LncRNA DBH-AS1 facilitates the tumorigenesis of melanoma by targeting miR-233-3p *via* IGF-1R/Akt signaling

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Abstract. – OBJECTIVE: While Long Noncoding RNAs (LncRNAs) are well-known to modulate human cancer progression, the specific function of DBH-AS1 in melanoma remains to be fully established. The study will investigate the role of DBH-AS1 in melanoma cell.

PATIENTS AND METHODS: The expression profiles of DBH-AS1, miR-223-3p, and IGF-1R in melanoma tissues and cell lines were determined by RT-qPCR analysis. CCK-8 assay, colony assays and transwell assay were employed to analyze the effects of DBH-AS1 on the proliferation, migration, and invasion in GC cells. Bioinformatics analysis and Dual-Luciferase reporter assay determined the direct binding relation between DBH-AS1, miR-223-3p and IGF-1R in GC.

RESULTS: Herein, we observed significant reductions in DBH-AS1 expression in melanoma tumor tissues and cell lines. Knockdown DBH-AS1 in melanoma cells impaired their proliferative, migratory, and invasive potential. We determined that DBH-AS1 was able to modulate insulin growth factor receptor (IGF-1R) expression as a competing endogenous RNA for DBH-AS1. In line with this finding, the knockdown DBH-AS1 was associated with decreases in the expression of glucose transporter (GLUT)-1 and a consequent inhibition of glucose uptake, lactate production, and ATP generation by melanoma cells.

CONCLUSIONS: These findings therefore suggest that DBH-AS1 can enhance glycolytic activity in melanoma cells, thereby disrupting melanoma progression via miR-223-3p/EGFR/AKT axis. As such this signaling axis may be a viable therapeutic target for melanoma treatment in human patients.

Key Words:
Melanoma, LncRNA-DBH-AS1, MiR-223-3p, GLUT1, IGF-1R.

Introduction

Melanoma remains the most challenging form of skin cancer to treat, as it is associated with high rates of malignancy and there are few therapeutic options for patients with advanced disease[1]. Melanoma development is associated with both genetic and environmental factors, but the specific molecular basis for this form of cancer remains to be fully elucidated[2,3]. As such, it is vital that novel genetic targets associated with promoting or preventing melanoma progression be identified in an effort to develop novel therapeutic and diagnostic strategies for this disease[4].

Long noncoding RNAs (lncRNAs) are RNAs which are >200 nucleotides long and which generally lack the potential to code for protein[5]. Dysregulated lncRNA expression profiles have been shown to be associated with a range of pathological processes including aberrant proliferation[6], angiogenesis[7], and tumor metastasis[8]. Most lncRNAs are found within cellular nuclei[8], wherein they regulate a number of biological activities at the transcriptional and posttranscriptional levels[9]. Long non-coding RNA DBH-AS1 (DBH-AS1), transcribed from chromosome 9q34, was a newly identified lncRNA. Previously, it was found that the expression levels of DBH-AS1 were distinctly increased in HepG2 cells which were analyzed using microarray analysis[10]. Then, the overexpression of DBH-AS1 was also reported in colorectal cancer[11]. On the other hand, the oncogenic roles of DBH-AS1 were confirmed in hepatocellular carcinoma using gain-function and lost-function assays[12,13]. Up to date, to our best knowledge, the expression profiles of DBH-AS1 in melanoma remain largely unclear, and the potential effects of DBH-AS1 in

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this tumor have not been investigated. In our study it was identified that the expression of the DBH-AS1 was markedly increased in melanoma compared with the corresponding adjacent non-tumour samples; thus, the focus of the study centred on the roles and corresponding mechanisms of DBH-AS1 in the progression of melanoma.

MicroRNAs (miRNAs) are small RNA molecules that do not encode protein[14,15], yet are able to regulate diverse processes including proliferation, survival, and differentiation in cells[15,16]. Cancers and many other diseases are associated with profound miRNA dysregulation[17-20]. Indeed, several different miRNAs have been found to be dysregulated in melanoma and to thereby alter tumor development and progression[21,22]. Previous studies[23-27] have highlighted miR-223-3p as a miRNA that is downregulated in several cancer types due to its ability to regulate malignant progression. Tumor cells are often dependent upon aerobic glycolysis for energy production, relying on such glycolytic activity even when oxygen is available at levels sufficient to permit normal mitochondrial respiration to occur[28,29]. As a result, the overexpression of glucose transporters (GLUTS) and glycolytic enzymes is commonly observed in tumor cells, with such overexpression arising through a variety of mechanisms. So, enhanced insulin growth factor receptor (IGF-1R) signaling can lead to increased downstream Akt phosphorylation and consequent GLUT upregulation[30-32]. Any lncRNAs or miRNAs that target this IGF-1R/Akt/GLUT1 axis therefore have the potential to serve as therapeutically viable compounds due to their ability to disrupt glycolytic signaling in tumor cells.

