Int J Clin Exp Pathol 2019;12(2):477-487
www.ijcep.com /ISSN:1936-2625/IJCEP0089180

Original Article

Interference from LncRNA SPRY4-IT1 restrains the proliferation, migration, and invasion of melanoma cells through inactivating MAPK pathway by up-regulating miR-22-3p

Zhiqing Li[1], Xuefeng Tang[3], Song Duan[2]

[1]Department of Dermatology, Institute of Dermatology and Venereal Diseases, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, Chengdu, Sichuan, China; [2]Department of Pathology, Chongqing Three Gorges Central Hospital, Chongqing, China; [3]Department of Pathology, Xinqiao Hospital of AMU, Chongqing, China

Received November 29, 2018; Accepted December 22, 2018; Epub February 1, 2019; Published February 15, 2019

Abstract: Melanoma is a common malignancy with a low survival rate worldwide. Long non-coding RNA Sprouty4-Intron 1 (SPRY4-IT1) is correlated with various cancers, including melanoma. Herein, the underlying molecular mechanisms of SPRY4-IT1 in melanoma were characterized. We found that SPRY4-IT1 level was upregulated in melanoma cells lines compared to the normal skin cells, while miR-22-3p was downregulated. According to of bioinformatics analysis, SPRY4-IT1 was a hypothetic target of miR-22-3p, and knockdown SPRY4-IT1 by sh-RNA (sh-SPRY4-IT1) markedly elevated the miR-22-3p level. Also, the target relationship was further confirmed by dual luciferase reporter assay. In addition, low-expression of SPRY4-IT1 impeded cell proliferation, invasion, migration, and epithelial-mesenchymal transition. Furthermore, western blot assay indicated that the enhanced miR-22-3p further decelerated the phosphorylation of p38MAPK, MAPKAPK and Hsp27, which indicates that miR-22-3p could inactivate the p38MAPK/MAPKAPK/Hsp27 signaling pathway. Overall, our results show that sh-SPRY4-IT1 inhibits cell proliferation and motility through inactivating MAPK signaling by up-regulating miR-22-3p. Therefore, designing targeted drugs against SPRY4-IT1 provides a new direction for the treatment of melanoma.

Keywords: Melanoma, SPRY4-IT1, miR-22-3p, MAPK pathway, proliferation, motility

Introduction

Melanoma is one of the most common malignancies with high aggressive and metastatic worldwide [1]. Nowadays, the incidence of melanoma has increased with an annual growth rate of about 4.1% [2]. Generally, melanoma constitutes only 5% of all skin cancers but accounts for over 65% of skin cancer-related deaths due to the abnormal proliferation and early metastasis [3]. Localized melanomas can be cured by surgical excision. Patients with distant metastases might be treated with chemotherapeutic, radiotherapeutic and immunotherapies [4, 5]. However, the clinical application of these methods faces many limitations and challenges. Therefore, it is imperative to further explore the underlying molecular mechanisms and discover some new effective therapeutics to melanoma.

Sprouty4 (SPRY4) is one of the genes (SPRY1-4) which has been reported to be involved in various human cancers [6, 7]. Long non-coding RNA Sprouty4-Intron 1 (SPRY4-IT1) is a 708 bp lncRNA that originated from an intron of SPRY4 gene. It encodes an endogenous inhibitor of the receptor-transduced mitogen-activated protein kinase (MAPK) pathway [7, 8]. Initially, SPRY4-IT1 was reported to be a carcinogen in melanoma [9]. Much research has shown that abnormal expression of SPRY4-IT1 occurs in many other diseases, such as preeclampsia [10], osteosarcoma [11], testicular germ cell tumor [12], lung cancer [13], pancreatic cancer [14], and gastric cancer [15]. Although the role of SPRY4-IT1 was first discovered in melanoma,

the underlying molecular mechanism remains unclear.

miR-22-3p is a noncoding RNA consisted of 22 nucleotides. It was originally identified as an anti-oncogene in HeLa cells [16, 17]. A mass of studies indicated that miR-22-3p was involved in various diseases, including the liver cancer [18], pancreatic cancer [19], cervical carcinoma [20] and gastric cancer [21]. However, the roles of miR-22-3p in various cancers are inconsistent. In previous studies, miR-22-3p was reported to act as an oncogene, promoting malignancy in breast cancer [22-24], cervical cancer [20] and multiple myeloma [25]. However, several studies found it was an anti-oncogene in liver cancer [18] and hepatocellular carcinoma [26]. Nevertheless, the role of miR-22-3p in melanoma remains unknown.

The p38/mitogen-activated protein kinase (p38/MAPK) is a crucial signal pathway which is shown to modulate multifarious biological processes such as proliferation, differentiation, and survival [27]. Both stress and inflammatory cytokines can induce activation of p38/MAPK pathway. As the primary substrate for p38/MAPK, MAPK-activated protein kinase-2 (MAPKAPK-2; MK2) can momentarily phosphorylate the ubiquitously expressed heat shock protein 27 (HSP27), a protein in response to osmotic stress, reactive oxygen species, and inflammatory cytokines. Previous studies showed that HSP27 plays an important role in cell migration, apoptosis, and actin cytoskeleton organization [28]. Numerous studies exhibited that the activation of p38MAPK pathway involved in various diseases, such as epithelial cells [29], intrahepatic cholangiocarcinoma [30] and colorectal cancer [31]. However, whether p38MAPK pathway is correlated with melanoma is still unclear.

This study aimed to investigate the underlying molecular mechanism of SPRY4-IT1 in melanoma in vitro. In present study, we revealed a novel mechanism of SPRY4-IT1 in melanoma and assert that designing targeted drugs against SPRY4-IT1 is a new direction for the treatment of melanoma.

Materials and methods

Cell lines and cell culture

The human melanoma cell lines (A375, A875) and normal (fibroblast-like) skin cell lines (TE 353.SK) from the same individual were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). COLO-679 was from Deutsche Sammlung von Mikroorganismen (Braunschweig, Germany). All cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, GIBCO, Carlsbad, CA), 1% penicillin (GIBCO, Carlsbad, CA), and 1% streptomycin (GIBCO, Carlsbad, CA) at 37°C under 5% CO_2.

