Original Article

METTL3-induced UCK2 m^{6}A hypermethylation promotes melanoma cancer cell metastasis via the WNT/β-catenin pathway

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Background: Melanoma is a highly aggressive, malignant skin tumor with a statistically high mortality rate. N6-methyladenosine (m^{6}A) modification is involved in a variety of biological processes, including tumorigenesis. m^{6}A modifications regulate the fate and functions of RNA, such as mRNA stability, nuclear processing, transport, localization, translation, primary microRNA (miRNA) processing, and RNA-protein interactions. Several members (including METTL3, METTL14, FTO, ALKBH5, and YTHDF2) are actively involved in a variety of human cancers. However, the basic mechanism of the involvement of uridine cytidine kinase 2 (UCK2) in melanoma metastasis has not been studied. UCK2 is upregulated in a variety of malignancies. However, the complex molecular mechanisms and therapeutic effects of UCK2 in melanoma remain unclear.

Methods: The expression of UCK2 was evaluated by qRT-PCR. The effects of UCK2 on the biological characteristics of PC cells were investigated on the basis of loss-of-function analyses. Immunoprecipitation-qPCR (MeRIP-qPCR) was performed to identify the m^{6}A targeted effect of UCK2 in melanoma cancer.

Results: Based on the bioinformatics analysis in this study, up-regulation of UCK2 could be essential in melanoma cancer, and associated with poor survival. Furthermore, the m^{6}A modification regulated by METTL3 led to UCK2 increased messenger RNA (mRNA) stability in melanoma cancer. Functional and mechanistic experiments indicated that UCK2 enhanced the metastasis of melanoma cancer cells through the WNT/β-catenin pathway.

Conclusion: In this study, we found that m^{6}A-METTL3 axis induced abnormal UCK2 expression plays a role in melanoma metastasis by enhancing the Wnt/β-catenin pathway, which may provide new clues for melanoma metastasis. It also provides a potential target for the prevention and treatment of melanoma.

Keywords: Uridine-cytidine kinase 2 (UCK2); database; m^{6}A; metastasis; melanoma cancer

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Introduction

Melanoma is one of the most harmful skin cancers, usually originating from ultraviolet exposure from sunshine or tanning beds (1,2). In recent years, more than 150,000 people worldwide have been diagnosed with melanoma cancer annually, of which 30% of cases have been invasive and dangerous (3,4). Therefore, early diagnosis and suitable treatment of melanoma is critical and urgently required.

The N^6-Methyladenosine (m^{6}A) modification is implicated in diverse biological processes including tumorigenesis. The

m^{6}A modification regulates RNA fate and functions such as mRNA stability, nuclear processing, transport, localization, translation, primary microRNA (miRNA) processing, and RNA-protein interactions (5-7). Several members (including METTL3, METTL14, FTO, ALKBH5, and YTHDF2) actively participate in multiple human cancers. Recently, studies have reported that METTL3 plays a key role in melanoma cancer (8); however, the mechanism of METTL3 in melanoma has not been fully elucidated.

Uridine-cytidine kinase (UCK), an enzyme which can limit the rate of the salvage pathway of pyrimidine-nucleotide biosynthesis, has the ability to convert cytidine and uridine to cytidine 5'-monophosphate and uridine 5'-monophosphate on the condition of mono-phosphorylation, at the same time, the process requires nucleoside triphosphate (NTP) as a phosphate donor (9). Both *UCK1* and *UCK238* are UCK human genes, and have similar catalytic capabilities to UCK2. However, the ability of UCK2 to convert cytidine and uridine into cytidine 5'-monophosphate and uridine 5'-monophosphate is 15–20 times higher than that of UCK1(10), indicating that UCK2 plays an irreplaceable role in the salvage of pyrimidine-nucleotide biosynthesis. It has been found that UCK2 is upregulated in various cancers, like pancreatic tumor, colorectal cancer, neuroblastoma, breast cancer, and hepatocellular carcinomas (HCCs) (11-15), whereas the fundamental mechanisms of UCK2 involvement in melanoma cancer metastasis are essentially unknown. We present the following article in accordance with the MDAR reporting checklist (available at https://dx.doi.org/10.21037/atm-21-2906).