In the present study, we determined that DBH-AS1 is increased in melanoma tissues and cells, wherein it targets this pro-glycolytic pathway. When DBH-AS1 was knocked down, we found that this resulted in a significant decreased in IGF-1R expression and consequent reductions in glycolysis and melanoma cell proliferation.

Patients and Methods

Patients and Sample Collection

We analyzed 62 melanoma tissues and their corresponding healthy tissue (≥5 cm surgical margins) in samples collected from Zhejiang University School of Medicine (between 2013 and 2017). None of the patients received anti-cancer therapy before the surgical resection. Histological diagnosis of melanoma was evaluated according to criteria established by the World Health Organization (WHO)[33]. Part of the tissue samples was preserved in liquid nitrogen and part of them were fixed with 10% formalin. After routine dehydration, the tissues were preserved by paraffin embedding. The ethics committee of Zhejiang University School of Medicine approved the study protocols and all participants provided informed consent. Patient information that could lead to patient identification remained confidential throughout the study.

qRT-PCR Analysis

TRIzol was added to cell/tissues for RNA extraction which was quantified on a nanodrop. cDNA synthesis was then performed and gene expression was quantified through qRT-PCR analysis[34]. Briefly, cDNA was generated using a miScript II RT kit (Qiagen, Shanghai, China) from 1 μg of input RNA or miRNA. RT-PCR parameters were as follows: 37°C for 60 min, 95°C for 5 min. The miScript SYBR Green PCR kit was used for qRT-PCR. The reactions were performed as follows: 95°C for 15 min; 40 cycles of 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s on a 7500 Fast Real-Time PCR platform. Values were normalized to GAPDH and U6 and quantified. All reactions were performed in triplicate. The primers were shown in Table I.

Cell Based Assays

Human melanoma cell line A375 and keratinocyte cell line HaCaT were cultured in Dulbecco's Modified Eagle's Medium (DMEM), and the melanoma cell line A875 was cultured in Roswell Park Memorial Institute-1640 medium. Media were supplemented with 10% fetal calf serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA). All cells were cultured at 37°C in an incubator with a humified atmosphere of 5% CO_2. HaCaT cell lines were obtained from Kunming Cell Bank (Kunming, China), and A375 and A875 cell lines were obtained from the Shanghai Institute of Biological Sciences (Shanghai, China). siRNAs targeting DBH-AS1, agomiR-223-3p were synthesized by Gene Pharma Co. (Gene Pharma Co., Shanghai, Shanghai, China).

Cell Proliferation

Cell viability was assessed using Cell Counting Kit-8 (Thermo Fisher Scientific, Waltham, MA, USA) as per the manufacturer's protocols. Briefly, 5000 cells were seeded in 96 well plates, transfected for 48 h, and CCK-8 was added for 1 h. Absorbance's was read at 450 nm.

Table I. Sequence of primers for qRT-PCR.

Primer	Sequence (5' to 3')
IGF-1R forward primer	GAGGTGGCTCGGGAGAAGAT
IGF-1R reverse primer	TTCACCACACCCTTGGCAAC
MiR-223-3p forward primer	CGCUAUCUUUCUAUUAACUGACCAUAA
MiR-223-3p reverse primer	CGCUAUCUUUCUAUUAUGACUCC AUAA
U6 forward primer	CTCGCTTCGGCAGCACA
U6 reverse primer	AACGCTTCACGAATTTGCGT
Actin forward primer	CTCCATCCTGGCCTCGCTGT
Actin reverse primer	GCTGTCACCTTCACCGTTCC

Colony Formation Assay

Each group of treated cells (1×10^3 per well) was seeded into 10 cm culture dish, and cultured for 2 weeks. Finally, the colonies were stained using 1% crystal violet and the number of cell colonies was counted.

Glucose Uptake Assay

The Glucose Uptake Colorimetric Assay kit (BioVision, Waltham, MA, USA) was used to assess glucose uptake based on provided instructions, as in previous reports[28].

Invasiveness and Migratory Ability

The detection of the invasive capacity of indicated treated cells was done using transwell chambers coated with Matrigel (Corning, Corning, NY, USA). The chamber on top was filled with indicated treated cells (2×10^4 cells) in 200 µL volume and culture medium (600 µL) and to the chamber beneath; FBS (20%) was added. After 18 hours, staining (crystal violet) of indicated treated cells that were invaded was done and enumeration using an inverted microscope from CarlZeiss (Germany). Data shown are the averages of three independent experiments.

Western Blotting

RIPA buffer (Thermo Fisher Scientific, Waltham, MA, USA) was used to lyse cells, after which a bicinchoninic acid assay (BCA) kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify protein levels and 20 µg of each sample was separated through 10% SDS-PAGE prior to transfer to a polyvinylidene difluoride (PVDF) membrane. These blots were then blocked with 5% non-fat milk for 2 h, after which they were probed overnight with antibodies specific for ACTIN (Rabbit, 1:5000t, Abcam, Cambridge, MA, USA), (GLUT)-1 (Rabbit, 1: 2000t, Abcam, Cambridge, MA, USA), AKT (Rabbit, 1: 2000t, Abcam, Cambridge, MA, USA), and p-AKT (Rabbit, 1: 2000t, Abcam, Cambridge, MA, USA) at 4˚C. Next, blots were incubated for 1 h with HRP-conjugated goat anti-rabbit (1:5,000t, Abcam, Cambridge, MA, USA) secondary antibodies, and were then washed prior to development *via* enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA) together with a ChemiDoc Imaging Platform (Bio-Rad, Hercules, CA, USA).

