Long Non-Coding RNA *LINC00518* Induces Radioresistance by Regulating Glycolysis Through An miR-33a-3p/HIF-1 α Negative Feedback Loop in Melanoma

Ke Cao (✉ csucaoke@163.com)

Department of oncology

Liu yan

Central South University Third Xiangya Hospital

He Dong

the second Hunan Provincial People's Hospital

Xiao Mengqin

Central South University Third Xiangya Hospital

Xiang Liang

Central South University Third Xiangya Hospital

Zhu Yuxing

Central South University Third Xiangya Hospital

Research

Keywords: LINC00518, Negative feedback loop, Glycolysis, Radioresistance

Posted Date: July 2nd, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-37465/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Background: The long non-coding RNA (lncRNA), *LINC00518*, is highly expressed in many human cancers and is involved in cancer progression. However, the potential function and regulatory mechanism of *LINC00518* in cutaneous malignant melanoma (CMM) remain unclear.

Methods: Short hairpin RNA (shRNA) was used to silence *LINC00518* and HIF-1 α , and real-time PCR was performed to determine mRNA expression. Then, cell proliferation, colony formation, flow cytometric, scratch, and transwell assays were used to examine the influence of *LINC00518* silencing on cellular radiosensitivity. Dual luciferase reporter system, CHIP and COIP was used to verify the target relationship between *LINC00518*, miR-33a-5b and HIF-1 α . Glycolysis assays were conducted to examine cell glycolysis process. Western blotting was performed to explore the expression of HIF-1 α and LDHA. Finally, animal experiments were performed to demonstrate the effect of *LINC00518* silencing on the radiosensitivity of melanoma in vivo.

Results: *LINC00518* expression was significantly upregulated in CMM samples, and *LINC00518* levels were associated with poor prognosis of patients with CMM. Knockdown of *LINC00518* in CMM cells significantly inhibited cell invasion, migration, proliferation, and clonogenicity. *LINC00518*-mediated invasion, migration, proliferation, and clonogenicity were negatively regulated by the microRNA, miR-33a-3p, *in vitro*, which intensified sensitivity to radiotherapy via inhibition of the hypoxia-induced factor 1 α (HIF-1 α)/lactate dehydrogenase A (LDHA)-glycolysis axis. Additionally, HIF-1 α recognized the miR-33a-3p promoter region and recruited histone deacetylase 2 (HDAC2), which decreased the expression of miR-33a-3p and formed an *LINC00518*/miR-33a-3p/HIF-1 α negative feedback loop. Furthermore, signalling initially activated glycolysis and radioresistance in CMM cells was recovered by Santacruzamate A (a histone deacetylase inhibitor) and 2-deoxy-D-glucose (a glycolytic inhibitor). Lastly, knockdown of *LINC00518* expression sensitized CMM cancer cells to radiotherapy in an *in vivo* subcutaneously implanted tumour model.

Conclusion: *LINC00518* was confirmed to be an oncogene in CMM, which induces radioresistance by regulating glycolysis through an miR-33a-3p/HIF-1 α negative feedback loop. Our research may provide a potential strategy to improve the treatment outcome of radiotherapy in CMM.

Background

Cutaneous malignant melanoma (CMM) is one of the commonest skin malignancies, with rapid progression and extremely poor prognosis, and is responsible for nearly 75% of all skin tumour mortalities [1,2]. Despite improvements in treatment, such as surgery, radiotherapy, chemotherapy, immunotherapy, and molecular-targeted treatment, CMM recurs in approximately 75% of patients a year after treatment, and the three-year overall survival rate of terminal CMM patients is no more than 30% [3-5]. The National Cancer Comprehensive Network guidelines (2018) point out that radiotherapy is the main available treatment for some primary CMMs, and adjuvant radiotherapy is necessary for

infiltrating neutrophilic melanoma, metastatic encephaloma, and bone metastases [6,7]. In recent years, some studies have found that palliative radiotherapy significantly eased symptoms such as

bone pain and central nervous system dysfunction produced by MM metastases [8,9]. However, radioresistance limits the clinical application of radiotherapy to treat CMM, leading to poor prognosis. Thus, identification of approaches to overcome

radioresistance CMM could provide more effective therapeutic strategies and improve outcomes of patients with CMM.

Hypoxia, a common phenomenon in animal and human solid tumours, is an important contributor to radioresistance and can act as a prognostic indicator of the outcome of radiotherapy [10,11]. Conceptual progress in the last decade has indicated that reprogramming of energy metabolism is a newly emerging hallmark of cancer and that hypoxia plays a significant role in radioresistance because of reduced cell viability and fixation of DNA damage [12,13]. In a hypoxic microenvironment, cancer cells obtain energy mainly by inhibiting aerobic respiration and promoting glycolysis [14]. Many glycolysis- and metabolism-related proteins participate in the molecular mechanisms that regulate radioresistance. The hypoxia-induced factor 1 α (HIF-1 α), is an important factor that aggravates the transformation of normal melanocytes into melanoma, and HIF-1 α activity in melanoma tissues and cell lines even exhibited an increase in atmospheric oxygen levels [15,16]. The HIF-1 α pathway is involved in a tumour-protective response to radiotherapy, which is achieved mainly by increasing cancer antioxidation by launching glycolytic tumour metabolism [17]. Targeting tumour glucose metabolism and HIF-1 α could change the tumour microenvironment, leading to metabolic alterations and sensitization of multiple solid cancers to radiotherapy [18]. Additionally, several studies have found that the levels of certain glycolysis-related proteins are closely connected to radiotherapy resistance; for instance, higher expression of glucose transporter 1 (GLUT1) in breast and liver cancers indicated poor prognosis of patients treated with radiotherapy [19,20]. HK2 (Hexokinase 2) is a critical rate-limiting enzyme in the glycolytic pathway. Multiple regression analysis models implied that HK2, which facilitates glycolysis and inhibits cancer cell apoptosis, was an independent negative prognostic factor for cervical squamous carcinoma [21].

Long non-coding RNAs (lncRNAs) are an important class of non-coding RNAs of >200 bases in length that show limited protein coding capability but are closely correlated with the occurrence and development of human diseases [22,23]. lncRNAs participate in a series of modifications of the glycolysis pathway in cancer cells: lncRNA *IDH1-AS1* links the functions of MYC proto-oncogene, BHLH transcription factor (c-MYC), and HIF-1 α via isocitrate dehydrogenase (NADP(+)) 1, cytosolic (IDH1), to regulate mitochondrial respiration and glycolysis in cervical carcinoma cells. Restoring *IDH1-AS1* expression might provide a potential metabolic approach to treat cervical carcinoma [24]. lncRNA-*p21* is a hypoxia-responsive lncRNA that causes HIF-1 α accumulation by integrating HIF-1 α and Von Hippel-Lindau tumour suppressor (VHL), thus destroying the VHL–HIF-1 α interaction, which promotes glycolysis under hypoxic conditions in colorectal cancer cells [25]. Studies have shown that lncRNAs, such as

LncRNA-TUG1 and *LncRNA-MIF*, participate in the glycolytic pathway and regulate glycometabolism via competing endogenous RNA (ceRNA) networks [26-29].

Although recent research has clarified that some lncRNAs participate in regulating radiotherapy resistance; till date, no studies have shown that lncRNAs play significant roles in regulating CMM radiosensitivity. In the present study, we analysed two CMM data sets, GSE46517 [30] and GSE4587 [31], which were published in the GEO database, to investigate dysregulated lncRNAs in CMM. Long intergenic noncoding RNA 518 (*LINC00518*) was significantly upregulated in CMM tissues compared with that in adjacent tissues. The Cancer Genome Database (TCGA) data set showed that high expression of *LINC00518* indicated a worse relapse-free survival (RFS) and overall survival (OS) of patients with CMM. Furthermore, we verified that *LINC00518* could form a negative feedback loop with HIF-1 α and increase radiation resistance of CMM by modulating the glycolytic pathway. The results of the present study provide a new perspective concerning the role of lncRNAs in the radiation sensitivity of CMM and may assist in identifying new biomarkers or targeted therapies for CMM.