Cell transfection

sh-SPRY4-IT1, Ad-SPRY4-IT1, miR-22-3p inhibitor, miR-22-3p mimic and negative control were provided by Gene Pharma (Shanghai, China). Transfection of A375 cells was conducted in accordance to Lipofectamine 2000 transfection reagent (Invitrogen, Grand Island, NY, USA).

Quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA was extracted from human melanoma cell lines (A375, A875, COLO-679), normal (fibroblast-like) skin cell lines (TE 353.SK) and tumor tissues with TRIzol reagent (Invitrogen, USA) and reverse-transcribed to cDNA by RevertAid First Strand cDNA Synthesis kit (Thermo Fisher, USA). RT-qPCR was conducted by SYBR-Green PCR Master Mix kit (Takara, Japan) and ABI 7500 Real-Time PCR System (Applied Biosystems, USA). The primers of GAPDH were 5'-GTCAGGATCCACTCATCACG-3' (sense) and 5'-GATCGGACTTACGGACTCACATC-3' (antisense); The primers of SPRY4-IT1 were 5'-GCTTATCGATCCGGATGGCGTAG-3' (sense) and 5'-GGCTAAATCGGCTGAGCTAGGC-3' (antisense); The primers of miR-22-3p were 5'-TGCCATGCAAGGGCTTTACGCC-3' (sense) and 5'-CCGTAAGGCTTAAACGTAGGCTCC-3' (antisense). GAPDH was used as an internal reference. Fold changes were calculated by the equation 2-ΔΔCt. The experiments were independently repeated in triplicate.

Dual luciferase reporter assay

Targetscan7.0 (http://www.targetscan.org) was employed to speculate the target of miR-22-3p on the lncRNA SPRY4-IT1 3'untranslated region (3'UTR). The 3'UTR segments of SPRY4-IT1 (wt and mut) was amplified and inserted into the luciferase reporter vector (pmirGLO; Promega, USA). A375 cells were transfected with Luc-SPRY4-IT1-wt or Luc-SPRY4-IT1-mut or combi-

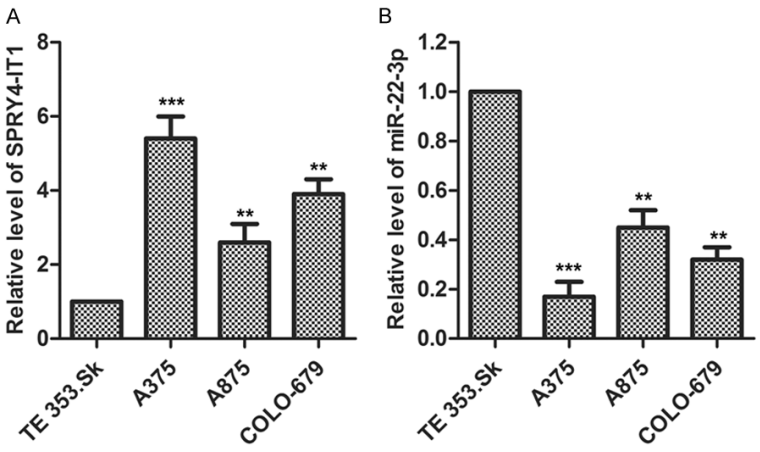

Figure 1. Increase of SPRY4-IT1 and decrease of miR-22-3p in human melanoma. A. The expression level of SPRY4-IT1 was measured by RT-qPCR in human melanoma cell lines (A375, A875 and COLO-679) and normal skin cells (TE 353.SK). B. The expression level of miR-22-3p was detected by RT-qPCR in human melanoma cell lines (A375, A875 and COLO-679) and normal skin cells (TE 353.SK). (**$P < 0.01$ vs. control; ***$P < 0.001$ vs. control).

nation with miR-22 mimic in accordance to Lipofectamine 2000 transfection reagent (Invitrogen, 11668019, USA). The luciferase activities were monitored by Dual-Luciferase Reporter Assay kit (Promega, USA). The experiments were independently repeated in triplicate.

BrdU staining assay

A375 cells transfected with sh-SPRY4-IT1 or miR-22-3p inhibitor and in combination with miR-22-3p inhibitor were seeded on coverslips in 96-well plates and cultivated overnight. After adding BrdU (10 μg/mL) to culture medium, the cells were further incubated for another 1 h, and then fixed in 4% paraformaldehyde for 10 minutes. Finally, cells were stained with anti-BrdU antibody (Biocompare, South San Francisco, CA) in accordance to the manufacturer's instructions. The coverslips were counterstained with DAPI and photographed with fluorescence microscopy (Olympus, Tokyo, Japan). Results were expressed as the cell number per field. The experiments were independently repeated in triplicate.

Western blot analysis

A375 cells and tumor tissues were ground in liquid nitrogen and lysed in lysis buffer (Beyotime, China). After separated by 8% SDS poly-acrylamide gel, proteins were moved to polyvinylidene difluoride (PVDF) membranes (Millipore, IPFL00010, Billerica, MA). After probed with primary antibodies, the samples were incubated with corresponding secondary antibodies (Santa Cruz Biotechnology, USA) and detected with an enhanced chemiluminescence (ECL) substrate kit (Amersham Biosciences). The primary antibodies and secondary antibodies specific to Ki-67 (sc-23900, 1:1000), PCNA (sc-71858, 1:1000), E-cadherin (sc-8426, 1:1000), Vimentin (sc-80975, 1:1000), p-p38 (sc-7973, 1:1000), p-MAPKAPK (sc-293140, 1:1000), p-HSP 27 (sc-81498, 1:1000), GAPDH (sc-66163, 1:1000) were all from Santa Cruz Biotechnology (USA). The relative protein level was analyzed by ImageJ software. The experiments were independently repeated in triplicate.

Transwell assay

To determine invasive capacity of cells, Transwell assay was conducted. After diluted by serum-free DMEM medium, matrigel was coated on the upper surface of the apical chamber. Air drying at room temperature for later use. After 24 h of starving culture with serum-free medium, cell suspension was added into the apical chambers (200 ml per chamber). The lower chambers were filled with 600 μl medium containing 10% FBS. After incubation at 37°C for 48 h, the chambers were pulled out and swashed with PBS for twice. A cotton bud was utilized to expunge the residual cells on the upper membranes. After fixed with 95% alcohol and stained with crystal violet, cells were examined by microscope (Leica, Germany) and the average number of invasive cells was recorded. The experiments were independently repeated in triplicate.