Bioinformatics Analysis

StarBase 3.0 (http://starbase.sysu.edu.cn/) was used to predict potential binding targets for lncDBH-AS1

RNA Immunoprecipitation (RIP)

A Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was used based on provided directions in order to assess interactions between miR-223-3p and lncDBH-AS1. Briefly, cellular lysates were mixed at 4°C overnight with magnetic beads coated with a human AGO2 antibody or control IgG (Millipore, Billerica, MA, USA). These beads were then collected, and qRT-PCR was used in order to analyze RNA enrichment in the precipitated fraction.

Luciferase Reporter Assay

We obtained lncDBH-AS1 constructs containing wither wild-type (WT) or mutated (MUT) versions of the putative miR-223-3p binding site from GenePharma (Gene Pharma Co, Shanghai, Shanghai, China), and we then inserted these fragments into the pmirGLO Dual-Luciferase Vector (Invitrogen, Carlsbad, CA, USA) in order to produce the WT- and MUT-DBH-AS1 reporter constructs. This same strategy was used for EGFR reporter plasmid preparation. Prior to transfection, cells were added to 24-well plates overnight and were allowed to

grow until reaching 60-70% confluence. Cells were then co-transfected with reporter plasmids along with either agomir-223-3p or agomir-NC. Following additional 48 h incubation, the Dual-Luciferase Reporter Assay System (Invitrogen, Carlsbad, CA, USA) was utilized according to provided instructions, with Renilla Luciferase activity being used as a normalization control.

Statistical Analysis

GraphPad Prism (GraphPad Software, La Jolla, CA, USA) was utilized for all statistical testing Data are means ± SEM. Data were tested using two-tailed Student's *t*-tests, Wilcoxon signed-rank tests, Pearson chi-squared tests, Pearson correlation analyses, Log-rank tests, Fisher's exact tests, nonparametric Mann-Whitney U tests, and Cox proportional hazards regression models as appropriate, with p-value < 0.05 as the significance threshold.

Results

Melanoma is Associated With Increased DBH-AS1 Expression

We first assessed DBH-AS1 expression in melanoma patient tumor samples, revealing a significant increase in the expression of this miRNA in these samples relative to levels in normal control tissues (Figure 1A and B). Consistent with this, we also detected increased DBH-AS1 levels in melanoma cell lines (A375 and A875) relative to control HaCaT skin cells (Figure 1C). This therefore suggested that DBH-AS1 is increased in melanoma, and that it may thus play a role in regulating tumor development or progression.

Knockdown DBH-AS1 Suppresses Melanoma Proliferative, Migratory, and Invasive Activity

To further examine the role played by DBH-AS1 in melanoma, we next transfected sh-DBH-AS1 into A375 and A875 cells and confirmed that DBH-AS1 levels were consequently decreased (Figure 2A). We found that knockdown DBH-AS1 significantly impaired tumor cell growth (Figure 2B and C) and colony formation (Figure 2D) in both tested cell lines. This thus indicated that DBH-AS1 functions in a pro-proliferative capacity in melanoma. We further found that the knockdown DBH-AS1 in melanoma cells resulting in a significant reduction in their migratory and invasive activity in a transwell assay (Figure 2E and F). As such, these results show that DBH-AS1 played an oncogene in melanoma.

MiR-223-3p Was Regulated by DBH-AS1 in Melanoma Cells

In many cases, lncRNAs have been shown to act as competing endogenous RNAs (ceRNAs)

