Original Paper

ZNF23 Suppresses Cutaneous Melanoma Cell Malignancy via Mitochondria-Dependent Pathway

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Key Words

Cutaneous melanoma • ZNF23 • Mitochondria • Prognosis • Malignancy

Abstract

Background/Aims: Cutaneous melanoma is one of the leading causes of cancer deaths with an increasing incidence worldwide. A KRAB-containing zinc finger protein member, zinc finger 23 (ZNF23), was reduced in some types of tumors and inhibited cell growth by inducing cell cycle arrest. However, the role of ZNF23 expression is still poorly understood in melanoma.

Methods: The level of ZNF23 expression was detected in cutaneous melanoma, adjacent normal skin tissues and cutaneous melanoma cell lines using immunohistochemistry and western blotting. The correlations between ZNF23 expression and other clinicopathologic parameters were analyzed in melanoma patients. Ectopic expression of ZNF23 plasmid was transfected into melanoma cells, SK-MEL-1 and SK-MEL-28. MTT, flow cytometry and transwell assay were used to measure cell proliferation, apoptosis, invasion and migration abilities, respectively. Mitochondrial functions and structures were detected by mitochondrial membrane potential assay and Transmission electron microscopy (TEM) method in melanoma cells transfected with overexpressing ZNF23 plasmid or empty vector. Western blotting was performed to detect the levels of ZNF23, p53, p27, Bcl-2 and cleaved caspase-3 after overexpressing of ZNF23 in melanoma cells. **Results:** ZNF23 was elevated in adjacent normal skin tissues compared with melanoma tissues. Patients with low level of ZNF23 expression exhibited higher incidence of lymphoid metastasis, thicker size of tumors and worse outcome. By using Cox's regression analysis, ZNF23 expression, tumor thickness and lymph node metastasis were the independent prognostic factors for overall survival ($p < 0.05$). Results from cellular experiments indicated that ectopic expression of ZNF23 induced cell apoptosis by activation of caspase-3, p27, p53 expression and down-regulation of Bcl-2 through mitochondria-dependent pathway.

Conclusions: Decreased ZNF23 was contributed to melanoma progression and poor survival with mitochondria-dependent pathway. It indicated that ZNF23 could be a promising therapeutic biomarker candidate for cutaneous melanoma.

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Introduction

Cutaneous melanoma has been steadily increasing worldwide with the poor 5-year survival rate. Surgery, radiotherapy and chemotherapy are considered to be the regular strategies in patients with melanoma [1, 2]. However, it can't improve the overall survival obviously owing to the melanoma recurrence and horrible metastasis. Hence, exploring the early diagnostic biomarkers or prognostic indicators is necessary in melanoma [3, 4].

The human zinc finger protein 23 (ZNF23) gene is located at chr16q22.2, belonging to the Kruppel-associated box zinc-finger proteins (KRAB-ZFPs) [5]. The KRAB-finger genes encode putative regulatory proteins involved in transcriptional regulation [6]. KRAB-ZFPs constitute the largest individual family of transcriptional repressors encoded by the higher organisms' genome, representing approximately over 1/3 of about 800 different zinc finger proteins. The 75-amino acid N-terminal KRAB domain provides a site for interaction with components of the transcriptional machinery resulting in activation or repression of gene expression [7]. KRAB-ZFPs family play an important role in regulating embryonic development, cell differentiation, proliferation, apoptosis, cell cycle and malignant transformation [8]. Few evidences have been reported that ZNF23 expression was downregulated in human hepatocellular carcinoma, cervical cancer and endometrial carcinoma [9, 10]. Moreover, ZNF23 inhibited cell proliferation and cell cycle arrest in SKOV-3 and HEK293 cells after transfection of ZNF23 plasmid in melanoma cells [9].

