



MicroRNA-153-3p sensitizes melanoma cells to dacarbazine by suppressing ATG5-mediated autophagy and apoptosis

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Background: Dacarbazine is one of the most commonly used chemotherapeutic agents for the treatment of melanoma; however, only 5–10% of patients benefit from this treatment. MicroRNA-153-3p (miR-153-3p) has a tumor-suppressive effect in melanoma. In the present study, we found that miR-153-3p was downregulated in melanoma cell lines (A357 and M14).

Methods: The target relationship between miR-153-3p and Autophagy-related gene 5 (ATG5) was confirmed by Dual-Luciferase Reporter Assay. Cell Counting Kit-8, flow cytometry, immunofluorescence, and Western blot were used to examine cell viability, apoptosis, and autophagy, respectively.

Results: miR-153-3p overexpression decreased the half-maximal inhibitory concentration value of dacarbazine, while increasing the apoptotic rate in both A357 and M14 cells. Moreover, miR-153-3p enhanced dacarbazine-induced autophagy in melanoma cells. Our bioinformatics study revealed that ATG5 is one of the potential targets of miR-153-3p. The overexpression of ATG5 decreased dacarbazine sensitivity and promoted proliferation, as well as inhibited apoptosis and autophagy in melanoma cells. miR-153-3p exhibited suppressive effects via directly binding and downregulating ATG5 expression, which subsequently increased sensitivity to dacarbazine and inhibited proliferation, and enhanced apoptosis and autophagy in melanoma cells.

Conclusions: The results of the present study showed that miR-153-3p sensitizes melanoma cells to dacarbazine by suppressing ATG5-mediated autophagy and apoptosis, and provided a basis to explore the functions of miRNAs on drug resistance in the treatment of melanoma.

Keywords: MicroRNA-153-3p (miR-153-3p); melanoma; dacarbazine; autophagy related gene 5 (ATG5); autophagy

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Introduction

Melanoma is a malignant tumor deriving from melanocytes, and has a high mortality rate (1). In the USA, melanoma was considered to account for 5% of all cancers in 2013, which indicates its high prevalence (2,3). Although in China the incidence of melanoma is not such high as that

of the USA, the increasing morbidity and mortality rates over past years have attracted widespread concern (4). In the early stage, melanoma can be surgically removed, and the 5-year survival rate of melanoma patients is as high as 98% (5). Nevertheless, melanoma can easily metastasize to regional lymph nodes and result in poor prognosis, with a 5-year survival rate of only 16% (6,7). Various methods

are used for the treatment of melanoma, including surgery, chemotherapy, radiation therapy, and immunotherapy (8,9). Dacarbazine is one of the most commonly used chemotherapeutic agents for the treatment of melanoma (10); however, only 5–10% of patients benefit from this treatment (11). The main reason is the drug resistance of dacarbazine due to various reasons including hypoxia exposure (12), BRAFV600E targeting (13), advanced melanoma (14). Melanoma is still a malignant tumor that is insensitive to traditional treatment methods such as chemotherapy drugs, so it is urgent to explore new treatments (15).

Autophagy is a self-cannibalization process that occurs in both normal and cancer cells. The cells reuse damaged proteins and organelles by autophagosomes to maintain cell homeostasis (16). Autophagy plays a dual role in cancer development and treatment. Previous studies have reported that autophagy is linked to chemotherapy resistance in cancer cells (17,18). *In vivo* experiments identify autophagy ATG7 to be essential for autophagy activation. Mechanistically, inhibition of the autophagic flux in dormant breast cancer cells leads to the accumulation of damaged mitochondria and reactive oxygen species (ROS), resulting in cell apoptosis (19). The inhibition of apoptosis enables the survival and proliferation of tumors and contributes to resistance to conventional chemotherapy agents (20). However, the mechanism underlying the relationship between autophagy and dacarbazine resistance in melanoma cells remains unclear and requires further elucidation.