Materials And Methods

Data mining and analysis

To identify functional lncRNAs in CMM, the gene expression patterns of two different cohorts of CMM, derived from the Affymetrix GeneChip® Human Genome U133 Plus 2.0 Array, GSE46517 and GSE4587, were downloaded from the Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>). The Significant Analysis of Microarray (SAM) mode was used to analyse the different expression levels of lncRNAs between adjacent and CMM tissue samples in these two data sets. The critical value for the expressed lncRNAs was set at ≥ 2.0 -fold change and a false discovery ratio (FDR) of < 0.05 .

Cell lines and tissue

Human malignant melanoma cell lines (WM35, WM451, and A375) and human melanocytes (HM) were purchased from the ATCC (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin under a humidified atmosphere of 5% CO₂ at 37 °C. Human CMM tissues and corresponding adjacent non-cancerous skin tissues were collected from patients who underwent surgery and were pathologically diagnosed with MM at the Third Xiangya Hospital of the Central South University and Hunan Cancer Hospital from January 2015 to December 2017. Informed consent was obtained from all the participants.

Cell transfection

MiR-33a-3p-mimics, and-inhibitors, LINC00518-shRNAs, HIF-1 α -shRNAs, HIF-1 α overexpression plasmid, and negative controls (NC) were purchased from GeneChem Biotechnology Company. Cells were

transfected as previously described [32] . CMM cells were cultured in 6-well plates and allowed to reach 70% confluence after 24 h. Lipofectamine™ 2000 (Invitrogen Life Technologies) was used to transfect cells with DNA complexes following the manufacturer's instructions. The cells were harvested after 24 h and 36 h, and RNA and protein were extracted after transfection, respectively. Real-time PCR was used to confirm the efficiency of transfection.

Quantitative real-time reverse transcription PCR

Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The PARIS kit (Ambion) was used to isolate RNA from the cytoplasm and nuclei of transfected cells. Real-time PCR was performed using RNA isolated from the cytoplasmic and nucleic fractions. RNA purity was assessed by spectrophotometry ($A_{260}/A_{280} > 1.8$). To perform quantitative real-time reverse transcription PCR (qRT-PCR), 1 µg of RNA from the samples was reverse transcribed using a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas #K1631; Thermo Fisher Scientific, Waltham, MA, USA). qRT-PCR was performed to assess the expression of *LINC00518* and miR-33a-3p using a SYBR Green PCR Master Mix (ABI 4309155) employing the CFX96 Real-Time PCR Detection System (ABI 7900; Applied Biosystems, Foster City, CA, USA). PCR amplifications were performed in triplicates. The sequences of the qRT-PCR primers were as follows:

LINC00518: forward:5'-TGCAATTCAGGTCGGTTGTA-3';

reverse:5'-GTGGAGCTCCCTGAAGACAG-3'.

miR-33a-3p: forward:5'-ACACTCCAGCTGGGCAATGTTTCCACAGTG-3';

reverse:5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGGTGATGCA-3'.

β-actin:forward:5'-GTGGGGCGCCCCAGGCACCA-3';

reverse:5'-CTCCTTAATGTCACGCACGATTTC-3'.

ACTB (encoding β-actin) was used as an internal reference.

Western blotting

Phosphate-buffered saline (PBS) was used to wash the cancer biopsy specimens and cancer cells, and later the cells were lysed on ice using the radioimmunoprecipitation assay (RIPA) buffer containing 10% protease inhibitor cocktail (Roche Applied Science, Basel, Switzerland) for 30 min. Proteins were quantified using a Total Protein Extraction Kit (ProMab, Richmond, CA, USA). The proteins were separated using a 10% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes. The membranes were incubated with the following primary antibodies: anti-HIF-1α (1:1000, Bioss, bs-20398R), anti-lactate dehydrogenase A (LDHA) (1:50, Proteintech, 19987-1-AP), anti-Histone H3 (acetyl K4) (1:500, Abcam, ab232931), anti-Histone H3 (acetyl K9) (1:2000, Abcam, ab4441), anti-Histone H3 (acetyl K27) (1:200, Abcam, ab177178), anti-Histone H3 (1:1000, CST,#4499), and anti-β-Actin (1:2000, CST, # 3700S)

at 4 °C overnight, followed by the addition of HRP-conjugated secondary antibodies at 37 °C for 60 min. The signals of the immunoreactive protein bands were assessed using densitometric analysis software and quantified by densitometry using the ImageJ system. β -actin served as a loading control.

Invasion and migration assays

The migration capacity of CMM cells was assessed using a wound-healing assay. Cells (1×10^5 per ml) were cultivated in 6-well plates and grown to 85–90 % confluence. A wound was scratched in the middle of the monolayer using a 10- μ l pipette tip, and the cells were washed twice with 10% PBS to dislodge cellular debris. A photograph was taken at 0 h under a microscope. The plates were placed in a humidified atmosphere of 5% CO₂ at 37 °C, and the wound was photographed again at 48 h. The difference between the two measured widths on the images represented the extent of cell migration.

CMM cell invasion capacity was detected using a Transwell assay. Cells (1×10^5) in 100 μ L of serum-free medium were added to the apical chamber of a Transwell plate that was pre-coated with Matrigel matrix, and 500 μ L of 10% FBS-containing medium was added to the lower chamber. After incubation for 48 h at 37 °C, the invading cells that traversed the membrane were fixed with methanol and stained with 0.1% crystal violet. Cells that remained on the upper chamber surface of the Transwell membrane were wiped off using a cotton bud. Six visual fields at 20 $\times$ magnification were randomly captured and the numbers of invaded tumour cells were counted.

Cell proliferation bioassay

CMM cells treated with a short interfering RNA (siRNA) or plasmid were cultivated in 96-well plates (500 cells per well). Cell proliferation after irradiation or transfection was determined using a commercial 3-(4,5-dimethylthiazol-2-yl)-2,5-

Diphenyltetrazolium bromide (MTT) assay kit (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. First, 50 μ L of MTT was added to the cells in the wells and incubated for 4 h. Later, 150 μ L of dimethyl sulfoxide was added to dissolve the MTT crystals. The light absorption value of each well was measured using a spectrometer at 570 nm. All the assays were repeated no less than three times in triplicate wells.

Cell cycle and apoptosis assays

Cell cycle and apoptosis assays were performed using flow cytometry. CMM cells transfected with siRNA or vector were cultivated in 6-well plates. Cells were treated with or without irradiation or additional processing for 48 h, and then harvested by centrifugation. An annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining kit (Mebchem) and cell cycle detection kit (Sigma) were used to stain the cells, according to the manufacturers' protocols. All the tests were performed in triplicates.

Clonogenic assay

A clone formation assay was used to assess the proliferation activity and cellular radiosensitivity of MM cells. Cells transfected with the siRNA or plasmid were seeded in 6-well plates at 1000 cells per well. The cells were treated with or without irradiation or additional processing and then incubated for 14 days. The culture medium was replaced at intervals of 2–3 days. MM cells were washed twice with 10% PBS before being harvested and immobilized using 1 mL of poly-formaldehyde for 45 min. The surviving fractions (>50 cells) were stained with haematoxylin and counted under a microscope. All the clone formation assays were performed in triplicates.

Dual-luciferase experiment

For the luciferase reporter experiment, the *LINC00518*-WT (wild-type), *LINC00518*-MUT (mutant), HIF-1 α -3'-UTR-WT, and HIF-1 α -3'-UTR-MUT vectors were used. WM451 and A375 cells were seeded in 6-well plates and co-transfected with hsa-miR-33a-3p mimics or empty plasmids for 48 h. The dual luciferase reporter system (Promega) was used to assess firefly and sea pansy luciferase activities. The relative luciferase activities were calculated, and control cells were used for normalization.

Chromatin Immunoprecipitation (ChIP)

WM451 and A375 cells were treated with or without Santacruzamate A (an HDAC inhibitor) and underwent two rounds of dual crosslinking. The protein-DNA complexes were precipitated using anti-H3K27AC antibodies (ab177178). Signal intensity of the promoter region of the target gene was determined from PCR results. The signal strength of the miR-33a-3p promoter was measured by PCR using a SYBR Green PCR Master Mix Kit (ABI 4309155). According to the promoter region of miR-33a-3p, containing HIF-1 α -binding sites (AGACGTGA and GGGCGTGG), primers were designed to analyse the purified DNA. The following primer sequences for qRT-PCR were used:

miR-33a-3p forward: 5'-CTTAGCAGCAGACGTGATGG-3';

reverse: 5'-GAGTCGAGAGGCAGGTCACT-3'.

