European Journal of Inflammation

Letter to the Editor

European Journal of Inflammation
Volume 17: 1–13

DOI: 10.1177/2058739219837050
journals.sagepub.com/home/eji
SAGE

LncRNA UCA1/miR-143 miR-216b/HK2 /MAPK signaling pathway is involved in the regulation of endothelial cell proliferation via the modulation of glycolysis in melanoma

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Abstract

Long noncoding RNAs (lncRNAs) and microRNAs (miRNAs/miRs) are noncoding RNAs that function as regulators of tumor suppressors and oncogenes. The aim of the present study was to investigate the potential mechanism associated with the involvement of urothelial cancer associated 1 (UCA1) in melanoma. Reverse transcription-quantitative polymerase chain reaction and western blot analysis were performed in order to determine the expression levels of UCA1, miR-143, miR-216b, and hexokinase 2 (HK2) in the melanoma and control groups, as well as the influence of UCA1, miR-143, and miR-216b on the expression of HK2, and the effect of lactate and UCA1 on the phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK). Bioinformatics algorithm analysis and a luciferase assay were performed in order to predict miRNA targets. In addition, an MTT assay was performed in order to determine the effect of lactate and UCA1 expression on cell proliferation. A total of 39 participants, consisting of 18 patients with melanoma and 21 healthy control subjects, were included in the present study. The present study demonstrated that the expression levels of UCA1 mRNA, and HK2 mRNA and protein were enhanced in patients with melanoma compared with healthy controls; whereas the expression levels of miR-143 and miR-216b mRNA were suppressed in patients with melanoma compared with healthy controls. Furthermore, it was revealed that UCA1 negatively modulated the expression of miR-143 and miR-216b, and that miR-143 and miR-216b directly targeted the HK2 protein by binding to the HK2 3′ untranslated region (UTR). In addition, it was demonstrated that miR-143 and miR-216 suppressed the luciferase activity exhibited by wild-type HK2 3′-UTR. Furthermore, it was revealed that transfection with UCA1 small interfering RNA, and miR-143 and miR-216b mimics markedly suppressed HK2 mRNA and protein expression levels as well as lactate levels in human umbilical vein endothelial cells; however, O_2 consumption was revealed to be enhanced post transfection. By contrast, transfection with UCA1 enhanced HK2 mRNA and protein expression levels as well as lactate production; however, O_2 consumption was revealed to be suppressed post transfection. Lactate-induced phosphorylation of p38 MAPK was revealed to occur in a concentration-dependent manner, and UCA1 enhanced the phosphorylation level of p38 MAPK via the inhibition of miR-143 and miR-216b expression. Lactate and UCA1 were demonstrated to enhance cell proliferation. In conclusion, the present study demonstrated that the lncRNA UCA1/miR-143 miR-216b/HK2/lactic acid/MAPK axis may be involved in the pathogenesis of melanoma via the modulation of endothelial cells, and thus, lncRNA UCA1 may serve as a potential therapeutic target for melanoma treatment.

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Keywords
endothelial cells, hexokinase 2, lactic acid, long noncoding RNAs, melanoma, microRNA-143, microRNA-216b, mitogen-activated protein kinase, proliferation, urothelial cancer associated 1

Date received: 14 November 2018; accepted: 18 February 2019

Introduction

Melanoma is a major public health issue worldwide and particularly affects Caucasian populations. Melanoma is the third most prevalent cancer affecting women and men in Australia; the fifth most prevalent cancer affecting women and men in the United States; and the sixth most prevalent cancer affecting women, and the twelfth most prevalent cancer affecting men, in the United Kingdom.[1] There are numerous studies that have demonstrated that angiogenesis is necessary for melanoma progression.[2] In response to such studies, a range of antiangiogenic agents have been tested in clinical trials; however, when administered as a monotherapy, none of the tested agents demonstrated an increase in the survival time of patients with malignant melanoma.[3] Clinical trials are ongoing in order to determine whether antiangiogenic treatment combined with conventional chemotherapy or immunotherapy can increase the survival of patients with malignant melanoma.[3] Angiogenesis is the process by which novel blood vessels are formed from preexisting blood vessels via budding and sprouting of new capillaries. Angiogenesis represents an important process in numerous physiological and pathological mechanisms, including tumor growth and development, wound healing, and reproduction.[4] Endothelial cells (EC) are important in all stages of angiogenesis and are involved in a number of associated processes, such as anastomosis of endothelial sprouts and lumen formation.[5]

The expression of hexokinase 2 (HK2) is suppressed in numerous types of healthy tissues; however, it is frequently enhanced in cancerous tissues.[6] Enhanced glycolysis is frequently exhibited by cancerous cells and may be attributable to the increased expression of key enzymes involved in glycolysis, such as 6-phosphofructo-1-kinase, glyceraldehyde 3-phosphate dehydrogenase, and HK2.[7] HK2, the most prevalent hexokinase isoform, can bind to the outer membranes of mitochondria and its expression is significantly enhanced in malignancies.[8] HK2 facilitates the synthesis of adenosine triphosphate (ATP) and the phosphorylation of glucose in mitochondria, thus providing cancer cells with ample quantities of biosynthetic precursor as well as a highly glycolytic phenotype.[8] Aerobic glycolysis has previously been revealed to be induced via the phosphoinoside 3-kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase-1 (ERK) signaling pathways.[9] The results of the suppression of the AKT and ERK pathways suggest that IMQ activates PI3K/AKT in order to induce aerobic glycolysis; however, IMQ does not affect MAPK/ERK signaling. Phosphorylation of p38 MAPK is a stress-sensitive mechanism inducible by oxidative stress, hyperglycemia, angiotensin II, and diabetes. It has previously been revealed to be associated with the pathogenesis of vasculopathy via the enhancement of EC proliferation, lesion development, and impairment of endothelial function.[10]

