

RESEARCH ARTICLE



MiR-873, as a suppressor in cervical cancer, inhibits cells proliferation, invasion and migration via negatively regulating ULBP2

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Abstract

Background Cervical cancer (CC) remains a large burden in the developing countries. The tumor inhibitory role of miR-873 has been verified in a variety of cancers, however, whether miR-873 has a suppressive effect on CC remains unclear.

Objective The purpose of this study was to investigate the functional role of miR-873 in CC, as well as explore the underlying molecular mechanism.

Methods The prognostic values of miR-873 were assessed by Kaplan–Meier methods and cox regression models using the data which were downloaded from TCGA database. The expression of miR-873 was measured by RT-qPCR. Cell counting Kit-8, clone formation, and Transwell assays were used to assess the cell viability and metastasis, appropriately. The targeting relationship between miR-873 and ULBP2 was predicted by biological software and confirmed by dual luciferase reporter assay. Rescue assays were conducted to investigate whether miR-873 affects the phenotype of CC cells via regulating ULBP2.

Results We observed that miR-873 was low-expressed in CC. Up-regulation of miR-873 notably restrained the proliferation, invasion and migration of C33a cells. Meanwhile, down-regulation of miR-873 in SiHa cells presented the opposite outcomes. ULBP2 was forecasted and certified as a target of miR-873. The results of rescue assays showed that overexpression of ULBP2 could restore the proliferation and motility of CC cells that inhibited by miR-873.

Conclusion MiR-873 suppressed the CC cells proliferation, invasion and migration via negatively regulating ULBP2, suggesting that miR-873 could serve as a valuable therapeutic target for CC therapy.

Keywords miR-873 · Cervical cancer · ULBP2 · Proliferation · Migration and invasion

Introduction

Even with the increase in human papillomavirus (HPV) vaccination and cancer-preventing screening, cervical cancer (CC) still remains the second leading cause of death in young female aged 20–39 years, only behind breast cancer (Siegel et al. 2018). Largely owing to high rates of HPV infection, CC is the most recent common of infection-related cancer in Africa (Parkin et al. 2019). And in China, because the development of geography and socioeconomic are unequal, there is still a large burden of CC (Di et al. 2015). Cervical adenocarcinoma is prone to lymphatic metastasis,

and the prognosis is relatively poor. One paper reported that metastatic CC patients only have a median survival of 8–13 months (Menderes et al. 2016). Currently, targeted therapies have been considered to be an attractive option for these patients and have achieved positive outcomes (van Meir et al. 2014). Therefore, it is necessary to identify valuable therapy targets and develop new strategies for CC.

MicroRNAs (miRNAs), non-coding single-stranded RNA molecules encoding by endogenous genes with a length of approximately 22 nucleotides, are involved in the regulation of post-transcriptional gene expression in plants and animals (Bartel 2004; Acunzo et al. 2015). Lots of investigations have found that its aberrant expression is commonly in connection with cancer. In CC, it is reported that 20 miRNA families are participated in reaction and progression of carcinogenesis (Granados-Lopez et al. 2017). Previous reports have illustrated that miR-215-3p (Liu et al. 2019), miR-101 (Wei et al. 2019), miR-299-3p (Yu et al. 2019) and miR-145 (Ma et al. 2019) expression

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levels are significantly down-regulated in CC tissues compared with adjacent tissues, and could suppress CC cells progression and metastasis. On the contrary, miR-93-5p is found to be highly expressed in CC cells when compared with normal cells, and it could promote CC cell progression (Sun et al. 2019). In addition, miR-454-3p could promote CC cell proliferation and induce apoptosis (Song et al. 2019). Via a comprehensive literature review and bioinformatics analysis, we focus on the low-expressed miRNA miR-873 in CC in our study. And many literatures have reported the abnormal expression of miR-873 in various diseases. In lung adenocarcinoma tissues, high expression of miR-873 was detected, and it could induce cell proliferation and migration (Gao et al. 2015). While in glioblastoma, miRNA-873 has been proven to inhibit tumorigenesis and metastasis, and could act as a sensitizer for cisplatin (Chen et al. 2015; Wang et al. 2015). Moreover, miR-873 was reported to have a connection with colon cancer (Zhu et al. 2019), colorectal cancer (Gao et al. 2019a, b; Li et al. 2019), ovarian cancer (Wu et al. 2016), Alzheimer (Shi et al. 2018), liver fibrosis and cirrhosis (Fernandez-Ramos et al. 2018). Herein, we set out to determine whether miR-873 plays a role in CC and, if so, the mechanism it works through.

Then we used miRNA target gene prediction site, and found that human cytomegalovirus glycoprotein UL16 binding protein 2 (ULBP2) was one of target genes of miR-873. ULBP2, a ligand of natural killer group 2, member D (NKG2D), is an important activating receptor on the surface of natural killer cells, and mainly expressed in intestinal mucosal epithelial cells and epithelial tumor cells under stress conditions (Cosman et al. 2001). In normal tissues, ULBP2 is not expressed or at a low level which can activate immune cells (Champsaur et al. 2010), and many reports have confirmed its abnormal expression in tumor cells. By Kaplan–Meier survival analysis, ULBP2, as well as TRM2 and PHLPP2, were found to be connected with prognosis in colon cancer (Gao et al. 2019a, b). Likewise, through immunohistochemistry, it has been revealed that ULBP2 has a heterogeneous expression in metastatic tumor from different samples, and soluble ULBP2 could signify a poor prognosis (Paschen et al. 2009). However, the relationship between ULBP2 and CC remains unclear and worthy for further investigation.

Thus, the purpose of this study is to detect miR-873 and ULBP2 expression in CC, and to explore their targeted regulatory relationship, as well as their influence on CC cells proliferation, invasion and migration. And we found that miR-873 expression negatively regulated ULBP2 to affect CC cells biological functions, which may be helpful to understand the depth molecular mechanism of CC pathogenesis and progression.

Materials and methods

Microarray data information

From The Cancer Genome Atlas (TCGA, <https://cancer.genome.nih.gov>), we got the RNA sequence (RNA-Seq) data and clinical materials of 306 CC-related samples and three normal samples. Perl language package was used to extract matrix files and converted ensemble ID (download from Ensembl site, <https://www.ensembl.org/index.html>) into gene expression profile. We adopted the Chi-square test to identify the correlations between the expression of miR-873 or ULBP2 and clinical symptoms of CC patients. And cox proportional hazards models were used to evaluate potential predictive factors for CC. Survival analysis were implemented by Kaplan–Meier methods, together with the log-rank tests.