Wound healing assay

Wound healing assay was performed to monitor migration ability in vitro. Mitomycin C (10 μg/ml) was added to the cell culture medium to inhibit cell replication [32]. A375 cells were

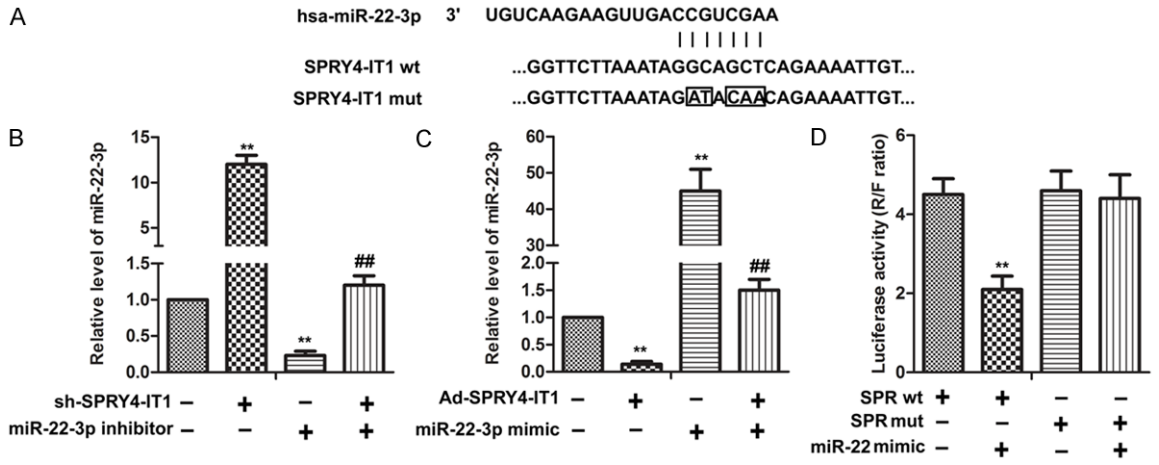

Figure 2. miR-22-3p was negatively regulated by SPRY4-IT1 in A375 cells. A. The target of miR-22-3p on SPRY4-IT1 3'UTR (wt and mut). B. The expression level of miR-22-3p was monitored by RT-qPCR in A375 cells transfected with sh-SPRY4-IT1 or miR-22-3p inhibitor and in combination with miR-22-3p inhibitor. (**$P < 0.01$ vs. control; ##$P < 0.01$ vs. miR-22-3p inhibitor group). C. The expression level of miR-22-3p was monitored by RT-qPCR in A375 cells transfected with Ad-SPRY4-IT1 or miR-22-3p mimic and in combination with miR-22-3p mimic. (**$P < 0.01$ vs. control, ##$P < 0.01$ vs. miR-22-3p mimic group). D. The activity of SPRY4-IT1 was monitored by dual luciferase reporter assay in A375 cells co-transfected with SPRY4-IT1 (wt and mut) and miR-22-3p mimic. (**$P < 0.01$ vs. control).

transfected with sh-SPRY4-IT1 or miR-22-3p inhibitor and in combination with miR-22-3p inhibitor and incubated at 37°C overnight. After that, cell monolayers were ripped by a sterile micropipette tip and photographed with inverted microscope (Nikon, Tokyo, Japan) at 0 h and 24 h. All scratch would formation, images were monitored at a magnification of 40 × and measured by Image Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA). The experiments were independently repeated in triplicate.

Immunofluorescent assay

To estimate the level of p38 in A375 cells transfected with sh-SPRY4-IT1 or miR-22-3p inhibitor and in combination with miR-22-3p inhibitor, immunofluorescent assay was manipulated. Briefly, A375 cells were seeded onto small glass dishes and transfected with sh-SPRY4-IT1 or miR-22-3p inhibitor and in combination with miR-22-3p inhibitor. After that cells were fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.5% TritonX-100 for another 30 min, and blocked with TBST containing 5% bovine serum albumin (BSA, Affymetrix, Cleveland, OH) at 37°C overnight. After being pretreated with primary antibodies against p38 (1:50, #9212, Cell Signaling Technology) at 4°C overnight, cells were incubated with Alexa Flour® 594 secondary antibody (1:2000, cat. no. Z-25307, Thermo Fisher) and stained with DAPI (1:1000, cat. no. D9564, Sigma-Aldrich) at room temperature for 3 h. Subsequently, the cells were washed with PBS and analyzed by confocal microscopy (LSM 510 Meta, Zeiss, Oberkochen, Germany). The experiments were independently repeated in triplicate.

Statistical analysis

The statistical analysis was conducted with SPSS 21.0 (SPSS, Inc, Chicago, IL, USA). Measurement data are presented as mean ± SD (x ± s), and data consistent with the normal distribution were analyzed by t-test. Multiple sets of data were analyzed by One-way ANOVA followed by Bonferroni post-hoc test. Enumeration data are presented as a percentage or ratio and verified by Chi-Square Test. $P < 0.05$ was considered as significant.

Results

Increase of SPRY4-IT1 and decrease of miR-22-3p in human melanoma

We firstly monitored SPRY4-IT1 and miR-22-3p in three melanoma cell lines (A375, A875 and COLO-679) and normal skin cells (TE 353.SK) by RT-qPCR. As shown in **Figure 1A**, SPRY4-IT1 level was significantly increased in three mela-

