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Research paper

miRNA-223 is a potential diagnostic and prognostic marker for osteosarcoma

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ABSTRACT

Background: MicroRNA-223 (miR-223) has been shown to be a potential diagnostic and prognostic marker for several cancers. In addition, miR-223 has been reported to suppress osteosarcoma cell proliferation *in vitro*. However, the clinical value of miR-223 is still unknown.
Methods: We detected the expression of miR-223 expression in the serum of osteosarcoma patients and in osteosarcoma cancer cells using RT-PCR. We compared the serum expression of miR-223 with the clinicopathological characteristics and survival of osteosarcoma patients. Finally, we explored the role of miR-223 on the invasion of osteosarcoma cancer cells using cell migration and invasion assays.
Results: We observed that the expression of miR-223 was significantly decreased in the serum of osteosarcoma patients and osteosarcoma cancer cells compared to healthy controls ($P < 0.01$). Moreover, a receiver operating characteristic (ROC) curve analysis indicated that serum miR-223 is a potential diagnostic marker of osteosarcoma with an area under the ROC curve (AUC) of 0.956. Importantly, the patients with a lower expression of miR-223 tended to have distant metastasis ($P < 0.001$) and a more advanced clinical stage ($P < 0.001$). In addition, the survival time of patients with low miR-223 expression was significantly shorter compared to patients with high miR-223 expression ($P < 0.001$). Furthermore, we found that miR-223 could inhibit the migration and invasion of osteosarcoma cells.
Conclusions: miR-223 might be related to the metastasis of osteosarcoma and could be used as a potential diagnostic and prognostic biomarker in osteosarcoma.

1. Introduction

Osteosarcoma is one of the most common primary bone malignancies, with an incidence of 4–5 cases per million people, mainly in adolescents and young adults [1]. Osteosarcoma localizes to the proximal tibia or the distal femur and has a highly malignant tendency to destroy the surrounding normal tissues and to metastasize [2,3]. Although there have been advancements in treatment therapies, including chemotherapy, radiotherapy and tumor excision strategies, it has been reported that approximately 50% of patients with osteosarcoma develop metastases, which results in a low cure rate and a low 5-year survival rate [4]. Therefore, it is important to develop new strategies for the early diagnosis of osteosarcoma to improve treatment strategies and the prognostic outcomes in these patients. Although previous studies have shown that some molecular targets are related to tumorigenesis, the molecular mechanism underlying osteosarcoma has not been fully elucidated. Consequently, it is difficult to develop strategies for the effective diagnosis and prognosis of osteosarcoma [5,6]. Therefore, it is important to understand the molecular mechanisms of osteosarcoma in order to identify novel diagnostic and prognostic markers that will improve the clinical prognosis of osteosarcoma patients.

MicroRNAs (miRNAs) are a class of small noncoding and endogenous regulatory RNA molecules that are approximately 19–25 nucleotides in length [7,8]. Studies have shown that miRNAs regulate target gene expression by either inducing messenger RNA (mRNA) degradation through perfect base-pairing or inhibiting mRNA translation *via* imperfect base-pairing in the seed sequence using the 3′-untranslated region (UTR) of target mRNAs [9,10]. Aberrant expression of miRNAs has been observed in human cancers and has been shown to be involved in a variety of critical cellular processes, including cell differentiation, proliferation and metabolism [11]. In addition, the expression profiles of miRNAs in plasma and serum samples have been used to accurately classify human cancers, which suggest that miRNAs could potentially be used as a diagnostic and prognostic marker of cancer [12]. MicroRNA-223 (miR-223) is a hematopoietic specific microRNA with crucial functions in myeloid lineage development [13]. Previous

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studies have found that miR-223 is repressed in several human cancers, such as hepatocellular carcinoma, acute lymphoblastic leukemia, gastric MALT lymphoma and recurrent ovarian cancer [14–17]. Recently, Xu et al. [18] demonstrated that miR-223 was a tumor suppresser in osteosarcoma and showed that miR-223/Ect2/p21 signaling played a role in osteosarcoma cell cycle progression and proliferation. However, the role of miR-223 expression in the diagnosis and prognosis of osteosarcoma has not been reported.