Methods

Cell lines

Melanoma cancer cell lines HS294T were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in humidified incubators with 5% CO_2 at 37 ℃.

Validation of differential expression in Gene Expression Profiling Interactive Analysis (GEPIA) and the UALCAN database

The GEPIA is a tool created by Dr. Zhang with his team at the lab of Peking University, adapting data from The Cancer Genome Atlas (TCGA) database. This paper used GEPIA to research the expression of UCK2 in a variety of tumors, and probe into the prognosis of melanoma expression-related UCK2.

Transwell invasion analysis

Transwell invasion analysis was performed as previously described to identify the migration and invasion abilities of cells (16). All cell culture reagents were incubated at 37 ℃.

To be measured cell culture to the logarithmic growth phase of digestion cells with and serum-free medium successively washed once.

Suspend cells with serum-free medium count, adjust the concentration to 2×10^5 cells/mL and add 600–800 μL medium containing 10 GH serum in the lower chamber, the bottom of the 24-well plate.

Add 100–150 μL cell suspension and incubate in incubator for 24 h.

Carefully remove the aspirated upper chamber liquid with tweezers and transfer it to the well chamber with about 800 μL methanol.

Set the temperature for 30 minutes .

Remove and blot the upper chamber fixator and transfer to the well with 800 μL of dye solution in advance. Staining at room temperature for 15–30 min.

Gently rinse with water and soak several times.

The cells in the upper chamber were removed by a cotton swab and the cells in the lower chamber were then stained with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA) for 10 min at room temperature. Finally, the invaded cells were counted and photographed under a light microscope (magnified ×400). Every experiment was independently replicated at least 3 times.

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

RT-PCR is a technology that combines reverse transcription (RT) of RNA and PCR of cDNA. First, cDNA is synthesized from RNA by the action of reverse transcriptase, and then the target fragment is amplified and synthesized by the action of DNA polymerase using cDNA as a template. RT-PCR technology is sensitive and versatile, and can be used to detect the level of gene expression in cells, the content of RNA viruses in cells, and the direct cloning of cDNA sequences of specific genes. In accordance with the SuperScript™ IV First-Strand Syn-thesis System kit operating instructions, TRIzol reagent was used to extract as much RNA as possible

from the sample (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Next, with the help of Prime Script™ RT Reagent kit (Takara Bio Inc., Kusatsu, Shiga, Japan), the reverse transcription from RNA (2 μg) to circular DNA (cDNA) was conducted, and the performance of quantitative polymerase chain reaction (qPCR) was finished using SYBR Green (Roche Diagnostics, Mannheim, Germany). The internal control and quantification of relative gene expression were performed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the $2^{-\Delta\Delta Cq}$ method, respectively.

Germany). The internal control and quantification of relative gene expression were performed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the $2^{-\Delta\Delta Cq}$ method, respectively.

GAPDH: Forward 5'-GGAGCGAGATCCCTCCAAAAT-3'; Reverse 5'-GGCTGTTGTCATACTTCTCATGG-3'; UCK2: Forward 5'-TTCCCAAGAAGGTTCGATTG-3'; Reverse 5'-TGCAGACTCCTGCTGTTGTT-3'; CTNNB1: Forward 5'-GATAGTTGTGATCGCCTCACC-3'; Reverse 5'-GTCCTCTGAGTCGAAGCTGTC-3'; MYC: Forward 5'-CCCCTGTGGCTAACAGTTACA-3'; Reverse 5'-AGGTAGCTTTTAAGGCTTGACTC-3'; MMP7: Forward 5'-GGGTGACATCGGGAGAACG-3'; Reverse 5'-CTGAACAGGCTTCGTAACTCAT-3'; SOX13: Forward 5'-CGATGGAACTTCGACTTTGTCA-3'; Reverse 5'-GCACAAGGGTACAAGACAGTG-3'; DKK1: Forward 5'-AACGTGCGAGTGTCTAACGG-3'; Reverse 5'-CCCTCTAGGGGTTTGTGATTCT-3'; HNF1A: Forward 5'-TCCAGCATAGCTTTAGCTTTGC-3'; Reverse 5'-GGTCATCGGCGCTCAGAATAG-3'.