Noncoding RNAs (ncRNAs) can be classified into two groups dependent upon their size: long noncoding RNAs (lncRNAs) consisting of >200 nucleotides and small ncRNAs consisting of <200 nucleotides.[11] Small ncRNAs include ribosomal RNA, transfer RNA, Piwi-interacting RNA, small nuclear RNA, small nucleolar RNA, small interfering RNA (siRNA), and microRNAs (miRNAs/miRs), which all exhibit a variety of cellular functions.[12] miRNAs are small ncRNAs consisting of ~21–23 nucleotides that can complementarily bind to sequences inherently expressed by mRNA molecules, and thus may be involved in RNA silencing.[13] Furthermore, enhanced expression of non-protein coding RNAs (lncRNAs and miRNAs) may function as early prognostic markers and therapeutic targets in numerous disorders; miRNAs in particular have received growing attention as a result of their potential involvement in tumorigenesis.[14,15] In addition, several genome-wide expression studies have suggested that numerous miRNAs and lncRNAs function as important modulators of melanoma development.[16]

The lncRNA urothelial cancer associated 1 (UCA1) has previously been reported to be involved in the modulation of EC behavior[17–19] and the present study revealed that miR-143 and

miR-216b function as endogenous competitive miRNAs binding to UCA1. Furthermore, the present study demonstrated that HK2 is a direct target of miR-143 and is a target gene of miR-216b as detailed in the online miRNA database.[19] HK2 has previously been demonstrated to promote aerobic glycolysis, which can activate the MAPK signaling pathway.[20,21] Furthermore, p38 MAPK may enhance the proliferation of ECs, which represents a major cornerstone of melanoma pathogenesis-associated angiogenesis development.[22] In the present study, samples from patients with malignant melanoma were collected, and the expression levels of UCA1, miR-216b, miR-143, and HK2 were determined and compared with the controls. In addition, the functions of UCA1, miR-216b, miR-143, and HK2 were investigated as well as their involvement in the proliferation of ECs.

Materials and methods

Sample collection

A total of 39 participants were recruited for the present study, including 18 patients with melanoma and 21 patients with normal skin to act as a control. All participants, or their first-degree relatives, provided written informed consent regarding participation prior to the beginning of the experiment. All samples were stored in liquid nitrogen for later analysis. This study was granted ethical approval by the Qilu Hospital Shandong University (Shandong, China; no. IRB2016002716). The present study was performed in conformity with the Declaration of Helsinki.

RNA isolation and reverse transcription-quantitative polymerase chain reaction

To determine the expression levels of UCA1, miR-143, miR-216b, and HK2 mRNA, the miRNA easy Mini kit (Qiagen, Inc., Valencia, CA, USA) was used, in accordance with the manufacturer's instructions, to extract total RNA from human umbilical vein endothelial cells (HUVECs) and tissue samples. Following this, 1% agarose gel was used in order to analyze the degradation and contamination of total RNA. A NanoPhotometer spectrophotometer (Implen, Inc., Westlake Village, CA, USA) was then used in order to determine RNA purity, and following this, a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to test all RNAs to control quality. The MiScript Reverse Transcription kit (Qiagen, Inc.) was then used to perform RT using a total of 1 μg RNA for the synthesis of HK2 cDNA. qPCR was performed in order to determine the relative expression levels of UCA1, miR-143, miR-216b, and HK2 mRNA using the miScript SYBR Green PCR kit (Qiagen, Inc.) on the Applied Biosystems Stepone Plus system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA U6 small nuclear 2 was chosen as the endogenous control. The $2^{-\Delta\Delta Cq}$ method and GraphPad Prism 4.0 software (GraphPad Software, Inc., La Jolla, CA, USA) were used in order to quantify the relative expression levels of UCA1, miR-143, miR-216b, and HK2 mRNA. Each experiment was conducted in triplicate.

HUVECs culture and transfection

RPMI 1640 (Gibco; Thermo Fisher Scientific, Inc.) containing 100 mg/mL streptomycin, 100 U/mL penicillin, and 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) was used for the incubation of HUVECs under an atmosphere of 5% CO_2 at 37°C. When the cells had reached 80% confluence, RPMI 1640 with 1% FBS was used for the seeding of cells in a low-serum condition in order to starve the cells. Following this, the cells were treated with lactate, and Lipofectamine 2000™ (Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect pcDNA3, pcDNA3-UCA1, miR-143, miR-216b, or UCA1 siRNA into HUVECs, in accordance with the manufacturer's instructions. Each experiment was conducted in triplicate.

Vector construction

The coding sequence of UCA1 was amplified via PCR, then the PCR products were purified, double digested, and cloned into pcDNA3 vectors. Direct Sanger sequencing was performed in order to confirm the presence of the cloned sequence. Each experiment was conducted in triplicate.

Cell proliferation assay

HUVECs were cultured in 24-well plates at a final concentration of 5×10^4 cells per well, and at 48 h post transfection, MTT (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into each

well, and the plates were then subjected to incubation at 37°C for 4 h. Following this, centrifugation was performed in order to remove the supernatant, and then dimethyl sulfoxide was added in order to dissolve the purple formazan. A microplate reader was used to measure the optical density of the cells based on their absorption value at a 490 nm wavelength. Each experiment was conducted in triplicate.

Dual-luciferase reporter assay

A luciferase reporter containing wild-type HK2 3′ untranslated region (UTR) was constructed by inserting HK2 3′-UTR into the pGL3-control vector (Promega Corporation, Madison, WI, USA), which is located downstream of the luciferase gene. PCR was then performed in order to amplify the 3′-UTR of HK2 containing the binding sites of either miR-216b or miR-143. Mutant HK2 3′-UTR was generated by mutation of either the miR-216b or miR-143 binding site, and then confirmed by Sanger sequencing. To perform the luciferase assay, HUVECs were seeded into 96-well plates at a density of 2×10^5 cells per well and then co-transfected using Lipofectamine 2000™ (Invitrogen; Thermo Fisher Scientific, Inc.) with luciferase constructs (50 ng) containing either wild-type or mutated HK2 3′-UTR, and either miR-216b/miR-143 mimics or inhibitors, in accordance with the manufacturer's instructions. A total of 48 h post transfection, the dual-luciferase reporter assay system (Promega Corporation) was used to measure the luciferase activity of *Renilla* luciferase and Firefly luciferase. Each experiment was conducted in triplicate.

