

Original Article

Higher expression of ZFAS1 is associated with poor prognosis in malignant melanoma and promotes cell proliferation and invasion

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Abstract: Long non-coding RNAs (lncRNAs) ZFAS1 had been reported in several tumors, however, the clinical role and biological function of ZFAS1 expression level in malignant melanoma (MM) is not well studied. In the study, quantitative real-time PCR (qRT-PCR) methods were used to assess the expression levels of ZFAS1 in 88 cases of malignant melanoma tissues and adjacent non-cancerous tissues in MM patients. The ability of MM cell proliferation was analyzed by CCK8 cell proliferation assay. Western-blot analysis detected the expression of cell proliferation associated protein Ki67, PCNA and Cyclin D1. Our results demonstrated that the expression levels of ZFAS1 were significantly increased in MM tissues compared to adjacent non-cancerous tissues and the higher ZFAS1 expression levels were correlated with tumor thickness, lymph node metastasis, and tumor stages in patients. Moreover, patients who had higher ZFAS1 expression predicted shorter disease-free survival time (DFS) or over survival (OS). Multivariate Cox analysis showed that tumor thickness, lymph node metastasis, tumor stages and ZFAS1 expression were independent risks for prognosis in MM patients. After knockdown of ZFAS1, the results showed that the cell proliferation was inhibited in sk-mel-1 and A375 cells. Thus, the results suggested that ZFAS1 could be used as a potential biomarker for prognosis and a therapeutic target for MM patients.

Keywords: ZFAS1, tumor prognosis, melanoma, cell proliferation

Introduction

Melanoma is the most dangerous type of skin cancer. In 2012, melanoma occurred in 232,000 people and resulted in 55,000 deaths in the world [1, 2]. Due to a rapid progression of malignant melanoma, metastasis to regional lymph nodes and distant organs and a limited efficiency of therapeutics, it causes more than 80% death in skin cancer patients [3, 4]. Therefore, to identify novel biomarkers and therapeutic targets is essential for prognosis and treatment of malignant melanoma.

lncRNAs are non-coding RNAs greater than 200 nucleotides in length and are emerging as important players in tumor biology and progression [5, 6]. lncRNAs are showed to regulate

gene expression in different biological processes, gene transcription and post-transcription in cancer progression including malignant melanoma [5, 7]. Such as, long noncoding RNA HOTAIR is associated with motility, invasion, and metastatic potential of metastatic melanoma [8]. Long non-coding RNA BANCR promotes proliferation in malignant melanoma by regulating MAPK pathway activation [9].

lncRNA ZFAS1 (zinc finger antisense 1), has been studied in some tumor as a tumor suppressor or oncogene. Knockdown of Zfas1 in a mammary epithelial cell line results in increased cellular proliferation and differentiation [10]. Amplification of long noncoding RNA ZFAS1 promotes tumor metastasis in hepatocellular carcinoma [11]. Long non-coding RNA ZFAS1 inter-

ZFAS1 is associated with poor prognosis in malignant melanoma

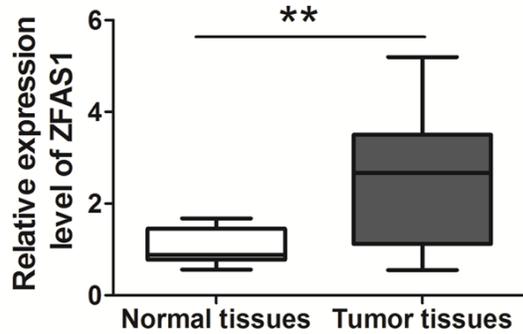


Figure 1. ZFAS1 is upregulated in human MM tissues. The mRNA levels of ZFAS1 in tumor tissues of MM patients and adjacent normal tissues. ** $P < 0.05$.

acts with CDK1 and is involved in p53-dependent cell cycle control and cell apoptosis in colorectal cancer [12]. Long noncoding RNA ZFAS1 promotes gastric cancer cells proliferation by epigenetically repressing KLF2 and NKD2 expression [13]. However, the clinical value of ZFAS1 in malignant melanoma is not reported so far.