A group of small non-coding RNAs with 20–22 of nucleotides are referred to as microRNAs (miRNAs). miRNAs can post-transcriptionally adjust various target genes by targeting their 3'-untranslated region (3'-UTR) (21). Previous research has shown that miR-153-3p suppresses cell proliferation and invasion by downregulating Snail family transcriptional repressor-1 (SNAI1) in melanoma (22). miR-153-3p restrains autophagy and intervertebral disc degeneration by downregulating the expression of autophagy related gene 5 (ATG5) (23). More importantly, long intergenic noncoding RNA 00641 (LINC00641) induces autophagic cell death by regulating miR-153-3p. Previous studies have demonstrated that some miRNAs are vital mediators of chemoresistance in cancer cells (24). Sun *et al.* reported that the downregulation of miR-374b-5p promotes chemotherapeutic resistance by upregulating multiple anti-apoptotic proteins in pancreatic cancer (25). miR-34a was reported to decrease

chemoresistance in ovarian cancer cells by targeting histone deacetylase-1 (HDAC1) (26). Importantly, the miR-31-SOX10 axis regulates tumor growth and dacarbazine resistance of melanoma via PI3K/AKT pathway (27). These results demonstrate that the dysregulation of miRNAs has an important role in the development of cancer cell chemoresistance.

In the present study, we explored the mechanism of miR-153-3p in the regulation of dacarbazine resistance of melanoma cells. The result revealed that miR-153-3p overexpression sensitizes melanoma cells to dacarbazine by suppressing ATG5-mediated autophagy and apoptosis, suggesting that targeting miR-153-3p may be a novel therapeutic strategy to treat melanoma patients.

We present the following article in accordance with the MDAR reporting checklist (available at <http://dx.doi.org/10.21037/tcr-20-2660>).

Methods

Cell culture and reagents

Normal human melanocytes (HEMn-LP) and melanoma cell lines (A357 and M14) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured with RPMI 1640 media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum at 37 °C in 5% CO₂. Dacarbazine was obtained from Sigma and dissolved in dimethyl sulfoxide. The dose of dacarbazine were selected according to this reference (28). Antibodies used in the present study were: anti-Beclin1 (#3738, 1:1,000), anti-LC3 I/II (#12741, 1:1,000), anti-ATG5 (#12994, 1:1,000), and anti-GAPDH (#5174, 1:1,000). All antibodies were obtained from Cell Signaling Technology.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. A RT kit (RR036A; Takara Biotechnology, Dalian, China) was used for cDNA production, cDNA amplification was detected using the SYBR Premix Ex Taq GC kit (Takara Biotechnology, Dalian, China), and GAPDH was used for normalizing. The relative level was calculated by relative quantification method.

Cell transfection

miR-153-3p mimic, miR-NC, and pcDNA3.1-ATG5 were designed and synthesized by GenePharma. Transfections were performed using the Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions.

Cell viability assay

Cell viability was detected by Cell Counting Kit-8 (CCK-8; Beyotime Institute of Biotechnology, Shanghai, China). Cells were added into 96-well plates and treated with different concentrations of dacarbazine for 24 hours. The culture medium was then replaced in each well with 100 μ L fresh medium containing 10% CCK-8 reaction solution and incubated for 2 hours. Absorbance were measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Carlsbad, CA, USA).

Flow cytometric analysis of apoptosis

The cell apoptotic rate was determined using the annexin V-fluorescein isothiocyanate/propidium iodide (PI) apoptosis detection kit (Multisciences). After washing with ice-cold phosphate-buffered saline three times, the cells were resuspended and incubated with 5 μ L annexin V-FITC and 10 μ L PI. A flow cytometer (BD Biosciences, US) was used to analyze the cell apoptotic rate. The flow cytometry excitation wavelength was 488 nm; a bandpass filter with a wavelength of 515 nm was used to detect FITC fluorescence, and another filter with a wavelength >560 nm was used to detect PI.

Immunofluorescence staining

The expressive levels of microtubule-associated protein 1 light chain 3 (LC3) was measured by immunofluorescence staining (29). Fluorescence was measured under confocal microscopy (C1SiR; Nikon, Japan).

Western blot assay

Proteins were extracted from cells using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). The protein concentration was tested by bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China),

following the manufacturer's instructions. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and then transferred to a polyvinylidene fluoride membrane (Bio-Rad). Membranes were blocked in 5% skim milk for 1 hour at room temperature, and then incubated with primary antibodies at 4 $^{\circ}$ C overnight. After incubating with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody for 1 hour at room temperature. The proteins were measured with an Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, UK).

Luciferase reporter assay

The 3'-UTR fragment of ATG5 containing the miR-153-3p binding sequence was cloned in a pMIR reporter vector, and a mutated plasmid was used as a control. The cells were then co-transfected with ATG5-WT or ATG5-MUT and miR-153-3p mimics using Lipofectamine 2000, according to the manufacturer's recommendation. Luciferase activity was tested using the Dual-Luciferase Reporter Assay System (Promega, Wisconsin, USA).

Statistical analysis

Data were presented as mean \pm standard deviation, and analyzed using analysis of variance test and Student's *t*-test in SPSS version 19.0 software. Values at $P < 0.05$ were considered statistically significant.