In the present study, we detected the expression of miR-223 in the serum of osteosarcoma patients and osteosarcoma cancer cells using RT-PCR. In addition, we analyzed the role of miR-223 in the diagnosis and prognosis of osteosarcoma. Moreover, we investigated the potential role of miR-223 in osteosarcoma metastasis.

2. Materials and methods

2.1. Patients and specimens

We collected the serum samples from 112 osteosarcoma patients who were recruited from the Department of Pathology at The First Affiliated Hospital of Zhengzhou University, from 2008 to 2011. None of the patients had received chemotherapy or radiation therapy prior to the surgery. The clinical stage of the osteosarcoma patients was classified according to the Tumor Node Metastasis (TNM) Classification of Malignant Tumors (Sixth edition) from the Union for International Cancer Control (UICC) [19]. All of the osteosarcoma patients received routine follow-up after surgery (every 4 months) until their death or the last follow-up period. The serum samples from the osteosarcoma patients and healthy controls were collected prior to surgery and then frozen and stored at −80 °C for RNA extraction.

2.2. Cell lines and cell culture

Osteosarcoma cancer cell lines (U2OS, HOS, MG-63) and the conditionally immortalized human fetal osteoblastic cell line hFOB were purchased from American Type Culture Collection. The U2OS, HOS and MG-63 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with fetal bovine serum in a humid atmosphere with 5% CO_2 at 37 °C. The hFOB1.19 cells were maintained in a 1:1 mixture of Ham's F12 Medium and Dulbecco's Modified Eagle's Medium supplemented with 2.5 mL of glutamine (without phenol red) and 10% fetal bovine serum.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

The serum levels of miR-223 in the osteosarcoma patients and healthy controls were detected using qRT-PCR assay. RNA extraction was performed using a mirVana PARIS kit (Ambion, Austin, TX) according to the manufacturer's instructions. Reverse transcription (RT) was performed with a total of 10 ng of total RNA using a TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The sequences of the primers were as follows: miR-223 forward, 5′-AGC CGT GTCAGTTTG TCA AAT-3′; reverse, 5′-GTGCAGGGTCCGAGG TC-3′ U6 forward 5′-CTCGCTTCG GCAGCA CA-3′ and reverse 5′-AACGCTTCACGA ATTTGCGT-3′. U6 sRNA was used as internal control. Real-time PCR reactions for miRNAs were performed using the TaqMan MicroRNA PCR Kit (Applied Biosystems, Foster City, CA, USA), and the fluorescent data from each sample was transformed using the 7500 SDS System software (Applied Biosystems, Foster City, CA, USA). The $2^{-\Delta\Delta Ct}$ method was used to calculate the quantity of miR-223. Each sample was examined in triplicate, and the relative expression level of miR-223 was normalized to the expression of U6 using the $2^{-\Delta\Delta Ct}$ cycle threshold method. The expression level of the target gene in the osteosarcoma patients and cancer cell lines is expressed as percentage relative to the expression level in healthy controls and the normal cell line, hFOB1.19 (normalized to 1).