In the study, we demonstrated that the expression levels of ZFAS1 were significantly increased in MM tissues. The higher ZFAS1 expression was correlated with tumor thickness, lymph node metastasis and tumor stages. Patients who had higher ZFAS1 predicted shorted prognosis. Besides, ZFAS1 promoted the cell proliferation in MM cells. Thus, our results suggested that ZFAS1 could be used as a potential predictor of prognosis and therapeutic target for MM.

Materials and methods

Patient tissue samples

The 88 cases human MM tissues and normal adjacent tissues were collected at Department of Dermatology, The Affiliated Hospital of Binzhou Medical University from January 2008 to March 2013. All of the tumor tissues were verified as MM by pathological diagnosis. The patients had no undergoing radiation therapy or chemotherapy before operation. The written informed consents were collected from the MM patients. Tissues were frozen in liquid nitrogen, and then stored at -80°C for further RNA analysis. The study was approved by the ethics committee of The Affiliated Hospital of Binzhou Medical University.

Cell culture

Two human malignant melanoma (MM) cell lines (sk-mel-1 and A375) were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (HyClone, USA) and 1% penicillin/streptomycin (Invitrogen, USA) in a humidified incubator at 37°C in a 5% CO_2 atmosphere.

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated from tissues and cells using Trizol Reagent (Thermo Fisher Scientific, Inc.) The RNA reverse transcription was performed using the miScript Reverse Transcription kit (Qiagen China Co., Ltd., Shanghai, China). Furthermore, quantitative PCR was performed using the QuantiNova SYBR Green PCR Kit (Qiagen China Co., Ltd., Shanghai, China). PCR was performed using the Applied Biosystem 7500 Sequence Detection System. (Applied Biosystems Life Technologies, Foster City, CA, USA). The primers selected were as follows: GAPDH-F: 5'-GGGAGCCAAAAGGGTCAT-3', GAPDH-R: 5'-GAGTCCTTCCACGATACCAA-3'. ZFAS1-F: 5'-ACGTGCAGACATCTACAACCT-3', ZFAS1-R: 5'-TACTTCCAACACCCGCAT-3'. The expression of ZFAS1 mRNA was normalized to the GAPDH expression and calculated using $2^{-\Delta\Delta\text{CT}}$ methods.

Cell transfection

The si-negative control and siRNA-ZFAS1 were purchased from Ribobio (Guangzhou, China). The sk-mel-1 or A375 cells was transfected with si-NC or siRNA-ZFAS1 using Lipofectamine 3000 transfection reagent accordingly to manufacturer's instructions (Invitrogen). The sequence against ZFAS1 was si-ZFAS1-1: 5'-CUGGUGAACCAGUCCACAAGGUU-3'. Si-ZFAS1-2: CACCAAGTGAAGATCTGGCTGAACCAGTTCGAAACTGGTTCAGCCAGATCTTCAC.

Cell counting kit 8 (CCK8) assay

Cell proliferation was performed to evaluate cell proliferation ability by Cell Counting Kit 8 (CCK8) kit (Dojindo, Japan) according to the manufacturer's instructions. 1×10^5 /well per well were seeded in 96-well plates and main-

ZFAS1 is associated with poor prognosis in malignant melanoma

Table 1. Relationship between ZFAS1 expression and clinicopathologic factors in 88 cases of malignant melanoma patients

Clinicopathologic factors	Cases	ZFAS1 expression		Chi-squared <i>P</i> -value
		Low (n=42)	High (N=46)	
Age (years)				0.640
≤55	48	24	24	
>55	40	18	22	
Gender				0.179
Male	56	16	30	
Female	32	16	16	
Tumor thickness (mm)				0.001**
<1	30	22	8	
>1	58	20	38	
Ulceration				0.080
Yes	48	27	21	
No	40	15	25	
Tumor subtype				0.992
Acral lentiginous melanoma	25	12	13	
Nodular melanoma	32	15	17	
Superficial spreading melanoma	31	15	16	
Lymph node metastasis				0.002**
No	28	20	8	
Yes	60	22	38	
Site				0.404
Sun exposed	42	22	20	
Sun protected	46	20	26	
Clinical stage				0.036**
I-II	32	20	12	
III	56	22	34	

