

MicroRNA-3662 targets ZEB1 and attenuates the invasion of the highly aggressive melanoma cell line A375

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Background: Cutaneous melanoma is the most aggressive form of skin cancer. It accounts for approximately 5% of all cutaneous malignancies and is currently responsible for the majority of skin cancer-related deaths. However, the exact mechanisms responsible for the occurrence of melanoma, in particular the invasive growth in normal skin or muscle tissue, remain unknown.

Materials and methods: miR-3662, a microRNA is a potential tumor suppressor targeting zinc finger E-box binding homeobox 1 (ZEB1), which functions as a key regulator of the epithelial-mesenchymal transition (EMT) process. This microRNA was identified using an online database (miRDB) and expression was confirmed by Western blot analysis. Quantitative polymerase chain reaction (qPCR) was used to examine whether miR-3662 inhibits the EMT process in the aggressive melanoma cell line, A375, through the modification of the expression of invasion-related genes in A375 cells. The effects of miR-3662 on the in vivo growth of A375 cells were examined in a nude mouse model.

Results: Using virtual screening of the miRDB database, miR-3662 was shown to target the 3' untranslated region (UTR) of the ZEB1 gene. Expression of miR-3662 via a lentivirus vector significantly decreased protein levels of ZEB1 and inhibited the growth of A375 cells in vitro and in vivo. The reduction in ZEB1 expression induced by miR-3662 resulted in EMT inhibition in A375 cells and decreased the relative expression of metastasis genes.

Conclusion: Down-regulation of ZEB1's expression via miR-3662 lentivirus vectors significantly decreased the in vitro and in vivo growth of the highly aggressive melanoma cell line A375.

Keywords: melanoma, microRNA, proliferation, invasive growth, zinc finger E-box binding homeobox 1

Introduction

Cutaneous melanoma is the most aggressive form of skin cancer, resulting in over fifty thousand deaths each year worldwide.^{1,2} Early stage diagnosis of cutaneous melanoma and effective primary care could significantly improve the incidence and mortality rates for this cancer.^{3,4} Cutaneous melanoma originates in melanocytes that colonize the skin.^{5,6} When exposed to risk factors, such as ultraviolet radiation (UV), mutations can accumulate within the cell genome of melanocytes, leading to their transformation to cutaneous melanoma.^{7,8} Researchers to date have focused largely on the alteration of tumor suppressors including *PTEN*, *CDKN2A* or *TP53*, or tumor promoters such as *TERT*, *Ras*, or *Raf*, in melanocytes or cutaneous melanoma cells.^{9,10} Few treatment options are available following the metastasis

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of cutaneous melanoma to a second site and consequently this stage of the disease then becomes life-threatening.^{11,12} Thus, it is important that we develop an increased understanding of the underlying mechanisms governing the invasive growth of cutaneous melanoma cells and their metastasis, to enable the development of more effective therapeutic strategies.

The human proto-oncogene zinc finger E-box binding homeobox 1 (ZEB1) can function as a transcription factor and plays additional important roles in the regulation of normal physiological functions, including cell fate decisions, conferment of cell stemness properties, and cell plasticity.^{13,14} ZEB1 is a key regulator of the epithelial-mesenchymal transition (EMT) process, which is responsible for the partial or complete transition of cancer cells with epithelial features to a mesenchymal state.^{15,16} High expression levels of ZEB1 have been identified in a variety of human cancers, such as lung cancer, hepatocellular carcinoma, and breast cancer, and research supports a role for ZEB1 in mediating the migration and invasion of cancer cells, via the induction of the EMT process.^{17,18} As such, ZEB1 is a promising potential therapeutic target, the inhibition of which may attenuate the proliferation or metastasis of cancer cells. Expression of ZEB1 has been identified in melanoma, though its function in the regulation of these cells remains unclear.¹⁹⁻²¹

A microRNA (miRNA) is a small noncoding RNA molecule that can mediate the silencing of targeted gene expression, by binding preferential sequences in the 3'-untranslated regions (UTRs) of target mRNAs. miRNAs exhibit regulatory functions in several important cellular processes, such as differentiation and development. The identification of miRNAs targeted to human cancer-related genes and their transfection may offer a promising approach for the treatment of such tumors. This work aimed to identify a miRNA targeting ZEB1, miR-3662, and further examine the effects of this miRNA on the proliferation and invasive growth of A375, a highly aggressive melanoma cell line used to mimic the spread of cutaneous melanoma.

Materials and methods

Clinical specimens

The methods and collection of clinical specimens in this study were approved by the review board in accordance with ethical norms (Ethics Committee) at the Peking Union Medical College Hospital with written informed consent of all patients. All clinical investigations were conducted according to the principles expressed in the Declaration of

Helsinki. This prospective study consisted of 80 paired tumor/non-tumor melanoma specimens between March 2014 and June 2017. Tumor pathology was confirmed and tumors staged²² as Clark I (8 cases); Clark II (12 cases); Clark III (17 cases); Clark IV (19 cases); or Clark V (24 cases).