Cell culture

From the Shanghai Cell Bank of Chinese Academy of Medical Sciences (CAMS), human CC cell lines SiHa, C33a, Caski, and HeLa were obtained. From the American Type Culture Collection (ATCC, Rockville, IN, USA), normal cervical cell line Ect1/E6E7 was purchased. All cells were cultured in RPMI-1640 (Gibco, MA, USA) and maintained at 37 °C under a 5% CO₂ atmosphere, and supplemented with 10% fetal calf serum, streptomycin (0.1 mg/ml) and penicillin (100 U/ml).

Transfection

MiR-873 mimic/inhibitor, negative control (NC), pcDNA3.1-ULBP2, pcDNA3.1, si-ULBP2, and si-con were all synthesized from Shanghai GenePharma Co., Ltd (<https>

Table 1 Primer sequences of target genes

Name	Sequence (5'–3')
MiR-873	
Forward	CAGGAAGTGTGAGTCTCC
Reverse	GAACATGTCTGCGTATCTC
snRNA U6	
Forward	CTCGCTTCGGCAGCAC
Reverse	AACGCTTACGAATTTGCGT
ULBP2	
Forward	GAGCAACTGCGTGACATTCAGC
Reverse	GCCCATCGAACTGAACTGCCA
GAPDH	
Forward	TAG ATGACACCCGTCCTGA
Reverse	ACCTCCACCTGCCTTAGTG

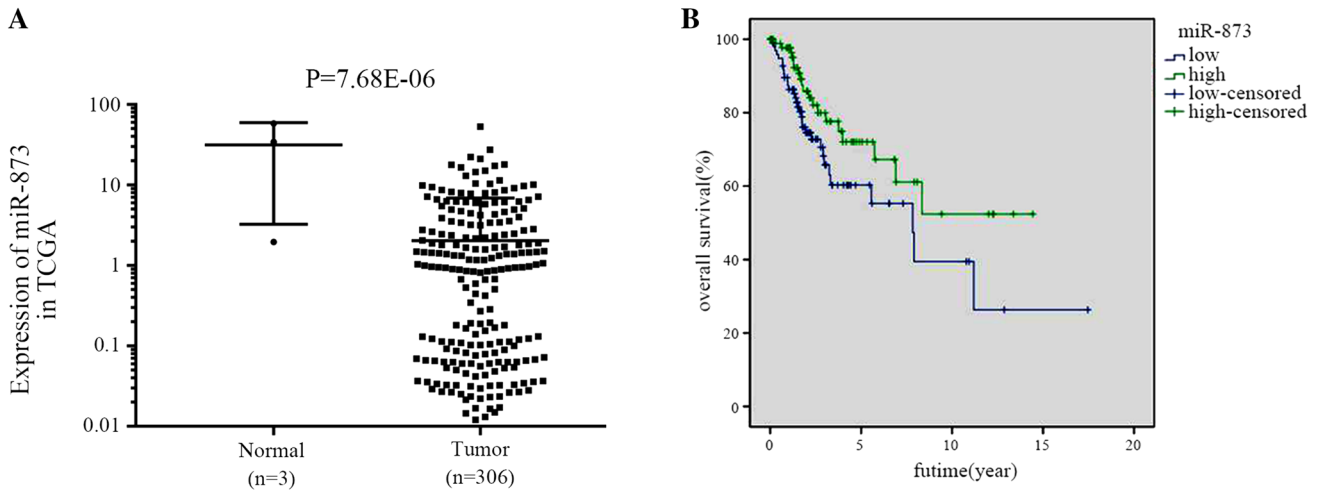


Fig. 1 miR-873 expression and its related survival curve by bioinformatics tools. **a** MiR-873 expression was significantly decreased in CC tissues (N=306) when compared with normal tissues in TCGA data-

base (N=3; **P=7.68E-06). **b** Patients with low miR-873 expression (N=102) had a poorer survival than those with high miR-873 expression (N=102) in TCGA database (*P=0.044)

Table 2 Prognostic value of miR-873 expression and other parameters in CC

Variables	Univariate analysis			Multivariate analysis		
	P value	HR	95% CI	P value	HR	95% CI
miR-873 expression (high/low)	0.047*	0.561	0.317–0.992	0.372	0.637	0.236–1.716
Clinical-stage (I + II/III + IV)	0.004*	2.292	1.293–4.061	0.125	0.330	0.080–1.362
Pathologic-T (T1 + T2/T3 + T4)	0.000*	4.305	2.029–9.134	0.018*	5.271	1.336–20.805
Pathologic-M (M0/M1)	0.688	1.521	0.196–11.789			
Pathologic-N (N0/N1 + N2 + N3)	0.008*	3.193	1.355–7.522	0.002*	4.344	1.717–10.990
Age (<60/≥60)	0.128	1.566	0.879–2.788			
Gender (female/male)	0.535	0.823	0.444–1.524			

I, II, III and IV represent different stages. T means size or direct extent of the primary tumor; N means degree of spread to regional lymph nodes; M means presence of distant metastasis

HR hazard ratio

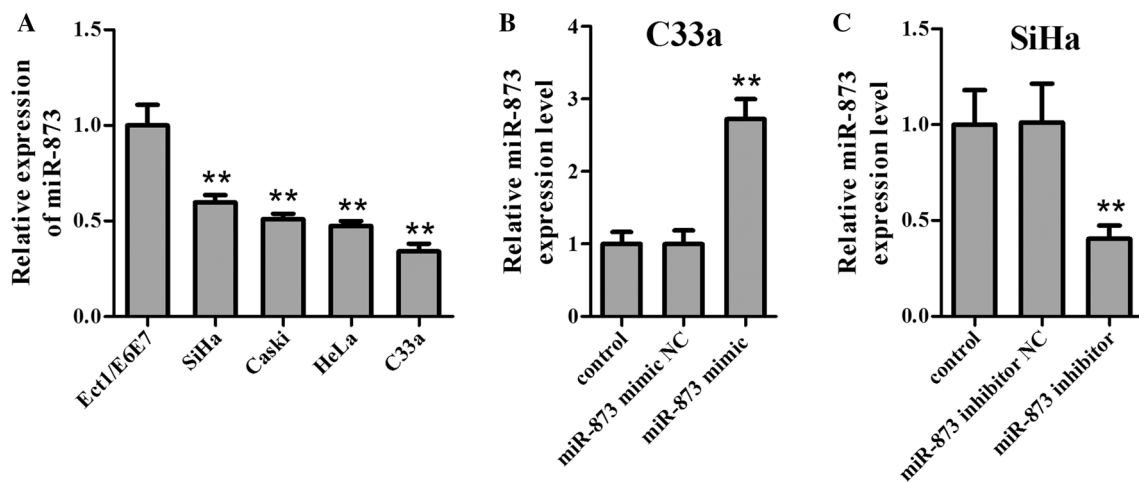


Fig. 2 Different miR-873 expression in different CC cell lines. **a** Compared with normal cell line Ect1/E6E7, miR-873 expression was significantly decreased in CC cell lines (**P<0.01). **b** In C33a cells, compared with miR-873 mimic NC or control, miR-873 mimic

expression level was significantly increased (**P<0.01). **c** In SiHa cells, compared with miR-873 inhibitor NC or control, miR-873 inhibitor expression level was significantly decreased (**P<0.01)