However, the role of ZNF23 in melanoma remains unclear. Thus, we will examine the level and significance of ZNF23 expression in matched cutaneous melanoma tissues and cell lines, and we will first illustrate the hypothesis that ZNF23 functioned as a tumor suppressor gene by suppressing cell malignant behaviors *via* mitochondrial pathway.

Materials and Methods

Melanoma cell lines and cell culture

The cutaneous melanoma cell lines, including SK-MEL-1, SK-MEL-2, SK-MEL-5, SK-MEL-28, were purchased from American Tissue Culture Collection (ATCC) of cell line resources (<https://www.atcc.org>), and the human immortal keratinocyte cell line HaCat derived from the Cell Bank of BioVector NTCC Inc (<http://www.biovector.net>). Cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in a humidified incubator containing 5% CO₂.

Melanoma specimens

72 primary malignant cutaneous melanoma and 16 adjacent normal appearance skin tissues were collected from patients between January 2006 and December 2011 (40 men and 32 women, range from 16 to 76 years, average age 58 years). These patients did not receive any radiation or chemotherapy before operation. The survival time was calculated from the operation day to death or until the last follow-up date. All tissue sections were reviewed by two pathologists and were assessed following the 2010 WHO Classification of Tumors: Pathology and genetics of skin tumors. The use of these specimens was approved by the Ethic Committee of General PLA Hospital.

Paraffin specimens were obtained from the archives of the department of pathology. Follow-up data were obtained from review of the patients' medical records. Selected demographic information, including gender, age, tumor anatomic site, tumor thickness, Clark level, lymph node metastasis, distant metastasis, and tumor ulceration were retrieved from the hospital cancer registry. The study has been approved by the Hospitals' Ethical Review Committee.

Immunohistochemistry

Paraffin sections with a thickness of 5 µm were prepared. After dewaxing in xylene and rehydrating stepwise in ethanol, antigen retrieval was carried out using 0.01 mol/L of citrate buffer (pH 6.0) for 1 min 45 s in a pressure cooker. Immunostaining was performed by the Ultrasensitive™ streptavidin-peroxidase (S-P) Kit (MaiXin Bio, Fuzhou, China). Hydrogen peroxide (3%) was applied to block endogenous peroxidase

activity, and normal goat serum was used to reduce nonspecific binding at 37°C for 30 min. Paraffin specimens were incubated with anti-ZNF23 antibody (ab68252, abcam, Cambridge, UK, dilution:1:200) at 4°C overnight. Biotinylated goat anti-mouse serum IgG was used as a secondary antibody. The peroxidase reaction was developed with DAB kit (Maixin Bio, Fuzhou, China), and nuclei were counterstained with hematoxylin. For negative control, the primary antibody was replaced by non-immune serum.

Two pathologists assessed the staining results on a semi-quantitative scale. Ten random views were examined per-slide, and 100 cells in each field were counted to evaluate the intensity and range of ZNF23 staining. Nuclear staining in tumor cells was considered positive staining. A negative result was given a score of 0 (no signal), faint yellow staining was recorded as a 1, yellow was scored as a 2, and deep brown staining was recorded as a 3. Percentage scores were assigned as 1, ≤25%; 2, 26-50%; 3, 51-75%; and 4, ≥76 %. The average scores of each tumor sample were multiplied to give a final score of 0-12, and the tumors were finally determined as negative (-), score 0; lower expression (+), score 1-4; moderate expression (++), score 5-8; and high expression (+++), score ≥9. Tumor sample scored (-) to (+) were considered "low expression", whereas scored (++) to (+++) were considered "high expression".

