Original Article
Long non-coding RNA PANDAR promotes melanoma cell invasion through regulating epithelial-mesenchymal transition

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Abstract: Melanoma is a malignant skin tumor and has a poor cure rate because of its high metastatic potential. Overexpression of long non-coding (Inc) RNA PANDAR has been observed in several kinds of cancer, but the function of PANDAR on melanoma is still unclear. Therefore, this study was to explore the mechanism of PANDAR on the occurrence and progression in malignant melanoma. We detected expression of PANDAR in malignant melanoma tissues and cell lines by qRT-PCR and analyzed correlation of PANDAR expression with the patients’ prognosis. Furthermore, we investigated the effects of PANDAR on cell viability, migration, invasion, tumorigenesis, and epithelial-mesenchymal transition (EMT) using CCK-8, Transwell, and nude mouse subcutaneous tumor formation model assays and Western blotting analysis, respectively. From the results, we discovered that the PANDAR expression is strikingly upregulated in melanoma tissues compared with paired-adjacent non-tumorous tissues and elevated PANDAR is positively correlated with short overall survival time. The results also demonstrate that knockdown of PANDAR inhibits cell viability, migration, invasion, tumorigenesis, and EMT, whereas overexpression of PANDAR gave opposite results by promoting cell viability, migration, invasion, tumorigenesis, and EMT of melanoma cells. These new findings all illustrate that PANDAR might play a pivotal oncogenic role in the occurrence and development of melanoma, and PANDAR might promote melanoma cell invasion through regulating EMT, providing a potential diagnostic and therapeutic target for melanoma.

Keywords: Long non-coding RNA PANDAR, melanoma, invasion, EMT

Introduction
Melanoma, derived from pigment cells, is responsible for the majority of skin cancer related deaths worldwide, as well as the most lethal cutaneous neoplasm [1, 2]. Currently, estimated new cases of melanoma are 160,000, and there are about 48,000 deaths caused by melanoma each year [3], supporting the significant threat posed to human health. Until now, there have been three primary pharmacological therapeutic options for patients with metastatic melanoma: (1) immunotherapy; (2) targeted therapy, focusing on the mutational status of melanoma cells; and (3) conventional chemotherapy, if the first two options are not suitable or available [4-6]. Although advanced improvements have been made in treatment options, unfortunately the incidence of melanoma is rising worldwide and metastatic melanoma is still associated with high rates of mortality and poor prognosis. Once metastasis occurs in patients with melanoma, the 5-year survival rate is 16% [7]. Therefore, the identifying possible molecular mechanisms of melanoma metastasis and tumorigenesis and researching biomarkers for early diagnosis and the effective therapeutic strategies for melanoma are urgently needed.

Long non-coding RNAs (IncRNA) represent a highly heterogeneous group of RNAs, which have an extensive variability in their cellular effects, and their molecular influences. They can be classified by their length (>200 nt) and by their lack of a functional open reading frame, meaning they encompass less than 100 amino acids [8-10]. However, more and more evidence has revealed that IncRNAs play irreplaceable roles in various pathophysiological processes and are frequently dysregulated in many can-
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cers including melanoma [11-14]. LncRNAs may act as oncogenes or tumor suppressor genes, with a specifically role in cancer metastasis and progression [15]. To date, many lncRNAs have been studied in melanomas such as lncRNAs HOXA11-AS [16], SPRY4-IT1 [17], HEIH [18], CCAT1 [19] and PVT1 [20], where increased expression and pro-oncogenic function was observed. Furthermore, lncRNAs GAS5 [21], NKILA [22] are decreased expression and exert a tumor suppressing function in melanoma. But there are still lots of lncRNAs that need to be explored and analyzed, providing the possibility for delivering them as therapeutic targets for melanoma.

PANDAR (GenBank accession ID: 101154753), a novel lncRNA consisting of 1506 nucleotides in length, is located at 6p21.2 and was first reported by Hung et al. [23]. PANDAR can interact with the transcription factor NF-YA, which is linked to the expression of pro-apoptotic genes. In addition, lncRNA PANDAR is overexpressed in many cancers and serves as an oncogene. For example, in bladder cancer, Zhang et al. [24] found that lncRNA PANDAR promoted cell proliferation and migration. Ma et al. [25], Peng et al. [26], Min et al. [27] and Xu et al. [28] all revealed that LncRNA PANDAR was up-regulated and associated with poor prognosis in gastric cancer, hepatocellular carcinoma, colorectal cancer, and cholangiocarcinoma, respectively. But studies of the effect of lncRNA PANDAR on melanoma progression are rare. Therefore, the objective of the present study was to investigate the role of IncRNA promoter of CDKN1A antisense DNA damage activated RNA (PANDAR) in melanoma. We explored the expression of lncRNA PANDAR in melanoma and the correlation between lncRNA PANDAR expression and clinical outcome of melanoma patients. We assessed the roles of lncRNA PANDAR in melanoma cell proliferation, migration, invasion, tumorigenicity, and epithelial-mesenchymal transition (EMT) progress, and our results demonstrate molecular mechanisms underlying the roles of lncRNA PANDAR in melanoma.