Cell transfection

Control small interfering RNA (siRNA, si-control), UCK2 siRNA (siUCK2 #1, #2, #3), and METTL3 siRNA (siMETTL3 #1, #2, #3) were designed and synthesized by Gene Pharma (Shanghai, China), who also supplied the lentivirus normal control (LV-NC) and UCK2-expressing lentivirus (LV-UCK2) To prevent cell transfection, Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used in line with the manufacturer's instructions. Cells for succeeding experiments were collected 48 h later.

The target sequence of siUCK2 #1: 5'-GCCCAAUAAUGCAUAUACUTT-3'; #2: 5'-GCUCUGGAUAUAGUAGCAATT-3'; #3: 5'-GCGGGUCCAUUACUAGUAATT-3'.

The target sequence of siMETTL3 #1: 5'-GGCAAGTACACAGATCTTAAC-3'; #2: 5'-AAUCUUAAGUUUCGUAAGUUA-3'; #3: 5'-GCAACACAACCGAAGATGACT-3'.

A scrambled shRNA targeting 5'-TTCTCCGAACGTGTCACGT-3' was used as a negative control.

TOP/FOP luciferase reporter assay

Transcriptional activity was assayed using the Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The luciferase activity was standardized for every sample and Renilla luciferase was used as an internal control to monitor the quality of transfecting. After transfection, the plasmids were allowed to stand for 24 h, followed by lysis, and the Dual-Luciferase Reporter (DLR) Assay Kit (Promega) was used to measure the luciferase activity. Finally, the Firefly and Renilla luciferase activity were determined with the help of a luminometer (Lumat LB9507, Berthold, Bad Wildbad, and Germany) and normalized.

m^6A-qPCR and RNA stability assays

The abovementioned assays were performed as previously reported (17).

Statistical analysis

Statistical analysis, Kaplan-Meier survival analyses, and independent sample *t*-tests were performed using the software SPSS version 23.0 (IBM Corp., Armonk, NY, USA). A P value <0.05 was considered statistically significant.

Results

UCK2 expression and survival analysis in melanoma cancer based on the GEPIA database

Firstly, we verified the difference of UCK2 expression by using the GEPIA tool database in melanoma, revealing that the mRNA of UCK2 was increased by a more than 1.5-fold change compared to normal tissues (*Figure 1A*). As shown in *Figure 1B* and *C*, the overall survival (OS) and disease free survival (DFS) curve confirmed that advanced UCK2 expression indicated a poor prognosis in melanoma.