Ectopic expression of ZNF23 plasmid

SK-MEL-2 and SK-MEL-28 melanoma cells were obtained from ATCC and cultured in DMEM (Hyclone, Logan, Utah, USA) containing 10% FBS (Hyclone), 100 IU/ml penicillin (Sigma, St. Louis, MO, USA), and 100 mg/ml streptomycin (Sigma). Cells were grown on sterile tissue culture dishes and passaged every 2 days using 0.25% trypsin (Hyclone). Ectopic expression of ZNF23 plasmid (Cat: RC203532) and empty vector (Cat: PS100001) were purchased from Origene Technologies, Inc. (Website, <http://www.origene.com>, Rockville, MD, USA). For transfections, SK-MEL-2 and SK-MEL-28 cells were seeded in a six-well plate 24 h before the experiment. The cells were transfected with overexpressing ZNF23 plasmid or empty vector mixed with Lipofectamin3000 reagent (Invitrogen, Carlsland, CA, USA) according to the manufacturer's protocol. Following transfections, the protein levels were assessed at 48 h.

Western blot analysis

Total proteins from cells and tissues were extracted in NP40 lysis buffer (Beyotime, Nanjing, China) and quantified using Bradford method. Fifty micrograms of protein was separated by 10% SDS-PAGE and transferred to the polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% BSA in Tris-buffered saline-Tween at room temperature for 2 h, membranes were incubated with primary antibodies for ZNF23 (1:800 dilution, Proteintech, Wuhan, Hubei, China), p27 (1:1000 dilution, Cell Signal, USA), p53 (1:500 dilution, Santa Cruz, USA), Bcl-2 (1:500 dilution, Beyotime Biotech, China), activate/cleaved caspase-3 (19/17 kD, 1:500 dilution, Beyotime Biotech, China) or β -actin (1:10000, Sigma, USA) overnight at 4°C. The membranes were incubated with peroxidase-coupled anti-mouse or rabbit IgG (Zhongshan Biotech, Beijing, China) at 37°C for 2 h, and were visualized using ECL (Thermo Fisher Scientific) and detected using a BioImaging System (UVP Inc., Upland, CA, USA). The relative protein expression was calculated based on β -actin as the loading control.

Cell viability assay

Cell proliferation was determined with 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H- tetrazolium bromide (MTT) (KeyGen, Nanjing, China) according to manufacturer's instructions. Briefly, 5×10^3 cells were plated into 96-well plates and grew in complete medium. At different time points, cells were incubated with MTT in serum free medium at 37°C for 4 h. Then the liquids were removed and 150 μ l DMSO was added. After 10 min in vortex, the absorbance was measured at 490 nm in a microplate reader (SUNRISERC, TECAN, Switzerland). Three independent experiments were repeated.

Mitochondrial Membrane Potential Detection

Mitochondrial membrane potential was visualized in melanoma cells with 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) following the kit's introduction.(Beyotime Co.Ltd.). Briefly describe, JC-1 is a dual-emission mitochondrial membrane potential (MMP) sensing dye, which can be accumulated and aggregates in polarized (normal) mitochondria, and aggregate JC-1 shifts its emission to red. When the mitochondrial depolarization (loss of MMP) suppress JC-1 entry into mitochondria, monomeric JC-1 remains in the cytosol and green fluorescence. Melanoma cells from different treatment

groups were washed and incubated with JC-1 at 37°C for 15 min, then washed and mounted on the confocal microscopy for imaging. The aggregate JC-1 (red) was detected at the emission wavelength of 590 nm, and the monomeric JC-1 (green) detected at 529 nm. The ratio of aggregates and monomeric JC-1 was used to quantify changes in MMP, and decreased JC-1 ratio represents the depolarization of mitochondria and the weakened MMP.

Flow cytometry

Cells were seeded into 6-cm tissue culture dishes. Cells were harvested and washed with phosphate-buffered saline (PBS), then fixed in cold 70% ethanol at 4°C overnight. After washing twice with PBS, cells were incubated with 100 µL RNase A (KeyGen, Nanjing, China) at 37°C for 30 min. After staining with propidium iodide (PI, KeyGen, Nanjing, China) for 30 min at 4°C, data were collected using BD FACSCalibur flow cytometer (BD Biosciences, USA). The data were analyzed using ModFit LT for Mac v3.0 (BD Biosciences, USA).