Western blot analysis

To determine the expression levels of HK2 and p38 MAPK, cell lysates were prepared using ice-cold lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 0.1% sodium dodecyl sulfate and 1% NP-40) containing protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). A total of ~40 μg protein samples were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). The protein on the membranes was then blocked using nonfat dry milk in TBS buffer with 0.1% Tween-20 (TBST) for 30 min. Following this, monoclonal antibodies against anti-HK2 or p-p38 MAPK (1:5000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-β-actin (1:8000; Santa Cruz Biotechnology, Inc.) were incubated with the membranes for 12 h at 4°C and the membranes were then washed three times with TBST (Biosharp, Hefei, China). Following this, horseradish peroxidase (HRP)-conjugated secondary antibody (1:15,000; Cell Signaling Technology, Inc., Danvers, MA, USA) in TBST was used to treat the membranes for a further 1 h, and the membranes were once again washed three times using TBST. An enhanced chemiluminescence-detection system (Santa Cruz Biotechnology, Inc.) was used in order to visualize the protein. LabWork 4.0 program (UVP, Inc., Upland, CA, USA) was used to determine the relative expression levels of HK2 and p38 MAPK.

Immunohistochemistry

The expression level of HK2 protein was determined using the streptavidin-peroxidase staining technique. The activity of endogenous peroxidase was blocked using 3% H_2O_2 for 10 min and following this, phosphate-buffered saline (PBS) was used as a washing reagent. Antibodies against HK2 (1:1000) were incubated with samples for 12 h at 4°C, and then the sample was washed using PBS. HRP-conjugated secondary antibodies (1:2000) were then incubated with the sample for 1 h at room temperature. Following this, the sample was treated with streptavidin-peroxidase for 30 min, washed with PBS, and then treated with 3′,3′-diaminobenzidine. Counterstaining was performed using hematoxylin stain. PBS substituting as a primary antibody served as a negative control. Two experienced pathologists independently scored the staining intensities as follows: 3, strong staining; 2, moderate staining; 1, weak staining; and 0, no staining. The final immunohistochemistry (IHC) score was determined by multiplying the staining intensity score with the percentage of positive cells.

Determination of the level of cellular ATP

A luciferin-luciferase reaction was performed in order to determine the cellular ATP level. Briefly, 1×10^5 per cells were collected for treatment with ES (BioVision, Inc., Milpitas, CA, USA), according to the manufacturer's instructions. Following this, a luminometer was used to determine the luminescence of the sample lysate (100 μL). Each experiment was conducted in triplicate.

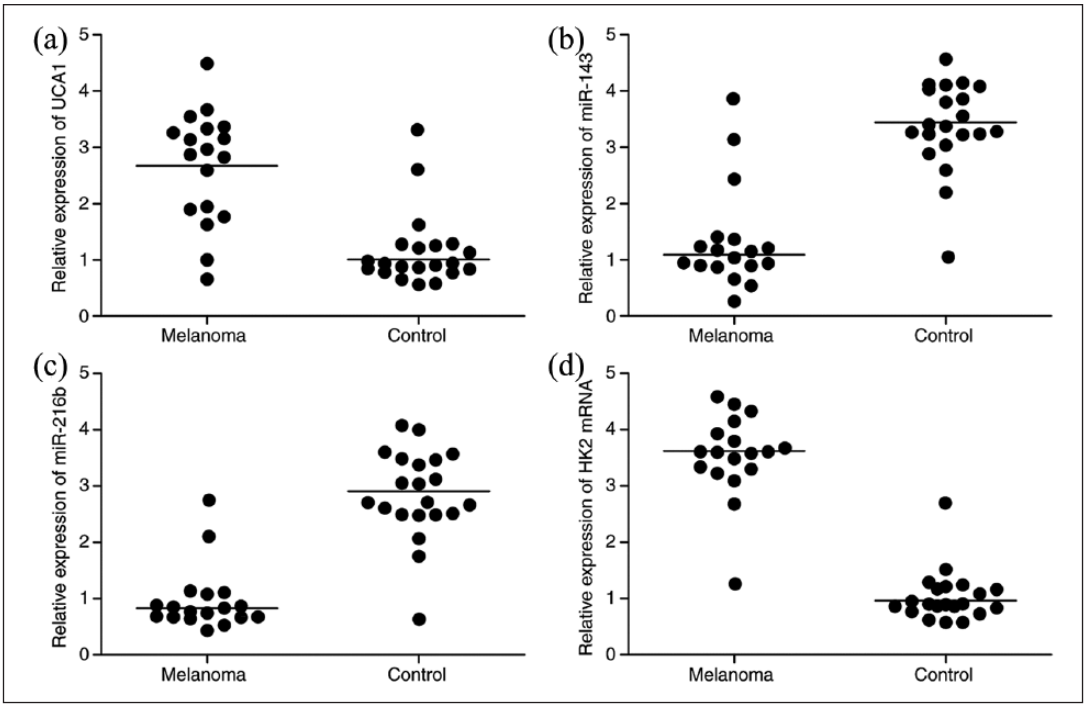

Figure 1. Reverse transcription-quantitative polymerase chain reaction was performed in order to determine the expression levels of UCA1, miR-143, miR-216b, and HK2 in patients with melanoma and in healthy control patients: (a) UCA1 expression was enhanced in the melanoma group compared with the control group, while (b) miR-143 and (c) miR-216b expression was suppressed in the melanoma group compared with the control group. (d) HK2 expression was enhanced in the melanoma group compared with the control group.
HK2: hexokinase 2; miR: microRNA; UCA1: urothelial cancer associated 1.

Determination of extracellular lactate levels

An assay buffer was used to prepare the sample using 1×10^6 cells, and the cell lysate was diluted to 1:10 for subsequent analysis. Lactate levels were determined using a glycolysis cell-based assay in accordance with the manufacturer's instructions (Cayman Chemical Company, Ann Arbor, MI, USA). A total of 2×10^4 cells per well were cultured in 24-well plates for 2 h, and then the cells were collected for colorimetric detection in a fresh 24-well plate. The Synergy 2 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) was then used to measure the absorbance at appropriate wavelengths. The level of lactate was then determined by comparing the absorbance of each replicate with the absorbance of the control. Each experiment was conducted in triplicate.

