present study, we investigated the expression and prognostic value of CHAC1 in UM based on the data got from Gene Expression Omnibus (GEO) and TCGA database. The function of CHAC1 in UM cell growth and migration was explored in vitro. From our results, we observed the up-regulation of CHAC1 in UM tissues and found that high expression of CHAC1 was associated with poor outcome of UM patients. Knockdown of CHAC1 dramatically decreased the growth and migration of UM cells. Our data evidenced that CHAC1 might exert a promoting function in UM progression.

Methods

The mRNA expression data and clinical data collection

The mRNA expression data of 35 UM tissues from patients with liver metastases (metastasis group) and 28 UM tissues from patients without metastases (non-metastasis group) were downloaded from GEO database (https://www.ncbi.nlm.nih.gov/gds) with the access number of GSE22138. And these data were used for analyzing the expression of CHAC1 gene. The mRNA expression data and clinical data of 78 UM patients were obtained from TCGA database (https://cancergenome.nih.gov/).

Cell cultivation

We bought the UM cell lines (SP6.5 and M23) and D78 corneal epithelial cell line from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in the Roswell Park Memorial Institute (RPMI)-1640 medium added with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. They were cultivated in an incubator at 37 °C with 5% CO_2.

Quantitative RT-PCR

The total RNA of the cells was isolated applying the RNAiso Plus kit (TaKaRa Biotechnology, Dalian, China). Next, the isolated RNA was used as template for reverse transcription making use of the HiFiScript cDNA Synthesis Kit (CwBio, Beijing, China) as described by the manufacturer. Then, the mRNA expression of CHAC1 was detected by qPCR making use of Applied Biosystems 7300 Sequence Detection System (Applied Biosystems). The primers for CHAC1 were:

CHAC1 F: 5′ CCCCATCCTGGAACTTGACC 3′;
CHAC1 R: 5′ CTATGGATGGCTGGGCTGAG 3′.
GAPDH was utilized as internal reference. The primer sequences for GAPDH were: GAPDH F: 5′ GGAGCGAGATCCCTCCAAAAT 3′;
GAPDH R: 5′GGCTGTTGTCATACTTCTCATGG 3′ [15].

The relative mRNA expression of CHAC1 was calculated using a $2^{-\triangle\triangle Ct}$ method [16].

Cell transfection

Two small interfering RNAs against CHAC1 (si-CHAC1-1: 5′-GATCATGAGGGCTGCACTT-3′; si-CHAC1-2: 5′-TCCGGCCACAACCTTGAAT-3′) and a scrambled si-RNA control (si-con: 5′-CGTCGGGAGTACTAGTTAC-3′) were synthesized by GENEWIZ (Suzhou, China). The siRNAs and si-con were transfected into M23 and SP6.5 cells utilizing Lipofectamine 2000 (Invitrogen) as the manufacturer's description. Then, qRT-PCR and western blot assays were performed to determine the knockdown efficiency.

MTT assay

The proliferation ability of M23 and SP6.5 cells was examined using MTT assays. Briefly, cells were added into 96-well plates (about 1×10^3 each well) after 24 h transfection. Then, the cells were cultured conventionally and the viability was tested at 0, 24, 48 and 72-h time points. When testing the viability, 20 µl of MTT (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. Then, the culture supernatant was removed and the purple-colored precipitates were dissolved in 150 µl DMSO. Then, a microplate reader (Bio-Rad) was utilized to detect the absorbance at 490 nm. Graph Pad Prism 5.0 software was used to draw the proliferation curve.

Wound healing assays

The migration ability of M23 and SP6.5 cell lines were detected by wound healing assays after 24 h transfection. A vertical wound was made on the cell monolayers by using a 200-µl pipette tip. The scratched cells were washed off using phosphate buffer saline (PBS). The cells remains on the plate were maintained in the incubator and grew for 24 h at 37 °C. The wounds were photographed and measured using an optical microscope (Olympus, Japan) after being scratched for 0 and 24 h. The relative migration distance was calculated by considering the distance in si-con group as 100%. All the tests were carried out in triplicate.