Invasion and migration assay

Transwell Migration and Matrigel-embedded Invasion Assay (Merck Millipore, Darmstadt, Germany) were used in our study following the protocol introduction. Cells were seeded at 1.5×10^5 /well in the upper chambers with 200 µl serum-free basic medium for migration or invasion assay, then, 500 µl complete endothelial cell growth medium (10% FBS) was added in the lower wells serving as a source of chemo-attractants. The cells were incubated for 24 h at 37°C. Cells migrated to the lower surface of the filter were fixed with 70% methanol and stained with 0.5% crystal violet solution. The number of migrated or invasive cells was determined by counting stained cells and the average cell number per field for each well was calculated. The data was designed for three independent experiments. For each experiment, three replicate wells were used and the representative images were taken from three randomly selected fields of each well.

Transmission electron microscopy (TEM)

Transmission Electron Microscopy (TEM) observations were performed by a Hitachi 7700, AT 100 kV (Hitachi High Technologies America Inc., Dallas, TX, USA). Melanoma Cell lines on coverslips were transfected with empty vector or ectopic expression of ZNF23 plasmid for 48 h, and fixed in 2.5% glutaraldehyde overnight, washed with 0.1 M phosphate-buffered saline (PBS, pH 7.2) and fixed in 2% osmium tetroxide for 2 h. The sections were then washed twice with distilled water and stained with 1% uranyl acetate for 1 min and air-dried. The specimens were prepared for TEM observations onto 400-mesh carbon-coated copper grid and drying slowly in air naturally.

Statistical analysis

All data were analyzed with SPSS 19.0 (SPSS, Chicago, IL, USA). The measurement data were shown as the mean $\pm$ SD. The differences between two groups were analyzed by Student's *t*-test. The impact of immunointensity of ZNF23 expression, tumor thickness and Clark level on overall survival was assessed by Kaplan-Meier analysis. The association between ZNF23 and clinicopathologic variables was analyzed using χ^2 -test method, and differences between groups were analyzed by the log-rank test. Multivariate Cox proportional hazard regression analysis was used to assess independent factors. *P*-values were based on the two sided statistical analysis, and *P* statistical significance was assessed with a *P* < 0.05.

Results

Decreased ZNF23 mostly occurred in melanoma tissues and cells

Western blot data indicated that ZNF23 was decreased in melanoma cell lines including SK-MEL-1, SK-MEL-2, SK-MEL-5, SK-MEL-28. While, high level of ZNF23 detected in the immortal keratinocyte cell line HaCat derived from adult human skin (Fig. 1A). Moreover, in seven fresh paired melanoma tissues, decrease or loss of ZNF23 was detected in melanoma tissues compared with the adjacent normal skin tissues (Fig. 1B). Furthermore, results from IHC staining exhibited that ZNF23 was specially expressed in the nuclei of cells and the high expression rate of nuclear ZNF23 were 54.2% (39/72) in melanoma, which was significantly

Fig. 1. Decreased ZNF23 occurred in melanoma cells and tissues by western blotting. (A) Decreased ZNF23 protein in most of melanoma compared with the immortal keratinocyte cell line HaCat derived from adult human skin; (B) The downregulation of ZNF23 protein in melanoma tissues compared with the adjacent normal skins.

Fig. 2. Immuno-staining with anti-ZNF23 antibody on epithelium and melanoma tissues. (A) Nuclear staining for ZNF23 in normal skin epithelial cells ($\times 100$); (B) Positive immunostaining for ZNF23 in basal epithelial cells ($\times 400$); (C) Strong positive staining of glandular epithelium while positive expression of melanoma ($\times 400$); (D) Low expression of ZNF23 in melanoma ($\times 400$); (E, F) Negative expression of ZNF23 in melanoma ($\times 400$).