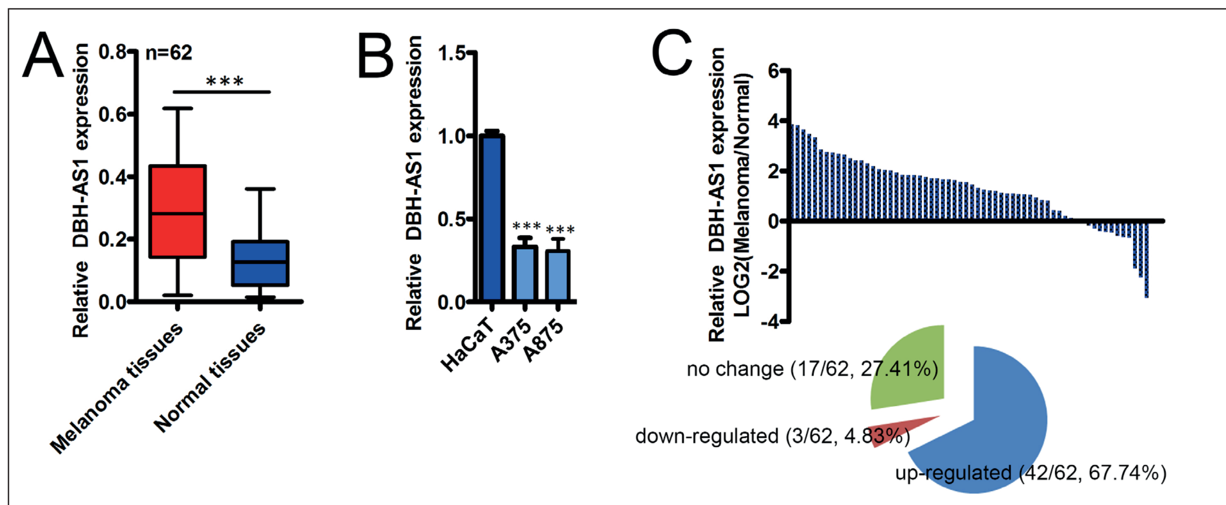

Figure 1. DBH-AS1 levels are increased in melanoma tissues and cells. **A**, Expression of DBH-AS1 was measured in melanoma and paired normal tissue samples via qRT-PCR. **B**, Histogram and pie chart of the proportions of melanoma samples in which DBH-AS1 expression was upregulated (42/62, 67.74%, blue), downregulated (3/62, 4.83%, red), or no change (17/62, 27.41%, green). Log2 (T/N) expression value > 1 as higher expression, which ≤1 as lower expression, and between -1 and 1 as no significant change. **C**, Expression of DBH-AS1 was measured in melanoma and control cell lines. p-value *<0.05; **<0.01; ***<0.001.

capable of binding to specific miRNAs and thereby interfering with their ability to suppress target gene expression. For figuring out if DBH-AS1 could be a ceRNA, its subcellular localization was firstly detected. Nuclear/cytoplasmic fraction assay results showed that, most DBH-AS1 presented a preferential localization in cytoplasm (Figure 3A). Therefore, it was assumed that DBH-AS1 could be a ceRNA. Using starBase 3.0, we were able to identify miR-223-3p as a putative binding target for lncDBH-AS1 based on sequence complementarity (Figure 3B). Using a Luciferase reporter assay system, we were able to confirm that a WT but not a MUT version of lncDBH-AS1 was able to bind to agomiR-223-3p such that this miRNA only altered Luciferase activity when