Materials and methods

Tissue specimens and patient characterization

Tissue were collected from patients who underwent surgical resections at First Affiliated Hospital of Bengbu Medical College after obtaining informed consent and included 62 melanoma tissues and 24 benign nevi tissues (considered as controls). The specimens were immediately frozen in liquid nitrogen and then stored at -80°C for analysis. Overall survival (OS) time was calculated from the date of the initial surgery to death. This study was carried out in accordance with the approved guidelines by the Ethics Committee of First Affiliated Hospital of Bengbu Medical College.

Cell culture

The normal human skin HACAT cells and five human melanoma cell lines, including CHL-1, A375, SK-MEL-1, WM-35, and WM-115, were purchased from American Type Culture Collection (Manassas, VA, USA), and cultured in DMEM medium (GIBCO-BRL; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% antibiotic/antimycotic solution in a humidified atmosphere containing 5% CO₂ at 37°C.

Cell transfection

Plasmid vector (pcDNA-PANDAR and pcDNA3.1-NC) were prepared using DNA Midiprep kits (Qiagen, Hilden, Germany), and were transfected into melanoma cells. shRNAs targeting PANDAR (sh-PANDAR) and negative control shRNA (sh-NC) constructed by GenePharma (China) were transfected into melanoma cells, respectively, using Lipofectamine™ 3000 (Life Technologies, San Diego, CA, USA). 48 hours post-transfection, PANDAR expression levels were measured. PANDAR shRNA-1: GCATACATACAGCTTTT, shRNA-2: GCCGATGCTCCCAGCTGAATA, shRNA-3: GCATAGCCATAGGGATTAGA.

qRT-PCR assay

Total RNA was extracted using Trizol Reagent (Invitrogen). 2 ng of total RNA was reverse transcribed into cDNA using the GoScript Reverse Transcription (RT-PCR) System (Promega, Madison, WI, USA). Then, SYBR Premix Ex Taq (TaKaRa, Dalian, China) was used to conduct qRT-PCR on an ABI 7900 system (Applied Biosystems, Foster City, CA, USA). GAPDH was used as internal reference. The relative expression was analyzed by the 2^(-ΔΔCt) method. The sequences of the primers are listed as follows: PANDAR (F): CCTGTTCGTCGATTCTGGCT; PAND-
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AR (R): GTCTGGCGTGAGATGTTTC; GAPDH (F): GAAGGTGAAAGTGGGAGT; GAPDH (R): GAAGATGGTGATGGGATTTC.

In vitro cell proliferation assays

Cell proliferation was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). The absorbance values of cells at various time points after transfection were measured using a microplate reader (Bio-Rad, Hercules, CA, USA). To perform colony formation assays, 400 cells were seeded per well in 6-well plates and cultured for two weeks. The colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Sigma, St Louis, MO, USA). Stained clones were observed under a microscope.

In vitro migration and invasion assays

Cell migration and invasion assays were performed in a 24-well transwell plate with 8-μm polyethylene terephthalate membrane filters.

In vivo tumorigenesis assay

Female athymic Balb/c nude mice (4-5 weeks of age, 18-20 g), obtained from Slac Laboratory Animal Co. Ltd. (Shanghai, China), were randomized to the control or experimental group (6 mice/group). Melanoma cells stably expressing sh-PANDAR or sh-NC were implanted subcutaneously into the back of each nude mouse. Tumors were measured with calipers every 3 days and the volume was calculated by the formula $V = 0.5 \times \text{length} \times \text{width}^2$. Over a five-week period, all mice were euthanized, and the tumors were excised and weighed. All experimental procedures were approved by the animal care and ethics committee of First Affiliated Hospital of Bengbu Medical College.