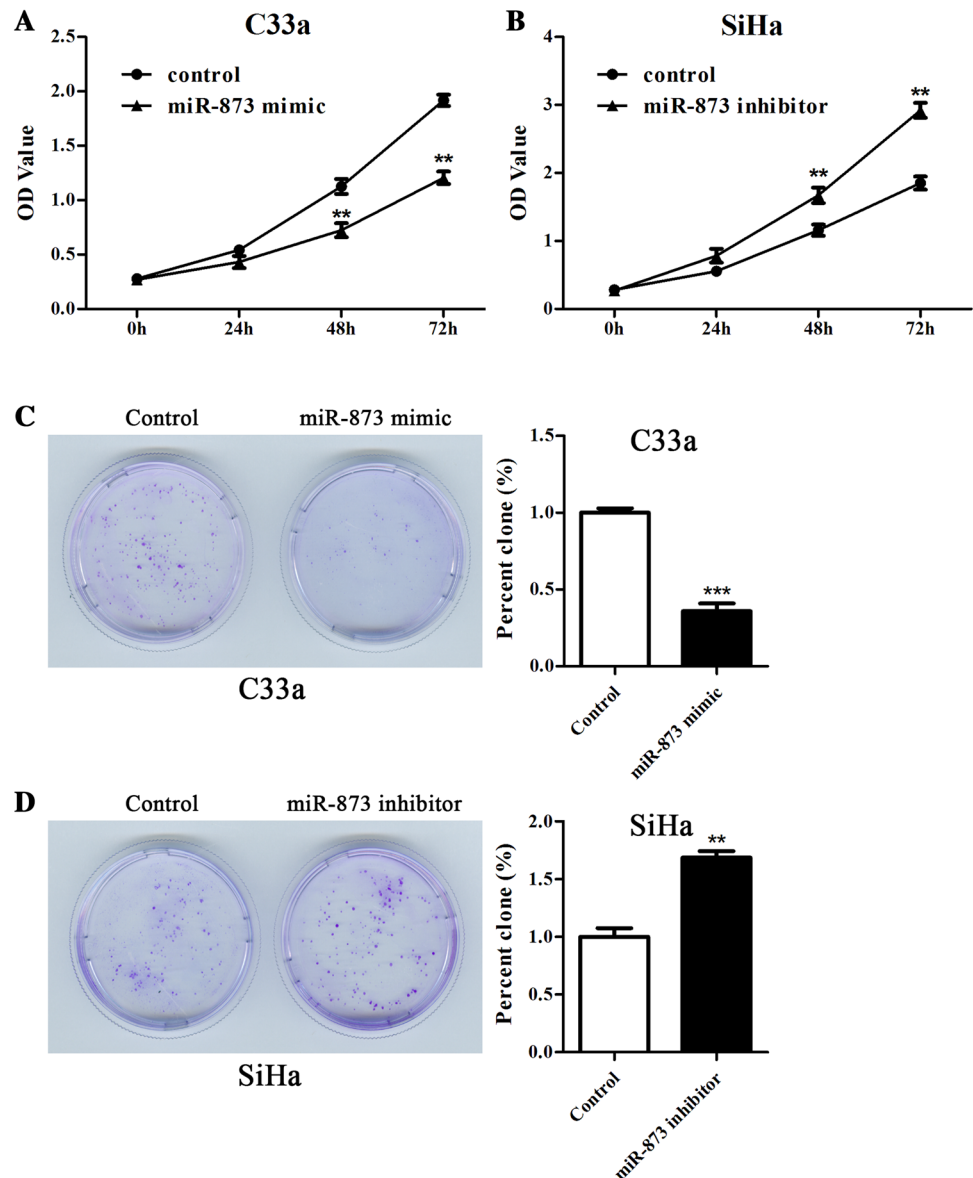
[://www.genepharma.com](http://www.genepharma.com)). Two hours before transfection, the culture medium without antibiotics were replaced in the 6-well plate when the cells confluence had reached 80%. The Lipofectamine 2000 kit (Thermo Fisher Scientific, MA, USA) was used to perform transfection following the manufacturer's instructions, and the transfection efficiency was detected 48 h later.

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted with RNA extraction kit TRIzol reagent (Thermo Fisher Scientific, MA, USA) from CC cell lines following the manufacturer's instructions. For miRNA analysis, RNA was reverse-transcribed to cDNA using

TaqMan miRNA Reverse Transcriptase Kit (Thermo Fisher Scientific, MA, USA). And the expression of miR-873 was measured with a TaqMan MicroRNA assay kit (Thermo Fisher Scientific, MA, USA). For ULBP2 mRNA analysis, cDNA was synthesized using PrimeScript RT Reagent Kit (TaKaRa, Dalian, China), and the mRNA levels of ULBP2 were tested by the SYBR Premix Ex Taq (TaKaRa, Dalian, China). Small clear RNA (snRNA) U6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal references to control with different expression of miR-873 and ULBP2, respectively. The primers list is shown in Table 1. The procedures of qPCR were: 95 °C for 5 min, 95 °C for 30 s, followed by 40 cycles, and 60 °C for 45 s, 72 °C for 30 min. Three duplication holes were set for each group. And cycle (Cq) method ($2^{-\Delta\Delta Cq}$) was used to calculate the miRNA and mRNA expression quantity.

Fig. 3 Altering miR-873 expression influenced CC cells proliferation. **a, b** CCK-8 assays showed that the CC cells proliferation ability of miR-873 mimic group was significantly decreased compared with control in C33a (** $P < 0.01$). Conversely, the CC cells proliferation ability was significantly increased in miR-873 inhibitor group compared with control in SiHa (** $P < 0.01$). **c, d** Clone formation assays showed that the CC cells colony formation ability was significantly decreased in miR-873 mimic group when compared with control in C33a (** $P < 0.01$). Conversely, the CC cells colony formation ability was significantly increased in miR-873 inhibitor group when compared with control in SiHa (** $P < 0.01$)



Western blot assays

In order to extract total protein, we added the Radio Immunoprecipitation Assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology, Nantong, China) with protease inhibitor to the 6-well plate on ice after transfecting 48 h. BCA Protein Assay Kit (cwbiotech, Beijing, China) was used to measure the protein concentration, and the SDS–polyacrylamide gel was used to separate the samples. Next, we transferred the samples onto polyvinylidene fluoride membranes. After blocking with 5% skimmed milk powder for 1 h, the membranes were incubated with the primary antibodies overnight at 4 °C. Subsequently, the membranes were washed 3 times for 5 min in Tris-buffered saline buffer with Tween-20 (TBST), and then incubated with secondary antibodies at room temperature for 1 h. Taken GAPDH as an internal reference. Finally, the protein luminescence effect was detected by an Electro-Chemi-Luminescence kit (ECL, Beyotime Institute of Biotechnology) and gray values were detected by QUANTITY ONE software. Relative expression quantity of proteins was the ratio of interest protein to internal reference.