Cell line, plasmids and reagents

Expression vectors with full length ZEB1 genes or ZEB1 gene cDNA sequences with mutated miR-3662 binding sites were purchased from Vigene Corporation (Jinan, China). Lentivirus particles expressing miR-3662 were also obtained (Vigene Corporation). miR-3662 examination kits (Cat. No. 478844_mir-A25576) and inhibitors (Cat. No. 4464066-AM17000-MH19546) were purchased from Thermo Fisher (Waltham, MA, USA). Cell-based experiments and the use of cell lines were approved by the Ethics Committee of Beijing Union Hospital. Both a highly aggressive melanoma cell line, A375, and a lowly aggressive melanoma cell line, OCM-1A, were obtained from the National Infrastructure of Cell Line Resource, (Chinese Academy of Medical Sciences, Beijing, China). The usage of these cell lines was approved by the Ethics Committee of the Chinese Academy of Medical Sciences & Peking Union Medical College. The cells were maintained in Dulbecco's modified eagle medium (DMEM; Thermo Fisher, Waltham, MA, USA) with 10% fetal bovine serum (FBS; Thermo Fisher, Waltham, MA, USA) at 37 °C with 5% CO₂. Regions of the ZEB1 mRNA 3'UTR (1561–1740, 1681–1860 or 1861–2040) containing miR-3662 targeting sequence (TCATTTT) were cloned into pGL4.26 plasmids to form Luciferase reporters ([Figure S1](#)).

Cell proliferation and metastasis

A375 cells were initially transfected with miR-3662 or a control miRNA (expression vector with a non-targeting random sequence) and then subject to either cell proliferation or metastasis analysis. To analyze cell proliferation, the transfected cells were analyzed via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For the analysis of cell metastasis, the transfected cells were analyzed by transwell experiments to measure the in vitro invasion and migration of cells.^{23,24}

Quantitative polymerase chain reaction (qPCR)

A375 cells transfected with miR-3662, miR-3662+ ZEB1^{Mut} (an expression vector containing a full length sequence of the

ZEB1 gene with mutated miR-3662 targets), miR-3662+ inhibitor (inhibitor of miR-3662), or control miRNA, were harvested 24 h after transfection and total RNA was extracted. qPCR experiments were performed using a kit following the manufacturer's instructions and methods described in references.^{23,24} Primers used in qPCR experiments are listed in Table 1.

Luciferase

A375 cells transfected with relevant plasmids were harvested for luciferase activation using a luciferase kit (Promega, Madison, WI, USA) following the protocol provided by manufacturer (Promega, Madison, WI, USA). Transfection was performed using Lipofection2000 transfection reagent (Thermo Fisher, Waltham, MA, USA). A total of 20µg of each plasmid was transfected into each well of a 24-well plate containing cells by using the standard transfection method. Luciferase activation was calculated as fold-improvement according to (treatment group luciferase activation)/(control group luciferase activation).

Antibodies and Western blot

Antibodies to ZEB1 (Cat. No. ab181451), E-cadherin (Cat. No. ab1416), N-Cadherin (Cat. No. ab98952), Vimentin (Cat. No. ab20346), MMP3 (Cat. No. ab137659), TIMP1 (Cat. No. ab61224), MMP9 (Cat. No. ab73734) and Glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) (Cat. No. ab8245) were obtained from Abcam (Cambridge, UK).

A375 cells transfected with miR-3662, miR-3662+ ZEB1^{Mut} or miR-3662+ inhibitor or control miRNA were harvested 48 h following transfection and total protein samples extracted for Western blot experiments. OCM-1A cells transfected with empty vector or ZEB1 vectors were also harvested for Western blot assays. Western blots were performed following a standard Western blotting protocol. Briefly, protein samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, proteins were transferred onto polyvinylidene fluoride (PVDF) membranes and blocked using 5% bovine serum albumin (BSA) diluted in tris-buffered saline with Tween 20 (TBS-T). Membranes were subsequently incubated with the relevant primary and secondary antibodies prior to detection by chemiluminescence.

Subcutaneous tumor model of melanoma cells

Animal experiments were approved by the Institutional Animal Care and Use Committee of the Chinese Academy of Medical Sciences & Peking Union Medical College. Animal studies were conducted in accordance with the UK Animals (Scientific Procedures) Act of 1986 and the associated guidelines. Nude mice (BALB/c nude) were purchased from Si-Bei-Fu Biotechnology (Beijing, China). For the subcutaneous tumor experiments,^{25,26} A375 cells that had been stably infected with miR-3662, miR-3662+ ZEB1^{Mut} or control miRNA or OCM-1A cells stably transfected with miR-3662 or control miRNA were injected into four- to six-week-old nude mice. Each nude mouse was seeded with 1×10^6 cells. After six weeks of growth subcutaneous tumors were harvested and the volume and weight of the tumors were measured.

In vivo invasive growth of melanoma cells in muscle tissue

A375 cells stably infected with miR-3662, miR-3662+ ZEB1^{Mut}, or control miRNA or OCM-1A cells stably transfected with ZEB1 vector or empty vector were mixed with biological-medical gel (Cai-Hong-Yi-Xue-She-Bei, Kunming, China) and injected into the muscle tissue in the legs of nude mice. Each mouse was seeded with approximately 5×10^5 cells. After four weeks' growth,

Table 1 Primers used in qPCR experimnts

Genes	Primer sequence (5'-3')
E-cadherin	Forward: 5'-AAGGCACGCCTGTGCGAAGCA-3' Reverse: 5'-ACGTTGTCCCGGTGTCATCCT-3'
N-cadherin	Forward: 5'-TGCGCGTGAAGTTTGCCAGT-3' Reverse: 5'-TGGCGTTCTTTATCCCGGCGT-3'
Vimentin	Forward: 5'-ACCGCACACAGCAAGGCGAT-3' Reverse: 5'-CGATTGAGGGCTCCTAGCGGT-3'
TIMP-1	Forward: 5'-ACTTCCACAGTCCCACAAC-3' Reverse: 5'-CACTGTGCATTCCTCACAGC-3'
MMP3	Forward: 5'-TGCTTTGTCCTTTGATGCTG-3' Reverse: 5'-GGAAAGAGATGGCCAAATGA-3'
MMP9	Forward: 5'-TGGGCTACGTGACCTATGAC-3' Reverse: 5'-CAAAGGTGAGAAGAGAGGGC-3'
ZEB1	Forward: 5'-GATGACCTGCCAACAGACCA-3' Reverse: 5'-CCCCAGGATTTCTTGCCCTT-3'
GAPDH	Forward: 5'-CTCTCTGCTCCTCCTGTTTCG-3' Reverse: 5'-ACGACCAAATCCGTTGACTC-3'