Western blot analysis

Cells were lysed with RIPA buffer supplemented with protease inhibitors. Protein concentration was measured using a BCA protein assay (Thermo Scientific, Rockford, USA). Proteins were separated using SDS-PAGE electrophoresis and transferred to PVDF membranes. The membranes were blocked and then probed with primary antibodies against E-cadherin, vimentin (Abcam, MA, USA), N-cadherin, and β-actin (Cell Signaling Technology, Danvers, USA). After incubation with a secondary anti-

Figure 1. Upregulated PANDAR predicts poor clinical outcome in patients with melanoma. A: Expression of PANDAR in melanoma tissues and benign nevi tissues was detected by qRT-PCR analysis. B: Expression of PANDAR in melanoma cells and the normal human skin HACAT cells were determined by qRT-PCR analysis. C: Survival curve was made of Kaplan-Meier analysis between the expression level of PANDAR and overall survival time in patients with melanoma. Data represent the mean ± SD from three independent experiments (*P<0.05, **P<0.001).
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Table 1. Relationship between PANDAR expression and clinicopathological characteristics of melanoma patients (n=62)

<table>
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<th>Characteristics</th>
<th>Total number</th>
<th>PANDAR expression</th>
<th>P value</th>
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<tr>
<td></td>
<td>Low (n=26)</td>
<td>High (n=36)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.951</td>
<td></td>
<td></td>
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<tr>
<td>Mean</td>
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<td>67.68</td>
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<tr>
<td>Median</td>
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<td>69.00</td>
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<tr>
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<td>46-83</td>
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<tr>
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<td>15</td>
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</tr>
<tr>
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<td>11</td>
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</tr>
<tr>
<td>III</td>
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<td>13</td>
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<tr>
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<td>0</td>
<td>1</td>
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body, the blots were visualized by enhanced chemiluminescence (Millipore, Billerica, MA). β-actin was used as a loading control.

Statistical analysis

All statistical analyses were performed using SPSS version 18.0 software (SPSS Inc., Chicago, IL, USA) and Graphpad Prism (version 6.01) software (GraphPad Software, Inc., La Jolla, CA, USA). Each experiment was performed in triplicate, and repeated at least three times. The data are expressed as the mean ± SD. Kaplan-Meier plots and log-rank tests were used for survival analysis. *P*<0.05 was considered to be statistically significant.

Results

Upregulated PANDAR predicts poor clinical outcome in patients

Here, we examined the expression profiles of PANDAR in 62 melanoma tissues and 24 benign nevi tissues. We observed that, as shown in Figure 1A, PANDAR was significantly highly expressed in the melanoma tissues than in benign nevi tissues. Also, the expression of PANDAR was remarkably higher in the melanoma cells than in the normal human skin HACAT cells (Figure 1B).

To further understand the significance of PANDAR in melanoma, we analyzed the correlation between PANDAR expression and the clinicopathological status of 62 melanoma patients. As indicated in Table 1, increased PANDAR expression level in melanoma tissues was closely correlated with tumor thickness (*P*=0.013), Clark level (*P*=0.002), histologic type (*P*=0.023) and TNM classification (*P*=0.029). Kaplan-Meier analysis revealed that high PANDAR expression was significantly associated with shorter overall survival (*P*=0.0) (Figure 1C).

PANDAR regulates the proliferation, migration, and invasion of melanoma cells

Since our clinical data showed that PANDAR expression levels negatively correlated with tumor growth and metastasis, we further explored the function of PANDAR in proliferation, migration, and invasion in melanoma cell lines. Cells were transiently transfected with sh-PANDAR, which efficiently silenced endoge-
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Ectopic expression of PANDAR (Figure 2A). Also, ectopic expression of PANDAR in cells by transfecting with pcDNA3.1-PANDAR markedly increased the PANDAR level than that in pcDNA3.1-NC-transfected cells. CCK-8 and colony formation assays were performed to assess the role of PANDAR in melanoma cell proliferation. The melanoma cells transfected with pcDNA3.1-PANDAR clearly grew faster compared to control cells (Figure 2B). The number and the mean size of the colonies formed by melanoma cells were significantly decreased following transfection with sh-PANDAR (Figure 2C), indicating that PANDAR exerts a promoting function in melanoma cell proliferation.

Furthermore, as shown in Figure 3, melanoma cells with reduced PANDAR expression showed increased ability of migration and invasion than that in control cells, whereas elevated PANDAR suppressed cell migration and invasion when compared to control cells. These data suggested that PANDAR inhibits melanoma cells migration and invasion.

PANDAR promotes melanoma tumorigenesis in vivo

To confirm the above data in vivo, melanoma cells transfected with sh-PANDAR or sh-NC were injected subcutaneously into nude mice,
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respectively. Xenograft tumor volumes were measured each week after a palpable tumor formed, and mice were sacrificed five weeks after tumor implantation. As shown in Figure 4, the final tumor volume and weight of sh-PANDAR group were markedly smaller than that of sh-NC group. Therefore, silencing of PANDAR could significantly inhibit tumorigenesis of melanoma cells in vivo.