Western blotting analysis

The effect of si-CHAC1 on the expression of CHAC1 and PI3K pathway-related proteins was analyzed by western blot as described previously [17]. The membranes were probed with the primary antibodies and then the appropriate HRP-conjugated anti-mouse/rabbit secondary antibodies (Cell Signaling Technology). Enhanced chemiluminescence (ECL) plus detection kit was applied for detecting the Immunoreactive bands. The primary antibodies used were as follows: CHAC1 (1:1000, 15207-1-AP, PTG), AKT (1:1000, 4691, CST), p-AKT (Ser473) (1:1000, 4060, CST), PI3K (1:1000, 4249, CST), p-PI3K (1:1000, 4228, CST), GAPDH (1:5000, 5174, CST). Image J program was used to quantify the protein expression. All the experiments were performed for at least three independent times.

Statistical analysis

The 78 samples (TCGA database) were separated into two groups on the basis of the median of CHAC1 expression. Kaplan–Meier method was applied for performing the prognostic analysis with log-rank test for comparison. The clinical relevance of CHAC1 expression in human UM was determined using Pearson's Chi-squared (χ^2) test. Univariate and multivariate cox analysis was conducted to analyze the independent prognostic value of CHAC1. All the experiment data were shown as mean ± standard deviation. The difference between two groups was compared by Student's t test. One-way ANOVA analysis together with a Tukey's post hoc test was applied for comparing the difference between more than two groups. These statistical analyses were carried out by using the SPSS 15.0 software (IBM, Chicago, IL, USA). It was considered as significantly different when p value was less than 0.05.

Results

The expression of CHAC1 is a potential independent prognostic factor for UM patients

We analyzed the mRNA expression of CHAC1 in UM tissues from patients with liver metastases (n = 28, metastases group) or without metastases (n = 36, non-metastases group) based on the data obtained from

GEO database (access number: GSE22138). The metastases group exhibited significant increased mRNA expression levels of CHAC1 compared with that of non-metastases group ($p = 0.0001$, Fig. 1a). The clinical relevance of CHAC1 expression in UM patients was then analyzed on the basis of the mRNA expression and clinical data of 78 UM patients downloaded from TCGA database (Table 1). We identified that CHAC1 expression is significantly correlated with histological-type ($p = 0.001$), pathologic-M ($p = 0.001$), recurrence ($p = 0.001$) and dead ($p < 0.001$), whereas CHAC1 expression did not correlate with age, gender, tumor basal diameter, pathologic-stage and pathologic-T ($p > 0.05$). Kaplan–Meier analysis demonstrated that high expression of CHAC1 correlates with the shortened overall survival time in UM patients ($p < 0.0001$, Fig. 1b).

To further determine the prognostic value of CHAC1, univariate and multivariate cox analyses were implemented (Table 2). Univariate analysis revealed that CHAC1 expression ($p < 0.0001$), pathologic-M ($p < 0.0001$) and recurrence ($p = 0.001$) can be seen as prognostic factors. Further, multivariate analysis suggested that CHAC1 expression ($p = 0.002$) and pathologic-M ($p < 0.001$) were independent prognostic factors for UM patients. Collectively, these data illustrated that CHAC1 expression is enhanced in UM tissues and has the potential to be an effective predictor for the prognosis of UM patients.

The viability of UM cells was suppressed after downregulation of CHAC1

At first, the mRNA expression of CHAC1 in SP6.5 and M23 cells was detected by qPCR. The results showed that CHAC1 expression is enhanced in UM SP6.5 and M23 cells compared with the normal D78 cells (Fig. 2a, $p < 0.01$). These data further proved the phenomena we observed by analyzing the public data. We then attempted to explore the biological functions of CHAC1 in SP6.5 and M23 cells in vitro using a RNA interference method. The effective depletion of CHAC1 in M23 cells was confirmed both at mRNA levels and protein levels (Fig. 2b–d, $p < 0.01$). Via MTT assays, we discovered that knockdown of CHAC1 significantly decreased the proliferation ability of SP6.5 cells at 48 and 72 h (Fig. 2e, $p < 0.01$). In M23 cells, the similar inhibitory effect was observed at 48 and 72 h after silencing CHAC1 (Fig. 2f, $p < 0.01$). These results implied that CHAC1 might possess the function of promoting UM cell proliferation and might be a forwarder in UM progression.