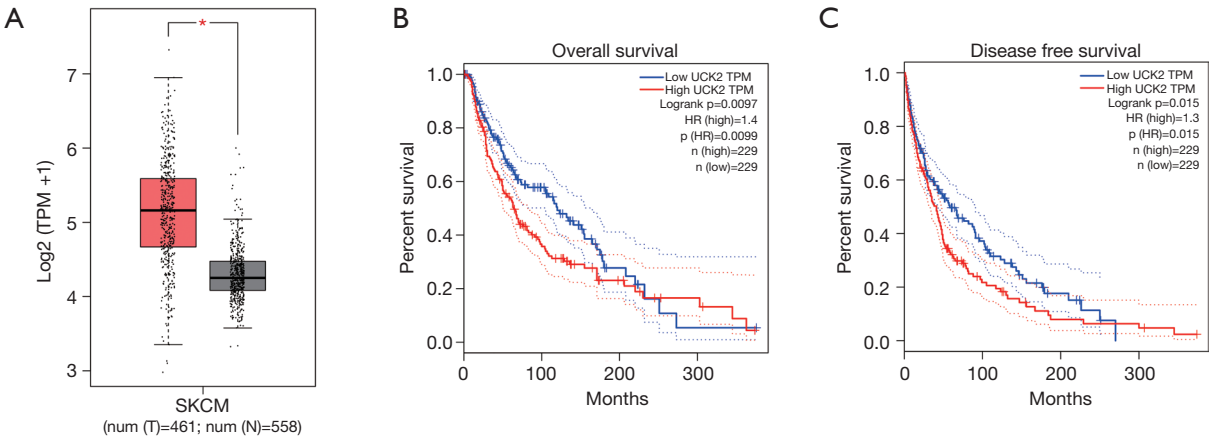

Figure 1 UCK2 expression and survival analysis in melanoma cancer based on the GEPIA database. (A) The expression of UCK2 was increased in melanoma tissues compared to normal tissues; (B,C) survival analysis of UCK2 in 458 samples in TCGA database demonstrated that high UCK2 expression was significantly associated with a reduced OS and DFS using Kaplan-Meier Plotter. *P<0.05, based on the Student's *t*-test. GEPIA, Gene Expression Profiling Interactive Analysis; TCGA, The Cancer Genome Atlas; OS, overall survival; DFS, disease free survival.

UCK2 enhanced Wnt/β-catenin signaling in melanoma

Based on GEPIA database analysis, the UCK2 expression was positively correlated with a couple of Wnt/β-catenin signaling-relative genes (*Figure 2A*), indicating that UCK2 might play key role in regulating Wnt/β-catenin signals within melanoma To address the effects of UCK2 regulating Wnt/β-catenin signals in melanoma, the siRNAs were adapted for silencing UCK2 expression within melanoma cancer cells (*Figure 2B*, left panel). Furthermore, LV-UCK2 was used to establish UCK2 stable cell lines in HS294T (*Figure 2B*, right panel). As shown in *Figure 2C* (left panel), after being stimulated by Wnt3a, UCK2 knockdown could remarkably inhibit the expression of Wnt targets, while increased UCK2 up-regulated the expression of Wnt targets inhibited by XAV939 (a Wnt inhibitor) (*Figure 2C*, right panel). To investigate the more latent effects of UCK2 within Wnt signaling, we actively determined the endogenous Wnt/TCF within melanoma cancer cells. As expected, UCK2 knockdown could remarkably inhibit endogenous Wnt/TCF luciferase reporter activity (*Figure 2D*, left panel) and overexpressed UCK2 enhanced endogenous Wnt/TCF luciferase reporter activity within HS294T cells (*Figure 2D*, right panel).

UCK2 promoted melanoma cell invasion through activating Wnt/β-catenin signaling

As shown in *Figure 3A*, XAV939 could strongly inhibit the signal transduction of Wnt/β-catenin, which then restrained the migration and invasion of melanoma cell lines which can express significant levels of UCK2. Additionally, we readily observed the activation of Wnt/β-catenin signal transduction via WNT3a incubation, which could remarkably restore the abilities of migration and invasion when the siUCK2 treatment was finished (*Figure 3B*). In conclusion, the above results showed that melanoma cell metastasis could be promoted by activation of Wnt/β-catenin signaling by UCK2.