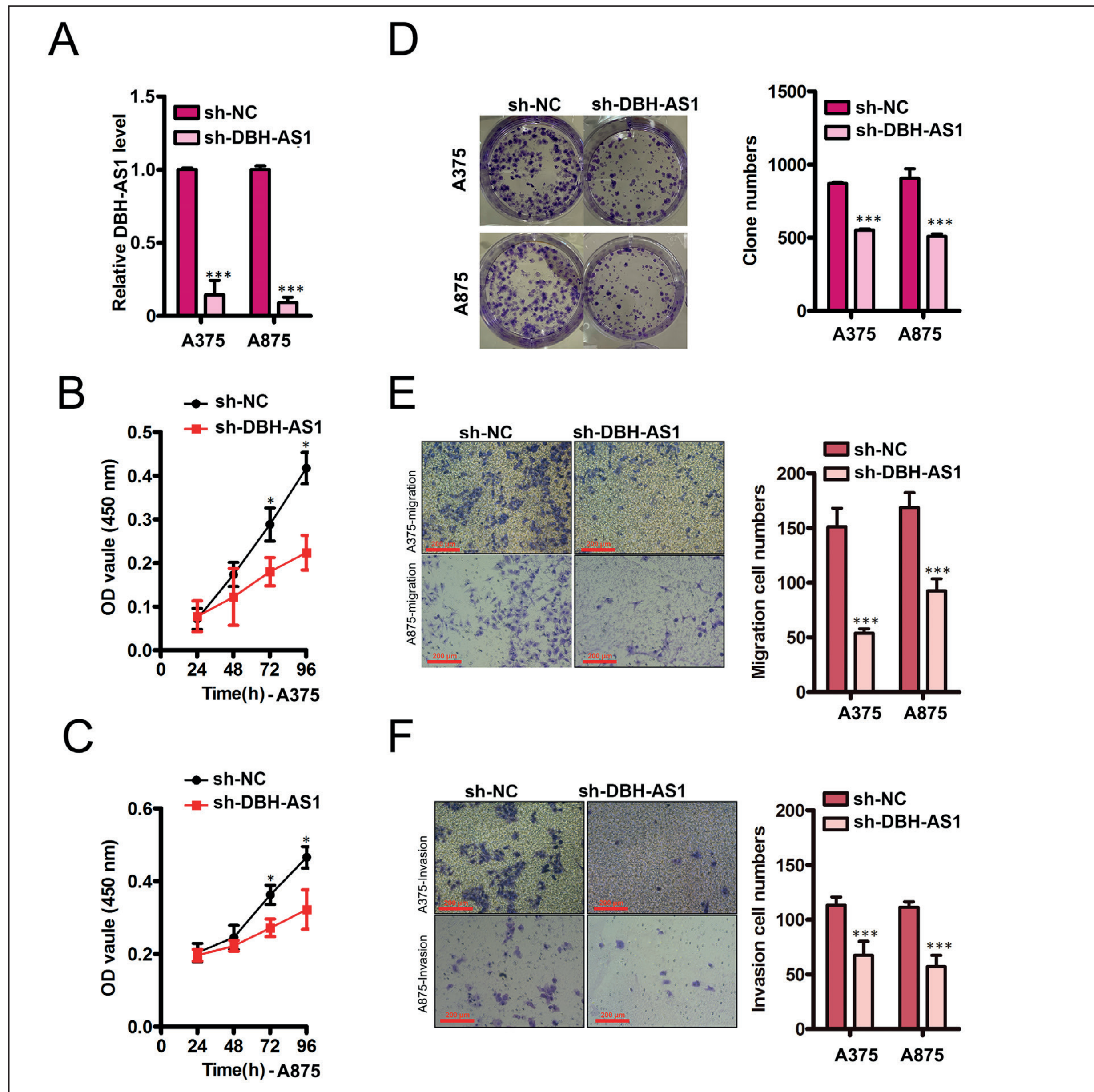

Figure 2. DBH-AS1 impairs the proliferative, migratory, and invasive activity of melanoma cells. **A**, The expression of DBH-AS1 was performed by qRT-PCR in melanoma cells. **B**, **C**, Changes in melanoma cell proliferation after DBH-AS1 knockdown was assessed via CCK-8 assay. **D**, Changes in melanoma cell proliferation following DBH-AS1 knockdown was assessed via a colony assay. The impact of DBH-AS1 knockdown on melanoma cell (**E**) migration and (**F**) invasion was assessed using a transwell assay system. The magnifications of Figure 2E and F, 200x, *p*-value *<0.05; **<0.01; ***<0.001.

transfected into cells that had been co-transfected with a WT-lncDBH-AS1 reporter construct (Figure 3C-D). We were further able to confirm a direct interaction between lncDBH-AS1 and miR-223-3p via a RIP assay which confirmed the significant enrichment of both of these RNAs in Ago2-containing immune-precipitates but not in precipitates prepared using control IgG (Figure 3E). These results therefore supported a model wherein lncDBH-AS1 functions as a ceRNA for DBH-AS1 in melanoma cells.

MiR-223-3p Suppresses IGF-1R Expression in Melanoma Cells

We next sought to identify potential miR-223-3p target genes using TargetScan, identifying IGF1R as one such target candidate (Figure 4A). We, then, used a Luciferase reporter assay approach to determine whether miR-223-3p was able to bind to its predicted target sequence in the IGF-1R 3'-untranslated region (UTR). We found that miR-223-3p mimic transfection was able to inhibit the activity of a wild-type (WT) IGF-1R 3'-UTR Luciferase reporter, whereas it had no effect on a reporter in which its putative binding site had been mutated, consistent with the ability of miR-223-3p to directly bind to this 3'-UTR region (Figure 4B and C). We further found that miR-223-3p overexpression was associated with marked decreases in IGF-1R mRNA and protein level expression as measured *via* qRT-PCR and Western blotting (Figure 4D and E). As such, these findings confirmed the ability of miR-223-3p to inhibit IGF-1R expression.

DBH-AS1 Regulated Glycolytic Activity Via IGF-1R/Akt/GLUT1 Signaling Axis

Previous studies have provided clear evidence that IGF-1R signaling can drive Akt phosphorylation and consequent GLUR1 upregulation, thus enhancing the glycolytic activity of tumor cells. As we had identified IGF-1R as a miR-223-3p target in melanoma, we next measured DBH-AS1 could regulate Akt activation and GLUT1 expression by Western blotting in our melanoma cell lines. We found that knock down DBH-AS1 significantly