2.4. Overexpression of miR-223 mimics and cell migration and invasion assays

miR-223 mimics were designed as pre-miR miRNA precursor (hsa-miR-223–3p; P/N: AM12301; Applied Biosystems). The negative control RNA duplex consisted of non-specific sequences that are nonhomologous to any human genome sequences. The RNAs were incubated with Opti-MEM (Invitrogen) and Lipofectamine RNAiMAX transfection reagent. The cell transwell invasion assay was performed using 24-well transwell plates (8.0 μm, BD BioCoat). The HOS and MG-63 cells were transfected with miR-223 mimics and incubated for 24 h. We added 5×10^4 cells in serum-free media to the upper chamber and filled the transwell chamber with 750 μl of 10% fetal bovine serum and then incubated at 37 °C for 24 h. The cells on top of the upper chamber were removed using a cotton-tipped swab. The cells on the membrane were stained for 5 min using crystal violet, and then, microscopic images were taken of 5 randomly selected fields. The relative cell invasion rate (%) was calculated as the number of cells in the treatment group/control group × 100%. For the wound closure assay, HOS and MG-63 cells were plated onto 6-well plates and transfected with miR-223 mimics. The cells were cultured until they formed a 100% confluent monolayer. Then, the monolayer cells were scratched to form a 100 mm "wound" using a sterile pipette tip. The cells were incubated for an additional 24 h. The migration area was calculated as the wound area at T0 mins the wound area after 24 h.

2.5. Statistical analysis

Statistical analyses were performed using SPSS Software (version 18.0) and GraphPad Software. The Mann Whitney *U* test was used to compare the differences in the serum levels of miR-223 between the osteosarcoma patients and the healthy controls. The receiver operating characteristic (ROC) curve was drawn to evaluate the diagnostic value of the serum miR-223 levels. The Chi-squared test was used to assess the relationship between the miR-223 expression and the clinicopathological features. The Kaplan–Meier method and log-rank test were used for the survival analysis. Statistical significance in this study was set at $P < 0.05$.

3. Results

3.1. The expression of miR-223 and its diagnostic value in osteosarcoma

The serum expression level of miR-223 was examined in 112 osteosarcoma patients and 50 healthy controls using qRT-PCR. As shown in Fig. 1(A), we observed significantly lower expression of miR-223 in the osteosarcoma patients (1.21 ± 0.13) compared to the healthy controls (3.9 ± 0.34, $P < 0.01$). Moreover, Fig. 1(B) shows that the expression of miR-223 was significantly downregulated in the osteosarcoma cell lines (U2OS, HOS and MG-63) compared to the hFOB control cell line. Fig. 1(C) shows the ROC curve analysis and indicates that serum miR-223 levels might be a potential biomarker for distinguishing osteosarcoma patients from healthy controls; AUC was 0.926. When the cut-off value was set to 1.76 according to Youdex, the sensitivity and specificity of discriminating miR-223 in osteosarcoma patients was 89.5% and