**PANDAR facilitates EMT in CRC cells**

EMT is an important factor in cell invasion. Thus, we next determined whether EMT mark-
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ers were altered in melanoma cells with deregulation of PANDAR. The expression of E-cadherin, N-cadherin, vimentin, and fibronectin protein level was analyzed by Western blot. As shown in Figure 5, we found that expression of N-cadherin and vimentin was increased while E-cadherin expression was decreased, in melanoma cells with overexpressed PANDAR, whereas opposite results were obtained when PANDAR was knocked down in melanoma cells.

Discussion

Melanoma is one of the most aggressive malignant carcinomas and generally has a poor cure rate because of its metastasis and invasive behavior [29]. Many IncRNAs, such as IncRNAs CCAT1 [19], GASS [30], SLNCR1 [31], were discovered to be dysregulated in melanoma tissues and play a vital role in the metastasis or invasion of melanoma. However, the function of IncRNA PANDAR in melanoma has not been previously clarified.

In the present study, we show for the first time that PANDAR is upregulated in melanoma tissues more than in normal tissues based on qRT-PCR analysis. Consistently, similar results were obtained from melanoma cell lines. Also, we investigated the association of PANDAR expression with clinicopathological characteristics of patients with melanoma and the results showed higher expression level of PANDAR, higher Clark level, thicker tumor, and advancer TNM classification. Also, we determined the relationship between PANDAR expression and clinical outcomes. Kaplan-Meier analysis showed that patients with high levels of PANDAR expression had remarkably shorter survival time than those with low levels. All data suggested that PANDAR might involve in the tumorigenesis and the development of melanoma. Furthermore, in vitro, we performed CCK-8 and colony formation assays to investigate the biological function of PANDAR in melanoma cells. PANDAR knockdown showed low cell viability compared with the control group, while overexpressed PANDAR showed high cell viability. In addition, Transwell assay and Western blotting analysis showed PANDAR could increase cell migration and invasion abilities, as well as regulate EMT. In vivo, we carried out a melanoma-bearing model to determine the tumorigenesis of PANDAR and PANDAR knockdown showed low tumorigenesis compared with the control group.

Cancer metastases represent a multistep biological process that is driven by the acquisition of genetic and/or epigenetic alterations within tumor cells. Many studies showed that cancer metastasis is related to the cancer cell EMT program [32-34]. EMT refers to the biological process of epithelial cells transformed into interstitial phenotype cells through specific procedures, which play an important role in embryonic development, chronic inflammation, tissues reconstruction, fibrosis disease, as well as cancer metastasis. Metastasis is characterized by reduce expression of cell adhesion molecules (such as E-cadherin) and increased expression of cells cytoskeleton protein (Vimentin) and N-cadherin. The epithelial cells...
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will lose epithelial phenotype of connection with the basement membrane, and obtain the mesenchymal phenotype with higher migration and invasion, anti-apoptosis, and the ability of degradation of extracellular matrix. Meanwhile, IncRNAs were reported to have close relationship with EMT progression, serving as a promoter or an inhibitor [35-37]. For example, IncRNA-ROR played an important role in the development of gallbladder cancer and mediates the EMT in gallbladder cancer [38]. Long noncoding RNA LINC01133 inhibited EMT in colorectal cancer by interacting with SRSF6 [39]. BRAF-activated IncRNA contributed to colorectal cancer migration by inducing epithelial-mesenchymal transition [40]. Additionally, high expression of IncRNA PANDAR indicated a poor prognosis for colorectal cancer and promoted metastasis by EMT pathway [27]. However, the function of PANDAR on EMT in melanoma still remained unknown. Thus, to further seek the molecular mechanism through which PANDAR promoted the metastasis of melanoma, we determined the expression level of EMT-related markers following knockdown/overexpression of PANDAR. Our results show that knockdown of PANDAR significantly weaken the expression of N-cadherin, vimentin, and increases the expression level of E-cadherin, however overexpression of PANDAR increased expression of N-cadherin, vimentin, and decreased the expression level of E-cadherin. These results demonstrate that PANDAR might promote melanoma metastasis and invasion through regulating EMT.

In conclusion, we first discovered that the PANDAR expression was strikingly upregulated in melanoma tissues compared with paired-adjacent non-tumorous tissues and elevated PANDAR showed a positive correlation with short overall survival time. These results indicate that PANDAR might play a pivotal oncogenic role in the occurrence and development of melanoma. Also, we identified PANDAR might serve as an indicator of poor survival rate and a negative prognostic factor for patients with melanoma. We also illuminated that knockdown of PANDAR could inhibit cell viability, migration, invasion, tumorigenesis, and EMT, whereas overexpression of PANDAR presented the opposite results, by promoting cell viability, migration, invasion, tumorigenesis, and EMT. These new findings suggested that PANDAR might promote melanoma cell invasion through regulating EMT and it might be used as a potential diagnostic and therapeutic target of melanoma.

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Disclosure of conflict of interest

None.

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References

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