METTL3 induced UCK2 m^{6}A to enhance its mRNA stability

Based on GEPIA database analysis, UCK2 expression was positively correlated with METTL3 (*Figure 4A*), indicating that UCK2 might be regulated by METTL3 in a m^{6}A pattern. Through qRT-PCR, it was indicated that knockdown of METTL3 led to a decrease of UCK2 in melanoma cells (*Figure 4B,C*). Further, m^{6}A-qPCR indicated that METTL3-induced the hypermethylation of UCK3'UTR m^{6}A

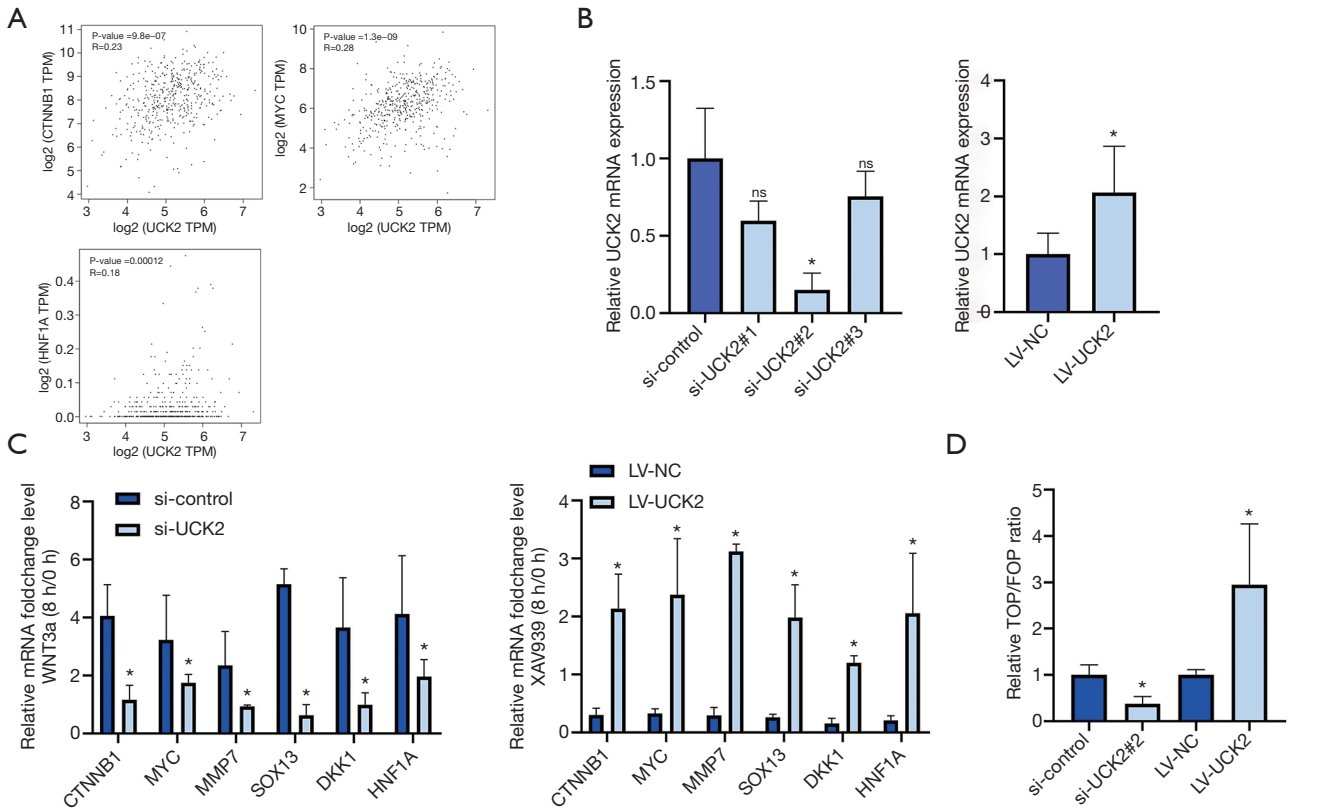

Figure 2 UCK2 Enhanced Wnt/β-catenin signaling in melanoma. (A) The correlation the Wnt-related genes and UCK2 based on GEPIA database; (B) qRT-PCR analysis of the level of UCK2 in indicated melanoma cancer cell lines; (C) the differences between the si-control treatment and silencing UCK2 on Wnt target expression after 8 h induction of Wnt3a (1.5 ng/mL), and LV-NC and LV-UCK2 on Wnt target expression after 8 h Wnt3a (2 nM) of induction in HS294T cell lines; (D) the changes in endogenous Wnt/TCF reporter activity in HS294T cell lines after silencing UCK2 or overexpressed UCK2 expression. *P<0.05, based on the Student's *t*-test. GEPIA, Gene Expression Profiling Interactive Analysis; qRT-PCR, quantitative real-time polymerase chain reaction; LV-NC, lentivirus normal control; LV-UCK2, UCK2-expressing lentivirus.