GAPDH: forward: 5'-TACTAGCGGTTTTACGGGCG-3';

reverse: 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'.

RNA pull-down assay

An RNA pull-down assay was performed using the Magnetic RNA-Protein Pull-Down Kit (Pierce™). *In vitro* biotin-labelled (Bio)-miR-NC, Bio-miR-33a-3p, and Bio-miR-33a-3p-Mut were transfected into WM451 and A375 cells, and the cells were for 48 h. The cell lysates were collected and incubated with streptavidin magnetic beads, forming protein-Bio/RNA-magnetic bead complexes. High salt elution was used to obtain the protein-bio/RNA from the magnetic bead-protein-bio/RNA mixture. The TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to purify the protein-bio/RNA, and PCR was used to measure the relative expression of *LINC00518*.

Co-immunoprecipitation assay

WM451 and A375 cells transfected with an siRNA or a plasmid overexpressing HIF-1 α were seeded in a 10 cm petri dish. The cells were washed twice with pre-chilled PBS and disrupted using RIPA lysis buffer (1 mL for 10⁷ cells). Protein A+G Agarose (Bioss, P1012) was used to precipitate protein complexes. The collected complexes were subjected to SDS-PAGE followed by western blotting. Anti-histone deacetylase 1 (HDAC1) (1:100, SAB, 32034), anti-HDAC2 (1:100, Abcam, ab12169), and anti-HIF-1 α (1:200, Bioss, bs-20398R) were used as detection antibodies; anti-IgG (1:150, Bioss, bs-0297P) was used as a loading control.

Measurement of glucose consumption and lactate production

Glucose consumption and lactate production were estimated as previously described [33]. Briefly, cells (5 $\times$ 10⁵) were recovered and cultured in 6-well plates. After incubation for 10 h, the cell culture medium was removed and the cells further incubated with fresh medium for 8 h. Glucose and lactate levels were measured (Automatic Biochemical Analyzer, 7170A, HITACHI, Tokyo, Japan) at the Clinical Biochemical Laboratory of Third Xiangya Hospital. The relative rates of glucose consumption and lactate production were normalized using consistent amounts of protein.

X-ray irradiation

X-ray irradiation was performed using an X-ray generator (Varian, USA), emitting at a fixed dose rate of 4 Gy/min. The energy of the X-rays used to irradiate

the cells was graded as 0, 2, 4, 6, and 8 Gy.

Xenograft Mouse Model

Male BALB/c nude mice (5 weeks old, 18 $\pm$ 0.75 g) were purchased from the Laboratory Animal Center of Central South University (Changsha, China) and were maintained under specific pathogen-free conditions (IVC) in the Experimental Animal-feeding Centre of the Xiangya Medical College of Central South University (Changsha, China). To generate tumours *in vivo*, WM451 and A375 cells (1 $\times$ 10⁶, 0.2 ml) were treated with shRNA-mock1, shRNA-mock2, shRNA-LINC00518-transfected, or Santacruzamate A, co-cultured, and injected into mice that were divided into four groups: A, B, C, and D (n = 3 animals per group, 5 weeks old); knockdown assays were performed as previously described [32]. The cells were injected subcutaneously on the superior border of the right upper limb, and isoflurane anaesthesia machine was used to administer inhalational anaesthesia to the animals. The procedure was simple and painless (Induction: 2-4%, ZS, Beijing, ZS-MV-HR). When the mice developed a 100-mm³ tumour, a single 2 Gy dose of irradiation was delivered to the B, C, and D groups. The tumour volume was estimated every 2 days by measuring the tumour length (L) and width (W). The tumour volume (V) was calculated using the formula $V = 1/2(L \times W^2)$. After 40 days, all the mice were sacrificed by cervical dislocation at the Laboratory Animal Center of the Xiangya Medical College of Central South University and the tumours were collected. All

the animal experiments were approved by the Ethics Committee of the Third Xiangya Hospital of Central South University.

Statistical analysis

The statistical software package GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used for all statistical analyses. All experiments were performed at least in triplicates. All data are shown as mean $\pm$ SD, and $P < 0.05$ was considered to be statistically significant. Differences between the groups were analysed using analysis of variance (ANOVA) followed by comparison between specific groups using Student's *t* test.

Results

***LINC00518* is overexpressed in CMM and indicates poor prognosis in patients with CMM**

To identify novel lncRNAs active in CMM, we downloaded two cohorts of gene expression data sets for CMM from the GEO database (GSE46517 and GSE4587). The SAM software was used to analyse differences in lncRNA expression in biopsy samples of CMM and adjacent non-cancerous skin tissues. We identified 12 lncRNAs that showed increased expression in CMM compared with normal skin tissues (Fig. 1A). Among the differentially expressed lncRNAs, *LINC00518* was overexpressed in the CMM samples from both the data sets (Fig. 1B) and had never been reported in human CMM. Based on data from the TCGA database, we found that the expression level of *LINC00518* was higher in CMM than in non-CMM samples (Fig. 1C). Patients with CMM who showed high expression of *LINC00518* had a shorter OS ($P = 0.0009$, Fig. 1C) and RFS ($P = 0.038$, Fig. 1C). In conclusion, *LINC00518* shows increased expression in patients with CMM, and this high expression is associated with poor survival of these patients.

***LINC00518* knockdown suppressed cell proliferation, colony formation, migration and invasion, and induced apoptosis.**

LINC00518 was upregulated in CMM tissues compared with normal tissues in GSE46517 and GSE 4587 data sets (Fig. 2A). Subsequently, *LINC00518* expression was validated in 12 paired CMM tissues and their corresponding adjacent non-cancerous skin tissues using qRT-PCR (Fig. 2B). To investigate the function of *LINC00518* in CMM, we analysed *LINC00518* expression in several CMM cell lines (HM, WM35, WM451, and A375). *LINC00518* expression was higher in WM451 and A375 cells, which have a high invasive and metastatic tendency, and lower in HM and WM35 cells, which are poorly invasive (Fig. 2C). Therefore, we knocked down *LINC00518* expression using a *LINC00518*-targeting small hairpin RNA (shRNA) in CMM cell lines, WM451 and A375 (Fig. 2D). Next, we conducted phenotypic and functional analyses of the effects of *LINC00518* knockdown in WM451 and A375 cell lines after establishing shRNA efficacy. MTT assay showed that the proliferation of *LINC00518*-silenced cells was significantly decreased compared to that of control CMM cells (Fig. 2E). Flow cytometry demonstrated that silencing of *LINC00518* expression in WM451 and A375 cells increased apoptosis

(Fig. 2F). Colony formation assays indicated that the cell survival capability of *LINC00518*-silenced cells decreased significantly (Fig. 2G). The effect of *LINC00518* on the migration and invasion of CMM cells were measured by wound healing assays and Transwell Matrigel invasion assays, respectively. *LINC00518* shRNA-transfected CMM cells displayed lower migratory and invasive capabilities than the control cells (Fig. 2H, Fig. 2I).