the mice were harvested and the muscle tissue containing A375 cells was taken for analysis by hematoxylin-eosin (H&E) staining.²⁷ Images of the H&E staining were quantitatively analyzed via Image J software according to previously detailed methods.^{28,29} Results were shown as relative invasive growth of A375 cells: (treatment group's total area of tumor tissues)/(control miRNA group's total area of tumor tissues). The inhibition rate of each group was calculated as follows: [(control group's relative invasive growth of A375 cells) - (treatment group's relative invasive growth of A375 cells)]/(control group's relative invasive growth of A375 cells) x 100%.

Statistical analysis

Western blot bands were analyzed using Image J software (version 1.51j8; National Institutes of Health, Bethesda, MD, USA). The relative expression of the targeted proteins was calculated as follows: (the indicated group intensity of the targeted protein bands)/(the indicated group intensity of the loading control bands). Statistical analysis was performed using the Bonferroni correction with or without two-way analysis of variance, with SPSS software (version 16.0; IBM, Armonk, New York, USA). *P*-values <0.05 were considered statistically significant.

Results

miR-3662 targets the 3' UTR of the ZEB1 mRNA

To investigate the potential role of ZEB1 in regulating melanoma, the expression of ZEB1 mRNA in clinical specimens was examined via qPCR. Results from 80 paired tumor and non-tumor specimens indicated that ZEB1 was expressed at higher levels in melanoma compared to non-tumor specimens (Figure 1A). Moreover, a higher level of ZEB1 expression was detected in the more aggressive stages of melanoma (eg Clark V or Clark IV) compared to the less aggressive stages (Clark I or Clark II) (Figure 1B).

A miRNA potentially targeted to ZEB1 mRNA's 3' UTR, was identified by screening the online database, miRDB (<http://www.mirdb.org/>). Analysis of the expression of miR-3662 in both tumor and non-tumor tissues revealed a higher level of miR-3662 expression in non-tumor specimens than that in tumor specimens (Figure 1C). Additionally, a higher level of miR-3662 expression was detected in the less aggressive stages of melanoma (Clark I or Clark II) compared to the more aggressive stages of melanoma (such as Clark IV or Clark V) (Figure 1D).

The expression of ZEB1 and its associated miRNA indicate the potential involvement of this protein in regulating the invasive growth of melanoma tumors. To

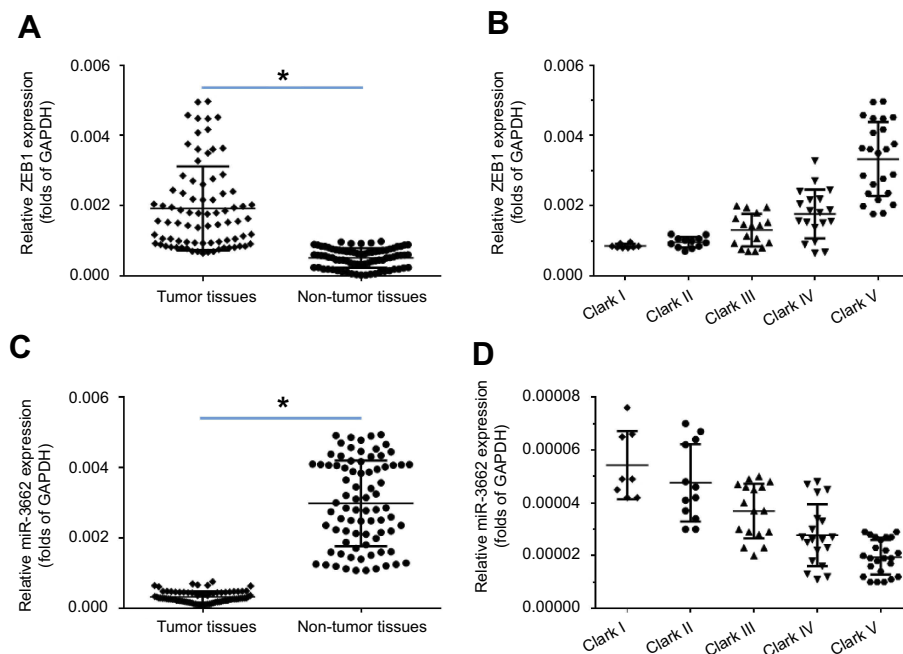


Figure 1 The expression of ZEB1 mRNA in melanoma specimens. A total of 80 paired melanoma and non-tumor clinical specimens were harvested for qPCR experiments. (A) ZEB1 mRNA levels in 80 paired melanoma and non-tumor clinical specimens. (B) ZEB1 mRNA levels in samples at different melanoma stages (Clark I-V). (C) miR-3662 levels in 80 paired melanoma and non-tumor clinical specimens. (D) miR-3662 levels in samples at different melanoma stages (Clark I-V). **P*<0.05.

understand the role of ZEB1 in proliferation or metastasis of melanoma, MTT and transwell experiments were performed to reveal that the knockdown of ZEB1 inhibited the proliferation, in vitro invasion and migration of A375 cells (Figures S2–S4), demonstrating a role for ZEB1 in the function of melanoma cells.