(*Figure 4D*). A previous study reported that m^{6}A modification could enhance mRNA stability (10). Our results confirmed that silencing of METTL3 reduces the mRNA stability of UCK2 in melanoma (*Figure 4E*). The results above indicated that METTL3 induces UCK2 m^{6}A hypermethylation to enhance its mRNA stability in melanoma.

Discussion

Over the past years, the prognosis of melanoma patients has largely improved. Unfortunately, few patients are able to achieve a sustained response, and most will eventually succumb to these diseases (18). Therefore, further research is urgently needed to identify more effective therapeutic schedules in order to achieve better clinical outcomes in melanoma. While a number of genes have been identified as having a relationship with melanoma cancer progress, the molecular mechanism has still not been clearly explained.

The pyrimidine ribonucleotide kinase UCK2 can catalyze phosphorylation of uridine-to-uridine monophosphate and cytidine-to-cytidine monophosphate. If UCK2 is overexpressed, it can lead to poor prognosis of several cancers, including those of the liver, pancreas, breast, and lung (19-23). Research by Zhou *et al.* showed that UCK2 has the ability to promote metastasis by up-regulating the expression of MMP2/9 and activating the signal transduction of STAT3 (24). However, the detailed molecular mechanisms and therapeutic role of UCK2 in melanoma cancer cells invasion remain to be elucidated. In existing studies, based on the GEPIA database, it was not