***P*<0.05.

Table 2. Multivariate analysis of disease-free survival time (DFS) for prognosis in MM patients (*n*=88)

Variables	HR	95% CI	<i>p</i> -value
Tumor thickness	2.488	0.926-4.206	0.001**
Lymphnode metastasis	2.985	1.365-4.986	0.001**
TNM stage	2.518	1.114-4.623	0.001**
ZFAS1	2.602	1.289-4.439	0.001**

***p*-value <0.05 was considered statistically significant, CI, confidence intervals.

Table 3. Multivariate analysis of the over survival (OS) time for prognosis in MM patients (*n*=88)

Variables	HR	95% CI	<i>p</i> -value
Tumor thickness	2.655	1.066-4.897	0.001**
Lymph node metastasis	3.238	1.438-5.369	0.001**
TNM stage	2.612	1.353-4.646	0.001**
ZFAS1	2.735	1.245-4.969	0.001**

***p*-value <0.05 was considered statistically significant, CI, confidence intervals.

tained at 37°C for 24 h. After transfection at 0, 24 h, 48 h, 72 h and 96 h, 10 µL of CCK-8 reagent was added to each well, and then incubated at 37°C for another 3 hours. The absorbance was determined at 450 nm with microplate reader (Bio-TEK).

Western blot assay

Western blot assay was performed as previous describe [14]. The cells were washed and cell protein lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the concentration of protein was evaluated by bicinchoninic acid protein assay kit (Pierce) and transferred to PVDF (Millipore) membranes. The membranes were incubated with blocking solution (5% Milk in TBS-T) for 2 hour at room temperature, and then blotted with specific anti-

bodies against Ki67 (1:1000, CST, USA), PCNA (1:1000, CST, USA), CyclinD1 (1:1000, CST, USA) and GAPDH (1:1000, CST, USA) overnight at 4°C. The membranes were then incubated with a HRP-conjugated secondary antibody (CST, USA) and were detected by an enhanced chemiluminescence (ECL) assay (GE Healthcare).

Statistical analysis

All data in the study were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation. The differences between two groups were detected by using Student's *t*-test. Kaplan-Meier curve and log-rank test was performed to assess the association between ZFAS1 expression and DFS or OS. Multivariate Cox analysis was also performed. *P*<0.05 was considered as statistically significant difference.

ZFAS1 is associated with poor prognosis in malignant melanoma

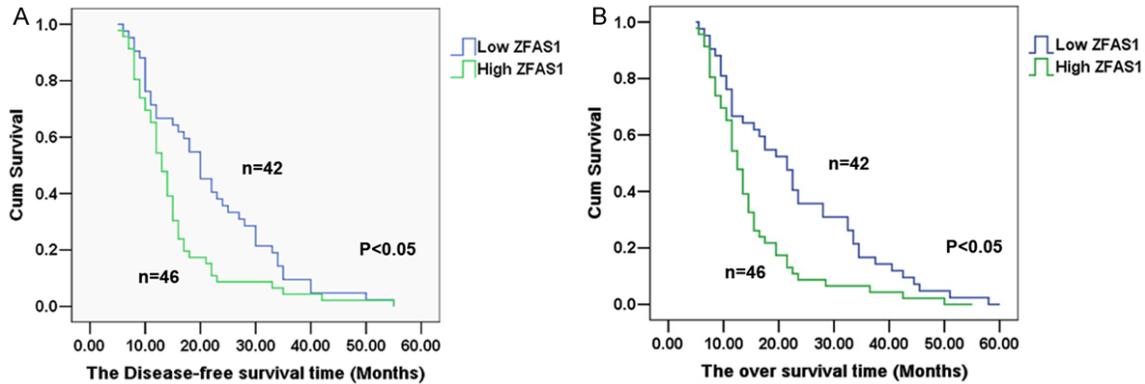


Figure 2. Higher ZFAS1 predicted a poor disease-free survival time (DFS) or over survival (OS) in MM patients. A. Kaplan-Meier curve and log-rank test was performed to assess the association between ZFAS1 expression and disease-free survival time (DFS) in MM patients. B. Kaplan-Meier curve and log-rank test was performed to assess the association between ZFAS1 expression and over survival (OS) in MM patients.