Cell proliferation assays

Cell counting Kit-8 (CCK-8) assays were used to evaluate cells proliferation activity. C33a and SiHa cells (at a density of 1000 cells/well) were seeded into 96-well plate and conventionally cultivated in a carbon dioxide incubator. Each well was added with 10 μ l CCK8 reagent and incubated at

37 °C for 1.5 h before detecting the cells viability (every 24 h). Finally, the OD value was recorded.

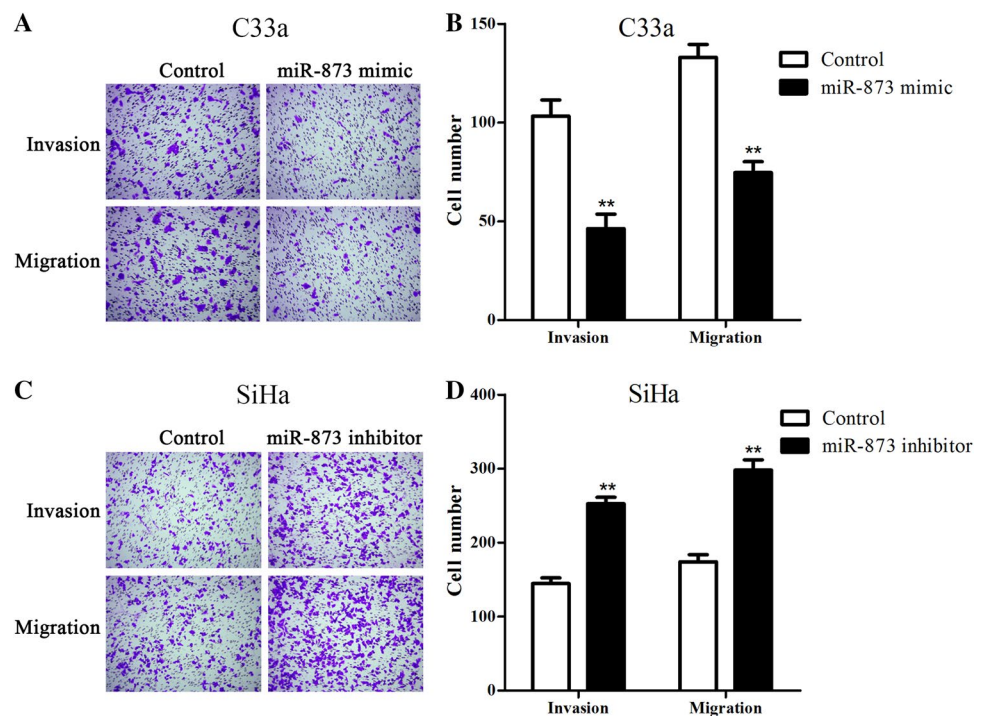
Plate clone formation assays

The trypsin was used to digest the exponential cells to prepare cell suspension. At a density of 400 per culture dish, the cells suspension was decanted into 60 mm dishes which containing 5 ml preculture medium. After rotating dishes gently, the cells was cultivated in a incubator at 37 °C with 5% CO₂ and saturated humidity for 1–2 weeks. The incubation process was terminated when visible communities appeared. Discarded the supernatant and carefully washed twice with phosphate buffered saline (PBS), the cells were fixed with 4% paraformaldehyde for 30 min, and also stained by 0.1% crystal violet for 30 min. Finally, the communities' size and number were evaluated.

Dual-Luciferase reporter assays

According to the predicted result of the website miRWalk (<https://mirwalk.umm.uni-heidelberg.de>), we conducted luciferase reporter vectors (ULBP2-wt and ULBP2-mut) which had been inserted with the wild type and mutant type ULBP2 3'-UTR sequences, respectively. Then the plasmid vectors were co-transfected with miR-873 mimic or mimic NC into HEK-293T cells using Lipofectamine2000 kit (Thermo Fisher Scientific, MA, USA). Transfected cells (at a density of 1×10^4 per well) were seeded onto 96-well plates,

Fig. 4 Altering miR-873 expression affected CC cells invasion and migration. **a, b** Transwell assays showed that, in miR-873 mimic groups, fewer C33a cells invaded and migrated when compared with control (** $P < 0.01$). **c, d** Transwell assays showed that, in miR-873 inhibitor groups, more SiHa cells invaded and migrated when compared with control (** $P < 0.01$)



and kept cultivation 48 h. Luciferase activity was calculated by the dual-luciferase reporter assay system (Promega).

Invasion and migration assays

Cells invasion and migration assays were performed by 24-well Transwell chambers (Millipore, USA). Experimental steps of the two were similar, except that the latter wasn't carried out the gelatinizing treatment. Dissolved Matrigel (1:6 dilution of serum-free medium, 100 μ l) was added to the upper of Transwell chambers and kept cultivation 4–6 h in a CO₂ incubator at 37 °C. Serum-free medium (500 μ l) was added to the lower of Transwell chambers and standing 30 min. Using serum-free medium re-suspended the transfected cell, the upper chambers supplemented with 100 μ l re-suspended suspension and the lower chambers applied with 500 μ l complete medium. Next day, using cotton swabs wiped off the residual cells of the upper chambers.

After cleaning with PBS, cells on the lower chamber were immobilized by 4% paraformaldehyde for 30 min, and were stained by 0.1% crystal violet for 20 min. 5 fields were randomly selected under the microscope for observation and calculation.

Statistical analysis

GraphPad Prism version 5.0 (San Diegl, CA, USA) and SPSS version 22.0 (IBM SPSS, Armonk, NY, USA) were used for statistical analysis of this work. Student's t test and One-way ANOVA were used to determine the significant difference of double- and multiple-group, respectively, and the latter followed with a Turkey's post hoc test. Data are presented as mean value \pm standard deviation, and P value less than 0.05 is regarded as significant. All the experimentations were repeated three times independently.