Table 1. Differential expression of ZNF23 in melanoma tissues and adjacent normal skin tissues

Type	Total Number	ZNF23 expression		<i>P</i> -value
		High	Low	
Melanoma	72	39	33	0.008
Normal	16	15	1	

lower than the adjacent normal skin tissues (93.8%, 15/16) ($P = 0.008$, Fisher's exact test, Table 1 and Fig. 2). It indicated that decreased ZNF23 occurred in most of melanoma cells and tissues.

Decreased ZNF23 predicted poor prognosis in melanoma patients

In 72 melanomas, a significant correlation between ZNF23 expression and the survival of melanoma patients was shown in Table 2 and Fig. 3C (log-rank test, $P < 0.001$). The overall survival of patients with high level of ZNF23 expression (39 cases) was 62.18 ± 2.18 months and patients with low level of ZNF23 expression (33 cases) was 32.32 ± 2.32 months (Table 2). The overall survival was significantly lower in patients with low level of ZNF23 expression than patients with high level of ZNF23 expression.

Moreover, decreased ZNF23, age, distant metastasis, lymph node status, Clark level and tumor thickness was significantly associated with patients' survival using logistic-regressi-

Fig. 3. Kaplan-Meier survival analyses were used in patients with cutaneous melanomas. (A) Stratified according to tumor thickness (log-rank test); (B) Stratified according to Clark level (log-rank test); (C) Stratified according to ZNF23 expression (log-rank test).

on analyses, respectively. No significant difference was observed according to tumor anatomic site, gender, and ulceration (Table 2). The influence of melanoma thickness, ulceration, and Clark level, three well-recognized prognostic factors on overall survival for cutaneous melanoma, were also assessed in this study. As shown in Fig. 3 and Table 2, both tumor thickness ($P < 0.05$) and Clark level ($P < 0.01$) were also the significant prognostic factors in our patients with primary cutaneous melanoma, whereas tumor ulceration was not associated with the prognosis (Table 2 and Fig. 3).

Furthermore, the association on ZNF23 expression with other clinical features was shown in Table 3. ZNF23 expression was significantly associated with tumor thickness ($P = 0.015$), lymph node status ($P = 0.019$) and the final status (live or die, $P = 0.004$). ZNF23 expression rate in thick melanoma (tumor thickness > 4

Table 2. Logistic regression analysis of clinicopathologic features in melanoma

Clinical features	Num.	Average survival (month)	Median survival (month)	P-value
Gender				
Male	40	45.72 $\pm$ 5.72	28.00 $\pm$ 8.00	0.410
Female	32	50.36 $\pm$ 0.36	46.00 $\pm$ 6.00	
Age				
<58 years	35	38.03 $\pm$ 8.03	26.00 $\pm$ 6.00	0.010
≥ 58 years	37	58.05 $\pm$ 8.05	50.00 $\pm$ 5.00	
Anatomic				
Extremities	15	54.29 $\pm$ 4.29	50.00 $\pm$ 3.00	0.168
Trunk	24	35.61 $\pm$ 5.61	34.00 $\pm$ 4.00	
Head or neck	33	53.81 $\pm$ 3.81	30.00 $\pm$ 3.00	
Thickness				
≤ 1 mm	12	77.50 $\pm$ 7.50	87.00 $\pm$ 7.00	0.026
1-2 mm	26	51.88 $\pm$ 1.88	46.00 $\pm$ 6.00	
2-4 mm	25	32.84 $\pm$ 2.84	25.00 $\pm$ 5.00	
> 4 mm	9	14.06 $\pm$ 4.06	16.00 $\pm$ 6.00	
Clark level				
I	8	90.00 $\pm$ 0.36	87.00 $\pm$ 13.02	0.009
II	20	43.42 $\pm$ 3.42	42.00 $\pm$ 2.00	
III	23	42.03 $\pm$ 2.03	28.00 $\pm$ 8.00	
IV-V	21	26.05 $\pm$ 6.05	23.00 $\pm$ 3.00	
Lymph node status				
No	37	66.10 $\pm$ 6.10	83.00 $\pm$ 2.44	<0.001
Yes	35	26.06 $\pm$ 6.06	23.00 $\pm$ 3.00	
Ulceration				
Absent	34	43.95 $\pm$ 3.95	38.00 $\pm$ 8.00	0.836
Present	38	50.86 $\pm$ 0.86	36.00 $\pm$ 6.00	
Distant metastasis				
No	37	59.35 $\pm$ 9.35	50.00 $\pm$ 6.00	0.001
Yes	35	28.88 $\pm$ 8.88	26.00 $\pm$ 6.00	
ZNF23 expression				
High	39	62.18 $\pm$ 2.18	87.00 $\pm$ 7.00	<0.001
Low	33	32.32 $\pm$ 2.32	22.00 $\pm$ 2.00	