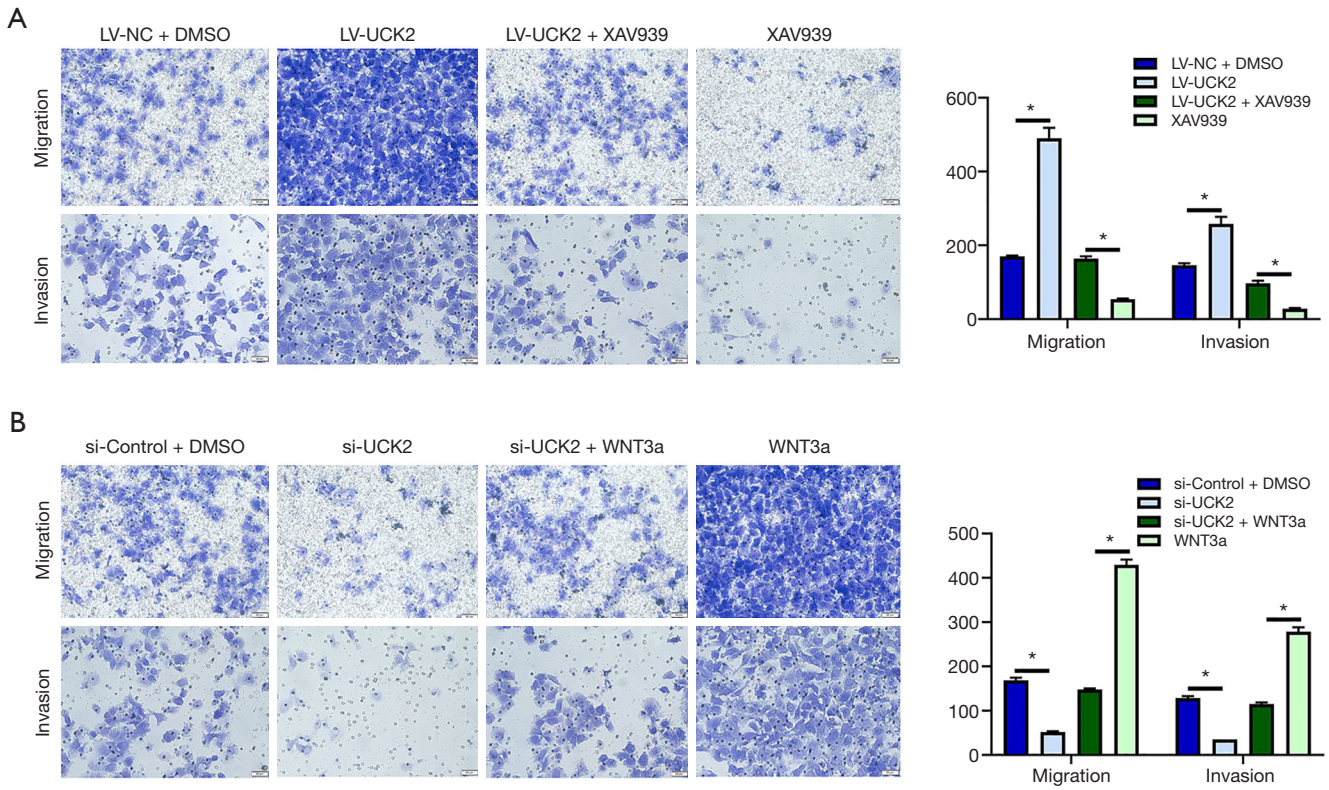

Figure 3 UCK2 promoted melanoma cells invasion through activating Wnt/β-catenin signaling. (A,B) Cell migration and invasion were determined by 0.1% crystal violet staining after 24 hours (magnified ×200). Results are shown as mean ± SD, n=3. *P<0.05, based on the Student's *t*-test. SD, standard deviation.

hard ascertaining an obvious increase in the expression of UCK2 in the melanoma specimens when compared with normal tissues. Meanwhile, there is an association between high UCK2 expression and dismal survival rate.

In the process of melanoma cancer, an aberrant activation of the Wnt/β-catenin signaling pathway can be found (25,26). Nevertheless, UCK2 has an uncertain relationship with the Wnt/β-catenin pathway in melanoma. In this study, we found that UCK2 was likely to be capable of activating the Wnt/β-catenin pathway in melanoma. Besides, UCK2 enhanced the migration and invasion of melanoma cells via the activation of Wnt/β-catenin pathway. Our subsequent study will focus on the precise mechanism by which UCK2 regulates the Wnt/β-catenin pathway in melanoma.

It is accepted that METTL3 is involved in all stages of the life cycle of RNA. It plays a pivotal role in pre-mRNA splicing, 3'-end processing, nuclear export, translation regulation, mRNA decay, and miRNA processing (27-31). Accordingly, METTL3 affects tumor formation via regulation of the m^{6}A modification in the mRNAs of critical oncogenes or tumor suppressors (32). In our study, it was indicated that METTL3 induced UCK2 3'UTR m^{6}A hypermethylation to enhance UCK2 mRNA stability in melanoma. This suggested that METLL3 could exhibit its oncogene roles in melanoma by regulating UCK2 mRNA levels via m^{6}A modification.

In summary, the present study revealed that abnormal expression of UCK2 induced by the m^{6}A-METTL3 axis plays a role in the metastasis of melanoma through enhancement of the Wnt/β-catenin pathway, which can provide novel clues for melanoma metastasis; at the same time, it can provide possibility targets for the prevention and treatment of melanoma cancer.