Results

Upregulation of miR-153-3p sensitizes melanoma cells to dacarbazine

We initially examined the relative expression of miR-153-3p in HEMn-LP and melanoma cell lines (A357 and M14), respectively. The relative expression levels of miR-153-3p were obviously downregulated in A357 and M14 cells compared with HEMn-LP cells ($P < 0.05$) (Figure 1A). The A357 and M14 cells were then transfected with miR-NC or miR-153-3p mimic, respectively, and the transfection efficiency was detected by qRT-PCR ($P < 0.05$) (Figure 1B). The upregulation of miR-153-3p was found to decrease the half-maximal inhibitory concentration (IC_{50}) values of dacarbazine in melanoma cells (Figure 1C,D). Furthermore, overexpressed miR-153-3p also notably increased cell apoptotic rates under dacarbazine treatment

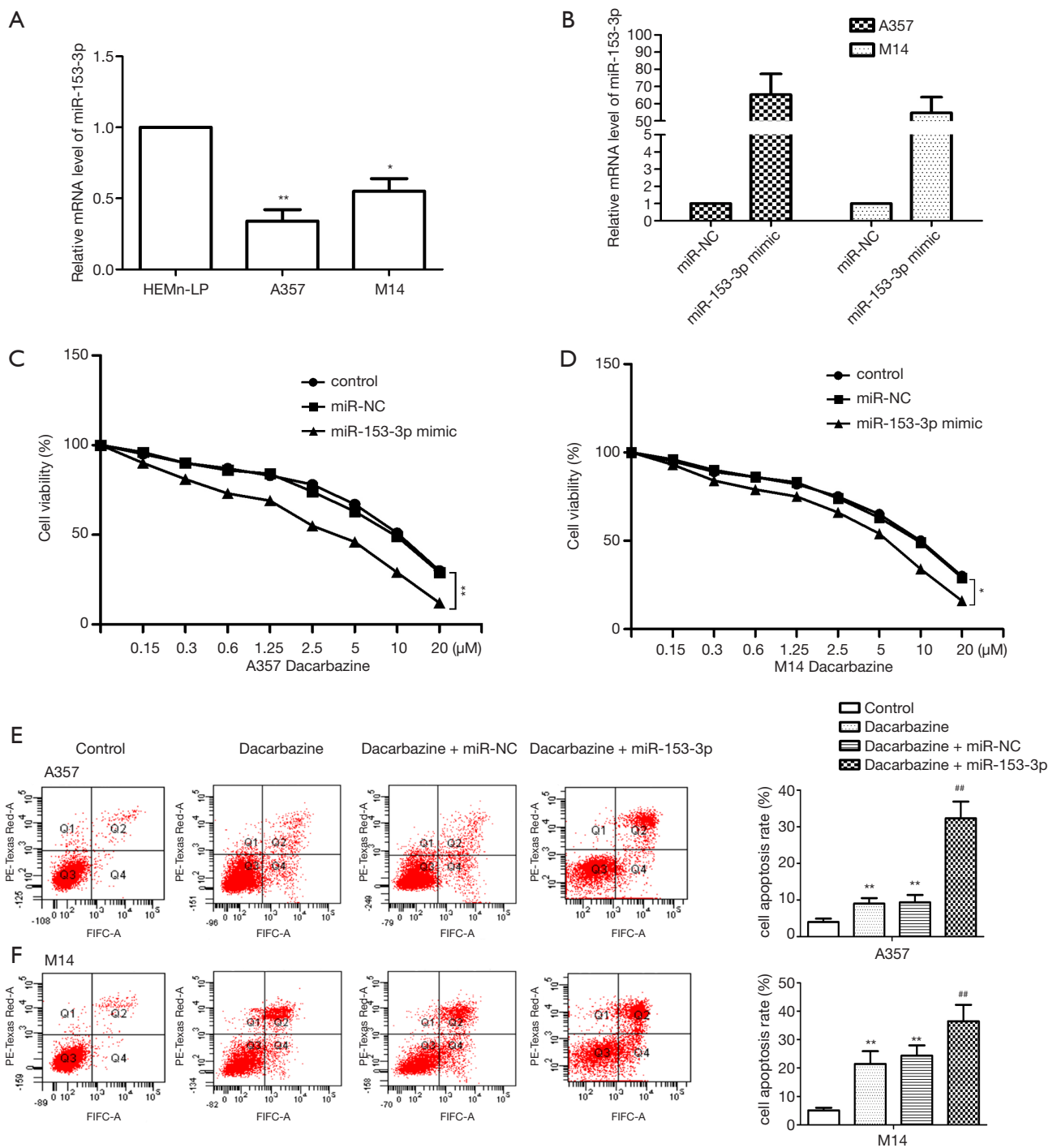


Figure 1 Upregulation of microRNA-153-3p (miR-153-3p) sensitized melanoma cells to dacarbazine. (A) Relative expression levels of miR-153-3p in melanoma cell lines (A357 and M14) and normal human melanocyte (HEMn-LP) were detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR). * $P < 0.05$, ** $P < 0.01$ compared with HEMn-LP cells. (B) After transfection with miR-153-3p mimic or miR-NC, miR-153-3p expression was detected by qRT-PCR. (C,D) Cell viability was measured by Cell Counting Kit-8 assay in both A357 and M14 cells. (E,F) Cell apoptosis was performed by flow cytometric analysis in both A357 and M14 cells. ** $P < 0.01$ compared with dacarbazine group; ## $P < 0.01$ compared with dacarbazine + miR-NC group.