mm group) was obviously lower (11.1%) than the thickness 2-4 mm group (48.0%), thickness 1-2 mm group (65.4%) and thickness < 1 mm (75.0%) groups ($P = 0.015$, Table 3). The expression rate of ZNF23 in patients with lymph node metastasis (67.6%) was higher than patients without lymph node metastasis (40.0%, $P = 0.019$, Table 3). These results indicated that the level of ZNF23 expression could serve as a molecular predictor for the prognosis of patients with primary cutaneous melanoma.

To investigate whether ZNF23 expression was an independent prognostic marker for cutaneous melanoma, we applied a multivariate Cox regression analysis for the overall

survival (Table 4). The variables taken into consideration included gender, age, tumor location, thickness, Clark level, lymph node status, distant metastasis, ulceration and status of ZNF23 expression. Such analysis demonstrated that ZNF23 expression (hazard ratio = 0.306, $P = 0.002$), tumor thickness (hazard ratio = 2.186, $P = 0.015$) and lymph node status (hazard ratio = 3.130, $P = 0.038$) were significant prognostic factors for cutaneous melanoma (Table 3). These data indicated that high level of ZNF23 expression was the potential independent protective factor with better outcome in melanoma patients.

Ectopic expression of ZNF23 inhibited the malignancy of melanoma cells

To determine whether ZNF23 influences proliferation and apoptosis of melanoma cells, overexpressing ZNF23 plasmid or empty vector was transfected into SK-MEL-2 and SK-MEL-28 melanoma cells. The ZNF23 expression was unaffected in cells transiently transfected with empty vector, whereas ZNF23-overexpressing plasmid considerably increased the protein level of ZNF23 expression at 48 h after transfection using western blotting assay (Fig. 4A). Results from MTT assay revealed that overexpressing ZNF23 suppressed cell proliferation ($P < 0.001$, Fig. 4B) and migration or invasion ability was suppressed after ectopic expression of ZNF23 in melanoma cells, respectively (Fig. 4C and 4D). Furthermore, the apoptotic rate was elevated after overexpressing ZNF23 in melanoma accompanied by upregulation of p27, p53, cleaved caspase-3 expression and downregulation of Bcl-2 expression (Fig. 4A and 4E).

ZNF23 mediated the mitochondrial pathway in melanoma cells

Based on the data showing that upregulation of ZNF23 promoted cell apoptosis and decreased cell migration and invasion, we examined some related proteins including p27, p53, activate caspase-3 and Bcl-2. Interestingly, most proteins were located at mitochondria. Hence, we further check the mitochondrial membrane potential variation after transfection with overexpressing ZNF23 plasmid or empty vector. As shown in Fig. 5A, decreased MMP was detected in ectopic expression of ZNF23 group apparently, and the mitochondrial cristae and microstructure were arranged in disorder after ZNF23 plasmid transfection (Fig. 5B).