The migration ability of UM cells was inhibited after downregulation of CHAC1

The results of wound healing assays showed that the migration distances of SP6.5 cells were dramatically shorter in si-CHAC1 group than that in si-con group (Fig. 3a). Measuring and calculating the migration distances, we found that the migration distance of SP6.5 cells in si-CHAC1 group was 42.3 ± 9.5% of that in si-con group (Fig. 3b, $p < 0.01$). Analogously, in M23 cells, the migration distance was also shorter in si-CHAC1 group than that in si-con group (Fig. 3c). The migration distance of M23 cells in si-CHAC1 group was 50.2 ± 8.9% of that in si-con group (Fig. 3d, $p < 0.01$). These observations revealed that knockdown of CHAC1 dramatically inhibited the migration ability of UM cells.

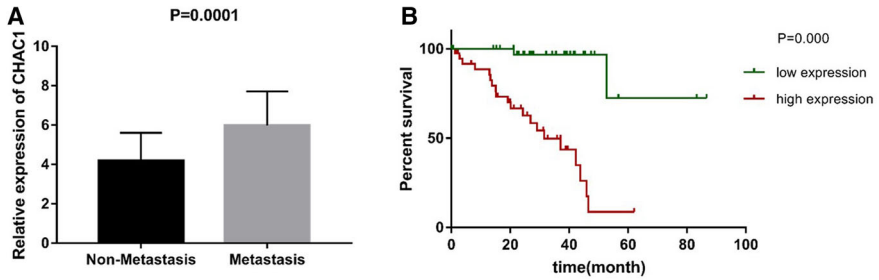

Fig. 1 Enhanced expression of CHAC1 is predictive of poor outcomes in UM patients. **a** Uveal melanoma from patients with liver metastases showed higher CHAC1 mRNA expression level than that from patients without liver metastases. **b** High expression of CHAC1 is correlated with lower overall survival rate in UM patients. *UM* uveal melanoma, *CHAC1* cation transport regulator-like protein 1

Table 1 Relationship between expression of CHAC1 and clinical characteristics

Characteristics	Expression of CHAC1		*p* value
	Low	High	
Age			0.361
< 60	19	15	
≥ 60	20	24	
Gender			0.819
Female	17	16	
Male	22	23	
Histological-type			0.001*
Epithelioid cell	3	9	
Spindle cell	23	7	
Spindle/epithelioid cell	13	23	
Tumor basal diameter			0.096
< 15 mm	17	10	
> 15 mm	22	29	
Pathologic-stage			0.113
II	23	16	
III + IV	16	23	
Pathologic-T			0.186
T2	10	4	
T3	15	16	
T4	14	19	
Pathologic-M			0.001*
M0	39	29	
M1	0	10	
Recurrence			0.001*
No	37	25	
Yes	2	14	
Dead			0.000*
No	37	19	
Yes	2	20	

CHAC1 cation transport regulator-like protein 1

*p < 0.05

PI3K signaling pathway was suppressed in UM cells with silenced CHAC1

PI3K signaling pathway is often disordered in many types of cancers, and it has been demonstrated to take vital parts in cell proliferation and maintains the biological features of tumor cells [18]. By western blotting, we observed that the protein expression levels of AKT and mTOR in M23 cells of si-CHAC1 group were similar with that in the si-con group. However, the levels of p-AKT and p-mTOR were lower in the si-CHAC1 group than that in si-con group (Fig. 4a). The ratios of p-AKT/AKT and p-mTOR/mTOR were significantly lower in the si-CHAC1 group than that in the si-con group (Fig. 4b, $p < 0.01$), implying that the PI3K signaling pathway was obviously suppressed when CHAC1 was silenced.