Statistical analysis

All results are presented as the mean ± standard deviation. Either the two-tailed Student's *t*-test or one-way analysis of variance was used for comparative analyses between groups. The value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

UCA1, miR-143, miR-216b, and HK2 are differentially expressed in patients with melanoma compared with control patients

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed in order to determine the expression levels of UCA1, miR-143, miR-216b, and HK2 in the melanoma and control groups. UCA1 (Figure 1(a)) and HK2 (Figure 1(d)) were revealed to exhibit enhanced expression in the melanoma group compared with the control group; however, the levels of miR-143 (Figure 1(b)) and miR-216b (Figure 1(c)) in the melanoma group were lower when compared with the control group, thus suggesting that UCA1, miR-143, miR-216b, and HK2 may be associated with the pathogenesis of melanoma. Furthermore, IHC was performed in order to determine the

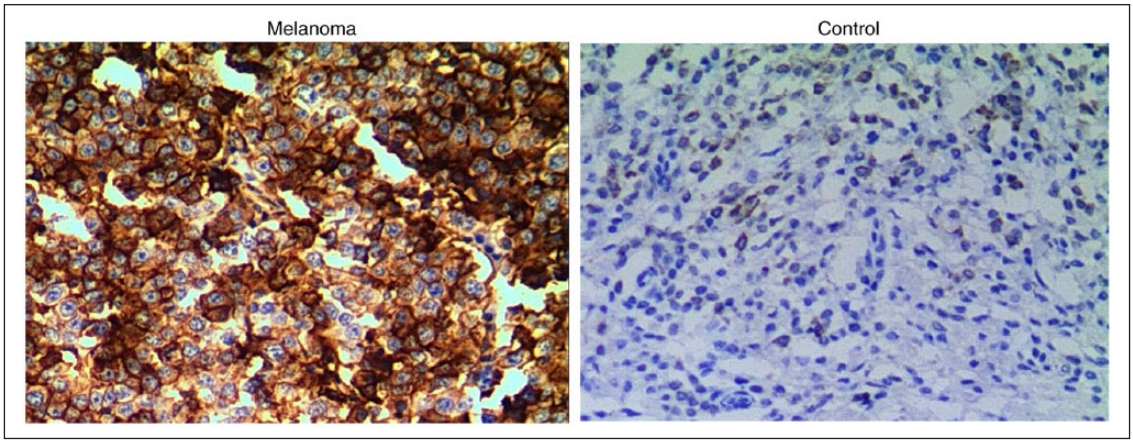

Figure 2. Immunohistochemistry was performed in order to determine the expression of the HK2 protein in melanoma and normal skin. The results demonstrated that the expression of HK2 was enhanced in patients with melanoma compared with healthy patients.
HK2: hexokinase 2.

expression levels of the HK2 protein in the melanoma and control groups. The results demonstrated that HK2 expression (Figure 2) was markedly enhanced in patients with melanoma compared with the control patients.

miR-143 and miR-216b directly target HK2

Bioinformatics algorithm analysis, such as TargetScan, was performed in order to predict miRNA targets, and two conserved sites present within the HK2 3′-UTR were identified as potential miR-143 (Figure 3(a)) or miR-216b (Figure 3(c)) target sites. The miR-143 and miR-216b binding sites within HK2 3′-UTR were mutated in order to generate mutant 1 and mutant 2 groups. Luciferase constructs were also generated via the insertion of wild-type, mutant 1, and mutant 2 HK2 3′-UTR into pGL3-control vectors in order to determine the association between miR-143/miR-216b and HK2. As revealed in Figure 3, luciferase activity exhibited by wild-type HK2 3′-UTR markedly decreased in miR-143 (Figure 3(b)) and miR-216b (Figure 3(d)) overexpressing cells; however, the luciferase activity exhibited by mutant 1 and mutant 2 HK2 3′-UTR in miR-143 (Figure 3(b)) and miR-216b (Figure 3(d)) overexpressing cells did not demonstrate a significant difference compared with the control. Furthermore, RT-qPCR was performed in order to investigate the interactions between UCA1 and miR-143/miR-216b. It was revealed that transfection with UCA1 siRNA significantly enhanced miR-143 (Figure 3(e)) and miR-216b (Figure 3(f)) expression levels compared with the scramble control. The results, therefore, suggest that UCA1 may negatively regulate miR-143 and miR-216b expression, and miR-143 and miR-216b may directly target HK2.

Transfection with UCA1, miR-143, and miR-216b affects HK2 expression, O_2 consumption, and lactate levels in HUVEC cells

HK2 expression, O_2 consumption, and lactate levels in HUVEC cells transfected with UCA1 siRNA, miR-143 mimics, or miR-216b mimics were investigated in order to determine the function of UCA1, miR-143, and miR-216b in aerobic glycolysis. As revealed in Figure 4(a), transfection with UCA1 siRNA, and miR-143 and miR-216b mimics markedly suppressed HK2 mRNA and protein expression levels in HUVEC cells compared with the scramble control. In addition, transfection with UCA1 siRNA, and miR-143 and miR-216b mimics markedly enhanced the O_2 consumption of HUVEC cells (Figure 4(b)). By contrast, there was a marked reduction in the extracellular lactate levels of HUVEC cells transfected with UCA1 siRNA, and miR-143 and miR-216b mimics compared with the scramble control (Figure 4(c)).