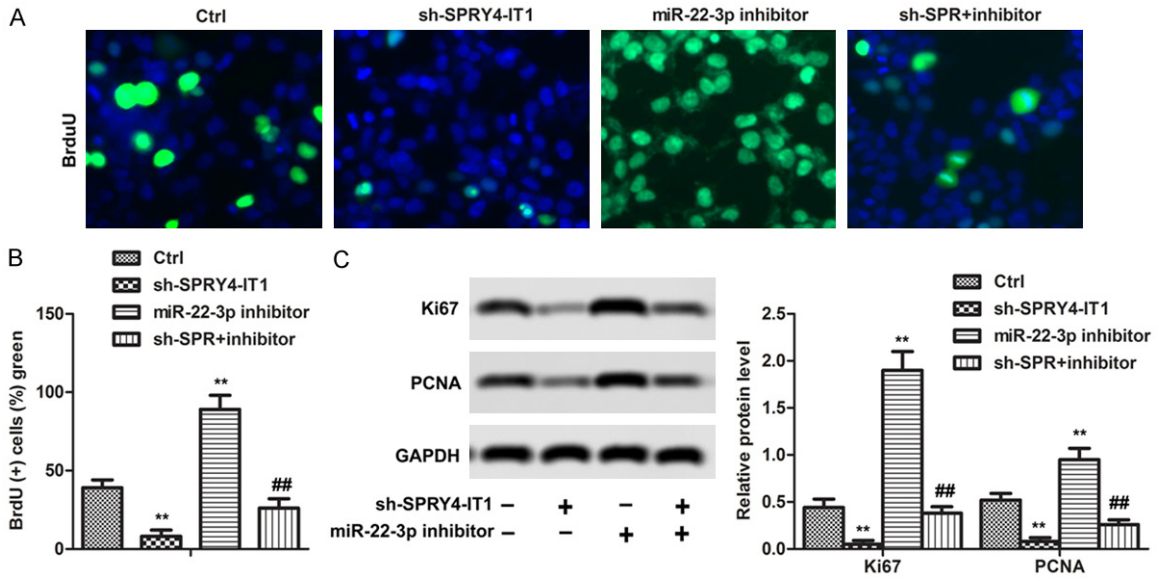

Figure 3. sh-SPRY4-IT1 suppressed proliferation by regulating miR-22-3p in A375 cells. A375 cells were transfected with sh-SPRY4-IT1 or miR-22-3p inhibitor and in combination with miR-22-3p inhibitor. A. The cell proliferation was monitored by BrdU staining assay. B. The bar graph showed the percentage of BrdU-positive cells. (**P < 0.01 vs. control, ##P < 0.01 vs. miR-22-3p inhibitor group). C. The expression of proliferation marker proteins Ki-67 and PCNA were monitored by western blot. (**P < 0.01 vs. control, ##P < 0.01 vs. miR-22-3p inhibitor group).

noma cell lines (A375, A875 and COLO-679) compared with normal skin cell lines (TE 353. SK). (P < 0.01, P < 0.001). Conversely, miR-22-3p level was significantly decreased (**Figure 1B**; P < 0.01, P < 0.001). Compared with TE 353. SK, A375 cell line showed greatly significant differences both in SPRY4-IT1 and miR-22-3p (P < 0.001). Thus, A375 cell line was used in the follow-up experiments (P < 0.001). Taken together, these results demonstrated that SPRY4-IT1 and miR-22-3p might be implicated in human melanoma with contrary effects.

miR-22-3p was negatively regulated by SPRY4-IT1 in A375 cells

Targetscan7.0 (http://www.targetscan.org) was employed to speculate the target of miR-22-3p on the lncRNA SPRY4-IT1 3'UTR (wt and mut). (**Figure 2A**). miR-22-3p level was measured by RT-qPCR. As shown in **Figure 2B**, knockdown SPRY4-IT1 by sh-RNA (sh-SPRY4-IT1) noticeably upregulated the expression of miR-22-3p in A375 cells (P < 0.01). A reverse result was observed after transfection with miR-22-3p inhibitor (**Figure 2B**; P < 0.01). On the contrary, transfection with Ad-SPRY4-IT1 significantly reduced the miR-22-3p level. (**Figure 2C**; P < 0.01). All data suggested that SPRY4-IT1 caused negative regulation of miR-22-3p. Luciferase report assay further confirmed the correlation between SPRY4-IT1 and miR-22-3p. Obviously, there was a significant decrease in A375 cells transfected with SPR wt and miR-22-3p mimic, (**Figure 2D**; P < 0.01) while no marked effect was observed in a combination of SPR mut and miR-548c mimic. (**Figure 2D**; P > 0.05). In short, these results indicated that SPRY4-IT1 negatively regulated miR-22-3p in A375 cells.

sh-SPRY4-IT1 suppressed proliferation by regulating miR-22-3p in A375 cells

BrdU staining assay was applied to investigate proliferation and western blot was utilized for further verification of cell proliferative capacity. As shown in **Figure 3A**, after transfection with sh-SPRY4-IT1, BrdU-positive cells were markedly decreased in the sh-SPRY4-IT1 group compared to control and miR-22-3p inhibitor group. (**Figure 3B**, P < 0.01). Western blot assays showed that the expression of proliferation marker proteins (Ki67 and PCNA) was down-regulated in the A375 cells sh-SPRY4-IT1 group (**Figure 3C**, P < 0.01) while a reverse result was observed in the miR-22-3p inhibitor group. (**Figure 3B**, P < 0.01). Taken together, the pres-