***LINC00518* directly targets miR-33a-3p and promotes HIF-1 α expression**

To explore the mechanisms of *LINC00518* in CMM tumorigenesis, we initially investigated the intracellular location of *LINC00518*. Using qRT-PCR assays, we found that *LINC00518* was mainly localized in the cytoplasm (Fig. 3A). Subsequently, miRNA target prediction databases (RegRNA and TargetScan) were used to predict miRNAs that *LINC00518* could sponge and the target genes that miRNAs could regulate (Fig. 3B). We observed that *LINC00518* could directly target miR-33a-3p, which in turn regulated HIF-1 α expression. HIF-1 α is a known oncogenic gene that we have previously described to be involved in the occurrence and development of CMM[34]. Next, presumptive binding sites for miR-33a-3p were identified in the *LINC00518* sequence and in *HIF1 α* 3' UTR by using RegRNA 2.0 website (Fig. 3C). Results of RNA pull-down demonstrated that the bio-miR-33a-3p group was enriched for larger amounts of *LINC00518* than bio-miR-NC and bio-miR-33a-3p-MUT groups (Fig. 3D). Expression of miR-33a-3p in CMM biopsy tissues compared with that in adjacent non-tumour tissues was assessed, and an inverse correlation between miR-33a-3p and *LINC00518* expression was observed (Fig. 3E). We employed dual-luciferase experiments to explore whether *LINC00518* could directly target miR-33a-3p. These assays demonstrated that miR-33a-3p mimics significantly inhibited the relative dual-luciferase activity of the *LINC00518*-WT group compared with that of control group, while they did not affect the reporter activity of *LINC00518*-MUT group, (Fig. 3F). These results indicated that *LINC00518* could directly target miR-33a-3p and reduce its expression. In addition, correlation analysis showed a negative correlation between miR-33a-3p and HIF-1 α expressions in CMM biopsy tissues (Fig. 3G). The significant inhibition of the relative dual-luciferase activity of the HIF-1 α -WT group by miR-33a-3p mimics indicated that miR-33a-3p could regulate HIF-1 α expression (Fig. 3H). To explore the effect of *LINC00518* knockdown and miR-33a-3p on glycolysis, we examined changes in the abundance of key proteins of the glycolysis pathway in CMM cell line. Western blotting analysis indicated that HIF-1 α and LDHA protein levels were regulated by *LINC00518* and miR-33a-3p (Fig. 3I and Fig. 3J). These results indicated that *LINC00518* increased the expression of HIF-1 α in CMM by targeting miR-33a-3p.

HIF-1 α negatively regulates miR-33a-3p expression in CMM cells

HIF-1 α is a recognized transcription factor and the JASPAR server predicted presumptive binding sites at positions 632 bp to 639 bp in a sequence upstream of the origin of miR-33a, with a score of 7.1 out of 10 (Fig. 4A). In addition, HDACs are highly expressed in CMM tissues and cell lines (Fig. 4B); therefore, we speculated that HIF-1 α might inhibit the transcription of miR-33a and decrease the relative expression level of miR-33a-3p by binding to the miR-33a promoter and recruiting HDACs. PCR assays demonstrated that miR-33a-3p expression was elevated after *LINC00518* and HIF-1 α knockdown in WM451 and A375

cell lines. We observed that enhanced HIF-1 α expression in WM451 and A375 cells could rescue the effect on miR-33a-3p expression by *LINC00518* knockdown (Fig. 4C,D). Furthermore, *LINC00518* expression was increased when HIF-1 α expression was enhanced in *LINC00518* knockdown CMM cells (Fig. 4E). The miR-33a-3p expression level was upregulated in WM451 and A375 cells after HDAC1-3 knockdown (Fig. 4F). Moreover, CHIP PCR was performed to verify that Santacruzamate A (an inhibitor of HDACs) could recover the relative expression levels of miR-33a-3p in WM451 and A375 cells (Fig. 4G). A co-immunoprecipitation assay showed that the HIF-1 α –HDAC immunoprecipitate was decreased after HIF-1 α knockdown (Fig. 4H). Western blotting analysis indicated that expressions of H3K4ac, H3K9ac, H3K27ac, and total Histone H3 increased after CMM cells were treated with Santacruzamate A (Fig. 4I). *LINC00518* knockdown decreased HIF-1 α and LDHA protein levels, while overexpression of HIF-1 α in *LINC00518* knockdown cells enhanced HIF-1 α and LDHA expression, suggesting that a *LINC00518*-HIF-1 α axis exists in CMM cells (Fig. 4J). These results indicated that HIF-1 α could provide a negative feedback to regulate miR-33a-3p expression. Specifically, it is probable that HIF-1 α binds to the miR-33a promoter and represses the transcription of miR-33a by accelerating histone deacetylation of miR-33a.

Overexpression HIF-1 α could reverse the regulatory effect of miR-33a-3p mimics on cell proliferation, apoptosis, and metastasis in CMM cells

LINC00518 regulates proliferation, apoptosis, autophagy, colony formation, migration, and invasion in CMM cells, and directly regulates the expression of miR-33a-3p; therefore, we determined whether miR-33a-3p could also regulate these phenotypes in CMM cells. The expression of miR-33a-3p assessed in CMM cell lines (HM, WM35, WM451, and A375) showed that miR-33a-3p expression was higher in HM and WM35 cells, which are poorly invasive, and lower in WM451 and A375 cells, which have high invasive and metastatic tendencies (Fig. 5A). Transfection of WM451 and A375 cells with miR-33a-3p mimics reduced cell proliferation and increased apoptosis, while enhancing miR-33a-3p overexpression of HIF-1 α in WM451 and A375 cells increased proliferation and reduced apoptosis (Fig. 5B,C). Additionally, colony formation assays demonstrated that the cell survival capability of miR-33a-3p – overexpressing cells was significantly decreased, while overexpression of HIF-1 α and miR-33a-3p reversed this negative effect on cell survival (Fig. 5D). Wound healing assays and Transwell Matrigel invasion assays suggested that miR-33a-3p is involved in regulating migration and invasion in CMM cells, and that HIF-1 α could reverse the regulatory effects of miR-33a-3p (Fig. 5E,F). The experimental outcome of enhancing miR-33a-3p expression in WM451 and A375 cells also demonstrated that miR-33a-3p participates in the regulation of proliferation, apoptosis, colony formation, migration, and invasion in CMM cells by targeting HIF-1 α .

Involvement of the *LINC00518*/miR-33a-3p/HIF-1 α negative feedback loop in glycolysis-mediated radiotherapy resistance of CMM cells

HIF-1 α has been reported to participate in regulating radiotherapy resistance by initiating glycolytic tumour metabolism in a variety of cancers [35]. Therefore, we explored whether the *LINC00518*/HIF-1 α signalling loop affects the radiosensitivity of CMM. To further study the function of the *LINC00518*/HIF-1 α signalling loop in CMM cell radioresistance, WM451 and A375 cells were treated with doses of 0, 1, 2,

4, and 6 Gy, and MTT and flow cytometry assays were performed to determine their radiosensitivity. The results indicated that after exposure to a radiation dose of 2 Gy, the proliferation capacity of CMM cell lines decreased significantly and the levels of glycolysis increased simultaneously (Supplementary Fig. 1A). Furthermore, the protein levels of HIF-1 α and LDHA were increased in cells treated with 2 Gy of radiation (Supplementary Fig. 1B). These results indicated that treatment of CMM cells with 2 Gy of radiation could inhibit their cell proliferation abilities while maintaining their radioresistance. Therefore, we selected a single dose of 2 Gy as the radiation dose for subsequent experiments.

Western blotting assays confirmed that knockdown of LINC00518 expression in WM451 and A375 cells decreased HIF-1 α and LDHA protein levels, while overexpression of HIF-1 α could reverse this effect. To identify the role of the LINC00518/HIF-1 α signalling loop in mediating radiosensitivity of CMM cells, the effects of the glycolytic inhibitor, 2-deoxyglucose (2DG), and HDAC inhibitor, Santacruzamate A, were investigated in WM451 and A375 cells. Both 2DG and Santacruzamate A decreased HIF-1 α and LDHA protein levels, which were inhibited by HIF-1 α overexpression or by upregulation of LINC00518 and HIF-1 α in CMM cells (Fig. 1C).

To investigate whether *LINC00518*/HIF-1 α plays a role in anaerobic glycolysis of CMM, we transfected WM451 and A375 cells with a *LINC00518* expression-silencing plasmid or a LINC00518 expression-silencing plasmid together with an HIF-1 α - overexpression plasmid, and biochemically tested glucose consumption, ATP levels, and lactic acid production. Knockdown of *LINC00518* expression in WM451 and A375 cells decreased glucose consumption, ATP levels, and lactic acid production in the cells, while overexpression of HIF-1 α significantly reversed the effects of *LINC00518* silencing of glycolytic tumour metabolism in WM451 and A375 cells (Fig. 6A–C). These results suggest that the *LINC00518*/HIF-1 α signalling loop could increase CMM cell glycolytic metabolism.