Fig. 5 ULBP2 was a target gene of miR-873, and analyzed their expression. **a** Bioinformatics analysis predicted that miR-873-5p binds to the 3'UTR of ULBP2. **b** Luciferase reporter assays showed that, in HEK-293T cells, co-transfection of miR-873 mimic and ULBP2-wt, the luciferase activity was significantly decreased compared with co-transfection of mimic NC and ULBP2-wt (**P < 0.01). Whereas no difference was observed between ULBP2-mut groups. **c** ULBP2 expression was significantly increased in CC tissues (N = 306) when compared with normal tissues in TCGA database (N = 3; **P = 3.53E-05). **d** Patients with high ULBP2 expression (N = 145) had a poorer survival than those with low ULBP2 expression (N = 146) (*P = 0.004). RT-qPCR assays (**e**) and western blot assays (**f**) showed that compared with control, the mRNA and protein expression levels of miR-873 mimic groups were significantly decreased (**P < 0.01), while were significantly increased in miR-873 inhibitor groups (**P < 0.01)

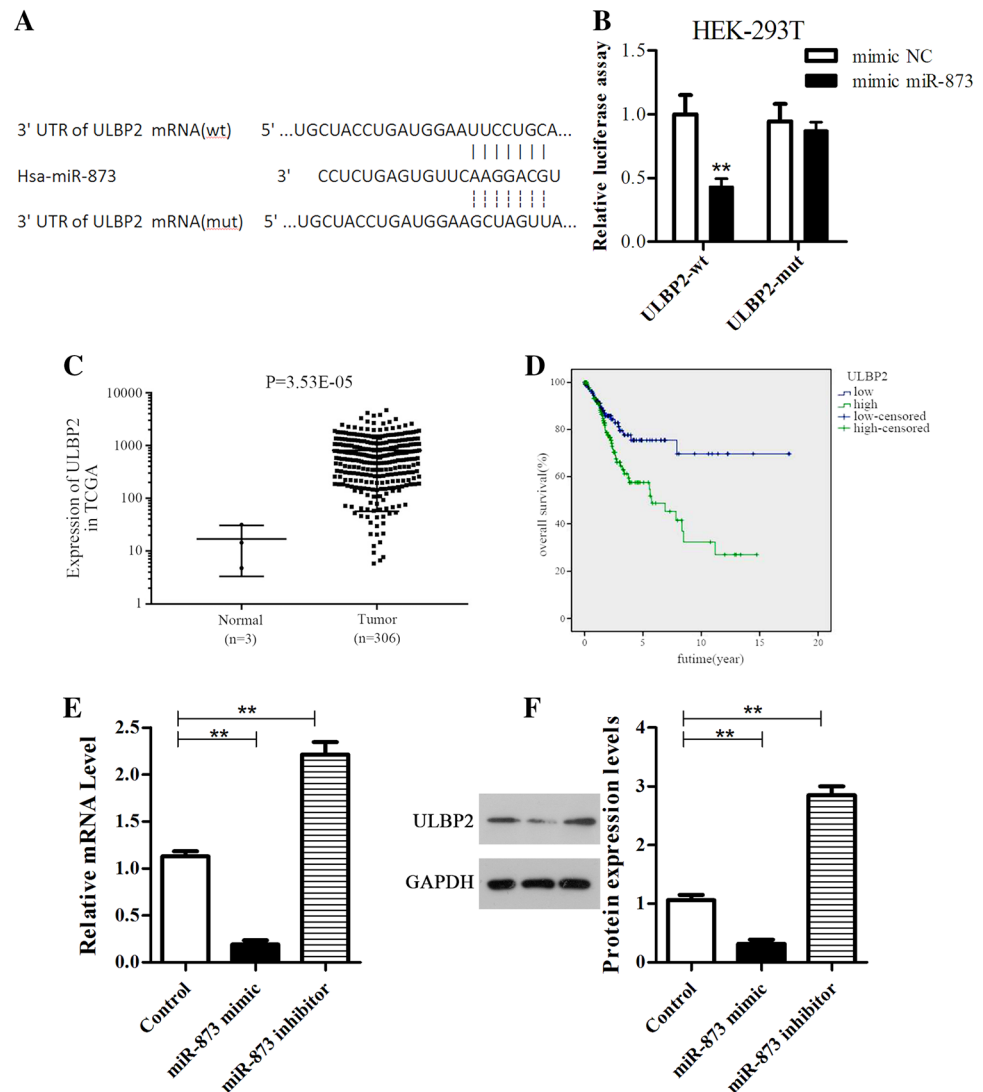


Table 3 Correlation between ULBP2 expression and clinicopathological features of CC (N = 309)

Characteristics	Expression of ULBP2		P value
	Low	High	
Age			0.795
< 60	115	116	
≥ 60	31	29	
Grade			0.119
G1 + G2	70	77	
G3	67	50	
Clinical-stage			0.847
I + II	112	111	
III + IV	32	30	
Pathologic-T			0.484
T1 + T2	109	95	
T3 + T4	12	14	
Pathologic-N			0.487
N0	70	58	
N1	27	28	
Pathologic-M			0.016*
M0	54	53	
M1	9	1	

I, II, III and IV represent different stages. T means size or direct extent of the primary tumor; N means degree of spread to regional lymph nodes; M means presence of distant metastasis

Results

miR-873 was downregulated in CC tissues and related to poor prognostic

First, we tested the miR-873 expression through bioinformatics tools in TCGA database, and uncovered that miR-873 exhibited a significantly lower expression in CC tissues when compared with normal tissues (**P = 7.68E−06,

Fig. 1a), indicating that miR-873 expression might have a useful effect on the development of CC.

For purpose of investigating the relationship between miR-873 expression and overall survival of CC patients, Kaplan–Meier methods and cox regression models were used. Kaplan–Meier plot demonstrated that the overall survival rate in patients with low expression of miR-873 was lower than that in patients with miR-873 high expression (P = 0.044; Fig. 1b). Then by univariate analysis, as shown in Table 2, miR-873 expression (P = 0.047), Clinical-Stage (P = 0.004), Pathologic-T (P = 0.000), Pathologic-N (P = 0.008) had significant connections with the overall survival of CC patients. At the further analysis of multivariate regression, we found that Pathologic-N (P = 0.002), Pathologic-T (P = 0.018) were the independent prognostic indicators of patients' overall survival. Noticeably, there was no significant results between miR-873 expression and the overall survival. All these analysis hinted that miR-873 couldn't be an independent predictor, but plays a role in CC cells growth.

miR-873 expression in CC cell lines in vitro

To detect the expression of miR-873 in CC cell lines, we selected 4 CC-related cell lines (Caski, HeLa, C33a and SiHa) and used normal cervical cell line Ect1/E6E7 as control. As shown in Fig. 2a, just like the bioinformatics results, all CC cell lines showed strongly low miR-873 expression compared with Ect1/E6E7 (**P < 0.01). Besides, C33a showed the lowest miR-873 expression level when compared with other CC cell line, so we selected C33a cell line to perform all miR-873 mimic experiments for the next steps. On the contrary, comparing to other CC cell lines, SiHa showed the highest miR-873 expression level, thereupon SiHa was chose to perform the next miR-873 inhibitor experiments. We found that, after transfection, the expression of miR-873 was significantly increased in miR-873 mimic group