miR-3662 target sequences within the ZEB1 transcript were identified and mutated to create control miRNA, with which to transfect cells (Figures 2 and 3A). The transfection of miR-3662, but not control miRNA, resulted in a significant decrease in the protein levels of ZEB1 in A375 cells (Figure 3B and C). Moreover, transfection of miR-3662's inhibitor or mutated ZEB1 was sufficient to restore the expression of ZEB1 within these cells. The interaction between ZEB1's 3'UTR sequence and miR-3662 was further confirmed by luciferase experiments (Figure S5). Western blot analysis revealed that the decrease in ZEB1 levels resulting from miRNA-3662 expression were able to be restored

with the over-expression of the mutated ZEB1 (Figure S6). Taken together, these data suggest that miR-3662 acts to repress the expression of ZEB1 protein by directly targeting the ZEB1 mRNA's 3' UTR in A375 cells.

miRNA-3662 inhibits the EMT indicators of melanoma cells and expression of invasion related factors

To investigate whether miR-3662 impacts on the invasive growth of A375 cells, the expression of EMT and invasion related factors was examined by qPCR and Western blot assays. Transfection of miR-3662 enhanced the expression of E-cadherin, an epithelial marker, and decreased the expression of N-cadherin and vimentin, two mesenchymal markers (Figure 4). Conversely, transfection of an inhibitor of miR-3662 or ZEB1^{Mut} decreased the observed inhibitory effect of miR-3662 on the EMT process in A375 cells



Figure 2 miR-3662 binding targets in ZEB1 mRNA's 3' UTR. The three binding sites of miR-3662 ZEB1 mRNA's 3' UTR.

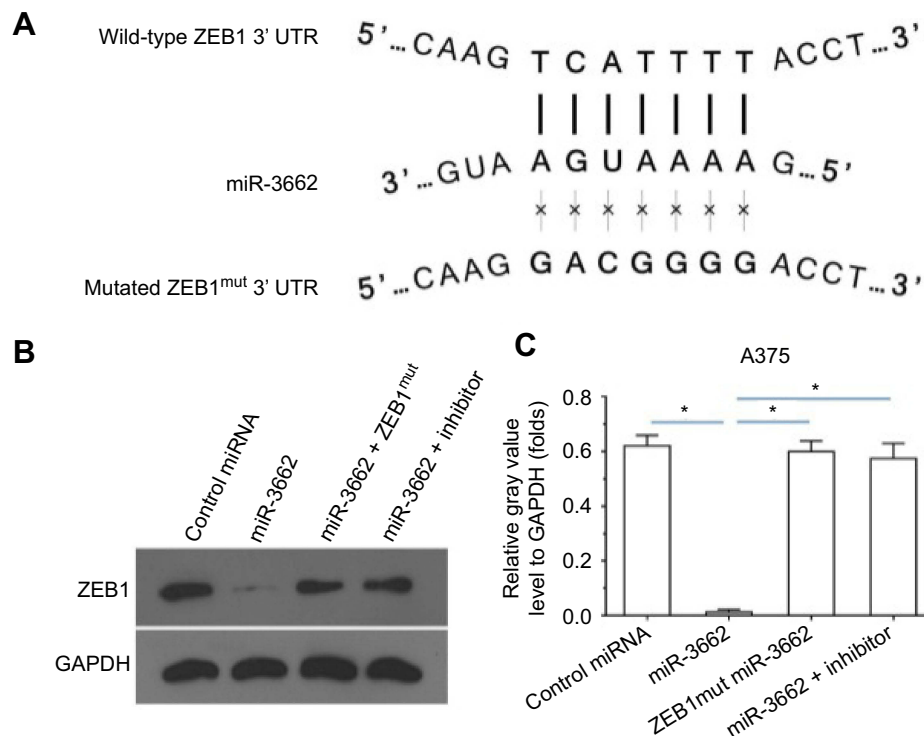


Figure 3 miR-3662 targets the 3' UTR of ZEB1 mRNA. (A) Sequences of potential binding sites of miR-3662 (bold) and the mutated binding site on 3' UTR of ZEB1 mRNA (italicized). (B) Western blot analysis of ZEB1 and GAPDH control levels in A375 cells transfected with control miRNA, miR-3662, miR-3662+ ZEB1^{Mut} or miR-3662+ miR-miR-3662 inhibitor. (C) Quantification of ZEB1 in A375 cells transfected with control miRNA, miR-3662, miR-3662+ ZEB1^{Mut} or miR-3662+ miR-miR-3662 inhibitor. *P<0.05.