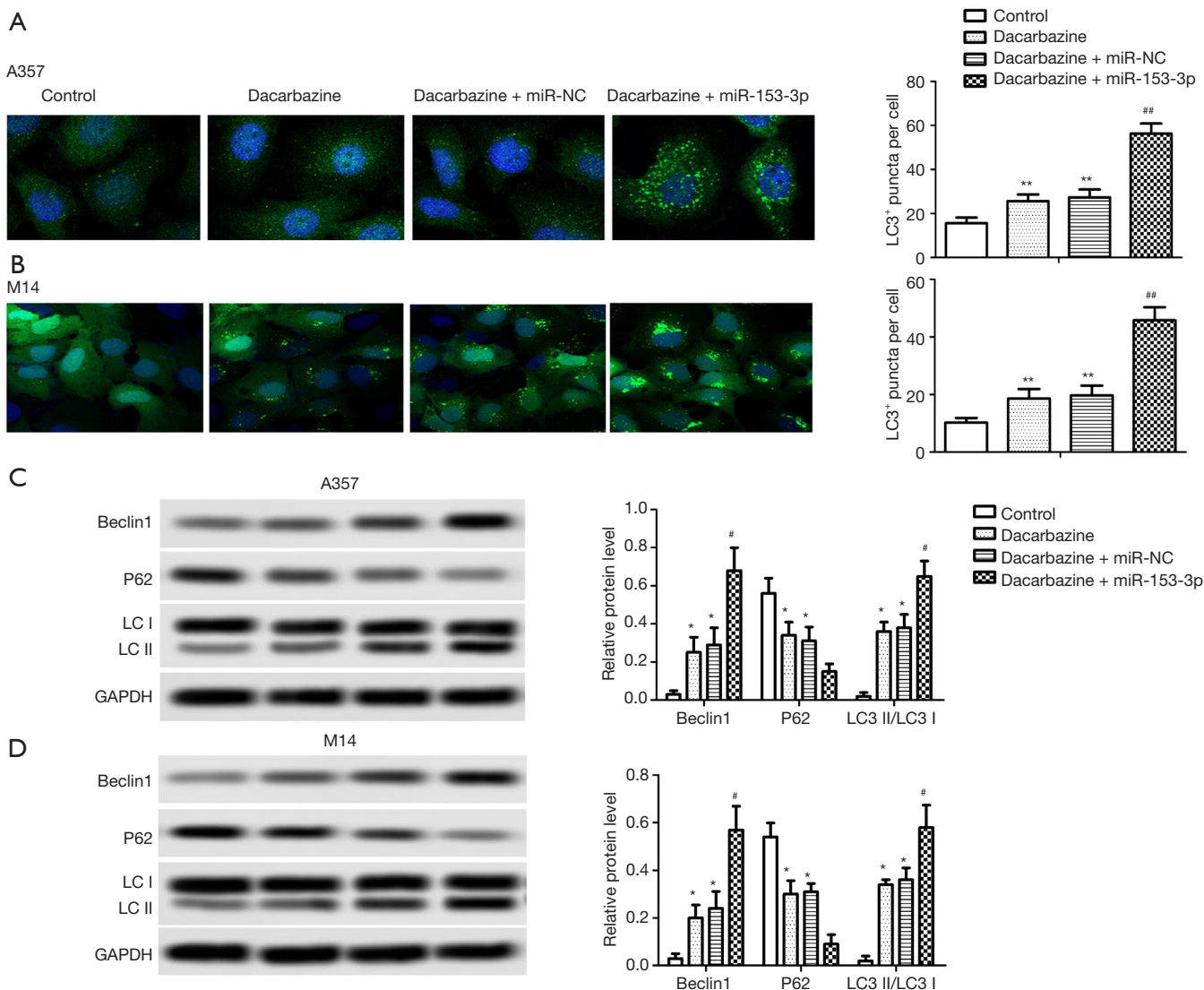


Figure 2 Upregulation of microRNA-153-3p (miR-153-3p) enhanced dacarbazine-induced autophagy in melanoma cells. (A,B) LC3⁺ puncta per cell was detected by immunohistochemistry in both A357 and M14 cells. (C,D) Protein expression levels of Beclin1, P62, and LC3 I/II were detected by Western blot in both A357 and M14 cells. *P<0.05, **P<0.01 compared with control group; #P<0.05, ##P<0.01 compared with dacarbazine + miR-NC group.

compared with the dacarbazine + miR-NC group (P<0.05) (Figure 1E,F). These results indicated that overexpressed miR-153-3p enhanced sensitivity to dacarbazine, inhibited cell proliferation, and increased apoptosis.

Upregulation of miR-153-3p enhanced dacarbazine-induced autophagy in melanoma cells

As shown in Figure 2, dacarbazine increased the number of LC3⁺ puncta per cell, Beclin1 expression levels, and

the LC3 II/I rate, with decreased p62 expressive levels significantly compared with the control group. The upregulation of miR-153-3p was found to increase the number of LC3⁺ puncta per cell, Beclin1 expression levels, and the LC3 II/I rate, and decreased p62 expressive levels compared with the dacarbazine + miR-NC group, indicating that overexpressed miR-153-3p enhanced dacarbazine-induced autophagy in melanoma cells (P<0.05). These findings suggested that overexpressed miR-153-3p enhanced cell autophagy.