Phosphorylation of p38 MAPK is induced by lactate and UCA1 administration

As revealed by Figure 5(a) and (b), lactate treatment (30 min) significantly enhanced the level of phosphorylated p38 MAPK in a dose-dependent

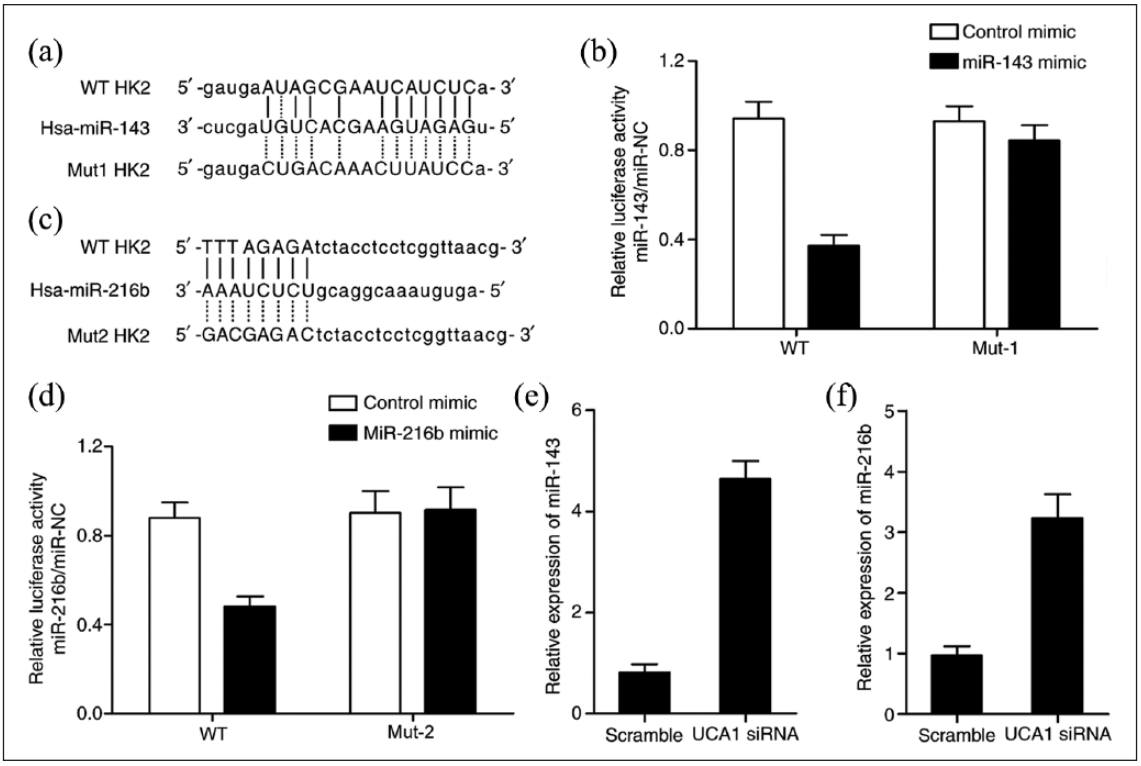

Figure 3. miR-143 and miR-216b were revealed to directly target HK2: (a) schematic representation of miR-143 and WT/mutant1 3′-UTR of HK2. (b) Luciferase activity of WT HK2 is suppressed via transfection with a miR-143 mimic. (c) Schematic representation of miR-216b and WT/mutant2 3′-UTR of HK2. (d) Luciferase activity of WT HK2 is suppressed following transfection with a miR-216b mimic. UCA1 siRNA upregulated (e) miR-143 and (f) miR-216b expression.
HK2: hexokinase 2; Hsa: human serum albumin; miR: microRNA; Mut: mutant; NC: negative control; siRNA: small interfering RNA; UCA1: urothelial cancer associated 1; UTR: untranslated region; WT: wild type.

manner, therefore suggesting that lactate induces the phosphorylation of p38 MAPK in a dose-dependent manner. As revealed in Figure 5(c) and (d), the level of phosphorylated p38 MAPK was markedly suppressed following transfection with UCA1 siRNA, and miR-143 mimics and miR-216b mimics, thus suggesting that UCA1 enhances p38 MAPK levels via the inhibition of miR-143/miR-216b.

UCA1 transfection affects HK2 expression, O_2 consumption, and lactate levels in HUVEC cells

HK2 expression, O_2 consumption, and lactate levels in HUVEC cells transfected with constructs containing UCA1 were investigated in order to determine the mechanism of UCA1 for aerobic glycolysis. The results demonstrated that overexpression of UCA1 enhanced HK2 expression (Figure 6(a)) and extracellular lactate levels (Figure 6(c)), while overexpression of UCA1 suppressed O_2 consumption (Figure 6(b)) compared with the control.

UCA1 induces the phosphorylation of p38-MAPK in HUVEC cells

The phosphorylation level of p38 MAPK in HUVEC cells transfected with constructs containing UCA1 was investigated using western blot analysis. As revealed in Figure 7, overexpression of UCA1 enhanced the phosphorylation level of p38 MAPK compared with the control.

Lactate and UCA1 enhance cell proliferation

An MTT assay was performed in order to determine the effect of lactate and UCA1 administration on cell proliferation. Various doses of lactate (15 and 25 nM) were administered to HUVEC cells, and following this, the proliferation of HUVEC cells was revealed to increase following lactate treatment in a dose-dependent manner (Figure 8(a)). In addition, the proliferation of HUVEC cells was markedly enhanced post transfection with UCA1 (Figure 8(b)), and markedly suppressed following transfection with UCA1 siRNA, and miR-143 mimics and miR-216b