Table 4 Prognostic value of ULBP2 expression and other parameters in CC

Variables	Univariate analysis			Multivariate analysis		
	P value	HR	95% CI	P value	HR	95% CI
ULBP2 expression (high/low)	0.005*	2.055	1.250–3.380	0.004*	9.026	2.011–40.515
Clinical-stage (I + II/III + IV)	0.001*	2.286	1.397–3.743	0.290	0.284	0.028–2.922
Pathologic-T (T1 + T2/T3 + T4)	0.000*	3.613	1.907–6.846	0.009*	20.186	2.091–194.885
Pathologic-M (M0/M1)	0.020*	3.671	1.229–10.962	0.987	0.000	
Pathologic-N (N0/N1 + N2 + N3)	0.003*	2.807	1.408–5.593	0.290	1.751	0.620–4.946
Age (< 60/≥ 60)	0.054	1.641	0.991–2.715			
Grade (G1 + G2/G3 + G4)	0.681	0.896	0.530–1.513			

I, II, III and IV represent different stages. T means size or direct extent of the primary tumor; N means degree of spread to regional lymph nodes; M means presence of distant metastasis

HR hazard ratio

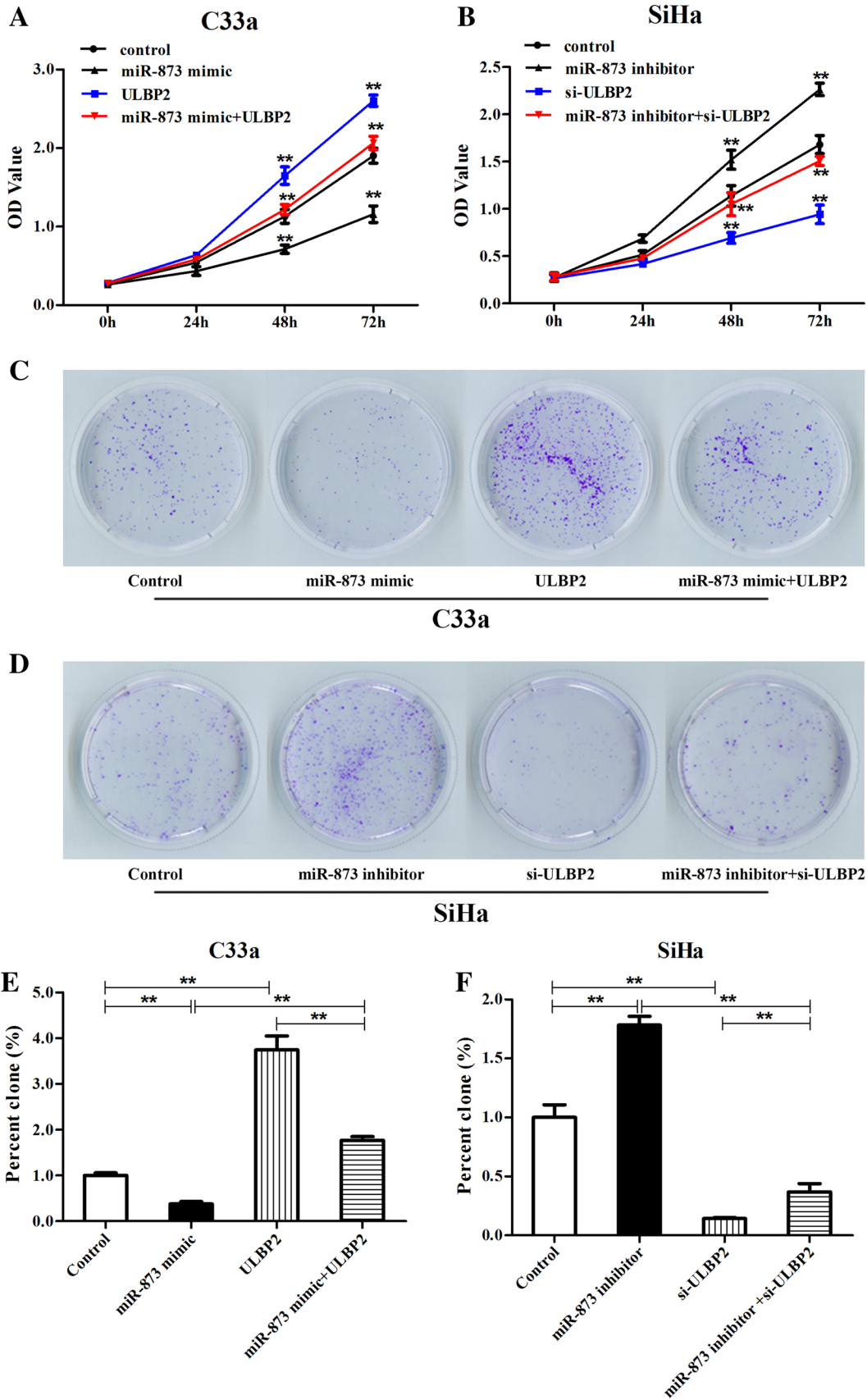


Fig. 6 miR-873 targeted ULBP2 to suppress CC cells proliferation. **a, c, e** In C33a, CCK-8 assays (**a**) and clone formation assays (C&E) showed that, compared with control, the OD values and clone efficiency were significantly reduced in miR-873 mimic groups (** $P < 0.01$), while notably enhanced in pcDNA3.1-ULBP2 groups (** $P < 0.01$). Co-transfection with miR-873 mimic and pcDNA3.1-ULBP2, the proliferation ability was reversed (** $P < 0.01$). In SiHa, CCK-8 assays (**b**) and clone formation assays (**d, f**) showed that, compared with control, the OD values and clone efficiency were notably enhanced in miR-873 inhibitor groups (** $P < 0.01$), while significantly reduced in si-ULBP2 groups (** $P < 0.01$). Co-transfection with miR-873 inhibitor and si-ULBP2, the proliferation ability was reversed (** $P < 0.01$)

by comparing with mimic NC group and control in C33a cell line (** $P < 0.01$, Fig. 2b). While miR-873 expression was significantly decreased in SiHa cells transfected with miR-873 inhibitor by contrast with inhibitor NC group and control (** $P < 0.01$, Fig. 2c). These results indicated that we successfully constructed the C33a cells with up-regulated miR-873 and SiHa with down-regulated miR-873.

MiR-873 expression influences CC cell proliferation

CCK-8 and plate clone formation assay were used to investigate the miR-873 function on CC cells proliferation. According to the results of CCK-8 assays in different time periods, the OD value of miR-873 mimic group was decreased, and significantly difference was presented at 48 h and 72 h when compared to control (** $P < 0.01$, Fig. 3a). In addition, through clone formation assays, miR-873 mimic group emerged a poorer clone efficiency as compared to control (** $P < 0.01$, Fig. 3c). These data indicated that the viability of CC cells was restrained when overexpression of miR-873.