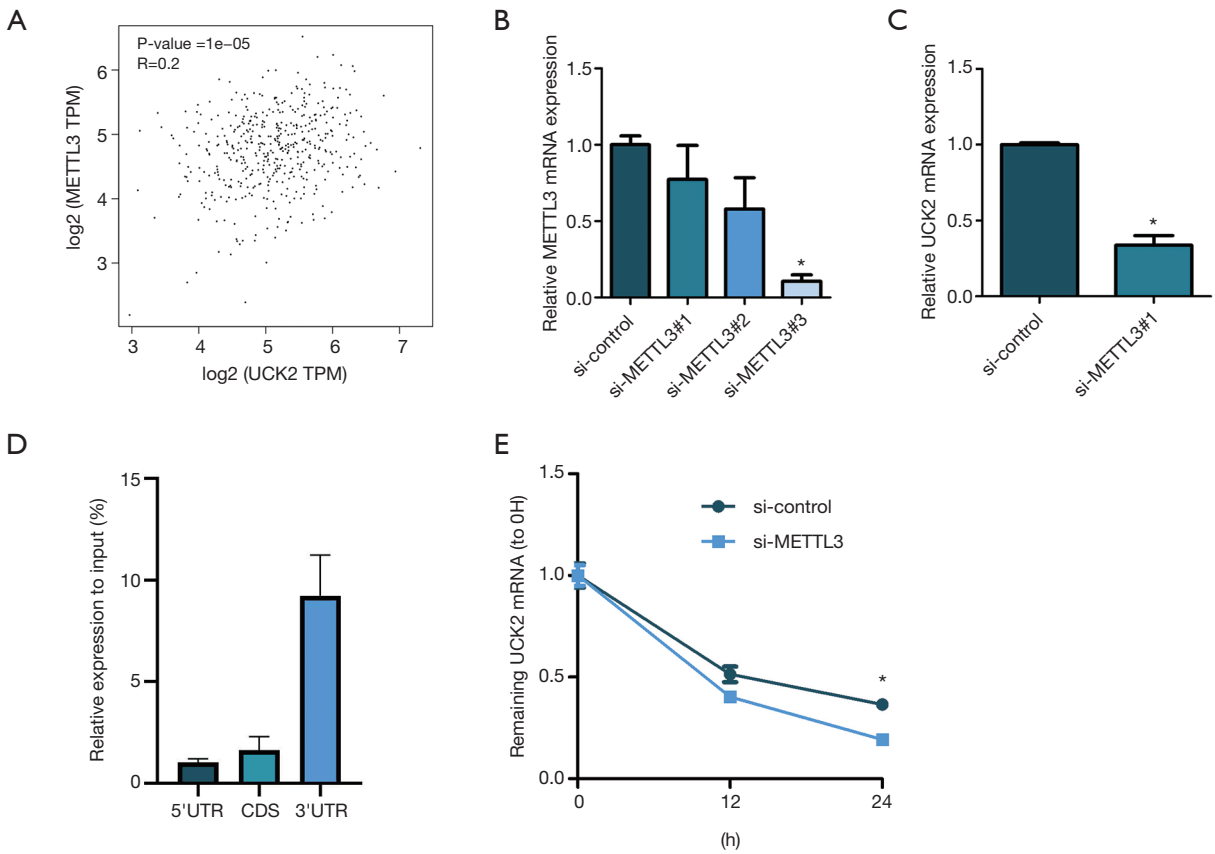

Figure 4 METTL3 induced UCK2 m^{6}A to enhance its mRNA stability. (A) The correlation of METTL3 and UCK2 based on GEPIA database; (B,C) qRT-PCR analysis of the level of METTL3 and UCK2 in indicated melanoma cell lines; (D) m^{6}A level in UCK2-5'UTR, 3'UTR, and CDS region in HS294T cell lines; (E) qRT-PCR analysis of UCK2 mRNA relative to GAPDH in the indicated treated melanoma. (B,C,E) *P<0.05, based on the Student's *t*-test. mRNA, messenger RNA; GEPIA, Gene Expression Profiling Interactive Analysis; qRT-PCR, quantitative real-time polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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Footnote

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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.

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