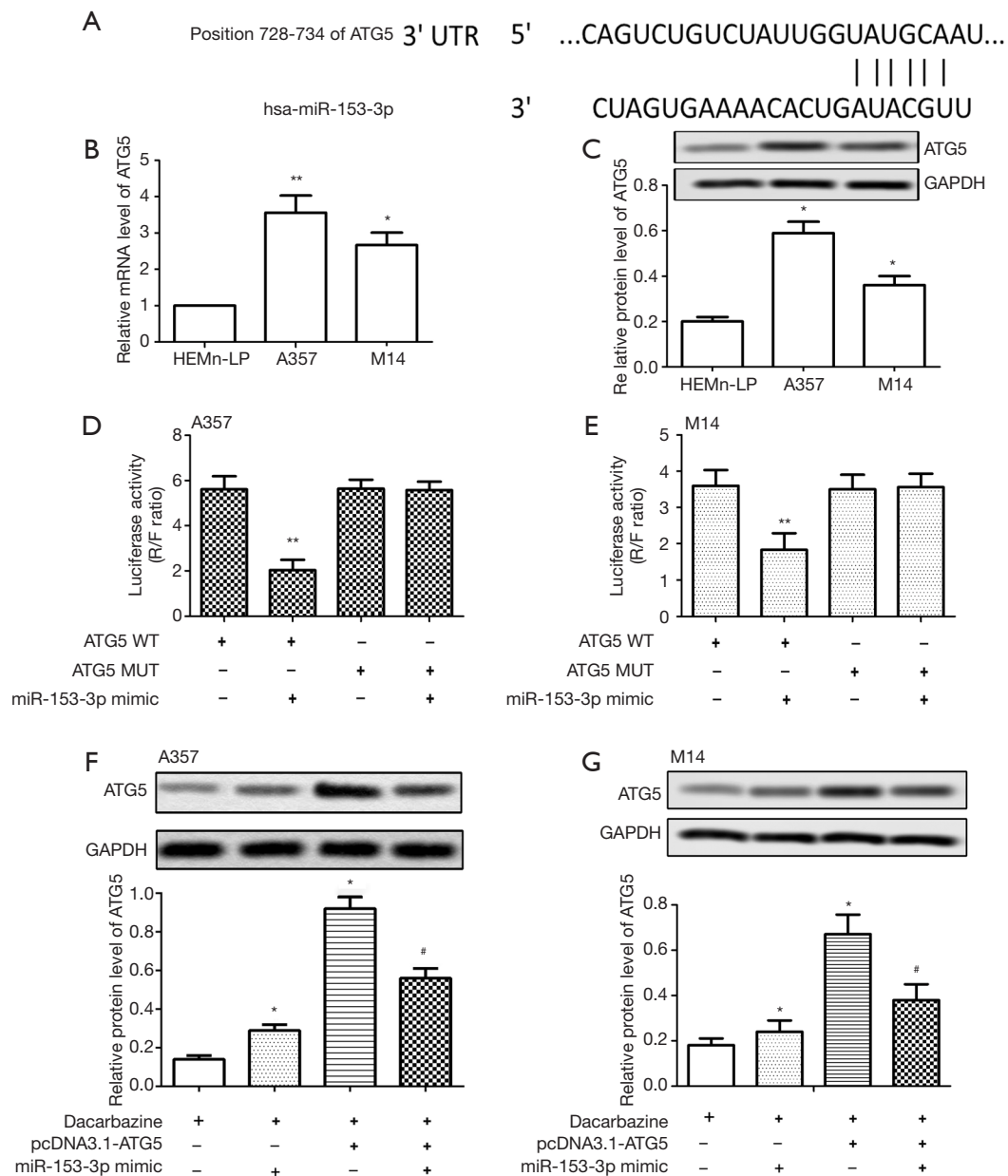


Figure 3 Autophagy related 5 (ATG5) was a target of microRNA-153-3p (miR-153-3p) in melanoma cells. (A) Results of the bioinformatics analysis. (B,C) Relative mRNA and protein expression levels of ATG5 were measured by quantitative reverse transcription polymerase chain reaction and Western blot in both A357 and M14 cells, respectively. (D,E) Results from the Dual-Luciferase Reporter Assay in both A357 and M14 cells. (F,G) Relative expression protein of ATG5 was detected by Western blot in both A357 and M14 cells in dacarbazine group, dacarbazine + miR-153-3p group, dacarbazine + ATG5 group, dacarbazine + ATG5 + mimic group. * $P < 0.05$, ** $P < 0.01$ compared with dacarbazine group; # $P < 0.05$ compared with dacarbazine + ATG5 group.

ATG5 is a target of miR-153-3p in melanoma cells

To detect the molecular mechanism underlying the miR-153-3p-regulated dacarbazine resistance in melanoma

cells, putative miR-153-3p targets were predicted through bioinformatics analysis. ATG5 was found to be one of the potential targets of miR-153-3p (Figure 3A). Relative expression mRNA and the protein of ATG5 were

significantly upregulated in A357 and M14 cells compared with HEMn-LP cells ($P < 0.05$) (Figure 3B,C). Moreover, the Dual-Luciferase Reporter Assay showed that co-transfection with miR-153-3p mimic and ATG5-WT caused a significant downregulation in luciferase activity, whereas co-transfection with miR-153-3p mimic and