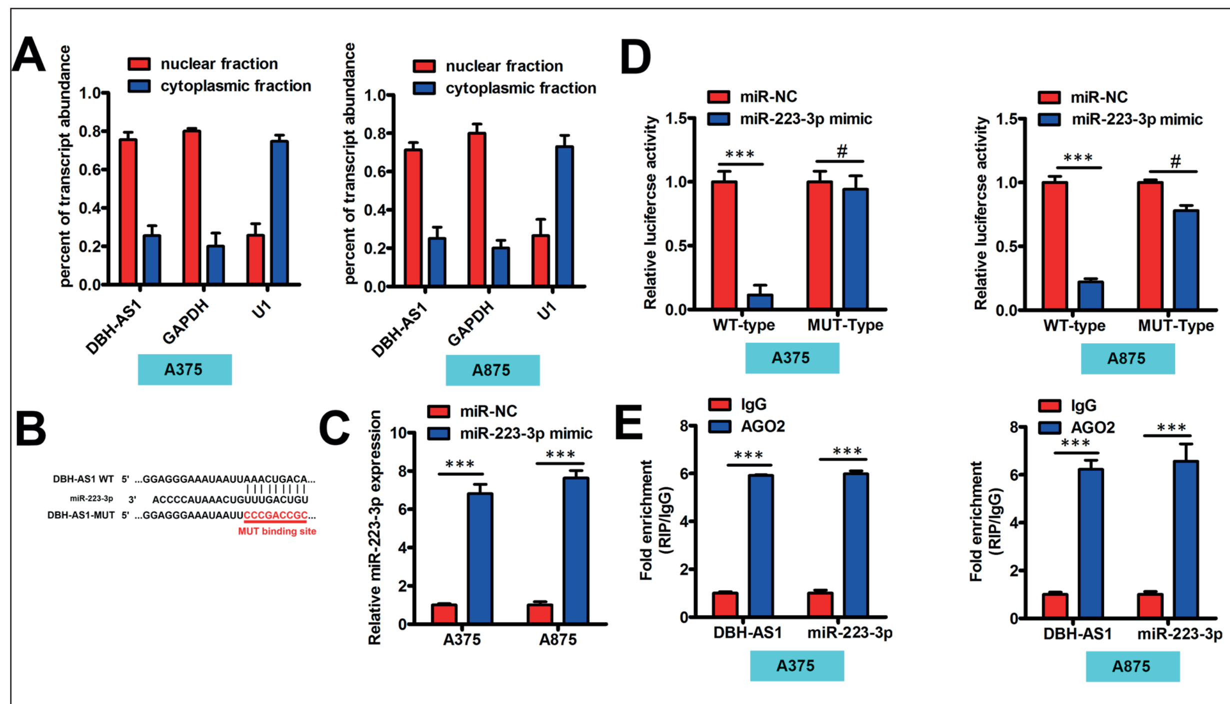

Figure 3. MiR-223-3p was regulated by DBH-AS1 in melanoma cells. **A**, As shown in nuclear RNA fractionation and cytoplasmic experiments, DBH-AS1 could be mainly found in cytoplasm of A375 and A875 cells. **B**, Bioinformatic analyses predicted an interaction between miRNA-223-3p and lncDBH-AS1. **C**, The expression of lncDBH-AS1 in A375 and A875 cells was assessed following agomir-223-3p or agomir-NC transfection. **D**, Wild type (WT)-lncDBH-AS1 or mutant (MUT)-lncDBH-AS1 were co-transfected into melanoma cells together with agomir-223-3p or agomir-NC. After 48 h, Luciferase activity was assessed. **E**, Increased DBH-AS1 and miR-223-3p levels were evident in Ago2-containing immune-precipitates relative to precipitates prepared using a control IgG. *p*-value *<0.05; **<0.01; ***<0.001.

decreased the levels of both phosphorylated Akt (S473) and GLUT1 in both analyzed cell lines (Figure 5A). We therefore next explored the ability of this lncRNA to influence glycolytic activity in these cells. Glucose uptake was found to be significantly decreased in both A375 and A875 cells following the knockdown DBH-AS1 (Figure 5B), and this coincided with a significant reduction in lactate production following knockdown DBH-AS1 (Figure 5C). As ATP generation is the primary output of glycolytic activity, we next assessed how DBH-AS1 impacted ATP production in melanoma