Discussion

CHAC1 was characterized as a new pro-apoptotic member of UPR pathway, which responds to endoplasmic reticulum stress [7, 19]. Previously, UPR pathway has been speculated to be associated with the failure of some patients to reply to chemotherapy. And it can provide a target for making the existing treatments better or help to discover new anti-tumor targets [20]. Our study is the first research that indicated a relationship between CHAC1 mRNA expression and the overall survival of UM patients. We discovered that the mRNA expression of CHAC1 was obviously higher in UM tissues from patients with metastases than that without metastases, suggesting the involvement of CHAC1 in UM metastases. Further, Kaplan–Meier analysis illustrated that high expression of CHAC1 is associated with the poor outcomes of UM patients. In the univariate analysis and multivariate analysis, CHAC1 overexpression is identified to be an independent prognostic indicator, indicating the important role it played in UM progression. Similar with our present results, high expression of CHAC1 has also been demonstrated as an independent factor in ovarian and breast cancer for high risk of recurrence [7].

Since the relationships discovered between enhanced CHAC1 mRNA expression and poor survival in UM patients, we supposed that CHAC1 may take part in UM cell growth and migration. By depleting CHAC1 in SP6.5 and M23 cells, we indeed found a dramatic reduction in cell proliferation and migration, implying that CHAC1 functions as a forwarder in UM progression. These results further confirmed our hypothesis based on the public expression data. Moreover, the results of our functional studies were consistent with the previous results obtained in breast and ovarian cancer cells by G Goebel et al. They have found that knockdown of CHAC1-repressed breast and ovarian cancer cell

Table 2 Identification of independent prognostic factors in UM by univariate and multivariate analysis

Variables	Univariate analysis			Multivariate analysis		
	p value	HR	95% CI	*p* value	HR	95% CI
CHAC1 expression	0.000*	16.041	3.713–69.304	0.002*	12.401	2.597–59.228
Age	0.131	1.963	0.819–4.703			
Gender	0.282	1.638	0.666–4.029			
Histological-type	0.783	1.096	0.570–2.110			
Tumor basal diameter	0.384	1.532	0.587–4.000			
Pathologic-stage	0.335	1.535	0.643–3.667			
Pathologic-T	0.163	1.602	0.826–3.110			
Pathologic-M	0.000*	26.028	8.411–80.544	0.000*	20.530	3.786–111.320
Recurrence	0.001*	4.308	1.850–10.030	0.360	0.502	0.115–2.197

UM uveal melanoma, *CHAC1* cation transport regulator-like protein 1, *HR* hazard radio, *CI* confidence interval