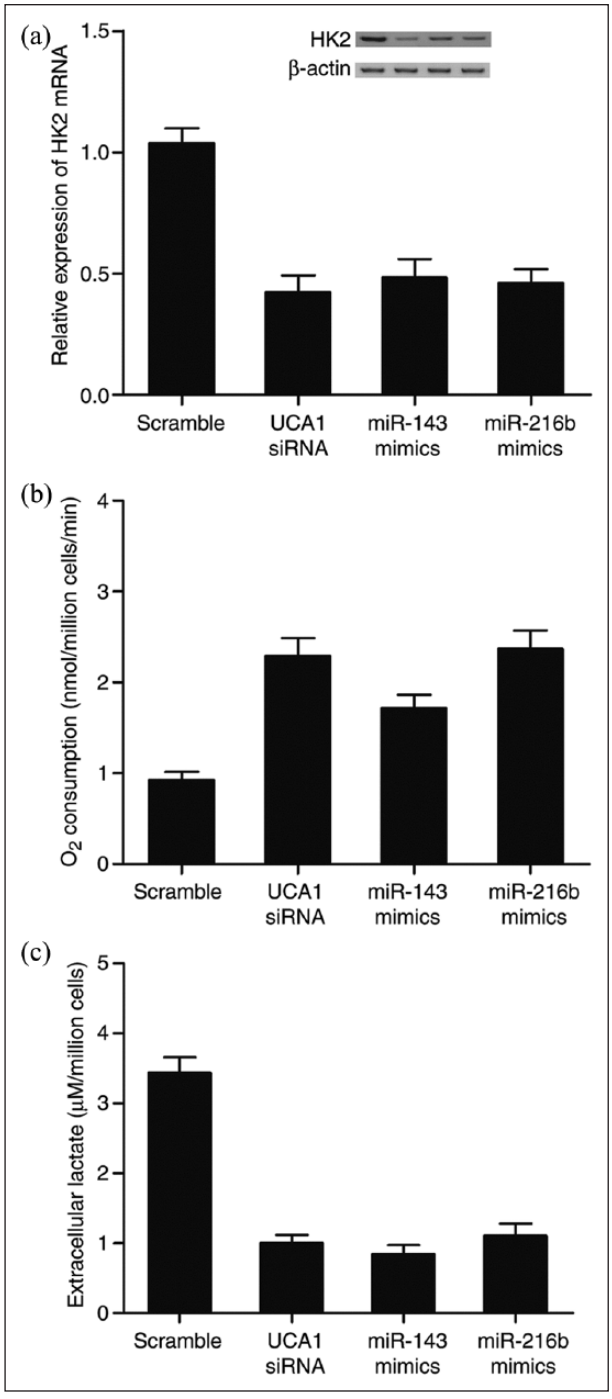

Figure 4. Effects of UCA1 siRNA, and miR-143 and miR-216b mimics transfection on the expression levels of HK2, the consumption of O_2, and the lactate level. UCA1 siRNA, and miR-143 and miR-216b mimics (a) suppressed HK2 mRNA and protein expression, (b) enhanced O_2 consumption, and (c) suppressed the level of lactate.
HK2: hexokinase 2; miR: microRNA; siRNA: small interfering RNA; UCA1: urothelial cancer associated 1.

mimics (Figure 8(c)) compared with the control. Therefore, the results suggest that lactate and UCA1 may enhance HUVEC cell proliferation.

Discussion

Melanoma progression is characterized by angiogenesis. The use of antiangiogenic agents has not been widely determined in patients with advanced melanoma.[23] Determination of the role of angiogenesis in the development of melanoma and its suppression may offer a foundation for the development of future cancer therapies.[24] Angiogenesis has been demonstrated to be an important process in the transformation of melanoma. Numerous angiogenic factors have previously been demonstrated to be released by host and/or melanoma cells within the tumor microenvironment.[25] The formation of blood vessels via angiogenesis is an integral process in the development of numerous vascular disorders as well as normal vascular development.[26] In response to angiogenic stimuli, ECs migrate, proliferate, and coalesce to form primitive vascular labyrinths that undergo remodeling and maturation, which is accompanied by the recruitment of smooth muscle cells to form mature blood vessels.[27] In the present study, an MTT assay was performed in order to determine the effect of lactate and UCA1 on cell proliferation, and it was subsequently revealed that lactate and UCA1 enhanced cell proliferation.

LncRNAs are noncoding transcripts of >200 nucleotides that exhibit important regulatory functions in tumorigenesis and tumor progression.[28] Tian et al.[29] investigated the roles of the abnormally expressed lncRNAs Malat-1 and UCA1 in melanoma metastasis, and revealed that the enhanced expression of lncRNAs Malat-1 and UCA1 was associated with the metastasis of melanoma. Furthermore, the function of *miR-143* as a tumor inhibitor associated with melanoma has been extensively investigated.[30] In addition, it has been demonstrated that the expression of miR-216b is markedly suppressed in melanoma cell lines compared with human keratinocytes, and that miR-216b modulates the migration and proliferation of ECs in melanoma; thus, this may represent a potential therapeutic target.[31] Recently, a study revealed that a 1.4 kb isoform of UCA1 can generate a complex with heterogeneous nuclear ribonucleoprotein I and hence function as an oncogene in breast cancer to reduce p27 expression.[32] However, it has not yet been determined whether additional mechanisms are involved in the modulation of breast cancer. Furthermore, it has also been determined that the 1.4 kB isoform of UCA1 directly interacts with miR-143.[33] miR-143 and