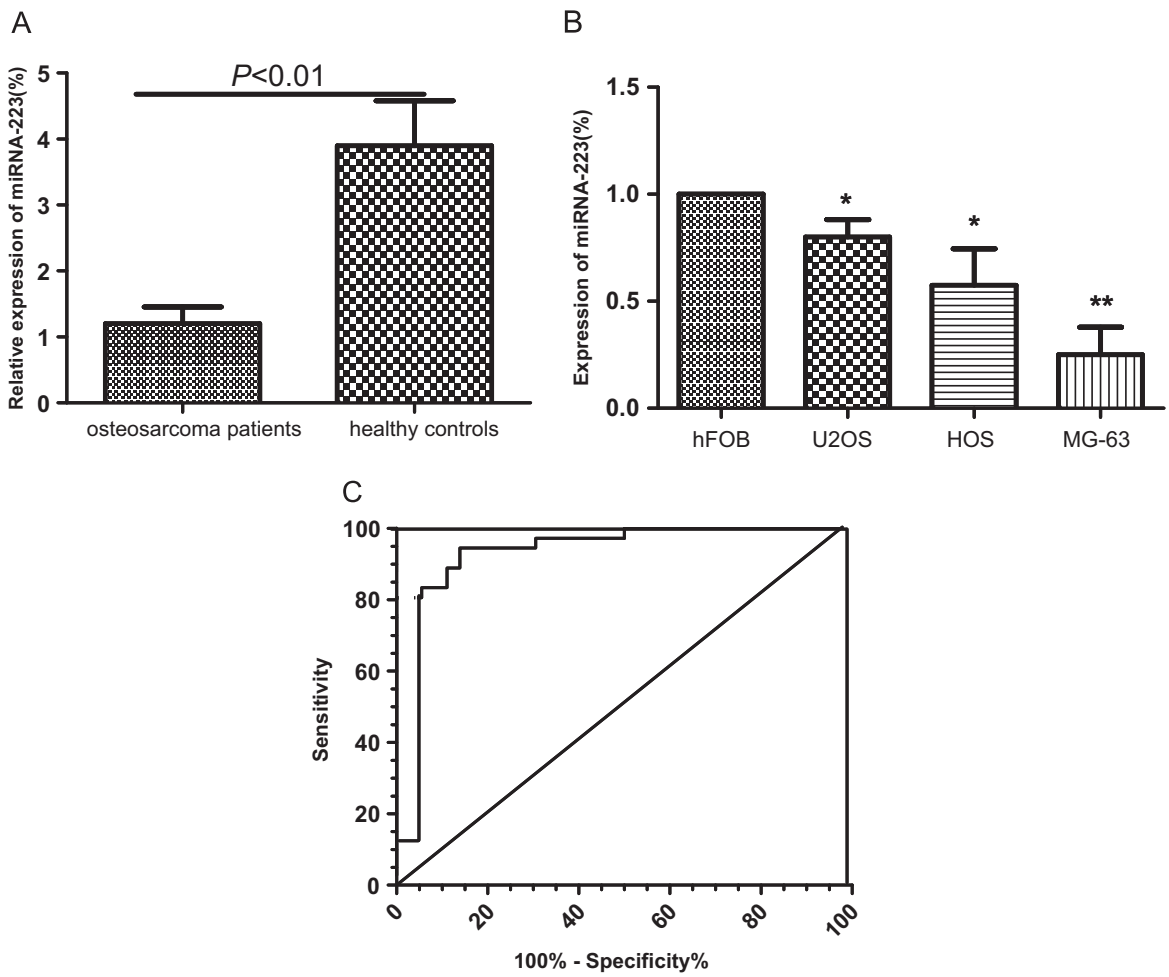

Fig. 1. (A) The serum expression levels of miR-223 in osteosarcoma patients (1.21 ± 0.13) were significantly lower compared to the healthy controls (3.9 ± 0.34, $P < 0.01$). (B) Decreased expression of miR-223 was found in three osteosarcoma cell lines (U2OS 0.80 ± 0.12, HOS 0.57 ± 0.26 and MG-63 0.25 ± 0.19) compared to a control cell line (hFOB 1.0 ± 0.01). (C) ROC analysis showed that serum miR-223 levels could distinguish osteosarcoma patients from healthy controls (AUC = 0.956).

97.2%, respectively.

3.2. Decreased expression of serum miR-223 is associated with clinicopathological characteristics in patients

We performed a preliminary analysis to identify whether serum miR-223 expression was associated with clinicopathological parameters in patients with osteosarcoma. The patients were divided into high and low miR-223 expression according to the median value of serum miR-223 expression (1.21). The results showed that low miR-223 expression occurred more frequently in osteosarcoma patients with distant metastasis ($P < 0.001$) and advanced clinical stage ($P < 0.001$). However, there was no significant association between serum miR-223 expression and age, gender, tumor site or tumor size in the osteosarcoma patients ($P > 0.05$) (Table 1).

Table 1
Association between serum miR-223 expression and clinical parameters in osteosarcoma patients.