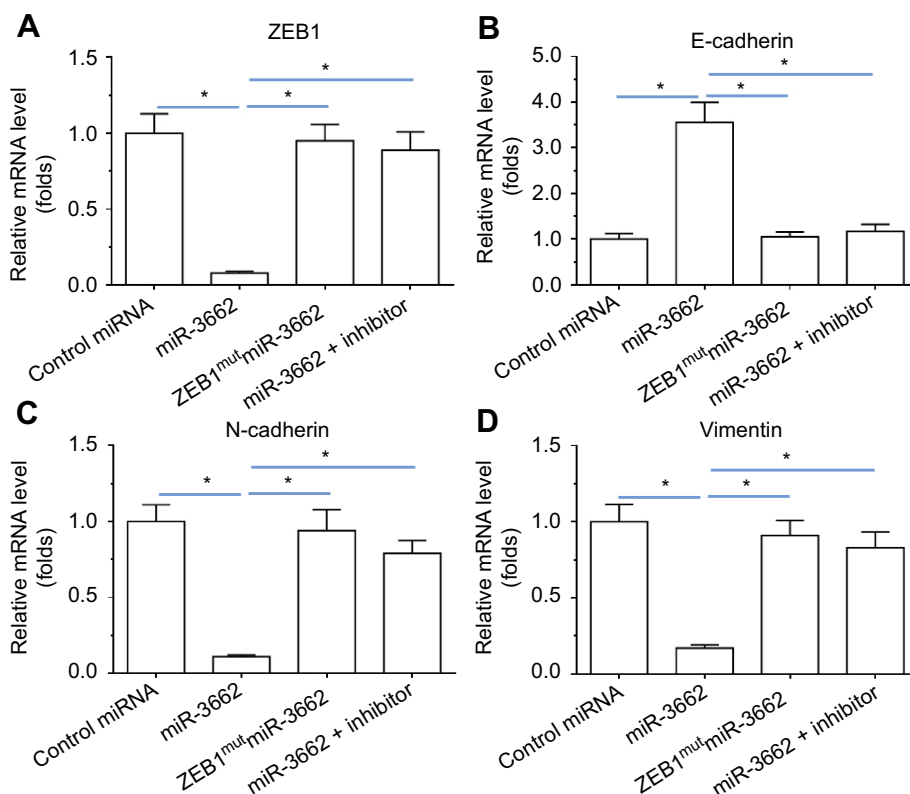


Figure 4 miR-3662 inhibits the EMT process of A375 cells. mRNA levels of (A) ZEB1, (B) E-cadherin, (C) N-cadherin, and (D) vimentin shown as mean \pm SD, in A375 cells transfected with control miRNA, miR-3662, miR-3662+ *zeb1*^{Mut}, or miR-3662+ miR-mi-3662 inhibitor. * $P < 0.05$.

(Figure 4). These results suggest that miR-3662 acts to inhibit the EMT process of A375 cells by targeting ZEB1.

qPCR analysis of the effect of miR-3662 on the expression of invasive growth-related genes in A375 cells, demonstrated that miR-3662 expression resulted in the inhibition of *mmp3* and *mmp9*, two matrix metalloproteinases that mediate the degradation of extracellular matrix (ECM) during the invasive growth of cancer cells (Figure 5). Furthermore, miR-3662 was also observed to increase the expression of *timp1* (tissue inhibitor of metalloproteinase 1), an inhibitor of matrix metalloproteinases in tissues (Figure 5). Transfection of the miR-3662 inhibitor or ZEB1^{Mut} inhibited the effects of miR-3662 on these invasive growth-related genes. Similar results were obtained from Western blot analysis (Figure 6), suggesting that miR-3662 functions to inhibit the expression of invasion related proteins in A375 cells by targeting ZEB1.

To further examine the effects of ZEB1 on melanoma cells, ZEB1 was overexpressed in OCM-1A cells, a lowly aggressive melanoma cell line, and the expression of EMT and metastasis-related factors subsequently examined (Figure S7). Overexpression of ZEB1 in OCM-1A cells enhanced the EMT process in these cells and increased the

expression of metastasis-related factors (Figure S7), confirming the effects of miR-3662 on ZEB1 function in melanoma cells.

miR-3662 inhibits the subcutaneous growth of melanoma cells in nude mice

The in vivo function of miR-3662 was investigated using a nude mouse model injected subcutaneously with A375 cells. In these animals the transfection of miR-3662 gave rise to a decrease in the subcutaneous growth of A375 cells, supporting the role of miR-3662 as an inhibitor of tumor progression. Indeed, transfection of ZEB1^{Mut} which is unresponsive to the miRNA, almost blocked the effects of miR-3662 on A375 subcutaneous cell growth (Figure 7). Analysis of subcutaneous tumors from the murine models demonstrated reductions in tumor volumes (Figure 7B) and tumor weights (Figure 7C) with the expression of miR-3662, supporting its role in decreasing the tumorigenic potential of ZEB1. Additionally, the growth inhibition rates calculated of tumor volume (Figure 7D) or tumor weight (Figure 7E), show a reduction in growth with ZEB1 inhibition by miR-3662.