Conversely, down-regulation of miR-873 in SiHa cells significantly inhibited cell proliferation, as we observed an effective higher OD value in the miR-873 inhibitor group compared with the control group (** $P < 0.01$, Fig. 3b). From clone formation assays, we found that the visible clone communities in the miR-873 inhibitor group was more than the control group (** $P < 0.01$, Fig. 3d). The findings mentioned above implied that the expression level of miR-873 could importantly react on the CC cells viability.

MiR-873 expression affects CC cell invasion and migration

Transwell invasion and migration experiments were executed to detect the effect of altering miR-873 expression on CC cells invasion and migration ability. For invasion, compared with control (103.33 ± 8.08), the invaded C33a cell number of miR-873 mimic group was significantly decreased (46.33 ± 7.37 , ** $P < 0.01$, Fig. 4a, b). Inversely, SiHa cells number of miR-873 inhibitor group (252.67 ± 8.5)

was obviously increased when compared with control (144.67 ± 7.64 , ** $P < 0.01$, Fig. 4c, d). Migration assays showed the same trend as the invasion assays. As shown in Fig. 4, compared with control (133.00 ± 6.56), the migrated C33a cell number of miR-873 mimic group was significantly decreased (74.67 ± 5.51 , ** $P < 0.01$, Fig. 4a, b). But SiHa cells number of miR-873 inhibitor group (298.00 ± 14.00) was remarkably increased when compared with control (174.00 ± 9.54 , ** $P < 0.01$, Fig. 4c, d). These outcomes demonstrated that miR-873 expression has a vital inhibitory impact on CC cells invasion and migration ability.

ULBP2 was a target of miR-873

We used the online bioinformatics analysis website miR-Walk (<https://mirwalk.umm.uni-heidelberg.de>) to predict the target genes of miR-873, and discovered that miR-873-5p sequence and ULBP2 3'-UTR sequences had a specific binding region (Fig. 5a). To confirm this bioinformatics result, luciferase reporter assays were used. The result revealed that luciferase activity was obviously decreased in cells that were co-transfected with ULBP2-wt and miR-873 mimic compared with that co-transfected with ULBP2-wt and mimic NC (** $P < 0.01$, Fig. 5b). However, no significant change was observed in ULBP2-mut and miR-873 mimic group compared with ULBP2-mut and mimic NC group. These findings indicated that ULBP2 is one of the target genes of miR-873.

Then we assessed the ULBP2 expression in CC tissues ($N = 306$) and normal tissues ($N = 3$) based on the data obtained from TCGA database. The results revealed that ULBP2 expression was significantly enhanced in CC samples when compared to normal controls, suggesting that ULBP2 might perform a key role in the progression of CC (** $P = 3.53E-05$, Fig. 5c).

Subsequently, we explored the relevance between ULBP2 expression and the overall survival of CC patients. Kaplan–Meier plot revealed that patients with high ULBP2 level appeared to be a worse prognosis survival status as a contrast with ULBP2 low-expressed group ($*P = 0.004$; Fig. 5d). And to ascertain the correlations between ULBP2 expression and clinical symptoms of CC patients, chi-square test was adopted. The results showed that there was no significant association between ULBP2 expression levels with Age, Grade, Clinical-Stage, Pathologic-T and Pathologic-N. But, it was noticeable that there have a positive relationship between ULBP2 expression and Pathologic-M ($*P = 0.016$, Table 3). By univariate analysis, we found that ULBP2 expression, Clinical-Stage, Pathologic-T, Pathologic-M and Pathologic-N had prognostic values ($*P < 0.05$, Table 4). Next, by further multivariate analysis, we found that ULBP2 expression and Pathologic-T were independently associated with the overall survival of CC patients ($*P < 0.05$, Table 4).

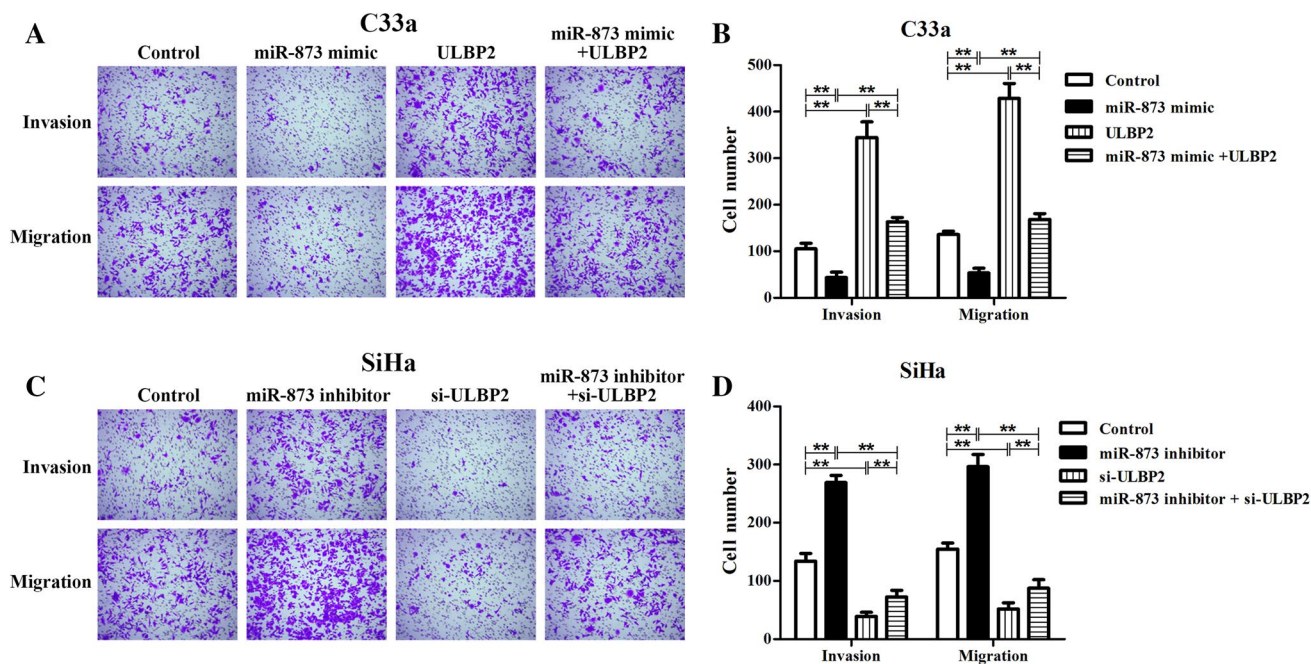


Fig. 7 miR-873 targeted ULBP2 to suppress CC cells invasion and migration. **a, b** Transwell assays showed that, compared to control, fewer C33a cells invaded and migrated in miR-873 mimic groups (** $P < 0.01$), but more cells appeared in pcDNA3.1-ULBP2 groups (** $P < 0.01$). Co-transfection with miR-873 mimic and pcDNA3.1-ULBP2, the number of invaded and migrated was more than that in miR-873 mimic groups (** $P < 0.01$), while fewer

than that in pcDNA3.1-ULBP2 groups (** $P < 0.01$). **c, d** Transwell assays showed that, compared to control, more SiHa cells invaded and migrated in miR-873 inhibitor groups (** $P < 0.01$), but fewer cells appeared in si-ULBP2 groups (** $P < 0.01$). Co-transfection with miR-873 inhibitor and si-ULBP2, the number of invaded and migrated cells was fewer than that in miR-873 inhibitor groups (** $P < 0.01$), while more than that in si-ULBP2 groups (** $P < 0.01$)

The above-mentioned results indicated that ULBP2 could be regarded as a useful independent predictor of CC.