Parameters	No. of cases	miR-223-high (n)	miR-223-low (n)	*P*
Gender				0.455
Male	55	27	28	
Female	57	32	25	
Age				0.664
< 18	60	31	29	
≥ 18	52	28	24	
Tumor site				0.111
Femur/Tibia	65	30	35	
Others	47	29	18	
Tumor size				0.792
< 8 cm	63	35	28	
≥ 8 cm	59	24	25	
Clinical stage				< 0.001
IIA	72	50	22	
IIB/III	50	10	40	
Metastasis				< 0.001
Absent	87	56	31	
Present	25	3	22	

3.3. Prognostic value of serum miR-223 in osteosarcoma patients

A Kaplan-Meier curve was performed to further assess the prognostic value of serum miR-223 expression for osteosarcoma patients. As shown in Fig. 2, the patients with low serum miR-223 expression had a shorter survival time compared to the patients with high serum miR-223 expression ($P < 0.001$). A multivariate Cox regression analysis was performed to determine whether serum miR-223 expression could be used as an efficient prognostic marker for the survival of patients with osteosarcoma. The results showed that serum miR-223 expression (RR = 4.59, 95% CI 1.84–11.45, $P = 0.001$), clinical stage (RR = 2.80, 95% CI 1.25–6.27, $P = 0.01$) and distant metastasis (RR = 2.11, 95% CI 1.02–4.36, $P = 0.04$) were independent prognostic factors for overall survival of osteosarcoma patients (Table 2).

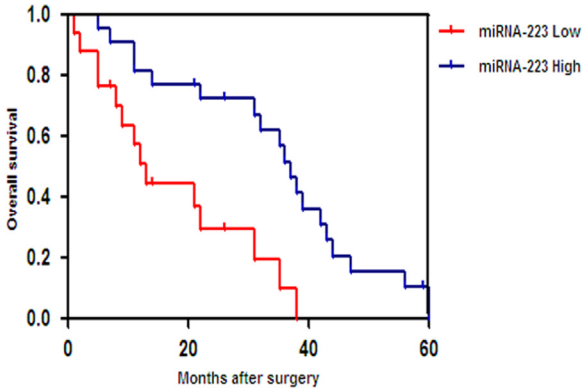

Fig. 2. A Kaplan–Meier survival curve showed the relationship between the overall survival of osteosarcoma patients and the serum miR-223 levels.

Table 2
Multivariate survival analyses for prognostic factors in osteosarcoma patients.

Variables	R Hazard ratio value	95% CI	*P*
MiR-223	4.59	1.84–11.45	0.001
Clinical stage	2.80	1.25–6.27	0.01
Metastasis	2.11	1.02–4.36	0.04

3.4. miR-223 inhibits the migration and invasion of HOS and MG-63 cells

We used a wound closure assay and transwell invasion assay to assess the potential role of miR-223 in osteosarcoma cancer metastasis. As shown in Fig. 3, the migration and invasion of the HOS and MG-63 cells that overexpressed miR-223 was inhibited compared to the negative control and native cells ($P < 0.05$ and $P < 0.001$, respectively).

4. Discussion

Osteosarcoma derives from primitive bone-forming mesenchymal cells and is the most common type of primary bone malignancy [20]. Over the past few decades, the introduction of combinatorial chemotherapy has improved the 5-year overall survival rate of patients with osteosarcoma to approximately 50–60% [21]. However, osteosarcoma-related morbidity remains high due to the difficulty of early diagnosis and the lack of efficient therapeutic approaches for osteosarcoma. Therefore, it is necessary to identify highly sensitive and specific diagnostic and prognostic biomarkers to diagnose osteosarcoma at an early stage and initiate aggressive therapy.

Studies have shown that miR-223 has a crucial role in cancer development. Additionally, miR-223 abnormal expression was found in several cancer types [22,23]. More importantly, serum miRNA expression has been shown to be a promising candidate for early detection and prognosis in cancer patients [24]. A recent study showed that miR-223 is involved in osteosarcoma cell cycle progression and proliferation [18]. However, the role of miR-223 as a serum diagnostic and prognostic biomarker has not been previously explored in osteosarcoma patients. In the present study, we quantified the serum expression levels of miR-223 in osteosarcoma patients and healthy controls and then assessed the potential value of miR-223 as a serum diagnostic and prognostic marker in osteosarcoma patients.