Results

The expression of ZFAS1 is upregulated in human MM tissues

We first examined the ZFAS1 expression levels in MM tissue samples and adjacent normal tissues by qRT-PCR assay. As shown in **Figure 1**, the results found that ZFAS1 was significantly higher in MM tissue samples compared to the adjacent normal tissues the mean expression level of ZFAS1 in MM tissues is 2.57 (**Figure 1**, $P < 0.05$). According the median expression, the patient was divided into two groups (higher expression and lower expression of ZFAS1). As shown in the **Table 1**, statistical analysis revealed that the expression level of ZFAS1 in the MM tissues was significantly association with tumor thickness ($P = 0.001$), lymph node metastasis ($P = 0.002$) and tumor stages ($P = 0.036$). But the expression level of ZFAS1 was not with the other clinicopathological features in MM patients including age, gender, tumor subtype, and so on.

Higher ZFAS1 predicted a poor disease-free survival time (DFS) or over survival (OS) in MM patients

Furthermore, we assessed the association between the expression levels of ZFAS1 and the disease-free survival time (DFS) or over survival (OS) time in MM patients. As shown in **Figure 2A, 2B**, Kaplan-Meier curve and log-rank test showed that higher ZFAS1 expression levels in MM patients had a significantly shorter DFS

(log-rank=6.898, $P < 0.01$) and OS (log-rank=9.298, $P < 0.01$). Furthermore, multivariate Cox analysis demonstrated that tumor thickness ($P = 0.001$, HR=2.488, 95% CI=0.926-4.206), lymph node metastasis ($P = 0.001$, HR=2.985, 95% CI=1.365-4.986), tumor stages ($P = 0.001$, HR=2.518, 95% CI=1.114-4.623) and ZFAS1 ($P = 0.001$, HR=2.602, 95% CI=1.289-4.439) were independent prognosis risks for the DFS in MM patients (**Table 2**). In addition, The multivariate Cox analysis also demonstrated that tumor thickness ($P = 0.001$, HR=2.655, 95% CI=1.066-4.897), lymph node metastasis ($P = 0.001$, HR=3.238, 95% CI=1.438-5.369), tumor stages ($P = 0.001$, HR=2.612, 95% CI=1.353-4.646) and ZFAS1 ($P = 0.001$, HR=2.735, 95% CI=1.245-4.969) were independent prognosis risks for the OS in MM patients (**Table 3**). Thus, these data showed that higher ZFAS1 predicted a poor disease-free survival time (DFS) or over survival (OS) for MM patients.

Higher ZFAS1 promoted the cell proliferation in MM

To investigate the effect of ZFAS1 expression on cellular proliferation ability, we knocked down ZFAS1 by two siRNA-ZFAS1, the results showed that ZFAS1 was significantly downregulated by transfecting with siRNA-ZFAS1-1 and si-ZFAS1-2 (**Figure 3A, 3B**). The si-ZFAS1-2 was higher efficiency for ZFAS1 knockdown experiment and was used in the following study. Next, CCK8 cell proliferation assays were used to monitor cell proliferation ability. The results