MTT, flow cytometry, colony formation, and immunofluorescence assays revealed that knockdown of *LINC00518* expression in WM451 and A375 cells increased radiosensitivity, while overexpression of HIF-1 α reversed this effect. Both 2DG and Santacruzamate A increased radiosensitivity, which was inhibited by HIF-1 α overexpression or by upregulation of *LINC00518* and HIF-1 α in CMM cells (Fig. 6D,E; Fig. 7A,B). The results demonstrated that the *LINC00518*/HIF-1 α signalling loop could regulate the HIF-1 α -LDHA pathway and promote glycolytic metabolism, thereby increasing the radioresistance of CMM cells.

Effect of the *LINC00518*/miR-33a-3p/HIF-1 α negative feedback loop on radiosensitivity in CMM cells *in vivo*

We employed a xenograft mouse model to validate the effect of *LINC00518*/HIF-1 α on the radiosensitivity of CMM cells *in vivo*. Repression of *LINC00518* expression using an sh-LINC00518 plasmid or treating the cells with Santacruzamate A reduced the tumorigenic ability of WM451 and A375 cells, and increased their radiosensitivity in the subcutaneous sarcoma model (Fig. 8A).

To analyse the relationship between radiosensitivity and glycolysis, immunohistochemistry and western blotting analysis were performed to measure HIF-1 α and LDHA protein levels in the xenograft tumours.

Immunohistochemistry showed that HIF-1 α , Ki67, and LDHA proteins were significantly downregulated after knockdown of *LINC00518* in WM451 and A375 cells or in cells treated with Santacruzamate-A (Fig. 8B). Western blotting assays also confirmed the above-mentioned results (Fig. 8C). The subcutaneous sarcoma model further confirmed that the *LINC00518*/miR-33a-3p/HIF-1 α negative feedback loop could increase radiotherapy resistance, accompanied by accelerated glycolysis. Schematic of this search (Fig. 8D)

Discussion

The long intergenic non-protein coding RNA 518 (*LINC00518*) is located on human chromosome 6p24.3 and shows low expression in a majority of normal and malignant human tissues. However, its expression is markedly upregulated in some tumour tissues [36-38]. *LINC00518* has been shown to regulate chemoresistance and promote epithelial cell growth and metastasis in breast cancer [37], promote cell proliferation, and to affect the invasion and metastasis of cervical cancer [38], indicating its significant role in the development and progression of malignant tumours. *LINC00518* overexpression as poor prognosis factor for melanoma have been reported previously[39], but its effect on cancer cell radioresistance is a relatively recent observation.

Recently, growing evidence has suggested that lncRNAs play an important role in the complex etiology and mechanism of carcinogenesis progression [40,41]. In the present study, we noted that *LINC00518* expression was significantly upregulated in CMM tissue compared with that in normal skin tissue, and that patients with CMM with high expression of *LINC00518* have a remarkably poor prognosis in terms of both RFS and OS. This indicates that *LINC00518* may be a molecular target in CMM cells and a potential biomarker for the diagnosis and prognosis of patients with CMM. Additionally, *LINC00518* was shown to increase the proliferation and clonogenicity of CMM cells, induce their migration and invasion, and decrease their apoptosis, suggesting that *LINC00518* acts as an oncogene in CMM cells.

LINC00518 is mainly localized in the cytoplasm, and this kind of lncRNA mainly functions by regulating the target genes of miRNAs by acting as miRNA sponges, thereby inhibiting their functions [42,43]. Therefore, we identified the miRNAs that bind to *LINC00518*. Further studies confirmed that *LINC00518* directly targets miR-33a-3p; miR-33a-3p targets HIF-1 α , a finding that was demonstrated in our previous studies[34]. *LINC00518* upregulates HIF-1 α expression by binding competitively to miR-33a-3p. An increasing number of studies have shown that transcription factors could promote lncRNA expression by binding to the promoter region of the gene [44-46]; however, the influence of transcription factors on the regulation of miRNA expression is rarely reported. Our study is the first to demonstrate that HIF-1 α could bind to the miR-33a-3p promoter region and recruit histone deacetylase in CMM cells and could restrain miR-33a-3p expression by promoting miR-33a histone deacetylation. HDAC1/2/3 are overexpressed in melanoma cell lines than in primary melanocytes. HDACs deacetylate histones and non-histone proteins, which are conducive to regulating gene transcription [47]. In cancer cells, inhibition of HDACs has been shown to influence the execution of extrinsic and intrinsic apoptosis pathways as well as DNA repair [48].

Our results also provide a new aspect and method to explore the interaction model between histone deacetylation and transcription factors. Besides, *LINC00518*, miR-33a-3p and HIF-1 α could comprise a negative feedback loop, providing the first evidence for the presence of a *LINC00518*–miR-33a-3p–HIF-1 α regulatory axis in CMM cells.

Next, we verified whether *LINC00518* and HIF-1 α could specifically inhibit miR-33a-3p expression. In some studies, miR-33a-3p is a recognized antioncogene that suppresses a malignant phenotype by inhibiting the expression of pre-B-cell leukaemia homeobox in hepatocellular cancer [49], and increased miR-33a-3p expression can promote the malignant phenotype of gastric carcinoma [50].

Metastasis is a multistep process that includes de-adhesion, migration, adhesion, and invasion; however, the influence of miR-33a-3p on the CMM phenotype has not been studied. To investigate the role of miR-33a-3p in CMM *in vitro*, malignant phenotype assays were performed after overexpressing miR-33a-3p in CMM cells. We found that miR-33a-3p acts as an antioncogene by inhibiting the activity of HIF-1 α in CMM. Our findings indicated that miR-33a-3p might be a tumour suppressor, which has an enormous value in diagnostic and prognostic evaluation and in the development of new targeted therapy of CMM.

Glycolysis is an important mechanism that confers a selective advantage to cancer cells by supporting uninterrupted growth, and a higher glycolytic rate in tumour cells is considered the main cause of radiotherapy failure due to radioresistance [51,52]. Thus, interrupting or disrupting tumour glycolysis will impact tumour growth via energy depletion as well as sensitization to therapeutics. Hypoxia and nutrient deficiency are common phenomena in advanced solid human tumours. As a hypoxia-induced factor, HIF-1 α can be stimulated by these stress conditions [53,54]. Our study found that the HIF-1 α -LDHA pathway regulates the activation of anaerobic glycolysis in tumour cells and suppresses cells that are sensitive to radiotherapy and chemotherapy. However, a significant correlation has not been established between HIF-1 α and the prognosis of malignant melanoma in public databases. Research has revealed two possible reasons for this: the first one is that HIF-1 α acts as a transcription factor to increase the transcription level of tumour suppressor genes, and the second is that HIF-1 α promotes transcription of oncogenes. Biochemical tests of glucose consumption, ATP levels, and lactic acid production suggested that the *LINC00518*–miR-33a-3p–HIF-1 α negative feedback loop plays a major role in the glycolysis of CMM cells. Thus, we hypothesized that this negative feedback loop might inhibit the radiotherapy sensitivity of CMM cells. To test this hypothesis, *in vitro* radio response assays were performed after knockdown of *LINC00518* in CMM cells. Considering that the endogenous expression of *LINC00518* was very high in these cells, overexpression of *LINC00518* was not performed. We found that the *LINC00518*–miR-33a-3p–HIF-1 α negative feedback loop could increase radiation resistance in CMM by regulating the HIF-1 α -LDHA glycolysis signalling pathway. Therefore, anticancer therapies that target the *LINC00518*–miR-33a-3p–HIF-1 α negative feedback loop might represent a potential therapeutic approach to enhance CMM cell radiosensitivity.

Conclusions

In summary, *LINC00518* is highly expressed in CMM tissues, and high expression of *LINC00518* is an indicator of poor prognosis in patients with CMM. The *LINC00518*–HIF-1 α –glycolysis axis can promote oncogenesis and the development of CMM cells by causing CMM cells to adapt to a state of tumour hypoxia. The *LINC00518*–HIF-1 α –glycolysis axis can also significantly upregulate HIF-1 α and LDHA, thereby inhibiting apoptosis and inducing proliferation of CMM cells in response to radiation, ultimately causing radioresistance. Our results demonstrated that *LINC00518* might be a prognostic biomarker and an actionable target for radiosensitization of CMM.