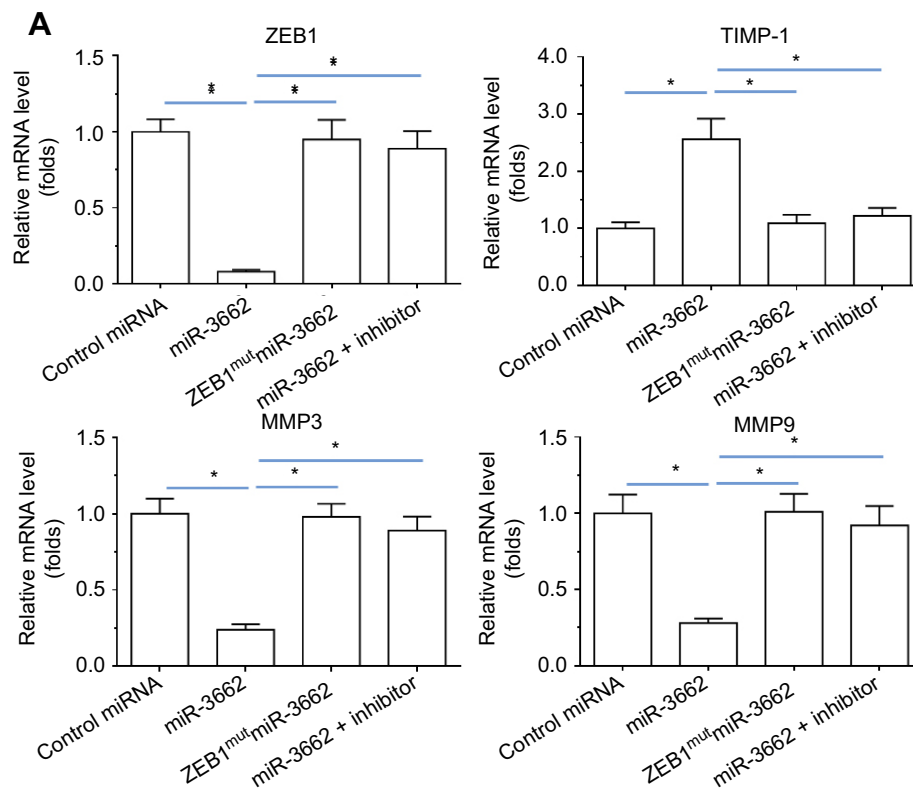


Figure 5 miR-3662 inhibits invasive growth-related genes' mRNA expression in A375 cells. mRNA levels of (A) ZEB1, (B) TIMP-1, (C) MMP3, or (D) MMP9 shown as mean \pm SD, in A375 cells infected with control miRNA, miR-3662, miR-3662+ ZEB1^{Mut}, or miR-3662+ miR-miR-3662 inhibitor. * P <0.05.

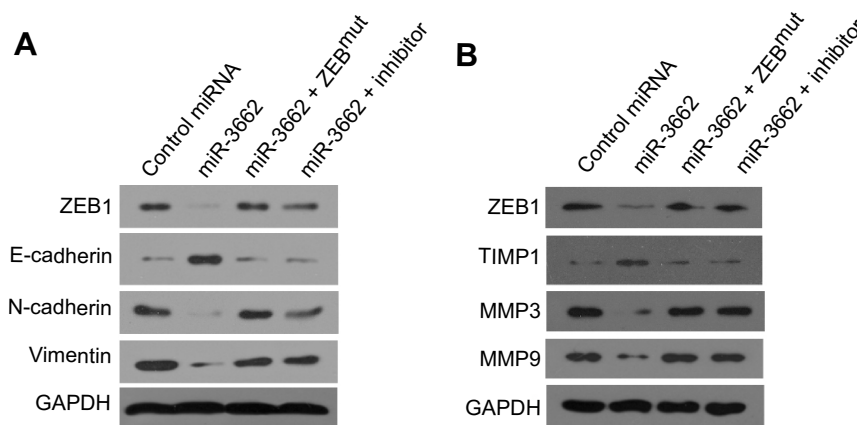


Figure 6 miR-3662 inhibits EMT-related or invasive growth-related gene protein expression in A375 cells. (A) ZEB1, E-cadherin, N-Cadherin and Vimentin protein levels expressed in A375 cells transfected with control miRNA, miR-3662, miR-3662+ ZEB1^{Mut}, or miR-3662+ miR-miR-3662 inhibitor determined by Western blot analysis. (B) ZEB1, TIMP-1, MMP3 and MMP9 protein levels expressed in A375 cells transfected with control miRNA, miR-3662, miR-3662+ ZEB1^{Mut}, or miR-3662+ miR-miR-3662 inhibitor determined by Western blot analysis. * P <0.05.

Expression of miR-3662 (Figure 7F) and ZEB1 (Figure 7G) in subcutaneous tumor tissues were determined by qPCR. The injection of a lowly aggressive tumor cell line, OCM-1A into the nude mouse model, was assessed to determine if the observed results were cell line dependent. Subcutaneous tumors were observed

in OCM-1A injected mice and these tumors showed increased size and weight in cells over-expressing ZEB1 (Figure S8).

These data demonstrate that miR-3662 acts to inhibit the subcutaneous growth tumor cells in vivo by targeting ZEB1.

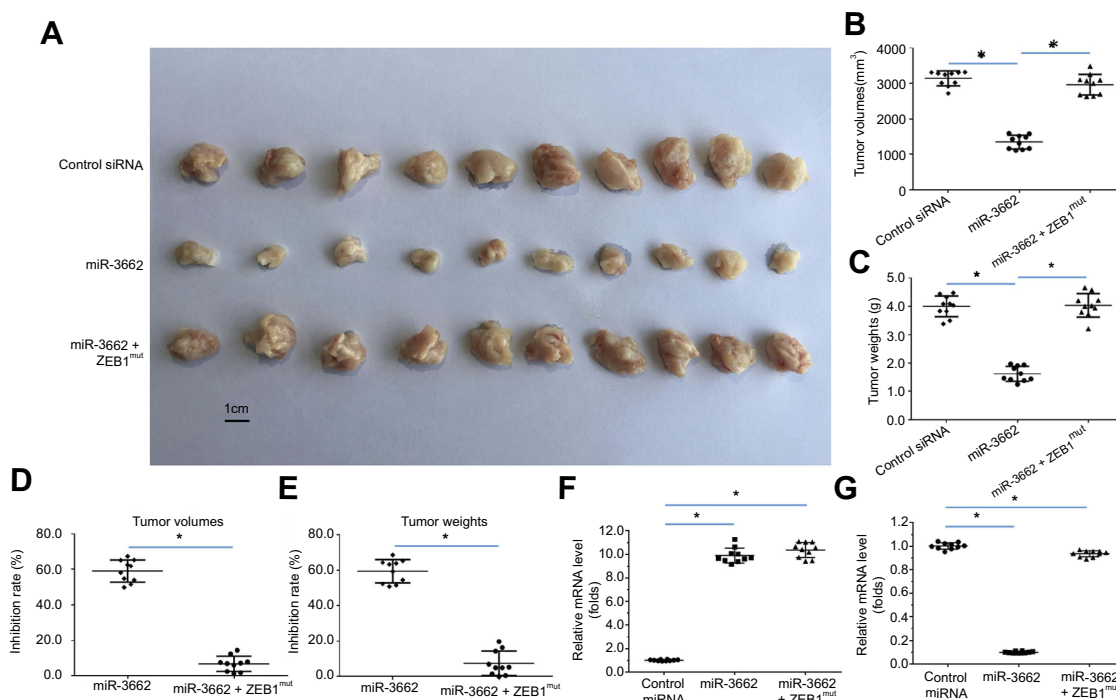


Figure 7 miR-3662 inhibits the subcutaneous growth of A375 cells. A375 cells transfected with control miRNA, miR-3662, or miR-3662 + ZEB1^{Mut} were seeded into nude mice to form subcutaneous tumors. (A) Representative image of subcutaneous tumors, (B) volume of subcutaneous tumors and (C) weight of subcutaneous tumors. (D, E) Inhibition rates were calculated by tumor volume (D) or tumor weight (E) RNA levels of miR-3662 and ZEB1 were shown in Figure 7G. The RNA levels of miR-3662 (F) and ZEB1 (G) were shown as scatter diagrams in Figure 7F and G. **P*<0.05.