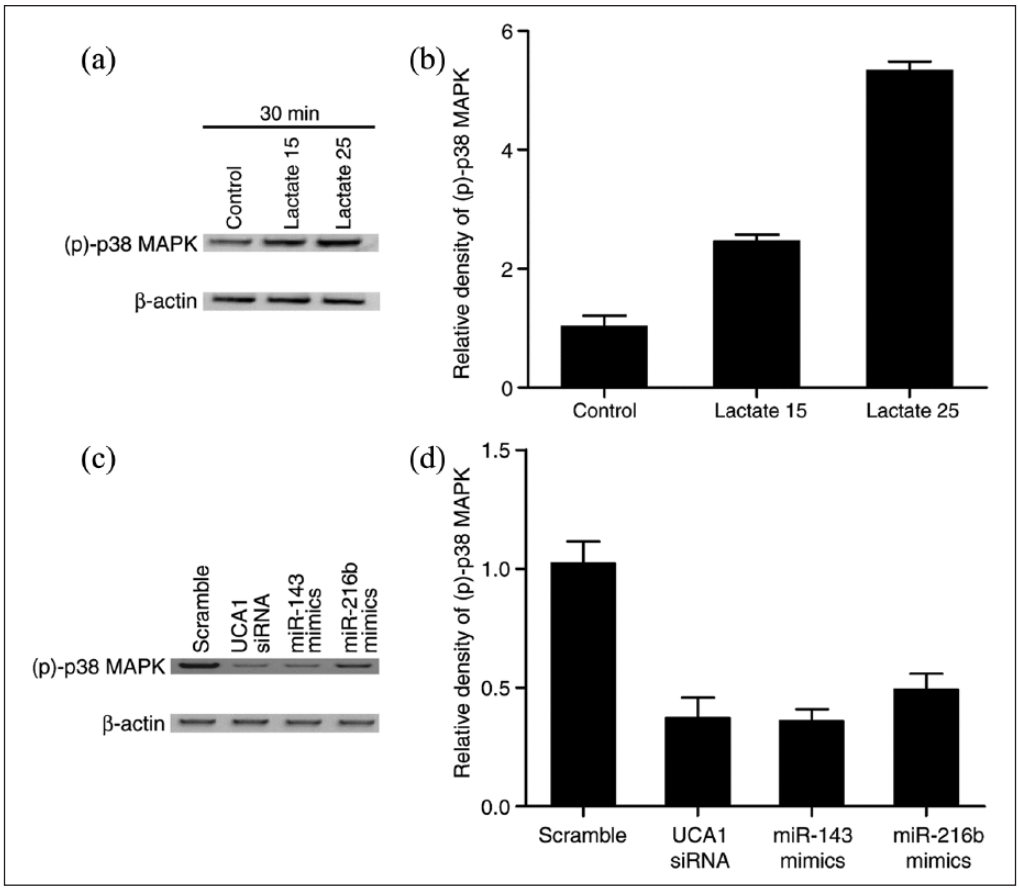

Figure 5. Effects of lactate, UCA1, miR-143, and miR-216b on the phosphorylation of p38 MAPK: (a and b) The level of MAPK phosphorylation increased with lactate administration in a dose-dependent manner. (c and d) Transfection with UCA1 siRNA, and miR-143 and miR-216b mimics suppressed the phosphorylation of p38 MAPK.
HK2: hexokinase 2; MAPK: mitogen-activated protein kinase; miR: microRNA; (p): phosphorylated; siRNA: small interfering RNA; UCA1: urothelial cancer associated 1.

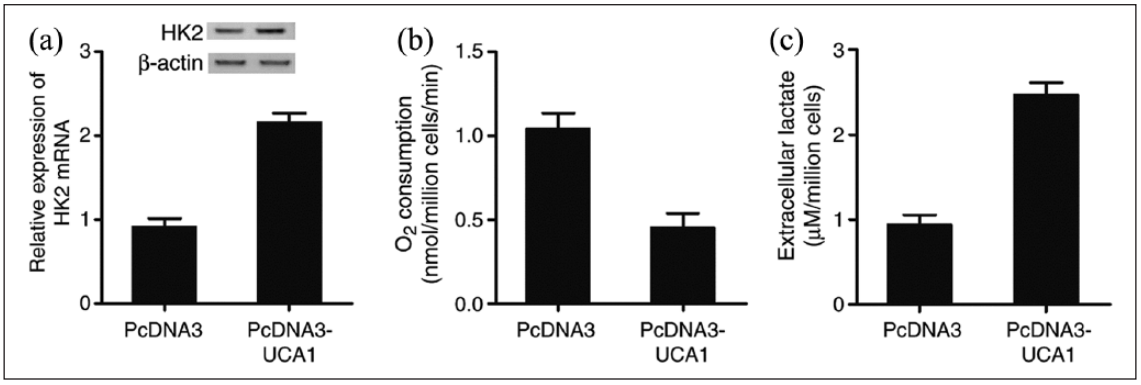

Figure 6. Effect of UCA1 administration on the expression level of HK2, the consumption of O_2, and the lactate level. Overexpression of UCA1 (a) enhanced HK2 mRNA and protein expression, and (b) suppressed O_2 consumption, and (c) transfection using constructs containing UCA1 enhanced the level of lactate.
HK2: hexokinase 2; UCA1: urothelial cancer associated 1.

UCA1 are in the identical complex of RNA-induced silencing complex.[33] Therefore, in breast cancer, suppression of miR-143 expression may enhance UCA1 expression.[33] In addition, it has been demonstrated that overexpression of UCA1 can result in the suppression of miR-216b expression in hepatocellular carcinoma cells in a dose-dependent manner, thus suggesting that UCA1, by functioning

as an endogenous antagomir, can suppress miR-216b expression.[18] In the present study, 39 subjects, including 18 patients with melanoma and 21 healthy patients, were enrolled, and it was demonstrated that UCA1 RNA, as well as HK2 mRNA and protein expression levels were markedly enhanced in patients with melanoma compared with the healthy control subjects. However, miR-143 and miR-216b mRNA expression levels were markedly suppressed in patients with melanoma compared with the healthy control patients. Furthermore, bioinformatics algorithm analysis was performed in order to investigate miRNA targets, and two conserved sites located on HK2 3′-UTR were identified as potential miR-143 or miR-216b target sites. In addition, the present study revealed that transfection with either miR-143 or miR-216b suppressed the luciferase activity exhibited by wild-type HK2 3′-UTR. It was also revealed that UCA1 administration suppressed the expression of miR-143 and miR-216b. In addition, the effects of UCA1, miR-143, and miR-216b on HK2 expression, O_2 consumption, and lactate levels were investigated, and it was demonstrated that treatment with UCA1 siRNA, and miR-143 and miR-216b mimics suppressed the expression of HK2 and extracellular lactate, while the consumption of O_2 was enhanced post treatment.

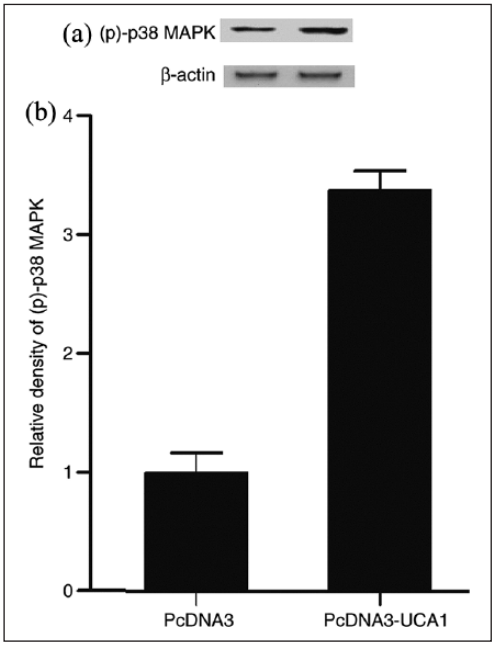

Figure 7. Overexpression of UCA1 enhanced the phosphorylation of p38 MAPK: (a) western blotting was performed on human umbilical vein endothelial cells transfected with constructs containing UCA1. (b) Overexpression of UCA1 was revealed to increase p38 MAPK phosphorylation.
MAPK: mitogen-activated protein kinase; UCA1: urothelial cancer associated 1.