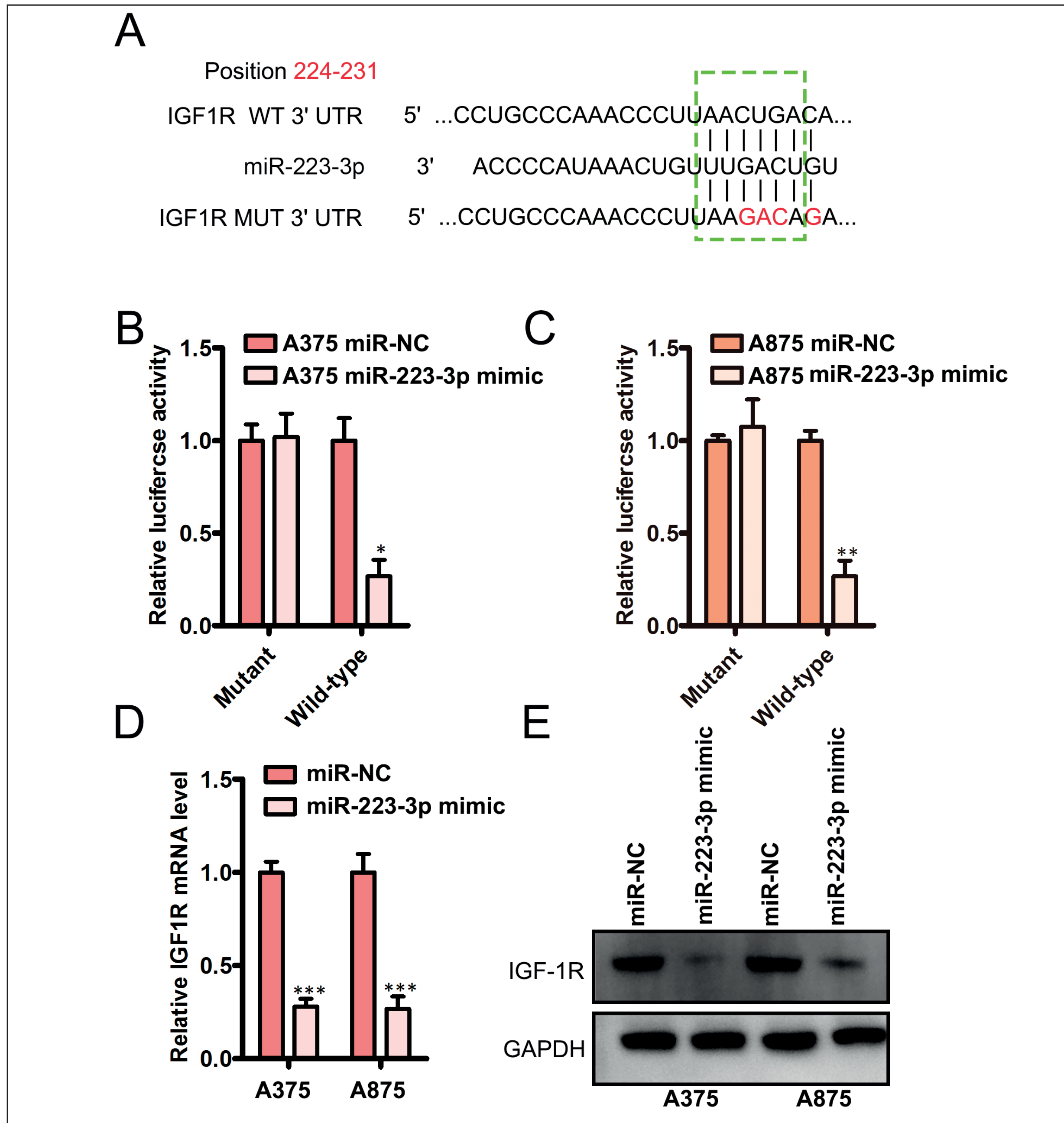

Figure 4. MiR-223-3p inhibits IGF-1R expression. **A**, The IGF-1R 3'-UTR miR-223-3p binding site. **B**, **C**, miR-223-3p mimic transfection reduced the activity of a WT IFG-1R 3'-UTR luciferase reporter in A375 and A875 cells. In contrast, no changes were observed upon miR-223-3p mimic transfection in cells co-transfected with a reporter in which these 3'-UTR binding sites had been mutated. Expression of IGF-1R in melanoma cells overexpressing miR-223-3p were quantified via (**D**) qRT-PCR, and (**E**) Western blotting. *p*-value *<0.05; ** <0.01; ***<0.001.

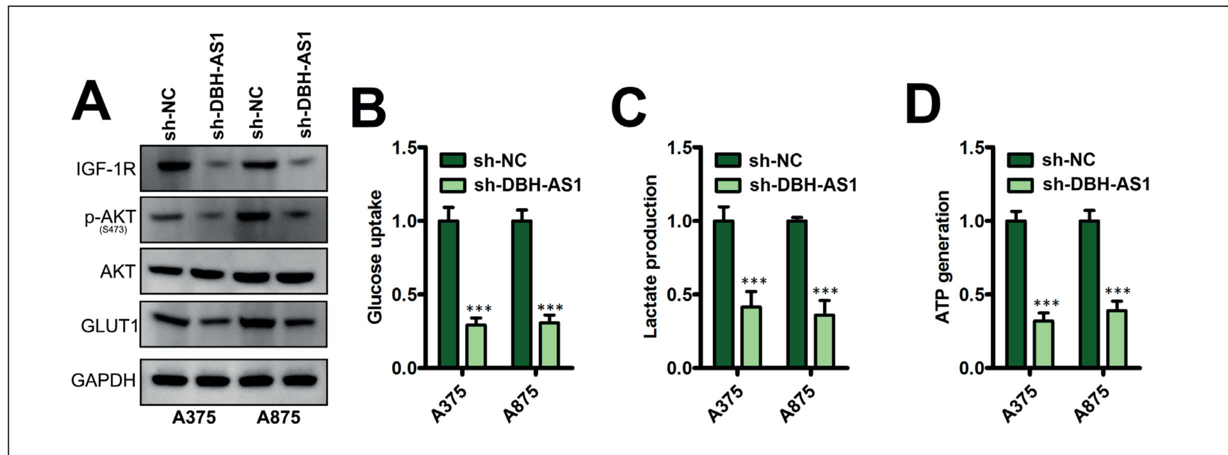

Figure 5. DBH-AS1 regulated glycolytic activity via IGF-1R/Akt/GLUT1 signaling axis. **A**, Lysates from indicated melanoma cells were used to analyze GLUT1 expression and Akt phosphorylation via Western blotting. **B**, **C**, Indicated melanoma cells were analyzed for both glucose uptake and lactate production. **D**, Levels of ATP generation were quantified in melanoma cells knockdown DBH-AS1 or controls. *p*-value *<0.05; **<0.01; ***<0.001.

cells. ATP levels were reduced in both tested melanoma cell lines following knockdown DBH-AS1 (Figure 5D), in line with the observed reductions in glucose and lactate levels. These results therefore suggested that DBH-AS1 may be able to positively regulate melanoma cell glycolytic activity.