*p < 0.05

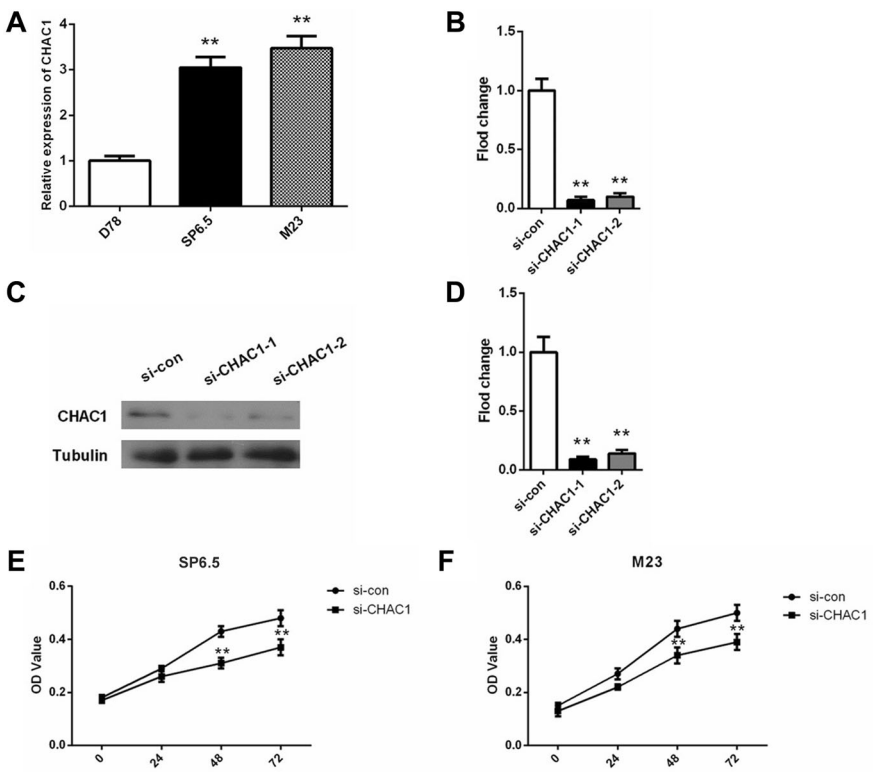

Fig. 2 Knockdown of CHAC1 decreased the proliferation ability of SP6.5 and M23 cells. **a** mRNA expression levels of CHAC1 in D78, UM SP6.5 and M23 cells determined by qRT-PCR. **b** mRNA expression levels of CHAC1 was inhibited in M23 cells transfected with si-CHAC1. **c** Protein expression level of CHAC1 in M23 cells detected by western blot. **d** Quantification of the protein expression levels of CHAC1. **e** Proliferation of SP6.5 cells was detected by MTT assays. **f** Proliferation of M23 cells was detected by MTT assays. $n = 6$, bars, SD. $^{**}p < 0.01$, versus si-con group

migration, while overexpression of CHAC1 exhibited the opposite effects [7]. However, in neuroblastoma cells, it was found that silencing LYAR gene up-regulated CHAC1 and suppressed cell proliferation [21]. The difference between these studies insinuated that CHAC1 possibly exhibited different functions in different tumor tissues.

In order to explore the possible mechanisms underlying CHAC1-involved cell proliferation and migration, we detected the changes of PI3K signaling pathway-related proteins in M23 cells after silencing CHAC1. The western blot results revealed that the activation of PI3K signaling pathway was dramatically inhibited in M23 cells with silenced CHAC1. The PI3K/AKT signaling pathway is a vital node that regulates cell proliferation, migration and metabolism in mammalian cells [18, 22, 23]. The activity of PI3K is stimulated by many growth factor receptors and oncogenes, and the up-regulated PI3K signaling is considered as a hallmark of tumor [24]. The