ZFAS1 is associated with poor prognosis in malignant melanoma

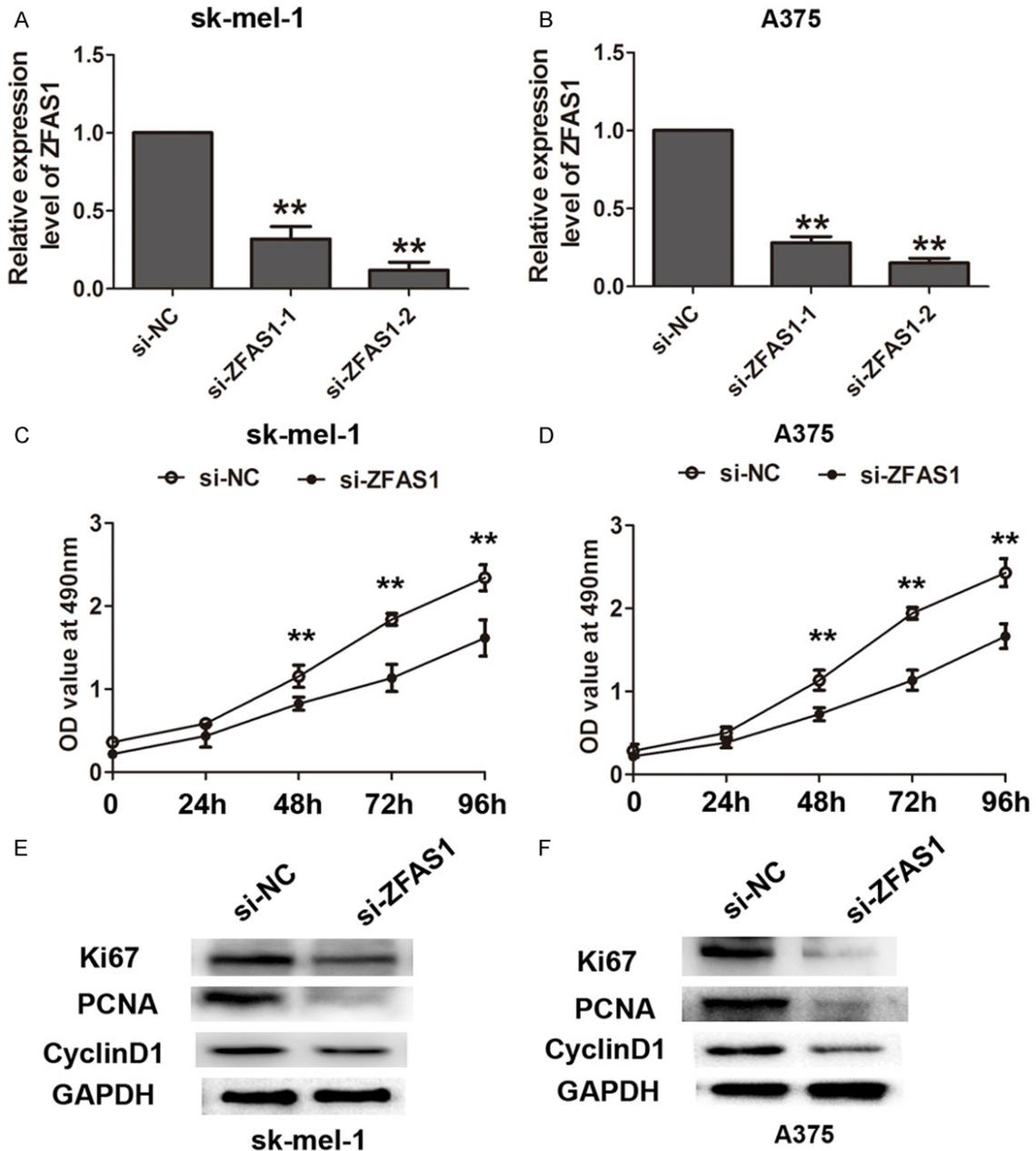


Figure 3. Higher ZFAS1 promoted the cell proliferation in MM (A, B). The expression of ZFAS1 was detected by qRT-PCR assays after transfecting with si-NC, si-ZFAS1-1, si-ZFAS1-2 in sk-mel-1 or A375 cells at 48 h. (C, D) The cell proliferation was monitored after knockdown of ZFAS1 in sk-mel-1 or A375 cells by CCK8 cell proliferation at 0, 24 h, 48 h, 72 h and 96 h, The absorbance was determined at 450 nm. (E, F) The protein expression of Ki67, PCNA, CyclinD1 were analyzed by western-blot assays after knockdown of ZFAS1 in sk-mel-1 or A375 cells at 48 h. **P<0.05.

showed that cells proliferation was significantly inhibited after ZFAS1 silencing in sk-mel-1 and A375 cells, compared with the control group (Figure 3C, 3D).