ATG5-MUT did not influence luciferase activity significantly, confirming the targeting relationship between miR-153-3p and ATG5 in both A357 and M14 cells ($P < 0.01$) (Figure 3D,E). When A357 and M14 cells were transfected with pcDNA3.1-ATG5, the expression of ATG5 increased with treatment with dacarbazine. When A357 and M14 cells were co-transfected with pcDNA3.1-ATG5 and miR-153-3p mimic, the expression of ATG5 decreased in the treatment with dacarbazine ($P < 0.05$) (Figure 3E,G). These results suggested that miR-153-3p could negatively regulate the expression of ATG5.

miR-153-3p increases sensitivity to dacarbazine in melanoma cells via the downregulation of ATG5

When A357 and M14 cells were transfected with pcDNA3.1-ATG5, cell viability increased in the treatment with dacarbazine compared with the control group. When A357 and M14 cells were co-transfected with pcDNA3.1-ATG5 and miR-153-3p mimic, cell viability decreased in the treatment with dacarbazine compared with the dacarbazine+ATG5 group ($P < 0.05$) (Figure 4A,B). As shown in Figure 4C,D,E,F, when A357 and M14 cells were transfected with pcDNA3.1-ATG5, cell apoptosis and autophagy were downregulated in the treatment with dacarbazine compared with the control group. When A357 and M14 cells were co-transfected with pcDNA3.1-ATG5 and miR-153-3p mimic, cell apoptosis and autophagy were upregulated in the treatment with dacarbazine compared with the dacarbazine+ATG5 group ($P < 0.05$) (Figure 4A,B).