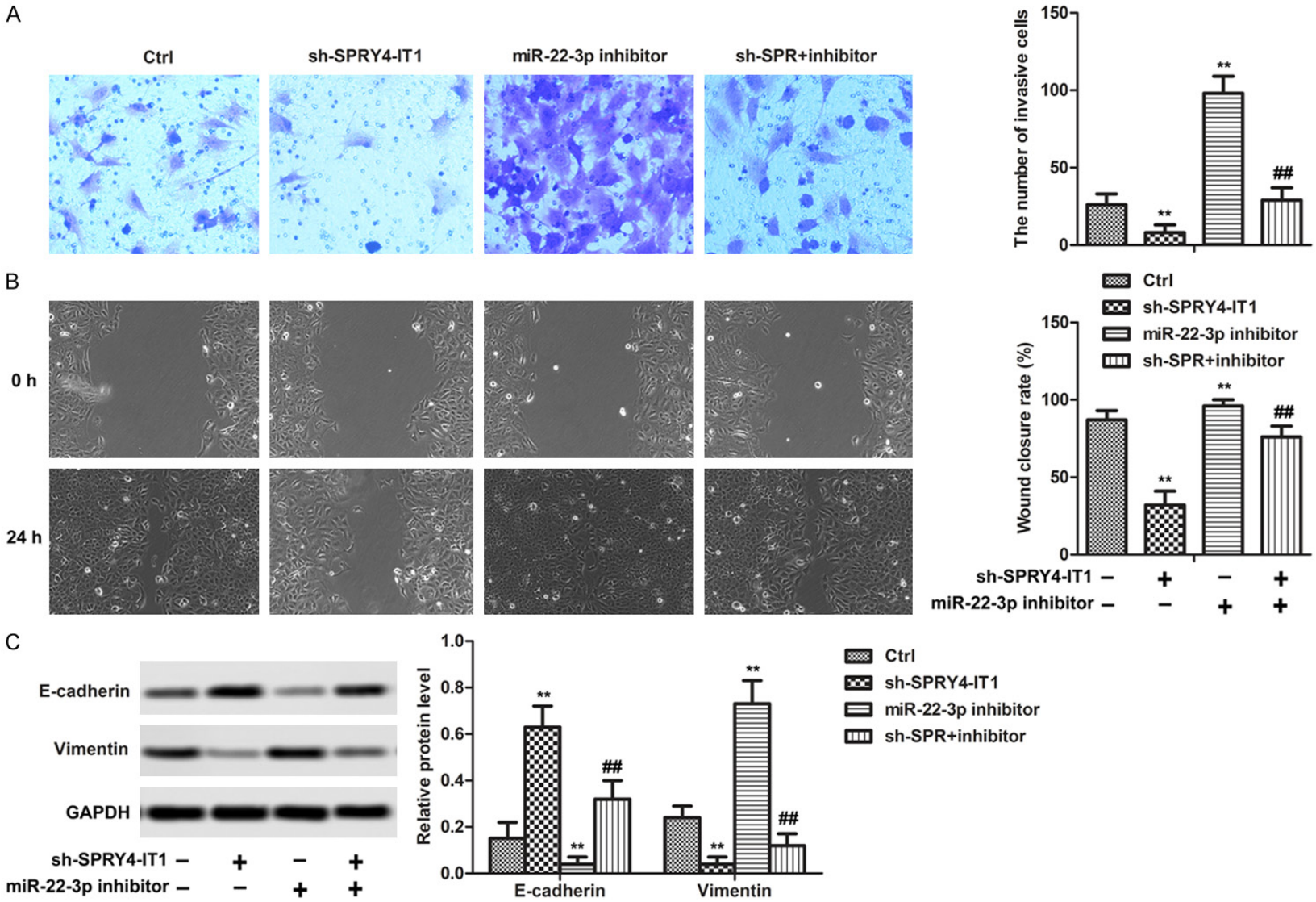

Figure 4. sh-SPRY4-IT1 restrained migration, invasion and EMT by regulating miR-22-3p in A375 cells. A375 cells were transfected with sh-SPRY4-IT1 or miR-22-3p inhibitor and in combination with miR-22-3p inhibitor. A. The invasive ability was monitored by transwell assay. B. Migration capacity was monitored by wound healing assay. (**$P < 0.01$ vs. control, ##$P < 0.01$ vs. miR-22-3p inhibitor group). C. The expression of EMT marker proteins (E-cadherin and Vimentin) were monitored by western blot. (**$P < 0.01$ vs. control, ##$P < 0.01$ vs. miR-22-3p inhibitor group).