We further detected the expression of cell proliferation related marker Ki67 and PCNA,

CyclinD1 after ZFAS1 silencing in sk-mel-1 and A375 cells. The results showed that after ZFAS1 silencing, the Ki67 and PCNA, CyclinD1 was significantly downregulated in sk-mel-1 and A375 cells after ZFAS1 silencing (Figure 3E, 3F). Thus, the results showed that higher ZFAS1 promoted the cell proliferation in MM.

Discussion

Long noncoding RNAs (ncRNAs) have attracted much attention in cancer research field worldwide [15]. Recent studies reveals lncRNAs are involved in the regulation of human melanoma progression. SPRY4-IT1 knockdown results in defects in cell growth, differentiation, and higher rates of apoptosis in melanoma cell lines [16]. Knocking down lncRNA linc23 remarkably suppresses the malignant property of YUSAC cells, accompanied by the repressed expression of proto-oncogene Rab23 [17]. Up-regulation of long noncoding RNA SPRY4-IT1 modulates proliferation, migration, apoptosis, and network formation in trophoblast cells HTR-8SV/neo [18].

In the study, we demonstrated that the expression levels of ZFAS1 were significantly increased in MM tissues than that in the adjacent non-cancerous tissues and higher ZFAS1 expression was correlated with tumor thickness, lymph node metastasis and tumor stages. The higher ZFAS1 in MM patients was significantly association with the shorter DFS or OS. Furthermore, multivariate Cox analysis demonstrated that tumor thickness, lymph node metastasis, tumor stages and ZFAS1 were independent prognosis risks for DFS or OS in MM patients. Thus, higher ZFAS1 expression was correlated with the poor overall survival in the MM patients.

Increased expression levels of UCA1 and Malat-1 lncRNAs were found to be correlation with melanoma metastasis [19]. Upregulating lncRNA GAS5 suppressed the migration and invasion ability of melanoma SK-Mel-110 cells, partially by decreasing the MMP2 expression and its activity [20]. The lncRNA SLNCR1 mediates melanoma invasion through a conserved SRA1-like region [21]. In our study, we found that after knockdown of ZFAS1, the results showed that the cell proliferation ability was inhibited in MM cells and the cell proliferation related protein Ki67, PCNA, CyclinD1 were significantly down-regulated in sk-mel-1 and A375 cells. Thus, these results indicated that ZFAS1 promoted the cell proliferation in MM.

In conclusion, our results showed ZFAS1 expression levels were higher in MM patients and predicted poorer prognosis. Furthermore, knockdown of ZFAS1 inhibited the cell prolifera-

tion ability in MM cells. Thus, the results suggested that ZFAS1 could predict the prognosis and act as a therapeutic target for MM patients.

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Disclosure of conflict of interest

None.

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References

- [1] Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; 55: 74-108.
- [2] Garbe C, Leiter U. Melanoma epidemiology and trends. *Clin Dermatol* 2009; 27: 3-9.
- [3] Baroudjian B, Pagès C, Lebbé C. [Melanoma, from diagnosis to treatment]. *Rev Infirm* 2016; 16-8.
- [4] Trotter SC, Sroa N, Winkelmann RR, Olencki T, Bechtel M. A global review of melanoma follow-up guidelines. *J Clin Aesthet Dermatol* 2013; 6: 18-26.
- [5] Maruyama R, Suzuki H. Long noncoding RNA involvement in cancer. *BMB Rep* 2012; 45: 604-11.
- [6] Wang J, Shao N, Ding X, Tan B, Song Q, Wang N, Jia Y, Ling H and Cheng Y. Crosstalk between transforming growth factor-beta signaling pathway and long non-coding RNAs in cancer. *Cancer Lett* 2016; 370: 296-301.
- [7] Esteller M. Non-coding RNAs in human disease. *Nat Rev Genet* 2011; 12: 861-74.
- [8] Tang L, Zhang W, Su B, Yu B. Long noncoding RNA HOTAIR is associated with motility, invasion, and metastatic potential of metastatic melanoma. *Biomed Res Int* 2013; 2013: 251098.
- [9] Li R, Zhang L, Jia L, Duan Y, Li Y, Bao L, Sha N. Long non-coding RNA BANCR promotes proliferation in malignant melanoma by regulating MAPK pathway activation. *PLoS One* 2014; 9: e100893.
- [10] Askarian-Amiri ME, Crawford J, French JD, Smart CE, Smith MA, Clark MB, Ru K, Mercer