HKs catalyze the first step of the glucose metabolism that phosphorylates glucose to produce glucose-6-phosphatase.[34] There are four isoforms (HK1, 2, 3, and 4) of the mammalian HK family. Numerous studies have demonstrated that overexpression of HK2 enhances cell proliferation and suppresses apoptosis in a number of cancer types, including prostate, lung, breast, brain, and ovarian cancer.[35–37] In the present study, the effects of UCA1 on HK2 expression, O_2 consumption, and lactate levels were investigated, and it was revealed that UCA1 enhances HK2 expression and lactate production, and suppresses O_2 consumption. One of the predominant metabolic characteristics of tumor cells is its reliance on aerobic glycolysis in which tumor cells, even with adequate oxygen, undergo aerobic glycolysis and convert pyruvate to lactate

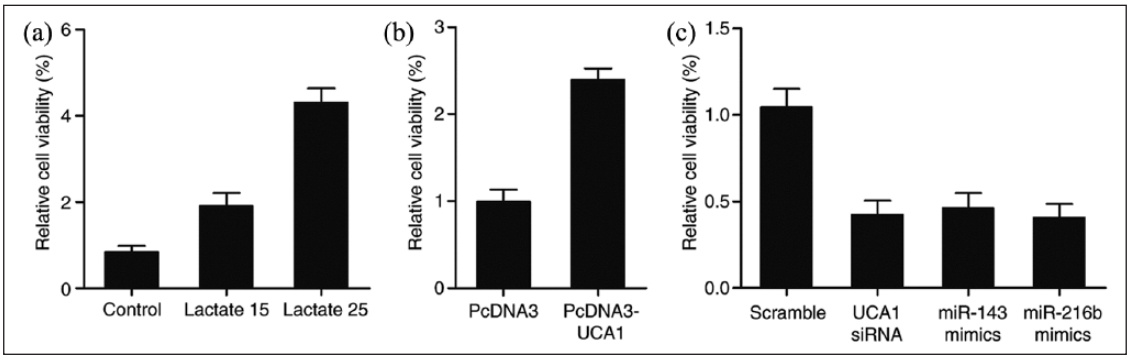

Figure 8. Administration of lactate and UCA1 enhanced HUVEC proliferation: (a) lactate administration enhanced the proliferation of HUVECs in a dose-dependent manner. (b) Overexpression of UCA1 enhanced the proliferation of HUVEC cells, and (c) UCA1 siRNA, and miR-143 and miR-216b mimics transfection suppressed cell viability.
miR: microRNA; HUVECs: human umbilical vein endothelial cells; UCA1: urothelial cancer associated 1.

acid.[38] Hence, glycolysis, the predominant pathway responsible for energy production, is fundamental for the growth and survival of cancer cells.[39] The mechanisms underlying the transition from aerobic respiration via the Kreb's cycle to aerobic glycolysis in cancer cells have been well established, and it has been suggested that HK2 may be involved in such mechanisms.[40] It has previously been demonstrated that incubating cells with lactate (20 mM) suppresses the phosphorylation of p38 MAPK and the trimethylation of histone H3 at lysine 4 (H3K4me3), which is associated with the suppression of the expression of muscle-specific genes in C2C12 myoblasts.[41] Reduced phosphorylation of p38 MAPK in C2C12 myoblasts following culture with lactate (20 mM) is consistent with the demonstrated suppression of H3K4me3 levels and muscle-specific gene expression. Myocyte-specific enhancer factor 2D is directly phosphorylated by p38 MAPK, which results in the formation of the active Ash2 histone methyltransferase complex subunit that in turn provides the H3K4me3 mark.[42] Vascular endothelial growth factors (VEGFs), particularly VEGF-A, are involved in the modulation of processes associated for angiogenesis: EC stimulation, proliferation, migration, and tubule formation.[43] p38 MAPK, a downstream effector of the VEGF signaling pathway, is an important regulator of the actin cytoskeleton remodeling necessary for endothelial proliferation.[44] Targeted gene deletion of either p38α is embryonically lethal; thus, the two are essential for angiogenesis and placental development.[45] In the present study, it was revealed that lactate administration induced the phosphorylation of p38-MAPK in a dose-dependent manner, and transfection with UCA1 siRNA, miR-143 mimics, or miR-216b mimics suppressed the phosphorylation of p38-MAPK. Furthermore, the effects of UCA1 on the phosphorylation of p38-MAPK were investigated, and it was demonstrated that UCA1 enhanced the phosphorylation of p38-MAPK.

The results of the present study suggested that the lncRNA UCA1/miR-143 miR-216b/HK2/lactic acid/MAPK axis may be involved in melanoma via the modulation of ECs. Furthermore, it was demonstrated that miR-143 and miR-216b function as endogenous competitive miRNAs for the binding of UCA1. In addition, it was also revealed that HK2 is a direct target of miR-143 and a potential target gene of miR-216b, as detailed in the online miRNA database. HK2 has been demonstrated to induce aerobic glycolysis, which may in turn activate the MAPK signaling pathway. It was also suggested that MAPK may enhance the proliferation of ECs, which is a major pathogenic mechanism involved in angiogenesis associated with melanoma. In conclusion, lncRNA UCA1 may serve as a potential therapeutic target for the treatment of melanoma.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

Written informed consent was obtained from all patients and the present study was approved by the Ethics Committee of Qilu Hospital Shandong University (ShanDong, China; No. SDUS20170124). The present study was performed in conformity with the Declaration of Helsinki and all the patients and their families were informed and they signed informed consent.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

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