The results from our study show for the first time that the expression of miR-223 was remarkably decreased in the serum of osteosarcoma patients and in osteosarcoma cancer cell lines. Furthermore, we showed that the expression of miR-233 could be used to discriminate osteosarcoma from healthy controls (AUC = 0.962), with a specificity of 86.4% and a sensitivity 97.2%. Notably, decreased serum miR-223 levels were found to be significantly associated with distant cancer metastasis and advanced clinical stage in osteosarcoma patients, which suggests that miR-223 might act as a tumor suppressor in the development of osteosarcoma. This finding is consistent with previous findings that miR-223 could inhibit osteosarcoma cell cycle progression and proliferation [18]. miRNAs can act as oncogenes or tumor suppressor in multiple types of cancers when abnormally expressed [25]. Similar to other miRNAs, the expression and role of miR-223 significantly varies in different types of cancer. Previous studies have shown that miR-223 is significantly downregulated in cancer tissues of patients with prostate cancer, suggesting that miR-223 may act as a tumor suppressor in prostate cancer [26]. Conversely, miR-223 has also been reported to be overexpressed in other types of cancers, such as esophageal carcinoma and gastric cancer [27,28]. Predicting the clinical outcomes of osteosarcoma patients is important for better treatment and therapeutic strategies. Previous studies have found that the abnormal expression of miR-223 was associated with the development and prognosis of lymphoma [29]. In the present study, we assessed the prognostic role of miR-223 in patients with osteosarcoma and showed that serum miR-221 is a potential independent and effective prognostic biomarker for osteosarcoma, particularly for patients with advanced clinical stage and distant metastasis. In addition, the transwell invasion assay showed that miR-223 mimics significantly inhibited the invasion of MG-63 cells, which suggests that miR-223 may play a role in osteosarcoma metastasis.

Previous studies have shown that miR-223/epithelial cell transforming sequence 2 (Ect2) signaling might be an important pathway regulating cell cycle progression and proliferation in osteosarcoma. Moreover, miR-223/Ect2 signaling was shown to be correlated with the recurrence and the poor response to chemotherapy in osteosarcoma [30]. In addition, inhibition of miR-223 expression promotes osteosarcoma cell proliferation by targeting Ect2 activation [31]. Furthermore, Ect2 expression is elevated in various tumor cell lines and tissues, including lung cancer, esophageal cancer, pancreatic cancer and oral cancer, which suggests that Ect2 may function as a proto-oncogene [32,33]. Therefore, we speculated that miR-223 may inhibit cell invasion by negatively regulating Ect2 expression during the progression of osteosarcoma. Future studies are necessary to determine the downstream functional targets of miR-223 in osteosarcoma. The results of the present study and other similar studies suggest that miR-223 is a potential biomarker in cancer development. However, it should be noted that all of these studies were collected in one center with relatively small number of samples. Therefore, future studies with a larger sample size are necessary to confirm these results.

In conclusion, decreased miR-223 expression was associated with advanced clinical stage and distant metastasis in osteosarcoma patients, which suggests that miR-223 has a suppressive role in osteosarcoma. In addition, we showed that miR-223 might be a potential diagnostic and prognostic marker in osteosarcoma. Most importantly, decreased miR-223 may be involved in the development of osteosarcoma by accelerating cell invasion. Furthermore, serum miR-223 could be potentially explored as a novel and efficient prognostic biomarker osteosarcoma in order to improve personalized therapeutic strategies.

Conflict of interest

None.