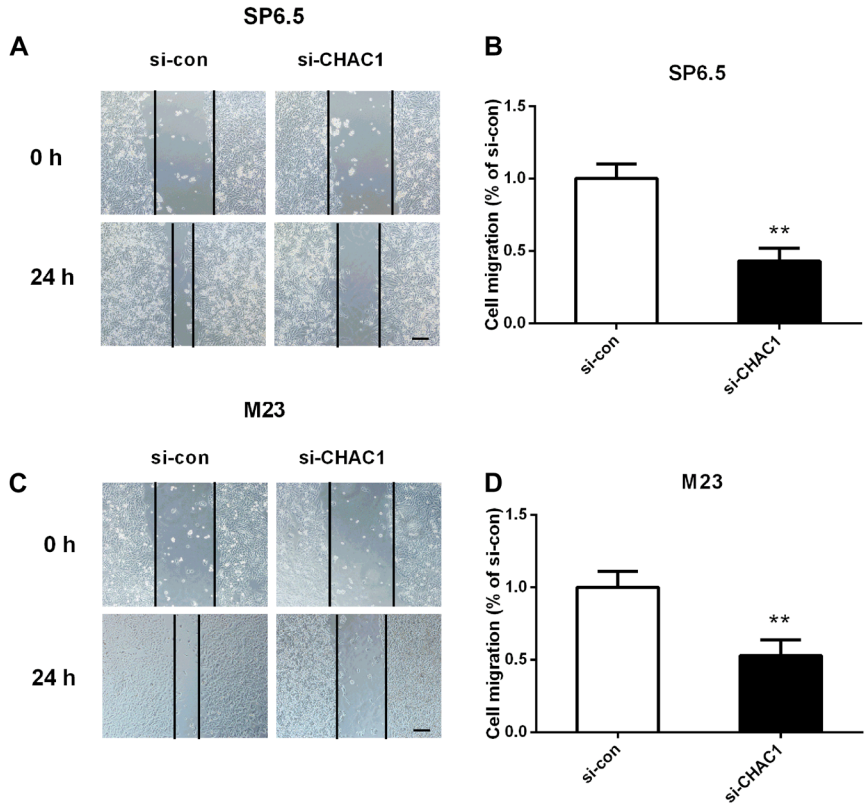

Fig. 3 Knockdown of CHAC1 inhibits the migration of SP6.5 and M23 cells. **a** Migration ability of SP6.5 cells was determined by wound healing assay. **b** Relative migration distance of SP6.5 cells transfected with si-CHAC1 to the cells transfected with si-con. **c** Migration ability of M23 cells was detected by wound healing assay. **d** Relative migration distance of M23 cells transfected with si-CHAC1 to the cells transfected with si-con. $n = 6$, $^{**}p < 0.01$ versus si-con group

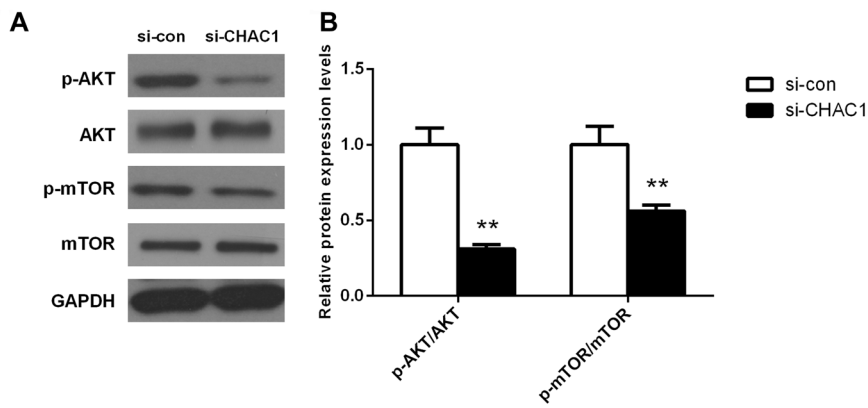

Fig. 4 Knockdown of CHAC1 reduced the ratios of p-AKT/AKT and p-mTOR/mTOR. **a** Protein expression of p-PI3K, PI3K, p-mTOR and mTOR in M23 cells were analyzed by western blot. **b** Ratios of p-PI3K/PI3K and p-AKT/AKT were computed according to figure **a**. $n = 6$, $^{**}p < 0.01$ versus si-con group

involvement of PI3K signaling pathway in UM progression has been reported by many researchers. It has been demonstrated that Ras-PI3K-AKT signaling facilitates UM cells viability and motility through negatively modulating H3 K56ac expression [25]. Ye et al. [26] have illustrated that the activation of the PI3K/AKT pathway induced by the HGF/c-Met axis takes a critical part in the migration of UM cells. Based on our present data, we supposed that CHAC1 possibly correlates to cell proliferation and migration partially through regulating the PI3K signaling pathway. However, how CHAC1 talk with PI3K signaling pathway remains to be elucidated.

There are still many deficiencies in our research as its preliminary nature, and many works need to be urgently conducted. Firstly, the current sample size is relatively small and lacks a larger dataset to support our results. This might partially because of the difficulty to obtain tissue specimens for such evaluations [2]. Secondly, our present experiments were

only conducted in UM cell lines, in vivo assays are eagerly needed to approve the present results. In addition, whether CHAC1 influences other signaling pathways in UM tissues are worth studying as well.

In conclusion, our analysis firstly illustrated that the high expression of CHAC1 in UM tissues was predictive of poor outcomes for patients with UM. The results of in vitro functional experiments suggested that CHAC1 acted as a facilitator in UM proliferation and migration. The present results lay the foundation for continued research on CHAC1 as a novel biomarker.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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