Discussion

Melanoma is one of the most aggressive malignant skin tumors worldwide (30). Although surgical resection can increase the 5-year survival rates of patients with early-stage melanoma, there is no gold-standard treatment for advanced melanoma. Dacarbazine is a standard antineoplastic agent commonly used for melanoma treatment. However, the resistance of melanoma cells to dacarbazine inhibits the efficiency of this agent; therefore, it is important to investigate the underlying mechanism of dacarbazine

resistance in melanoma cells. In the present study, we found that miR-153-3p overexpression sensitized melanoma cells to dacarbazine by suppressing ATG5-mediated apoptosis and autophagy, suggesting that targeting miR-153-3p may be a novel therapeutic strategy to treat melanoma patients.

miRNAs are involved in the post-transcriptional regulation of their target genes (31,32). Increasing evidence has demonstrated that various miRNAs are involved in the chemoresistance of many types of cancer cells, including melanoma cells. For example, miR-30a-5p confers cisplatin resistance by targeting insulin-like growth factor 1 receptor expression in melanoma cells (33). miR-579-3p ectopic expression was found to impair the establishment of drug resistance in human melanoma cells (34). Circular RNA 0084043 promotes malignant melanoma progression via the miR-153-3p/Snail axis (35). miR-153-3p was reported to affect tumor suppression, inhibiting cell proliferation and invasion by targeting SNAI1 in melanoma (22). However, whether miR-153-3p regulates dacarbazine resistance in melanoma cells has not been previously reported to the best of our knowledge, and requires further investigation. In the present study, the expression levels of miR-153-3p decreased in melanoma cells (A357 and M14), which was in agreement with Zeng *et al.*'s results that miR-153-3p expression levels decreased in melanoma tissues and melanoma cells (22). The upregulation of miR-153-3p decreased the IC_{50} value of dacarbazine, whereas it increased the cell apoptotic rate in both A357 and M14 cells, suggesting that the upregulation of miR-153-3p sensitized melanoma cells to dacarbazine.

20 It is well known that autophagy plays a regulatory role in the proliferation and death of cancer cells. Autophagy has also been linked to therapy resistance of cancer cells. Previous studies have demonstrated that chemotherapy might induce autophagy, which attenuates apoptosis, leading to drug resistance (36). Pei *et al.* reported that autophagy facilitates metadherin-induced fluorouracil resistance through the Adenosine monophosphate (AMP)-activated protein kinase (AMPK)/ATG5 pathway in gastric cancer (37). Autophagy is triggered during chemotherapy, and the inhibition of autophagy increases the chemosensitivity of castration-resistant prostate cancer cells (38). Besides LC3, Beclin1 is also important for the formation of autophagosomes (39). In the present study, dacarbazine treatment induced autophagy in melanoma cells by increasing autophagosomes, Beclin1 expression, and LC3 I to LC3 II conversion, and decreasing P62 expression. Moreover, miR-153-3p overexpression was observed to enhance dacarbazine-induced autophagy in melanoma cells.