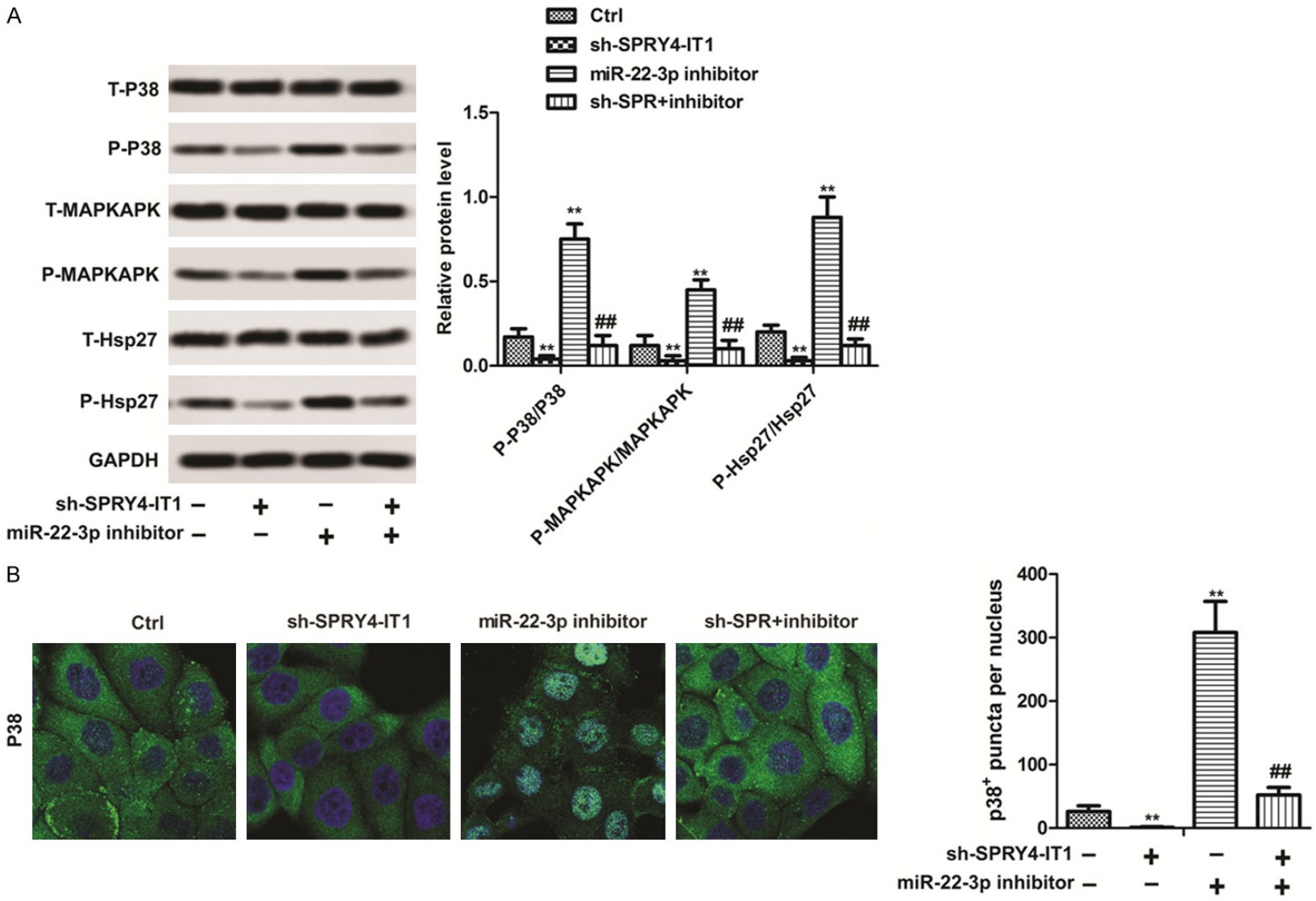

Figure 5. MAPK pathway is implicated in the inhibition of sh-SPRY4-IT1 on A375 cells proliferation and motility by regulating miR-22-3p. A375 cells were transfected with sh-SPRY4-IT1 or miR-22-3p inhibitor and in combination with miR-22-3p inhibitor. A. The phosphorylation levels of MAPK signaling related proteins were monitored by western blot. (***P* < 0.01 vs. control, ##*P* < 0.01 vs. miR-22-3p inhibitor group). B. The translocation of p38 in the nucleus was measured by immunofluorescence assay. (***P* < 0.01 vs. control, ##*P* < 0.01 vs. miR-22-3p inhibitor group).

ent study indicated that sh-SPRY4-IT1 suppressed proliferation by regulating miR-22-3p in A375 cells.

sh-SPRY4-IT1 restrained migration, invasion and EMT by regulating miR-22-3p in A375 cells

Transwell assay and wound healing assay were applied to monitor the migration and invasion levels, while western blot was utilized to investigate the EMT process of A375 cells. As shown in **Figure 4A** and **4B**, down-regulation of SPRY4-IT1 significantly inhibited A375 cell invasion, and migration capacity, whereas, miR-22-3p inhibitor counteracted the inhibitory effects. (**Figure 4A** and **4B**, $P < 0.01$). Generally, increase of E-cadherin and loss of vimentin are the fundamental events in EMT. As shown in **Figure 4C**, low expression of SPRY4-IT1 observably increased E-cadherin protein levels and decreased vimentin protein levels ($P < 0.01$), whereas the miR-22-3p inhibitor did the contrary ($P < 0.01$). Therefore, our research showed that sh-SPRY4-IT1 restrained migration, invasion and EMT by regulating miR-22-3p in A375 cells.

MAPK pathway is implicated in the inhibition of sh-SPRY4-IT1 on A375 cell proliferation and motility by regulating miR-22-3p

A375 cells were transfected with sh-SPRY4-IT1 or miR-22-3p inhibitors and in combination with miR-22-3p inhibitor. Western blot assays were used to investigate the molecular mechanism of sh-SPRY4-IT1 on A375 cells. As shown in **Figure 5A**, the phosphorylation levels of p38, MAPKAPK, and Hsp27 were restrained in sh-SPRY4-IT1 group. (**Figure 5A**, $P < 0.01$). However, after transfection with miR-22-3p inhibitors, phosphorylation levels of p38, MAPKAPK, and Hsp27 were significantly accelerated. (**Figure 5A**, $P < 0.01$). These results demonstrated that sh-SPRY4-IT1 suppressed the activation of MAPK signaling by up-regulating miR-22-3p. Furthermore, immunofluorescence analysis showed that sh-SPRY4-IT1 markedly reduced the p38-positive punctum. (**Figure 5B**; $P < 0.01$), and the decrease was counteracted with the transfection of miR-22-3p inhibitor. Collectively, we claimed that MAPK pathway was implicated in the inhibition of sh-SPRY4-IT1 on A375 cell proliferation and motility by regulating miR-22-3p.

Discussion

Melanoma has been a serious skin disease around the world due to the high mortality and the low survival rate. Despite a couple of novel targeted therapies available in melanoma treatment, as a result of the clinical limitations, the survival rates of patients suffered from malignant melanoma is remain low [33]. At present, no completely effective treatments are available. The abnormally expressed proteins associated with proliferation, invasion and migration were culprit in the formation of melanoma [3]. Therefore, it is the most intuitive way to investigate the underlying molecular mechanism by proliferation and invasion in melanoma.

Previous studies demonstrated that LncRNAs were identified as significantly abnormal expression in the melanoma. As an intronic lncRNA of the SPRY4 gene, SPRY4-IT1 plays significant roles in melanomagenesis. Knockdown of SPRY4-IT1 remarkably promoted cell death, invasion, and apoptosis of the melanoma cell lines A375 and WM1552C [9]. Further clinical studies showed that overexpression of SPRY4-IT1 was observed in melanoma patients, compared to low-expression in healthy controls. Additionally, the increase of SPRY4-IT1 markedly lowered overall survival rates of patients [34]. Consistent with these results, in our study, we found that SPRY4-IT1 was overexpressed in human melanoma compared to the normal skin cells. Knockdown of SPRY4-IT1 obviously inhibited cell proliferation, invasion and migration. These results suggest that SPRY4-IT1 is closely related to the formation of melanoma.

miR-22-3p is a pivotal small RNA molecule involved in cell proliferation, invasion, and tumorigenesis. Studies by Chen et al. showed that upregulation of miR-22-3p suppressed cell growth and induced apoptosis in hepatocellular carcinoma cells [18]. Guo et al. indicated that miR-22 acted as tumor suppressor by targeting the Sp1 gene and inhibiting gastric cancer cell migration and invasion [35]. Furthermore, Chen et al. found that miR-22-3p was regulated by berberine, thus suppressing cancer cell growth in HCC [36]. Likewise, in our study, we investigated the role of miR-22-3p in melanoma. The results showed that knockdown of SPRY4-IT1 obviously accelerated the

expression of miR-22-3p, and the enhanced miR-22-3p further inhibited the proliferation and metastasis of melanoma cells.