ZFAS1 is associated with poor prognosis in malignant melanoma

- TR, Thompson ER, Lakhani SR, Vargas AC, Campbell IG, Brown MA, Dinger ME, Mattick JS. SNORD-host RNA Zfas1 is a regulator of mammary development and a potential marker for breast cancer. *RNA* 2011; 17: 878-91.
- [11] Li T, Xie J, Shen C, Cheng D, Shi Y, Wu Z, Deng X, Chen H, Shen B, Peng C, Li H, Zhan Q, Zhu Z. Amplification of long noncoding RNA ZFAS1 promotes metastasis in hepatocellular carcinoma. *Cancer Res* 2015; 75: 3181-91.
- [12] Thorenoor N, Faltejskova-Vychytilova P, Hombach S, Mlcochova J, Kretz M, Svoboda M, Slaby O. Long non-coding RNA ZFAS1 interacts with CDK1 and is involved in p53-dependent cell cycle control and apoptosis in colorectal cancer. *Oncotarget* 2016; 7: 622-37.
- [13] Nie F, Yu X, Huang M, Wang Y, Xie M, Ma H, Wang Z, De W and Sun M. Long noncoding RNA ZFAS1 promotes gastric cancer cells proliferation by epigenetically repressing KLF2 and NKD2 expression. *Oncotarget* 2016; [Epub ahead of print].
- [14] Li J, Wang Q, Wen R, Liang J, Zhong X, Yang W, Su D and Tang J. MiR-138 inhibits cell proliferation and reverses epithelial-mesenchymal transition in non-small cell lung cancer cells by targeting GIT1 and SEMA4C. *J Cell Mol Med* 2015; 19: 2793-805.
- [15] Liz J, Esteller M. lncRNAs and microRNAs with a role in cancer development. *Biochim Biophys Acta* 2016; 1859: 169-76.
- [16] Khaitan D, Dinger ME, Mazar J, Crawford J, Smith MA, Mattick JS, Perera RJ. The melanoma-upregulated long noncoding RNA SPRY4-IT1 modulates apoptosis and invasion. *Cancer Res* 2011; 71: 3852-62.
- [17] Wu CF, Tan GH, Ma CC, Li L. The non-coding RNA linc23 drives the malignant property of human melanoma cells. *J Genet Genomics* 2013; 40: 179-88.
- [18] Zou Y, Jiang Z, Yu X, Sun M, Zhang Y, Zuo Q, Zhou J, Yang N, Han P, Ge Z, De W, Sun L. Up-regulation of long noncoding RNA SPRY4-IT1 modulates proliferation, migration, apoptosis, and network formation in trophoblast cells HTR-8SV/neo. *PLoS One* 2013; 8: e79598.
- [19] Tian Y, Zhang X, Hao Y, Fang Z, He Y. Potential roles of abnormally expressed long noncoding RNA UCA1 and Malat-1 in metastasis of melanoma. *Melanoma Res* 2014; 24: 335-41.
- [20] Chen L, Yang H, Xiao Y, Tang X, Li Y, Han Q, Fu J, Yang Y, Zhu Y. Lentiviral-mediated overexpression of long non-coding RNA GAS5 reduces invasion by mediating MMP2 expression and activity in human melanoma cells. *Int J Oncol* 2016; 48: 1509-18.
- [21] Schmidt K, Joyce CE, Buquicchio F, Brown A, Ritz J, Distel RJ, Yoon CH, Novina CD. The lncRNA SLNCR1 mediates melanoma invasion through a conserved SRA1-like region. *Cell Rep* 2016; 15: 2025-37.