miR-3662 inhibits in vivo invasive growth of melanoma cells in the muscle tissue of nude mice

Tumorigenic cells were injected into the muscle tissue of nude mice to assess the effects of miR-3662 on the in vivo invasive growth of these cells. Injection of A375 cells into the muscle tissue resulted in the formation of nodules and muscle degradation (Figure 8). The transfection of miR-3662 in these cells, inhibited their invasive capacity in murine muscle tissue (Figure 8); whereas transfection of ZEB1^{Mut} in these cells almost blocked the effect of miR-3662 on A375 cells' invasive growth (Figure 8). In contrast, the injection of OCM-1A cells resulted in an expected reduction in invasive capacity in the muscle tissue of the animals. However, over-expression of ZEB1 in these cells resulted in a promotion of the growth of invasive tumors (Figure S9), confirming the role of ZEB1 in promoting tumor invasion and the inhibition of this capacity by miR-3662.

Discussion

Melanoma arises through the malignant transformation of melanocytes into metastatic melanoma, a complex process

considered a neural crest neoplasia.³⁰ Although screening for melanoma by total body skin examination could benefit people at risk for melanoma, current regular screening approaches do not effectively diagnose thin melanoma and thus promote the development of invasive melanoma, negatively impacting upon patient survival.³¹ The invasive growth of advanced stage melanoma (Clark III to V) or recurrence of melanoma following surgery is a threat to patients and the subsequent therapeutic options are limited.^{32,33} Three BRAF-MEK inhibitor combinations are presently approved for BRAF^{Fv600}-mutated melanoma treatment: vemurafenib plus cobimetinib, dabrafenib plus trametinib, and encorafenib plus binimetinib.^{34,35} Oral administration of these small-molecule kinase inhibitors are considered first-line therapy for advanced and metastatic melanoma treatment. However, response rates of patients to small-molecule kinase inhibitors were reported to be as low as 16% of patients.^{1,36} Moreover, options for targeted therapies are scarce for patients suffering from BRAF wild-type melanoma.¹ Therefore, novel therapeutic approaches are urgently needed for these patients.

In the present work, the expression of ZEB1, which is confirmed as a key regulator of human cancer cells' EMT process, was found to be much higher in advanced