Discussion

Melanoma remains the most deadly skin cancer subtype, in addition to being among the most rapidly spreading forms of malignant tumors[3,4]. Melanoma treatment has traditionally been dependent on a combination of surgery, radiotherapy, and chemotherapy, although more recent immunotherapeutic options have been explored for the treatment of patients with advanced disease. Previous work has highlighted the dysregulation of lncRNAS that can occur in many tumor types, significantly impacting tumor progression[21,22]. Due to their ability to influence myriad downstream targets in a specific fashion, lncRNAs have been a recent focus of therapeutic research in the field of oncology. There is therefore clear value in identifying those lncRNAs capable of suppressing melanoma cell growth and/or migration.

Dysregulated lncNRAs expression patterns can significantly influence tumor growth and development, with many lncNRAs functioning as oncogenes or tumor suppressors[9]. Herein, we observed a significant increase in DBH-AS1 expression levels in melanoma tumor and cell samples. Knockdown DBH-AS1 was able to impair melanoma cell proliferative, invasive, and migratory activity. We found that DBH-AS1 functioned at least in part *via* directly targeting and suppressing the expression of miR-223-3p, thereby impairing the IGFR/Akt signaling axis in these tumor cells and disrupting tumor progression.

LncRNAs could interact with mRNA, proteins or miRNAs to induce gene expression[35,36]. In multiple types of tumorigenesis, some studies indicated that lncRNAs acted as competing endogenous RNAs (ceRNAs) for miRNAs[37-39]. Such as, in colorectal carcinoma obtained chemoresistance via KCNQ1OT1 enhancing ATG4B level through sponging miR-34[40]. Besides, MALAT1 played key role in cell stemness by enhancing Oct4 expression by sponging miR-20b-5p in colorectal carcinoma[41]. In addition, DGCR5 activated KLF1 to inhibit HCC progression via sponging miR-346[42,43]. Following, we wonder whether DBH-AS1 exerts roles through sponging miRNAs in melanoma cells. First, miR-223-3p was predicted to share a complementary binding site for DBH-AS1, and the interaction and binding between DBH-AS1 and miR-223-3p were further confirmed using luciferase reporter and RIP assays. Second, DBH-AS1 downregulation increased miR-223-3p expression. Taken together, DBH-AS1 could act as a ceRNA to sponge miR-223-3p in Melanoma cells.

Cancer cells rely on aerobic glycolysis far more so than do normal cells and as such they need to undergo significant metabolic reprogramming as a means of generating the energy required for their unrestrained proliferation[44]. The inhibition of glycolytic activity in tumor cells is thus an attracting therapeutic option, with multiple miRNAs hav-

ing been identified that target glycolysis-associated genes such as GLUT1, G6PD, and LDHA[45-48]. Aerobic glycolysis is an important characteristic of melanoma. In the normoxic state, melanoma cells with different heterogeneity usually exhibit a high glycolytic phenotype, in which 60-80% of glucose is metabolized as lactic acid[49]. IGF-1R functions as a transmembrane insulin family receptor with kinase activity[50-52], and its expression is significantly elevated in cancers such as renal cell carcinoma and melanoma[53,54]. Binding of IGF-1 to IGF-1R leads to the downstream activation of PI3K/Akt signaling[55-57], with Akt S473 phosphorylation serving to regulate the localization and activity of GLUT1 within cells as a means of controlling their glucose uptake. Tumor cell survival has been shown to be regulated at least in part *via* IKKβ, NF-κB, and Akt-mediated regulation of GLUT1 trafficking to the cell membrane[58]. Herein, we determined that DBH-AS1 knockdown resulted in a significant reduction in melanoma cell IGF-1R expression. As a result, Akt phosphorylation and GLUT1 expression was markedly suppressed in these cells, leading to significant decreases in the uptake of glucose and the generation of lactate and ATP. These data therefore indicate that DBH-AS1 can positively regulate glycolytic activity in melanoma cells.

Conclusions

In summary, the results of this study demonstrated that DBH-AS1 increased is common in melanoma cells, underscoring the fact that the overexpression of this lncRNA can significantly promote the migratory and proliferative activity of melanoma cells. From a mechanistic perspective, we found that DBH-AS1 was able to directly target miR-223-3p, increasing EGFR expression and thereby enhancing signaling through the Akt/GLUT1 axis. This in turn leads to the promoting of melanoma cell glycolytic activity. These results thus suggest that DBH-AS1 may represent a viable treatment strategy for melanoma.

Conflict of Interests

The authors declare that they have no conflict of interests.

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