Abbreviations

HIF-1 α hypoxia-induced factor 1 α ; LDHA lactate dehydrogenase A; HDAC2 histone deacetylase 2; Santacruzamate A a histone deacetylase inhibitor; 2DG 2-deoxy-D-glucose a glycolytic inhibitor; CMM Cutaneous malignant melanoma.

Declarations

Ethical approval and consent to participate

The design and methods of the research are in accordance with the requirements of related regulations and procedures (such as Ethical Review of Biomedical Research Involving Human Subject, ICH-GCP) as well as the ethical principles. The IRB has approved the research to be conducted in The Third Xiangya Hospital, Central South University.

Consent for publication

Not applicable.

Availability of data and material

Previously reported GSE4587 and GSE46517 data were used to support this study and are available at GEO datasets, the prognostic analysis of *LINC00518* were used to support this study and are available in [Gene Expression Profiling Interactive Analysis](#) datasets. These prior studies (and datasets) are cited at relevant places within the text as references.

Competing interests

The authors declare no conflicts of interest.

Funding

This work was supported by the National Natural Science Foundation of China (81874137), the Outstanding Youth Foundation of Hunan Province (2018JJ1047), the Huxiang Young Talent Project (2016RS3022).

Authors contributions

Yan Liu and Ke Cao designed the study, analyzed and interpreted the data, and wrote the manuscript. Dong He contribute to data acquisition and interpretation. Mengqin Xiao and Liang Xiang performed all bioinformatics analysis and carried out the experiments. Yuxing Zhu provide technical expertise and support. All authors have seen and approved the final version of the manuscript.

Acknowledgements

We thank our colleagues in the department of laboratory medicine for helpful discussions and valuable assistance.

References

- [1] Schadendorf D, Fisher DE, Garbe C, Gershenwald JE, Grob JJ, Halpern A, Herlyn M, Marchetti MA, McArthur G, Ribas A, Roesch A, Hauschild A. Melanoma. *Nature Reviews Disease Primers*. 2015;1(1):1-20.
- [2] Rastrelli M, Tropea S, Rossi CR, Alaibac M. Melanoma: epidemiology, risk factors, pathogenesis, diagnosis and classification. *In Vivo*. 2014 Nov-Dec;28(6):1005-11.
- [3] Lee CS, Thomas CM, Ng KE. An Overview of the Changing Landscape of Treatment for Advanced Melanoma. *Pharmacotherapy*. 2017 Mar;37(3):319-333.
- [4] Eggermont AM, Spatz A, Robert C. Cutaneous melanoma. *Lancet*. 2014 Mar 1;383(9919):816-27.
- [5] Balch CM¹, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB. Final Version of 2009 AJCC Melanoma Staging and Classification. *J Clin Oncol*. 2009 Dec 20; 27(36):6199-206.
- [6] Mendenhall WM, Amdur RJ, Morris CG, Kirwan J, Shaw C, Dziegielewski Adjuvant postoperative radiotherapy for cutaneous melanoma. *Acta Oncol*. 2017 Mar;56(3):495-6.
- [7] Beadle BM, Guadagnolo BA, Ballo MT, et al. Radiation therapy field extent for adjuvant treatment of axillary metastases from malignant melanoma. *Int J Radiat Oncol Biol Phys*. 2009;73:1376–1382.
- [8] Mahadevan A, Patel VL, Dagoglu N. Radiation Therapy in the Management of Malignant Melanoma. *Oncology*. 2015;29(10):743-51.
- [9] Maverakis E¹, Cornelius LA, Bowen GM, Phan T, Patel FB. Metastatic melanoma - a review of current and future treatment options. *Acta Derm Venereol*. 2015 May;95(5):516-24
- [10] Gilkes DM, Semenza GL, Wirtz D Hypoxia and the extracellular matrix: drivers of tumour metastasis. *Nat Rev Cancer*. 2014 Jun;14(6):430-9.
- [11] Barker HE, Paget JT, Khan AA, Harrington KJ. The tumour microenvironment after radiotherapy: mechanisms of resistance and recurrence. *Nat Rev Cancer*. 2015;15(7):409-25.

- [12] Macklin PS, McAuliffe J, Pugh CW, Yamamoto A. [Hypoxia and HIF pathway in cancer and the placenta](#). Placenta. 2017 Aug;56:8-13.
- [13] Parks SK, Cormerais Y, Pouysségur J. [Hypoxia and cellular metabolism in tumour pathophysiology](#). J Physiol. 2017 Apr 15;595(8):2439-2450.
- [14] Hanahan D, Weinberg R A. Hallmarks of cancer: the next generation. Cell., 2011;144(5):646-74.
- [15] Nys K, Maes H, Dudek AM, Agostinis P. Uncovering the role of hypoxia inducible factor-1alpha in skin carcinogenesis. Biochimica et biophysica acta 2011; 1816:1-12.
- [16] Kuphal S, Winklmeier A, Warnecke C, Bosserhoff AK.. Constitutive HIF-1 activity in malignant melanoma. Eur J Cancer 2010; 46:1159-69.
- [17] Ke Q, Costa M. [Hypoxia-inducible factor-1 \(HIF-1\)](#). Mol Pharmacol. 2006 Nov;70(5):1469-80.
- [18] Meijer TW, Kaanders JH, Span PN, Bussink J. Targeting hypoxia, HIF-1, and tumor glucose metabolism to improve radiotherapy efficacy. Clin Cancer Res. 2012 Oct 15;18(20):5585-94.
- [19] Zhao F, Ming J, Zhou Y, Fan L. Inhibition of Glut1 by WZB117 sensitizes radioresistant breast cancer cells to irradiation. Cancer Chemother Pharmacol. 2016 May;77(5):963-72.
- [20] Shimura T, Noma N, Sano Y, Ochiai Y, Oikawa T, Fukumoto M, Kunugita N. AKT-mediated enhanced aerobic glycolysis causes acquired radioresistance by human tumor cells. Radiother Oncol. 2014;112(2):302-7.
- [21] Huang X, Liu M, Sun H, Wang F, Xie X, Chen X, Su J, He Y, Dai Y, Wu H, Shen L. HK2 is a radiation resistant and independent negative prognostic factor for patients with locally advanced cervical squamous cell carcinoma. Int J Clin Exp Pathol. 2015;8(4):4054-63.
- [22] Quinn JJ, Chang HY. [Unique features of long non-coding RNA biogenesis and function](#). Nat Rev Genet. 2016 Jan;17(1):47-62.
- [23] Jarroux J, Morillon A, Pinskaya M. [History, Discovery, and Classification of lncRNAs](#). Adv Exp Med Biol. 2017;1008:1-46.
- [24] Xiang S, Gu H, Jin L, Thorne RF, Zhang XD, Wu M. LncRNA IDH1-AS1 links the functions of c-Myc and HIF-1 α via IDH1 to regulate the Warburg effect. Proc Natl Acad Sci USA. 2018 Feb 13;115(7):E1465-E1474.
- [25] Yang F, Zhang H, Mei Y, Wu M. Reciprocal regulation of HIF-1 α and LINC RNA-p21 modulates the Warburg effect. Mol Cell. 2014 Jan 9;53(1):88-100.
- [26] Leonardo Salmena, Laura Poliseno, Yvonne Tay, Lev Kats, and Pier Paolo Pandolfi. A ceRNA hypothesis: the Rosetta stone of a hidden RNA language? .Cell. 2011 Aug 5; 146(3): 353–358.