The p38 mitogen-activated protein kinase (MAPK) signaling pathway is activated by numerous inflammatory mediators and environmental stresses. Mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MAPKAPK-2 or MK2), a downstream substrate of the p38MAPK, is responsible for the signaling events influencing inflammation, cell division and differentiation, apoptosis, and cell motility. Previous studies demonstrated that p38/MAPK modulated cell proliferation and metastasis in various cancers. Jiang et al. indicated that inactivation of p38/MAPK induced by SIP-SII restrained migration and invasion of ovarian cancer cells [37]. Additionally, epithelial-mesenchymal transition (EMT) could also be regulated by the p38/MAPK/MAPKAPK/Hsp27 signaling pathway. According to the results of Kondo et al., after treating with flagellin and TGF-β1, the phosphorylation of p38MAPK, MAPKAPK and Hsp27 were all upregulated, and the levels of E-cadherin protein were reduced in intestinal epithelial cells [29]. Also, Ling et al. indicated that SB203580 was able to weaken cell migration, invasion, and vasculogenic mimicry forming abilities by blocking p38/MAPK signaling pathways [38, 39]. Similarly, in our studies, we found that knockdown SPRY4-IT1 by sh-RNA obviously impeded the phosphorylation of p38MAPK, MAPKAPK and Hsp27, and enhanced E-cadherin protein, which indicating that sh-SPRY4-IT1 could inhibit cell proliferation, invasion, and migration by inactivating the p38MAPK/MAPKAPK/Hsp27 signaling pathway.

In conclusion, our research revealed a novel mechanism of SPRY4-IT1 in melanoma. The primary mechanisms can be summarized as following: 1) SPRY4-IT1 was upregulated in melanoma, while miR-22-3p was downregulated. Low-expression of SPRY4-IT1 noticeably elevated miR-22-3p level in melanoma. 2) Knockdown of SPRY4-IT1 by sh-RNA (sh-SPRY4-IT1) noticeably suppressed proliferation and motility in melanoma. 3) sh-SPRY4-IT1 interacted with miR-22-3p to inhibit melanoma cell growth and motility by interfering with the MAPK pathway *in vitro*.

Disclosure of conflict of interest

None.

Address correspondence to: Song Duan, Department of Pathology, Chongqing Three Gorges Central Hospital, 165 Xincheng Road, Wanzhou District, Chongqing 404000, China. Tel: 86-13996669572; E-mail: xqsduany@sina.com

References

[1] Crawford AB, Nessim C, Weaver J and Walraven CV. Wait times for melanoma surgery: is there an association with overall survival? Ann Surg Oncol 2018; 25: 265-270.

[2] Wei C, Lu N, Wang L, Zhang Y, Feng Z, Yang Y, Qi F and Gu J. Upregulation of UHRF1 promotes the progression of melanoma by inducing cell proliferation. Oncol Rep 2018; 39: 2553-2562.

[3] Rajabi P, Bagheri M and Hani M. Expression of estrogen receptor alpha in malignant melanoma. Adv Biomed Res 2017; 6: 14.

[4] La Porta CA. Mechanism of drug sensitivity and resistance in melanoma. Curr Cancer Drug Targets 2009; 9: 391-397.

[5] Alexandrescu DT, Ichim TE, Riordan NH, Marincola FM, Nardo AD, Kabigting FD and Dasanu CA. Immunotherapy for melanoma: current status and perspectives. J Immunother 2010; 33: 570-590.

[6] Turnbull C and Rahman N. Genome-wide association studies provide new insights into the genetic basis of testicular germ-cell tumour. Int J Androl 2011; 34: e86-e97.

[7] Masoumi-Moghaddam S, Amini A, Morris DL. The developing story of sprouty and cancer. Cancer Metastasis Rev 2014; 33: 695-720.

[8] Kalderon D. Signaling pathways in cancer. Humana Press; 2008.

[9] Khaitan D, Dinger ME, Mazar J, Crawford J, Smith MA, Mattick JS and Perera RJ. The melanoma-upregulated long noncoding RNA SPRY4-IT1 modulates apoptosis and invasion. Cancer Res 2011; 71: 3852-3862.

[10] Zou Y, Jiang Z, Yu X, Sun M, Zhang Y, Zuo Q, Zhou J, Yang N, Han P and Ge Z. Upregulation of long noncoding RNA SPRY4-IT1 modulates proliferation, migration, apoptosis, and network formation in trophoblast cells HTR-8SV/neo. PLoS One 2013; 8: e79598.

[11] Li Z, Shen J, Chan MTV, Wu WKK. The long noncoding RNA SPRY4-IT1: an emerging player in tumorigenesis and osteosarcoma. Cell Prolif 2018; 51: e12446.

[12] Das MK, Furu K, Evensen HF, Haugen ØP and Haugen TB. Knockdown of SPRY4 and SPRY4-IT1 inhibits cell growth and phosphorylation of Akt in human testicular germ cell tumours. Sci Rep 2018; 8: 2642.

[13] Sun M, Liu XH, Lu KH, Nie FQ, Xia R, Kong R, Yang JS, Xu TP, Liu YW, Zou YF, Lu BB, Yin R,

Zhang EB, Xu L, De W, Wang ZX. EZH2-mediated epigenetic suppression of long noncoding RNA SPRY4-IT1 promotes NSCLC cell proliferation and metastasis by affecting the epithelial-mesenchymal transition. Cell Death Dis 2014; 5: e1298.

[14] Guo W, Zhong K, Wei H, Nie C and Yuan Z. Long non-coding RNA SPRY4-IT1 promotes cell proliferation and invasion by regulation of Cdc20 in pancreatic cancer cells. PLoS One 2018; 13: e0193483.

[15] Xie M, Nie F, Sun M, Xia R, Liu Y, Zhou P, Wei D and Liu X. Decreased long noncoding RNA SPRY4-IT1 contributing to gastric cancer cell metastasis partly via affecting epithelial-mesenchymal transition. J Transl Med 2015; 13: 250.

[16] Dang Y, Zhao S, Qin Y, Han T, Li W and Chen ZJ. MicroRNA-22-3p is down-regulated in the plasma of Han Chinese patients with premature ovarian failure. Fertil Steril 2015; 103: 802-807.

[17] Kaur K, Vig S, Srivastava R, Mishra A, Singh VP, Srivastava AK and Datta M. Elevated hepatic miR-22-3p expression impairs gluconeogenesis by silencing the wnt-responsive transcription factor Tcf7. Diabetes 2015; 64: 3659-3669.