Afterwards, for further verifying the interaction of miR-873 and ULBP2, we used RT-qPCR and western blot to detect the effect of altering miR-873 expression on the expression of ULBP2 at mRNA and protein levels in vitro. After transfected with miR-873 mimic, both ULBP2 mRNA and protein expression levels were significantly decreased in comparison with control (** $P < 0.01$, Fig. 5e, f). Inversely, both ULBP2 mRNA and protein expression were significantly increased after miR-873 inhibitor treatment in comparison with control (** $P < 0.01$, Fig. 5e, f). In summary, these findings provided strongly evidences that ULBP2 is a potential target gene of miR-873, and reflected that miR-873 reversely regulates the expression of ULBP2.

ULBP2 reverses the miR-873-mediated CC cells proliferation, invasion and migration

Rescue experiments were conducted to investigate whether miR-873 affects the phenotype of CC cells by regulating ULBP2. The results indicated that, after overexpression of miR-873 and ULBP2 in C33a cells, the OD values were upper than that in miR-873 mimic groups, while were lower than that in ULBP2 upregulation groups (** $P < 0.01$,

Fig. 6a). And clone formation assays revealed that, compared to miR-873 mimic group, ULBP2 overexpression and miR-873 mimic group showed a higher clone efficiency, while appeared a lower clone efficiency when compared with ULBP2 upregulation groups (** $P < 0.01$, Fig. 6c, e). Inversely, compared with miR-873 inhibitor group, the miR-873 inhibitor and si-ULBP2 groups showed that the OD value was significantly decreased in SiHa cells, but notably higher than that in si-ULBP2 groups (** $P < 0.01$, Fig. 6b). After co-transfection with miR-873 inhibitor and si-ULBP2, the number of visible colonies was more than si-ULBP2 groups, while was significantly lower than that in miR-873 inhibitor groups (** $P < 0.01$, Fig. 6d, f). These outcomes demonstrated that ULBP2 reverses the miR-873-mediated cell viability of CC.

About invasion and migration, as expected, rescue experiments indicated that, compared with miR-873 mimic group, the invaded and migrated cells of miR-873 mimic and ULBP2 group were significantly increased, but notably decreased in comparison with ULBP2 overexpression group (** $P < 0.01$, Fig. 7a, b). Conversely, the invaded and migrated cells of miR-873 inhibitor and si-ULBP2 group were significantly decreased compared with miR-873 inhibitor group, but increased compared with si-ULBP2 groups (** $P < 0.01$, Fig. 7c, d). These results indicated that ULBP2

reversed the miR-873-mediated cell invasion and migration capacity of CC. In all above, we had sufficient reasons to believe that miR-873 suppresses CC cells proliferation, invasion and migration via negatively regulating ULBP2.

Discussion

Previous articles have been reported that abnormal expression of miRNAs is in closely connection with CC cells occurrence and development (Granados-Lopez et al. 2017; Liu et al. 2019; Ma et al. 2019; Song et al. 2019; Sun et al. 2019; Wei et al. 2019; Yu et al. 2019). CC is still a heavy burden in developing countries (Di et al. 2015), so it is very helpful to explore potential molecular mechanisms for CC progression. This study aimed to explore miR-873 expression level in CC, investigate the relationship between miR-873 and ULBP2, and determine whether miR-873 expression could affect the biological functions of CC cell lines including proliferation, migration and invasion via affecting ULBP2 expression, hoping to provide a novel vision for CC therapy.

Using biological identification tools and in-vitro studies, we found that miR-873 expression in CC cells was obviously lower than that in normal cells. Up-regulation of miR-873 suppressed CC cells proliferation, migration and invasion. These findings were in agreement with some previous works in other cancers. Such as in colorectal cancer, it was reported that miR-873-5p could suppress cell invasion and migration (Li et al. 2019; Wang et al. 2019). MiR-873-5p expression was also detected at a low level compared with adjacent tissues and exerted an anti-cancer effect to inhibit tumorigenesis and metastasis in glioblastoma (Wang et al. 2015) and colorectal cancer (Wang et al. 2019). Collectively, these evidences suggest that miR-873 has a potentially inhibitory role in CC progression and metastasis.

MiR-873 targeting ULBP2 was first discovered through miRNA targeting prediction, and confirmed by Dual-Luciferase assays. ULBP2 acts as a bridge linking the two immune responses by simultaneously activating the body's natural immune effector cells and specific immune effector cells, enabling them to play a synergistic role (Cao et al. 2004; Eleme et al. 2004). Therefore, ULBP2 may play a very important role in anti-tumor immunity. In fact, some articles have also reported that it presents an abnormally expression in various types of cancer. ULBP2 expression was detected to be enhanced in ovarian cancer cells than that in normal ovarian epithelium, and could be a prognostic indicator (Li et al. 2009). Demirkol et al. discovered that combination of SEMA5A and ULBP2 could act as a prognosis biomarker for colon cancer (Demirkol et al. 2017). Lin et al. demonstrated that gemcitabine through down-regulated the soluble ULBP2 enhanced NK cells cytotoxicity and inhibited

immune escape of pancreatic cancer (Lin et al. 2016). In our work, we detected that ULBP2 was exhibited a higher expression in CC tissues when compared with normal tissues, and inferred that ULBP2 acts as an important role in CC cell development. Furthermore, through rescue assays, we demonstrated that miR-873 negatively regulated ULBP2 to suppress CC cells proliferation, migration and invasion.

In a conclusion, we demonstrated for the first time that miR-873 and ULBP2 expression was associated with the CC progression, and described for the first time the anti-tumor effects of miR-873 on CC. MiR-873 had the potential to inhibit the proliferation, invasion and migration of CC cells by negatively regulating ULBP2. According to the above information, we have sufficient evidences to illustrate that miR-873 plays an important role in the progression and metastasis of CC, however, whether miR-873 functions as a prognosis biomarker for CC needs to be further investigated.

Compliance with ethical standards

Conflict of interest Hai-Xia Liang and Yu-Hong Li declare that they have no conflict of interest.

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