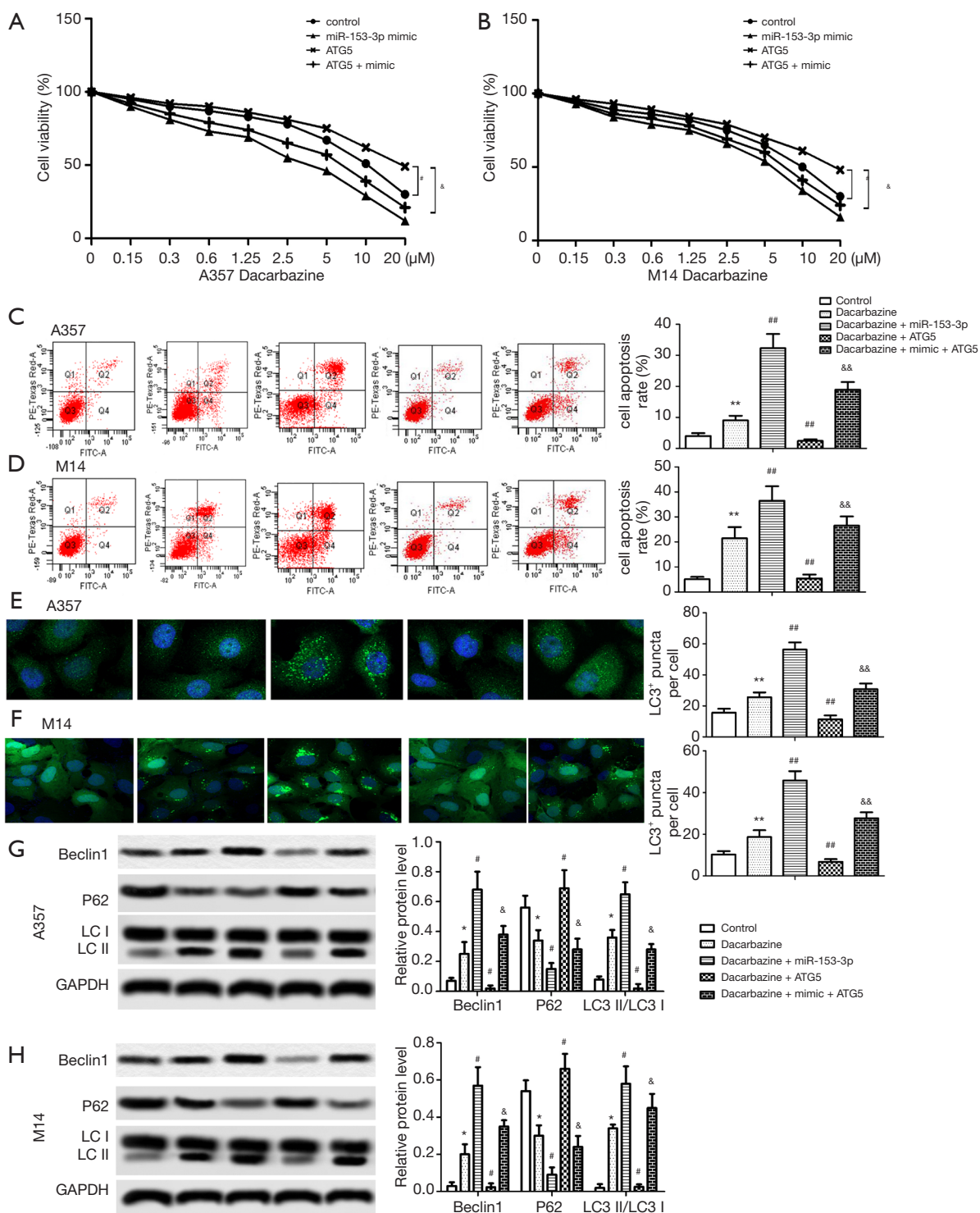


Figure 4 MicroRNA-153-3p (miR-153-3p) increased sensitivity to dacarbazine in melanoma cells via downregulation of autophagy related 5 (ATG5). (A,B) Cell viability was measured by Cell Counting Kit-8 assay in both A357 and M14 cells. (C,D) Cell apoptosis was detected by flow cytometric analysis in both A357 and M14 cells. (E,F) LC3⁺ puncta per cell was detected by immunohistochemistry in both A357 and M14 cells. (C,D) Protein expression levels of Beclin1, P62, and LC3 I/II were detected by Western blot in both A357 and M14 cells. *P<0.05, **P<0.01 compared with control group; #P<0.05, ##P<0.01 compared with dacarbazine group; &P<0.05, &&P<0.01 compared with dacarbazine + ATG5 group.

These results demonstrate that miR-153-3p overexpression enhances the sensitivity of melanoma cells to dacarbazine.

Previous studies have reported that ATGs play vital roles during autophagy. ATG5 is a key player in autophagosome formation (40). One study indicated that ATG5 promotes Wnt signaling and autophagy in melanoma (41). In our bioinformatics study, we revealed that ATG5 is a potential target of miR-153-3p. There is a negative relationship between ATG5 and miR-153-3p expression in melanoma cells. Furthermore, the overexpression of ATG5 decreases dacarbazine sensitivity and promotes proliferation, and inhibits apoptosis and autophagy in melanoma cells. miR-153-3p exhibits suppressive effects via directly binding and downregulating ATG5 expression, which subsequently increases sensitivity to dacarbazine and inhibits proliferation, and enhances apoptosis and autophagy in melanoma cells.

Conclusions

Taken together, the results of the present study show that the miR-153-3p/ATG5 axis plays a vital role in the regulation of dacarbazine resistance in melanoma cells. miR-153-3p was found to exhibit suppressive effects via directly binding and downregulating ATG5 expression, which subsequently increased sensitivity to dacarbazine and inhibited proliferation, as well as enhancing apoptosis and autophagy in melanoma cells, providing a basis to explore the functions of miRNAs on drug resistance in the treatment of melanoma.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <http://dx.doi.org/10.21037/tcr-20-2660>

Data Sharing Statement: Available at <http://dx.doi.org/10.21037/tcr-20-2660>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/tcr-20-2660>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are 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. Ethical approval was obtained from the Ethics Committee of Medical College of Shanxi Datong University (No. 20190123).

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