[18] Chen J, Wu FX, Luo HL, Liu JJ, Luo T, Bai T, Li LQ and Fan XH. Berberine upregulates miR-22-3p to suppress hepatocellular carcinoma cell proliferation by targeting Sp1. Am J Transl Res 2016; 8: 4932-4941.

[19] Hussein NA, Kholy ZA, Anwar MM, Ahmad MA and Ahmad SM. Plasma miR-22-3p, miR-642b-3p and miR-885-5p as diagnostic biomarkers for pancreatic cancer. J Cancer Res Clin Oncol 2017; 143: 83-93.

[20] Lv K, Liu Z, Feng J, Zhao W, Hao T, Ding W, Chu J and Gao L. MiR-22-3p regulates cell proliferation and inhibits cell apoptosis through targeting the eIF4EBP3 gene in human cervical squamous carcinoma cells. Int J Med Sci 2018; 15: 142-152.

[21] Guo MM, Hu LH, Chen P, Wang YQ, Lu N. miR-22 is down-regulated in gastric cancer, and its overexpression inhibits cell migration and invasion via targeting transcription factor Sp1. Med Oncol 2013; 30: 542.

[22] Pandey AK, Zhang Y, Zhang S, Li Y, Tucker-Kellogg G, Yang H and Jha S. TIP60-miR-22 axis as a prognostic marker of breast cancer progression. Oncotarget 2015; 6: 41290-41306.

[23] Koufaris C, Valbuena GN, Pomyen Y, Tredwell GD, Nevedomskaya E, Lau CH, Yang T, Benito A, Ellis JK and Keun HC. Systematic integration of molecular profiles identifies miR-22 as a regulator of lipid and folate metabolism in breast cancer cells. Oncogene 2016; 35: 2766-2776.

[24] Kong LM, Liao CG, Zhang Y, Xu J, Li Y, Huang W, Zhang Y, Bian H and Chen ZN. A regulatory loop involving miR-22, Sp1, and c-Myc modulates CD147 expression in breast cancer invasion and metastasis. Cancer Res 2014; 74: 3764-3778.

[25] Ahmad HM, Muiwo P, Ramachandran SS, Pandey P, Gupta YK, Kumar L, Kulshreshtha R and Bhattacharya A. miR-22 regulates expression of oncogenic neuro-epithelial transforming gene 1, NET1. FEBS J 2014; 281: 3904-3919.

[26] Li C, Ni J, Liu YX, Wang H, Liang ZQ and Wang X. Response of MiRNA-22-3p and MiRNA-149-5p to folate deficiency and the differential regulation of MTHFR expression in normal and cancerous human hepatocytes. PLoS One 2017; 12: e0168049.

[27] Kyriakis JM and Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. Physiol Rev 2001; 81: 807-869.

[28] Evans I, Britton G and Zachary I. Vascular endothelial growth factor induces heat shock protein (HSP) 27 serine 82 phosphorylation and endothelial tubulogenes is via protein kinase D and independent of p38 kinase. Cell Signal 2008; 20: 1375-1384.

[29] Kondo Y, Higa-Nakamine S, Maeda N, Toku S, Kakinohana M, Sugahara K, Kukita I and Yamamoto H. Stimulation of cell migration by flagellin through the p38 MAP kinase pathway in cultured intestinal epithelial cells. J Cell Biochem 2016; 117: 247-258.

[30] Zhang MX, Gan W, Jing CY, Zheng SS, Yi Y, Zhang J, Xu X, Lin JJ, Zhang BH, Qiu SJ. S100A11 promotes cell proliferation via P38/MAPK signaling pathway in intrahepatic cholangiocarcinoma. Mol Carcinog 2018; 58: 19-30.

[31] Li P, Li Q, Zhang Y, Sun S, Liu S and Lu Z. MiR-422a targets MAPKK6 and regulates cell growth and apoptosis in colorectal cancer cells. Biomed Pharmacother 2018; 104: 832-840.

[32] Milovic V, Teller IC, Murphy GM, Caspary WF and Stein J. Deoxycholic acid stimulates migration in colon cancer cells. Eur J Gastroenterol Hepatol 2001; 13: 945-949.

[33] Grossman D and Altieri DC. Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets. Cancer Metastasis Rev 2001; 20: 3-11.

[34] Liu T, Shen SK, Xiong JG, Xu Y, Zhang HQ, Liu HJ and Lu ZG. Clinical significance of long noncoding RNA SPRY4-IT1 in melanoma patients. FEBS Open Bio 2016; 6: 147-154.

[35] Guo MM, Hu LH, Wang YQ, Chen P, Huang JG, Lu N, He JH, Liao CG. miR-22 is down-regulated in gastric cancer, and its overexpression inhibits cell migration and invasion via targeting transcription factor Sp1. Medl Oncol 2013; 30: 542.

[36] Chen J, Wu FX, Luo HL, Liu JJ, Luo T, Bai T, Li LQ, Fan XH. Berberine upregulates miR-22-3p to suppress hepatocellular carcinoma cell proliferation by targeting Sp1. Am J Transl Res 2016; 8: 4932-4941.

[37] Jiang W, Cheng Y, Zhao N, Li L, Shi Y, Zong A and Wang F. Sulfated polysaccharide of sepiella maindroni ink inhibits the migration, invasion and matrix metalloproteinase-2 expression through suppressing EGFR-mediated p38/MAPK and PI3K/Akt/mTOR signaling pathways in SKOV-3 cells. Int J Biol Macromol 2018; 107: 349-362.

[38] Ling G, Ji Q, Ye W, Ma D and Wang Y. Epithelial-mesenchymal transition regulated by p38/MAPK signaling pathways participates in vasculogenic mimicry formation in SHG44 cells transfected with TGF-β cDNA loaded lentivirus in vitro and in vivo. Int J Oncol 2016; 49: 2378-2398.

[39] Kondo Y, Higa-Nakamine S, Noguchi N, Maeda N, Toku S, Isohama Y, Sugahara K, Kukita I and Yamamoto H. Induction of epithelial-mesenchymal transition by flagellin in cultured lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 2012; 303: 1057-1069.