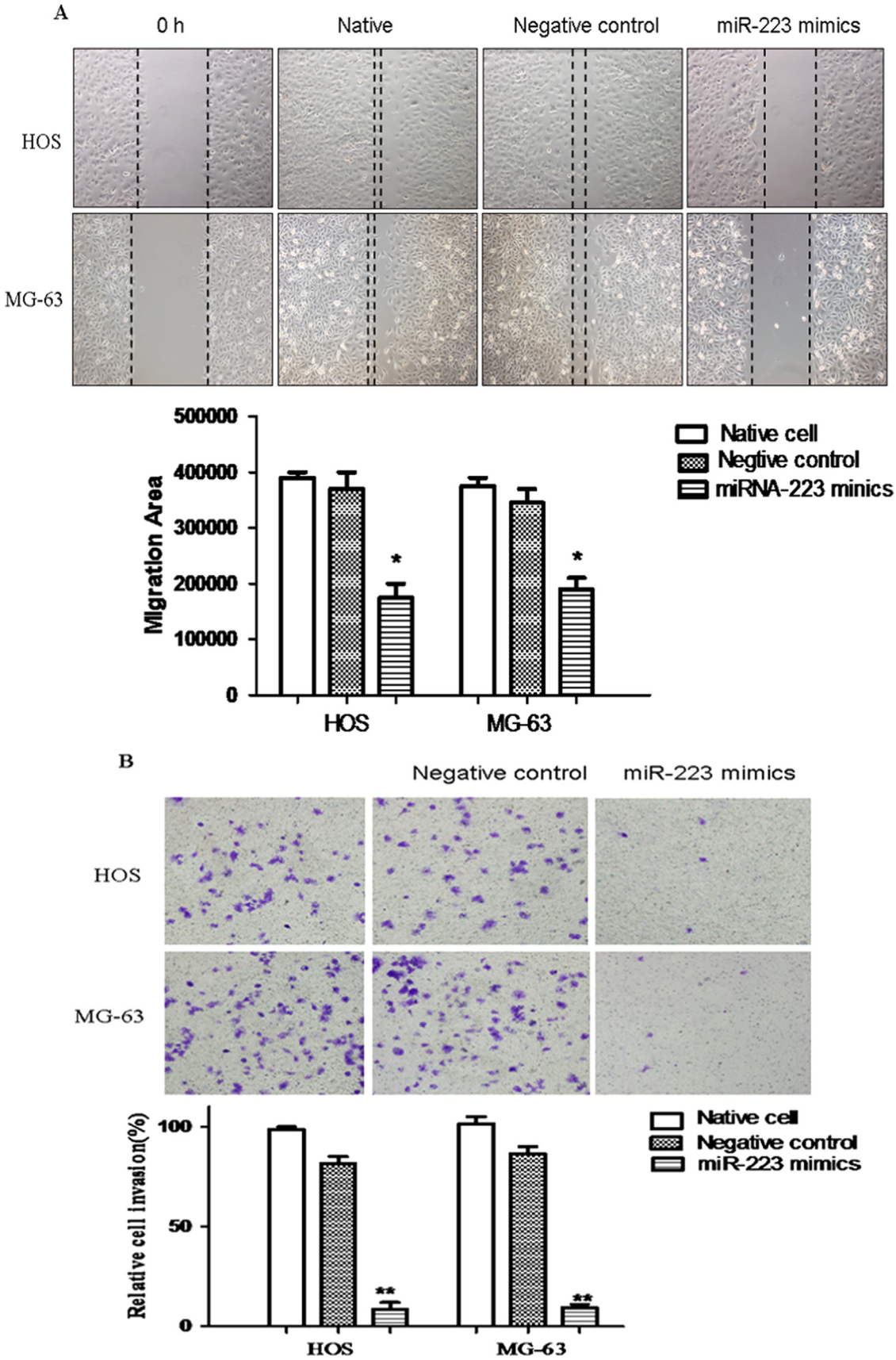

Fig. 3. (A) miR-223 mimics significantly inhibited the migration of HOS and MG-63 cells (*$P < 0.05$) compared to native HOS and MG-63 cells and negative control cells. (B) miR-223 mimics significantly reduced the invasion of HOS and MG-63 cells. The results are presented as the relative cell invasion rate (%) compared to native and negative control cells ($P < 0.001$).

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