Table 3. Relationship between ZNF23 expression and other clinical characteristics in melanoma patients

Features	Cases (n=72)	ZNF23 expression		P-value
		Low (%)	High (%)	
Gender				
Male	40	19 (47.5)	21 (52.5)	0.751
Female	32	14 (43.8)	18 (56.2)	
Age				
<58 years	35	18 (51.4)	17 (48.6)	0.354
≥58 years	37	15 (40.5)	22 (59.5)	
Anatomic				
Extremities	15	7 (46.7)	8 (53.3)	0.092
Trunk	24	15 (62.5)	9 (37.5)	
Head or neck	33	11 (33.3)	22 (66.7)	
Thickness				
≤1mm	12	3 (25.0)	9 (75.0)	0.015
1–2 mm	26	9 (34.6)	17 (65.4)	
2–4 mm	25	13 (52.0)	12 (48.0)	
>4 mm	9	8 (88.9)	1 (11.1)	
Clark level				
I	8	1 (12.5)	7 (87.5)	0.112
II	20	8 (40.0)	12 (60.0)	
III	23	11 (47.8)	12 (52.2)	
IV-V	21	13 (61.9)	8 (38.1)	
Lymph node status				
No	37	12 (32.4)	25 (67.6)	0.019
Yes	35	21 (60.0)	14 (40.0)	
Ulceration				
Absent	34	16 (47.1)	18 (52.9)	0.844
Present	38	17 (44.7)	21 (55.3)	
Distant metastasis				
No	37	13 (35.1)	24 (64.9)	0.061
Yes	35	20 (57.1)	15 (42.9)	
Status				
Alive	33	9 (27.3)	24 (72.7)	0.004
Dead	39	24 (61.5)	15 (38.5)	

Table 4. Cox regression analysis of clinicopathologic parameters and molecular signatures in melanoma

Features	Hazard Ratio (95%CI)	P-value
Gender	0.680 (0.310-1.495)	0.338
Age	0.746 (0.327-1.701)	0.486
Anatomic	1.008 (0.650-1.564)	0.972
Thickness	2.186 (1.166-4.098)	0.015
Clark level	0.974 (0.512-1.852)	0.936
Lymph node status	3.130 (1.066-9.189)	0.038
Distant metastasis	0.962 (0.383-2.416)	0.935
Ulceration	1.353 (0.657-2.788)	0.412
ZNF23 expression	0.306 (0.146-0.645)	0.002

Fig. 4. Ectopic expression of ZNF23 inhibited cell proliferation, migration and invasion in melanoma cells. (A) Validation of ZNF23, Bcl-2, p27, p53 and active caspase-3 protein expression levels transfected by ZNF23 overexpressing plasmid or empty vector using western blotting analysis. (B) ZNF23 inhibited cell viability by MTT assay. (C) The transwell assay was used to determine melanoma cell migration of overexpressing ZNF23 plasmid and vector-control transfectants. Data show the mean $\pm$ S.D. (three independent experiments); (D) The transwell assay was used to determine melanoma cell invasion of overexpressing ZNF23 plasmid and vector-control transfectants. Data show the mean $\pm$ S.D. (three independent experiments); (E) Cell apoptosis was determined by with Annexin-V/PI staining after transfection with control and ZNF23 overexpressing plasmids. The data shown were representative of three independent experiments. **P < 0.001.

Discussions

Human cutaneous melanoma is an aggressive disease with the highest increase in incidence worldwide. In the past decade, tremendous advances have been made in elucidating the mechanism of melanoma initiation and progression. The treatment of melanoma has greatly improved which includes surgery for localized melanoma and regional lymph node metastases, radiation, chemotherapy, or a combination of natural chemicals, gene therapy

Fig. 5. TEM was used to detect the mitochondrial ultramicrostructure in melanoma cells, and we found a marked difference in mitochondrial morphologies between two groups with transfection of ZNF23 overexpression plasmid or empty vector, respectively. (A) MMP was decreased in ZNF23-overexpressing melanoma cells. (B) The mitochondria were basically in a state of fusion and the mitochondrial cristae had a relatively regular arrangement in melanoma cells transfected with empty vector. However, after overexpression of ZNF23 in melanoma cells, most of the mitochondria presented short rod-like structures and spheroids, especially the mitochondrial cristae were arranged in disorder (bar = 500 nm).

and immunotherapy to suppress melanoma metastasis *in vitro* and *in vivo* [11-13]. However, the long term prognosis of metastatic melanoma remains unsatisfactory and the underlying mechanism in melanoma pathogenesis and progression is poorly understood. Therefore, identifying useful biomarkers and exploring novel therapeutic targets remain important.