- [27] Zhang P, Cao L, Fan P, Mei Y, Wu M. LncRNA-MIF, a c-Myc-activated long non-coding RNA, suppresses glycolysis by promoting Fbxw7-mediated c-Myc degradation. *EMBO Rep.* 2016 Aug;17(8):1204-20.
- [28] Lin YH, Wu MH, Huang YH, Yeh CT, Cheng ML, Chi HC, Tsai CY, Chung IH, Chen CY, Lin KH. Taurine up-regulated gene 1 functions as a master regulator to coordinate glycolysis and metastasis in hepatocellular carcinoma. *Hepatology.* 2018 Jan;67(1):188-203.
- [29] Leucci E, Coe EA, Marine JC, Vance KW. The emerging role of long non-coding RNAs in cutaneous melanoma. *Pigment Cell Melanoma Res.* 2016 Nov; 29(6) :619-626.
- [30] Kabbarah O, Nogueira C, Feng B, Nazarian RM et al. Integrative genome comparison of primary and metastatic melanomas. *PLoS One* 2010 May 24;5(5):e10770.
- [31] Smith AP, Hoek K, Becker D. Whole-genome expression profiling of the melanoma progression pathway reveals marked molecular differences between nevi/melanoma in situ and advanced-stage melanomas. *Cancer Biol Ther* 2005 Sep;4(9):1018-29.
- [32] Zeng Q, Liu J, Cao P, Li J, Liu X, Fan X, et al. Inhibition of REDD1 sensitizes bladder urothelial carcinoma to paclitaxel by inhibiting autophagy. *Clin Cancer Res.* 2018;24:445–59.
- [33] Li W, Peng C, Lee MH, Lim D, Zhu F, Fu Y, Yang G, Sheng Y, Xiao L, Dong X, et al. TRAF4 is a critical molecule for Akt activation in lung cancer. *Cancer Res.* 2013;73:6938–50.
- [34] Zhou J, Xu D, Xie H, Tang J, Liu R, Li J,, Xiong W, Cao P, Cao K. [miR-33a functions as a tumor suppressor in melanoma by targeting HIF-1α.](#) *Cancer Biol Ther.* 2015;16(6):846-55.
- [35] Magnon C, Opolon P, Schlumberger M. [Radiation and inhibition of angiogenesis by canstatin synergize to induce HIF-1α-mediated tumor apoptotic switch.](#) *J Clin Invest.* 2007 Jul;117(7):1844-55.
- [36] Gerami P, Yao Z, Polsky D, Jansen B, Busam K, Ho J, Martini M, Ferris LK. Development and validation of a noninvasive 2-gene molecular assay for cutaneous melanoma. *J Am Acad Dermatol.* 2017 Jan;76(1):114-120.
- [37] Chang L, Hu Z, Zhou Z, Zhang H. [Linc00518 Contributes to Multidrug Resistance Through Regulating the MiR-199a/MRP1 Axis in Breast Cancer.](#) *Cell Physiol Biochem.* 2018;48(1):16-28.
- [38] He J, Sun M, Geng H, Tian S. [Long non-coding RNA Linc00518 promotes paclitaxel resistance of the human prostate cancer by sequestering miR-216b-5p.](#) *Biol Cell.* 2019 Feb;111(2):39-50.
- [39] Ferris LK, Jansen B, Ho J, Busam KJ, Gross K, Hansen DD, Alsobrook JP , Yao Z, Peck GL, Gerami P. Utility of a Noninvasive 2-Gene Molecular Assay for Cutaneous Melanoma and Effect on the Decision to Biopsy. *JAMA Dermatol.* 2017 Jul 1;153(7):675-680.

- [40] Yu J, Liu Y, Guo C, Zhang S, Gong Z. Upregulated long non-coding RNA LINC00152 expression is associated with progression and poor prognosis of tongue squamous cell carcinoma. *J Cancer*. 2017 Feb 11;8(4):523-530.
- [41] Zhu Y, He D, Liu Y, Cao K. The MRVI1-AS1/ATF3 signaling loop sensitizes nasopharyngeal cancer cells to paclitaxel by regulating the Hippo-TAZ pathway. *Oncogene*. 2019 Aug;38(32):6065-6081.
- [42] Tay Y, Rinn J, Pandolfi PP. [The multilayered complexity of ceRNA crosstalk and competition](#). *Nature*. 2014 Jan 16;505(7483):344-52.
- [43] Qi X, Zhang DH, Wu N, Xiao JH, Wang X, Ma W. [ceRNA in cancer: possible functions and clinical implications](#). *J Med Genet*. 2015 Oct;52(10):710-8.
- [43] Chen H, Du G, Song X, Li L. [Non-coding Transcripts from Enhancers: New Insights into Enhancer Activity and Gene Expression Regulation](#). *Genomics Proteomics Bioinformatics*. 2017 Jun;15(3):201-207.
- [44] Moussaieff A, Rouleau M, , Aberdam D, Nahmias Y. Glycolysis-Mediated Changes in Acetyl-CoA and Histone Acetylation Control the Early Differentiation of Embryonic Stem Cells. *Cell Metab*. 2015 Mar 3;21(3):392-402.
- [45] Zhang E, Han L, Yin D, Shu Y, Chen J. H3K27 acetylation activated-long non-coding RNA CCAT1 affects cell proliferation and migration by regulating SPRY4 and HOXB13 expression in esophageal squamous cell carcinoma. *Nucleic Acids Res*. 2017, 45(6): 3086-3101.
- [46][Schoepflin ZR, Shapiro IM, Risbud MV](#). Class I and IIa HDACs Mediate HIF-1 α Stability Through PHD2-Dependent Mechanism, While HDAC6, a Class IIb Member, Promotes HIF-1 α Transcriptional Activity in Nucleus Pulposus Cells of the Intervertebral Disc. *J Bone Miner Res*. 2016 Jun;31(6):1287-99.
- [47] Krumm A, Barckhausen C, Küçük P, Tomaszowski KH, Loquai C, Fahrner J, Krämer OH. Enhanced Histone Deacetylase Activity in Malignant Melanoma Provokes RAD51 and FANCD2-Triggered Drug Resistance. *Cancer Res*. 2016 May 15;76(10):3067-77.
- [48] Han SY, Han HB, Tian XY, Sun H, Xue D, Zhao C. [MicroRNA-33a-3p suppresses cell migration and invasion by directly targeting PBX3 in human hepatocellular carcinoma](#). *Oncotarget*. 2016 Jul 5;7(27):42461-42473.
- [49] Liang M, Liu Z, Lin H, Shi B, Li M, Chen T. [High-throughput sequencing reveals circular RNA hsa_circ_0000592 as a novel player in the carcinogenesis of gastric carcinoma](#). *Biosci Rep*. 2019 Jun 28;39(6).
- [51] Akram M. [Mini-review on glycolysis and cancer](#). *J Cancer Educ*. 2013 Sep;28(3):454-7.

- [52] Ganapathy-Kanniappan S, Geschwind JF. [Tumor glycolysis as a target for cancer therapy: progress and prospects.](#) Mol Cancer. 2013 Dec 3;12:152.
- [53] Görlach A, Dimova EY, Petry A, Martínez-Ruiz A. [Reactive oxygen species, nutrition, hypoxia and diseases: Problems solved?](#) Redox Biol. 2015 Dec;6:372-385.
- [54] Nosrati N, Bakovic M, Paliyath G. [Molecular Mechanisms and Pathways as Targets for Cancer Prevention and Progression with Dietary Compounds.](#) Int J Mol Sci. 2017 Sep 25;18(10).

Figures

Figure 1
A

B

C

D

Figure 1

LINC00518 is overexpressed in CMM and indicates poor prognosis in patients with CMM. (A) Schematic overview of the workflow used to identify and validate dysregulated lncRNAs in two CMM microarray data cohorts. (B) Heatmap of 12 up-regulated probe sets representing 12 lncRNAs mined from the GSE4587 and GSE46517 data sets. (C) TCGA data showing that the Transcripts Per Million of LINC00518 in CMM was highest in all human cancers and that it is up-regulated in CMM compared with normal

tissues.(D) Kaplan–Meier curves of the OS and RFS of 436 patients with CMM with high or low LINC00518 expression. P value was computed by the log-rank test.