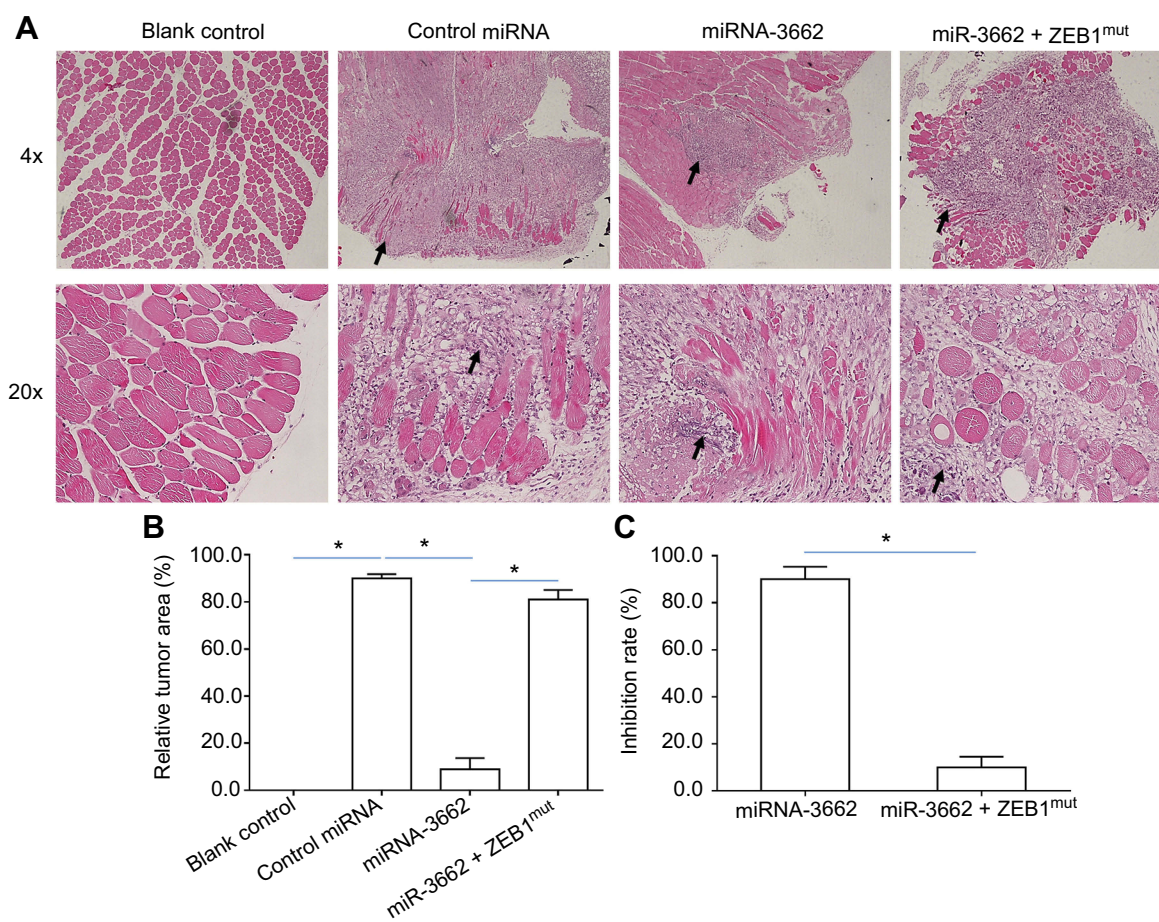


Figure 8 miR-3662 inhibits the invasive growth of A375 in the muscle tissue of nude mice. A375 cells transfected with control miRNA, miR-3662, or miR-3662 + ZEB1^{Mut} were seeded into the muscle tissue of nude mice. (A) Representative image of H&E stained muscle tissue with tumor-tissue formed by A375 cells, (B) relative invasive growth of A375 cells, (C) inhibition rates calculated from the relative invasive growth of A375 cells. The black arrows indicate the tumor lesions in muscle tissues formed by A375 cells. * $P < 0.05$.

melanoma (Clark IV or Clark V) than in the early stages (Clark I or Clark II) of melanoma. The identification of miR-3662 as potentially targeting ZEB1 through the 3'-UTR of this transcript was determined via an online database (miRDB.org). miR-3662 is a novel tumor suppressor with only 5 reports. Among them, Chen et al (2018) suggested that miR-3662 suppresses hepatocellular carcinoma growth through inhibition of the HIF-1 α -mediated Warburg effect.³⁷ Therefore, targeting the ZEB1 gene by miR-3662 is a promising approach to attenuate the invasive growth of melanoma cells. Here we confirmed the expression of miR-3662 in tumorigenic cell lines by qPCR and Western blot assays, indicating a potential role for this miRNA in these cells. Three 7^{mer} targeted sites of miR-3662 were identified in the ZEB1 mRNA 3' UTR (Figure 2). Consequently, transfection of miR-3662 was assumed to be an efficient strategy to decrease ZEB1 expression. Indeed, transfection of miR-3662 inhibited the EMT process of A375 cells,

decreased invasion-related gene expression, and inhibited the invasive growth of A375 cells in nude mice by decreasing the expression of ZEB1.

The BRAF mutation is widely used as an indicator or risk-related factor for melanoma's malignant progress. The results described in this study preliminarily reveal ZEB1 may be associated with the progress or invasion of melanoma and may serve as a potential biomarker, especially for BRAF wild-type melanoma.

Invasion is an important factor that influences the prognosis of patients with malignant tumors, especially in the metastatic or invasive stage of cutaneous melanoma.^{38,39} The EMT process is a physical process that mediates the loss of epithelial characteristics from cancer cells, such as polarity, and the development of mesenchymal characteristics.^{16,40} During the EMT process, cancer cells gain migratory properties. The ECM can function as an important barrier to confine cancer cells within their primary origin site.^{41,42} However, during the

EMT process ECM degradation is mediated by matrix metalloproteinases (MMPs), a family of zinc-dependent enzymes that proteolytically degrade various ECM components.^{43,44} In the present work, transfection of miR-3662 inhibited the EMT process and the expression of matrix metalloproteinases, MMP3 or MMP9. These results reveal a potential mechanism of action for miR-3662 in inhibiting A375 cell invasion.

A375 cells are a highly aggressive melanoma cell line.⁴⁵ Richard et al (2013), Pal et al (2016) and Caramel et al (2013) demonstrated that a high level of ZEB1 expression in epithelial cells is related to BRAF mutation in melanoma.^{46–48} Our results support this theory, indicating the invasive growth capacity of ZEB1-expressing cells in muscle tissues. The transwell invasion/migration assay is widely used to demonstrate the invasion of cancer cells by mimicking the degradation of ECM, however, it can only offer information on in vitro performance. This study, revealed both the in vitro and in vivo invasion of A375 cells by injecting cells into the muscle tissue of nude mice. Following the initial location of the A375 cells within the muscle tissue in these animals, tumor nodules were observed to form through degradation of the normal muscle tissue. The inhibitory activation of miR-3662 has been observed on A375 cells in muscle, where it is related to the decreased EMT process and reduced MMP activity. The tumor model utilized in this study mimics the invasion of melanoma in skin/muscle and highlights the potential application of miR-3662 in attenuating melanoma cells invasive growth.

In mammalian cells, miRNA is an important non-coding RNA serving as an epigenetic mechanism of gene expression silencing by post-transcriptional regulation.^{49–52} In this work, our results demonstrated that miR-3662 inhibited the invasion of A375 cells by targeting ZEB1, and offers a potential therapeutic target and treatment via lentiviral delivery. The expression of miRNAs in human cancer cells via the preparation of lentivirus particles is a promising new approach that may achieve significant patient benefits through antitumor activation.

Conclusion

The present work demonstrated that ZEB1 is targeted by miR-3662. The stable overexpression of miR-3662 in A375, a highly aggressive melanoma cell line, through lentiviral transfection, decreased ZEB1's expression and inhibited the invasion of A375 cells in the muscle tissue of nude mice.

Author contributions

All authors made substantial contributions to the design and conception; acquisition, analysis or interpretation of data. Authors took part in either drafting or revising the manuscript. At the same time, authors gave final approval of the version to be published; and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Disclosure

The authors report no conflicts of interest in this work.

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