In this study, we first discovered that ZNF23 was decreased in cutaneous melanoma tissues compared with normal skin. Decreased ZNF23 expression was associated with poor progression of cutaneous melanoma. Importantly, low level of ZNF23 expression was associated with tumor thickness, lymph node metastasis. The multivariate Cox regression analysis on the overall survival revealed that ZNF23 was a significant independent prognostic factor in melanoma patients. These data further support the hypothesis that ZNF23 is a potential tumor suppress gene in melanoma, and decreased ZNF23 was involved in the malignancy of melanoma.

ZNF23 was reduced generally in human endometrial carcinoma, hepatocellular carcinoma and ovarian adenocarcinoma cell lines and tissues [9, 10]. Our results were similar with other reports. ZNF23 was related to the cellular apoptosis in HepG2 cells with cisplatin treatment. Upregulation of p27 was detected in hepatocellular carcinoma after ectopic expression of ZNF23 plasmid with a dose-dependent manner [10]. Besides, SK-OV-3 cells with overexpressing ZNF23 induced apoptosis through elevation of p27 and activation

of caspase-3 compared with empty control [9]. In our study, upregulation of p27 and activation of caspase-3 induced by ZNF23 transfection were contributed for the inhibition of cell migration, invasion and proliferation in melanoma. The accumulating body of evidence provided the ZNF23/p53 signaling ample rationale in many types of cancer [14-16]. Loss of p27 upregulates MnSOD expression through STAT3-signaling pathway, with disruption of intracellular redox activity and enhances cell migration [17]. Cell-cycle related proteins such as p27, p16^{INK4a} and p14^{ARF} were frequently decreased in melanoma tissues and over-expression of these genes suppressed the proliferation and migration of human melanoma cells through Rb and p53 tumor suppressor pathways [18-21]. Interestingly, p53 tumor suppressor is central to the antineoplastic activity and exerts a direct pro-apoptotic role at the mitochondria by engaging in ROS-p53 interactions with anti- and pro-apoptotic Bcl-2 family members [22, 23]. In our study, the mitochondria structure was disrupted with decreased MMP after overexpressing ZNF23 in melanoma cells, accompanied with mitochondria-related apoptosis with downregulation of Bcl-2 expression and activation of caspase-3 and p53. Importantly, p53, the transcription factor, directly targets mitochondria for cell death induction in the majority of human cancers [24], and it indicates that mitochondria is a potential target for tumor therapy [25-28], because mitochondria as the cells powerhouses, are essential for maintaining cell life, and they also play a major role in regulating cell death, which occurs upon permeabilization of their membranes. Once mitochondrial membrane permeabilization (MMP) occurs, cells die either by apoptosis or necrosis by mobilization and targeting to mitochondria of Bcl-2 family members.

Collectively, we first reported that ZNF23 was the potential tumor suppressor gene in melanoma patients via mitochondrial dysregulation. ZNF23 was a candidate biomarker for the assessment of tumour prognosis in melanoma. Our results indicated that ZNF23-mitochondrial p53 pathway could become exploitable targets for new melanoma therapeutics.

Abbreviations

ZNF23 (Zinc finger 23); DMEM (Dulbecco's modified eagle's medium); MTT (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide); TEM (Transmission electron microscope); JC-1 (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethyl benzimidazolyl carbocyanine iodide); MMP (Mitochondrial membrane potential).

Disclosure Statement

The authors declare no competing financial interest.

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