Figure 2

Figure 2

LINC00518 knockdown suppressed cell proliferation, colony formation, migration and invasion, and induced apoptosis.(A) The gene chip data related to LINC00518 was up-regulated in T group (CMM tissues ,n = 73) compared with N group in GSE46517 data set(normal tissues,n = 17).(B) qPCR assay

showing that LINC00518 was overexpressed in 12 paired CMM tissues and their corresponding adjacent non-cancerous skin tissues obtained from tissue biopsy samples in our study.(C) qPCR assay showing that LINC00518 expression was higher in WM451 and A375 cells, which have high invasive and metastatic tendency; however, it was lower in HM (normal melanocyte cell lines) and WM35 (low invasive and metastatic tendency) cells. The expression data in HM was considered as a control.(D) qPCR assay demonstrating shLINC00518 knockdown of LINC00518 in WM451 and A375 cell lines.(E) MTT assay indicating that LINC00518 knockdown in WM451/A375 cells increased apoptosis significantly compared with that in control cells.(F) Flow cytometry examination of the effect on CMM cell apoptosis following LINC00518 knockdown. (G) Colony formation assay detection of the effect on CMM cell proliferation following LINC00518 knockdown.(H) Transwell assay showing the effect on CMM cell migration following LINC00518 knockdown. (I) Wound healing assay showing that LINC00518 knockdown in WM451/A375 cells can significantly inhibit cell metastasis compared with the control. The histogram data for each group are an average of three independent replicates; bars indicate SD; *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3

Figure 3

LINC00518 directly targets miR-33a-3p and promotes HIF-1α expression. (A) qPCR assay showing that LINC00518 was mainly localized in the cytoplasm of CMM cell; β-actin and U6, mainly located in the cytoplasm and nucleus, respectively, were used as references.(B) The Map showing that blue square frame represent LINC00518,red square frame represent the predicted miRNAs that LINC00518 could sponge and regulate the HIF-1αat the same time by using RegRNA 2.0 and TargetScan website ,gray

square frame represent the target gene HIF-1 α .(C) Presumptive binding sites for miR-33a-3p were identified in the LINC00518 sequence and in the HIF-1 α 3' UTR by using sequence comparison and RegRNA data.(D) RNA pull-down depicting that the bio-miR-33a-3p group was enriched for larger amounts of LINC00518 than the bio-miR-NC and bio-miR-33a-3p-MUT groups, thereby indicating that LINC00518 can target miR-33a-3p directly.(E) Negative correlation between LINC00518 and miR-33a-3p expressions in 12 cases of CMM tissues using Pearson correlation analysis.(F) A dual-luciferase assay showing that miR-33a-3p mimics directly and significantly inhibited the relative dual-luciferase activity of the LINC00518-WT group compared with that of control group and LINC00518 targeted miR-33a-3p. Luciferase activity was detected 48 h after transfection.(G) qPCR assay showing a negative correlation between miR-33a-3p and HIF-1 α expressions in 12 cases of CMM tissues.(F) Dual-luciferase assay showing a significant inhibition by miR-33a-3p mimics in the relative dual-luciferase activity of the HIF-1 α -WT group.(I,J) Western blotting analysis showing that HIF-1 α and LDHA protein levels were regulated by LINC00518 and miR-33a-3p. The histogram data for each group are an average of three independent replicates; bars indicate SD; *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4

Figure 4

HIF-1 α negatively regulates miR-33a-3p expression in CMM cells. (A) JASPAR predicted the binding sites of the HIF-1 α and LINC00518 promoter regions. (B) TCGA data showing that HDACs are highly expressed in CMM tissues and cell lines, indicating that the level of histone deacetylation was higher in CMM tissues. (C–F) qRT-PCR showing miR-33a-3p and LINC00518 expression levels after HIF-1 α knockdown or HIF-1 α overexpression and LINC00518 knockdown or HDAC knockout in WM451 and A375 cells indicates

that HDACs and HIF-1 α can inhibit miR-33a-3p expression and increase LINC00518 expression. (G) ChIP PCR showing that Santacruzamate A(inhibiting histone deacetylation) could recover the relative expression levels of miR-33a-3p in WM451 and A375 cells; immunoglobulin G (IgG) acted as the NC.(H) CoIP assay showed that the HIF-1 α -HDAC immunoprecipitate was decreased after HIF-1 α knockdown. (I,J) Western blotting analysis, indicating that the expression of H3K4ac, H3K9ac, H3K27ac, and total Histone H3 was increased after CMM cells were treated with Santacruzamate A; LINC00518 knockdown decreased HIF-1 α and LDHA protein levels, while overexpression of HIF-1 α in LINC00518 knockdown cells enhanced HIF-1 α and LDHA expression. The histogram data for each group are an average of three independent replicates; bars indicate SD; *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5

Figure 5

Overexpression of HIF-1 α could reverse the regulatory effect of miR-33a-3p mimics on cell proliferation, apoptosis, and metastasis in CMM cells. (A) Baseline expression of miR-33a-3p was detected in HM, WM35, WM451, and A375 by qRT-PCR. (B) WM451 and A375 cells were transfected with miR-33a-3p mimics, miR-33a-3p mimics, and HIF-1 α overexpression plasmid; the MTT method was used to detect cell proliferation. (C) The effects of overexpression of miR-33a-3p or miR-33a-3p mimics plus HIF-1 α on

apoptosis of WM451 or A375 cells were assessed using annexin V staining and flow cytometry analysis. (D) Colony formation assay showed that miR-33a-3p is involved in significantly decreasing cell proliferation, HIF-1 α could reverse the regulatory effects of miR-33a-3p. (E) Transwell assay indicated that overexpression miR-33a-3p inhibiting cell migration. (F) Wound healing assay indicated that miR-33a-3p is involved in significantly decreasing cell metastasis compared with the control, and that HIF-1 α could reverse the regulatory effects of miR-33a-3p. The histogram data for each group are an average of three independent replicates; bars indicate SD; *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 6

Figure 6

Involvement of the LINC00518–miR-33a-3p–HIF-1 α negative feedback loop in glycolysis-mediated radiotherapy resistance of CMM cells. (A-C) The levels of glucose consumption and lactate production were estimated in CMM cells. These levels indicated that knockdown of LINC00518 expression in WM451 and A375 cells decreased glucose consumption, ATP levels, and lactic acid production in the cells; by contrast, overexpression of HIF-1 α significantly reversed the effects of LINC00518 silencing on glycolytic tumour metabolism in WM451 and A375 cells. (D) Detection of CMM cell sensitivity to radiotherapy after LINC00518 knockdown using an MTT assay. LINC00518 knockdown in CMM cells could inhibit their proliferation after the use of radiotherapy and HIF-1 α could reverse this effect, while 2DG and Santacruzamate A offset the influence of HIF-1 α . (E) Flow cytometry examination of the effect of radiotherapy on CMM cell apoptosis following LINC00518 knockdown. LINC00518 knockdown in CMM cells could increase apoptosis after using radiotherapy and HIF-1 α could reverse this effect, while 2DG and Santacruzamate A offset the influence of HIF-1 α . The histogram data for each group are an average of three independent replicates; bars indicate SD; *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 7

Figure 7

Involvement of the LINC00518/miR-33a-3p/HIF-1α negative feedback loop in glycolysis-mediated radiotherapy resistance of CMM cells. (A) Colony formation showing that knockdown of LINC00518 expression in WM451 and A375 cells inhibited the invasion and proliferation of CMM cells, increased the radiosensitivity of cells, while overexpression of HIF-1α reversed this effect. Both 2DG and Santacruzamate A increased radiosensitivity, which was inhibited by HIF-1α overexpression or by

upregulation of LINC00518 and HIF-1α in the CMM cells. (B) Immunofluorescence assays indicating that knockdown of LINC00518 expression in WM451 and A375 cells could induce structural damage of their nuclei under radiotherapy; however, HIF-1α could reverse this effect, while 2DG and Santacruzamate A offset the influence of HIF-1α. The histogram data for each group are an average of three independent replicates; bars indicate SD; *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 8

Figure 8

Effect of the LINC00518/miR-33a-3p/HIF-1 α negative feedback loop on radiosensitivity in CMM cells in vivo. (A) Transplantation tumour experiments showing that repression of LINC00518 expression using the sh-LINC00518 plasmid or treating the cells with Santacruzamate A reduced the tumorigenic ability and tumour volume of WM451 and A375 cells, thereby increasing their radiosensitivity as observed by reduced colony formation in the subcutaneous sarcoma model. (B) Immunohistochemical assays showing that knockdown of LINC00518 expression or treating the cells with Santacruzamate A decreased HIF-1 α , Ki67, and LDHA protein levels in tumour tissues of mice. (C) Western blotting showing that LINC00518 expression or treatment of the cells with Santacruzamate A decreased HIF-1 α and LDHA protein expression in tumour tissues of mice. (D) Schematic of this search. The histogram data for each group are an average of three independent replicates; bars indicate SD; *P < 0.05, **P < 0.01, ***P < 0.001.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFigure1legend.pdf](#)
- [supplementaryFigure.pdf](#)