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A miR-124/ITGA3 axis contributes to colorectal cancer metastasis by regulating anoikis susceptibility

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ABSTRACT

Metastasis is the major cause for the death of patients with colorectal cancer (CRC). Anoikis resistance enhances the survival of cancer cells during systemic circulation, thereby facilitating secondary tumor formation in distant organs. miR-124 is a pleiotropically tumor suppressive small non-coding molecule. However, its role and mechanism in the regulation of cancer cell anoikis are still unknown. Here, we found that overexpression of miR-124 promotes anoikis of CRC cells *in vitro* and *in vivo*. In silico analysis and the experimental evidence supported that ITGA3 is a *bona fide* target of miR-124. Moreover, we identifies that ITGA3 plays a critical role in the regulation of anoikis sensitivity in CRC cells. Finally, our analysis in TCGA datasets demonstrates that high levels of ITGA3 are closely associated with poor prognosis in CRC patients. Collectively, we establish a functional link between miR-124 and anoikis susceptibility and provide that a miR-124/ITGA3 axis could be a potential target for the treatment of metastatic CRC.

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1. Introduction

Colorectal cancer (CRC) is one of leading causes of cancer-related morbidity and mortality worldwide. Distant metastasis remains major cause of CRC-related death [1]. Anoikis is an important barrier to distant metastasis. Anoikis occurring in detached cells prevents them from reattaching to inappropriate matrices and resuming growth. Resistance to anoikis enhances the survival of cancer cells during systemic circulation, thereby facilitating secondary tumor formation in distant organs [2–4]. The aberrant high activity of multiple signaling pathways, including integrin signal pathway has been demonstrated to be required to prevent anoikis in tumor cells. And blockage of anti-anoikis activity of tumor cells has been shown to be beneficial for the treatment of metastatic diseases [5].

MicroRNAs (miRNAs) are a family of 21–25 nucleotide small non-coding RNA molecules that negatively regulate gene expression in a sequence-specific manner. Increasing evidence indicates that they potently influence cellular behavior through the regulation of extensive gene expression networks [6]. High throughput techniques such as microarrays or next-generation sequencing show that miRNAs are dysregulated in almost all types of human cancer, including CRC [7,8]. MiRNAs function as either tumor suppressors or oncogenes, exerting a variety of roles in cancer development and progression [9]. Therefore, the modulation of a single miRNA may affect several pathways synchromously to achieve clinical benefit, and miRNAs are potential as therapeutic targets for the treatment of cancer [10,11]. MiR-124 has been reported as a pleiotropically tumor suppressor in glioblastoma (GBM), prostate cancer, liver cancer, breast cancer and CRC, involving multiple facets of malignant behaviors [12–18]. However, the function of miR-124 on the regulation of anoikis remains unknown.

In the present study, we investigated whether miR-124

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regulates anoikis susceptibility in CRC cells. Mechanistically, we explored in silico analysis and experimental study to identify new targets of miR-124 in CRC cells. Furthermore, we performed loss-of-function experiments to confirm the role of miR-124's downstream genes in the regulation of cancer cell anoikis. Finally, we assessed the prognostic value of miR-124-regulated genes in CRC patients. Collectively, the deeply understanding of miR-124 in the regulation of anoikis could help us to provide a promising treatment avenue of metastatic CRC.

2. Materials and methods

2.1. Kaplan-Meier plotter analysis

The prognostic value of the ITGB3 gene in cancers was analyzed using Kaplan-Meier Plotter (http://kmplot.com/analysis/), a database that integrates gene expression data and clinical data. Our analysis was focused on disease free survival (DFS) or overall survival (OS) patient information. The patient samples have been split into two groups. The two patient groups (higher and lower expression levels) were compared using a Kaplan-Meier survival plot. The hazard ratio with 95% confidence intervals and log rank p value was calculated. In order to reduce our false discovery rate, we selected $p < 0.01$ as a threshold.

2.2. Cell culture

293 T cells, colorectal cancer cell lines Lovo and SW620 were from the Type Culture Collection of the Chinese Academy of Sciences. 293 T cells were maintained in DMEM supplemented with 10% fetal bovine serum (Gibco, New York, USA), Lovo and SW620 cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin in humidified air with 5% CO2 at 37 C.

2.3. Dual luciferase reporter assay

For luciferase reporter assay, 293 T cells (1×10^5) were plated in a 48-well plate and then cotransfected with 200 ng of either pCDNA3-miR-124 or pcDNA3-EGFP control vector, 200 ng of either wild-type or mutant luciferase construct, and 5 ng of PRL-TK (Promega, Madison, WI, USA), using Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. Cells were collected 48 h after transfection and analyzed using the Dual-Luciferase reporter assay kit (Promega, Madison, WI, USA). The pRL-TK vector provided the constitutive expression of Renilla luciferase, and was used as an internal control to correct the differences in transfection and harvest efficiencies. Each experiment was done in triplicates and repeated in three independent experiments.

2.4. RNA isolation, reverse transcription and real-time polymerase chain reaction

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription (RT) for mRNA or microRNA was carried out using the PrimeScript™ RT Master Mix (TaKaRa, Japan) or the miScript Reverse Transcription Kit (Qiagen, Germany), respectively. The quantitative real-time PCR (qPCR) was conducted using the SYBR Green dye (TaKaRa, Japan). The following primers were as the following: ITGA3, forward primer, 5′- TCAACCTGGATACCCGATTCC-3′, reverse primer, 5′- GCTCTGTCTGCCGATGGAG-3’; GAPDH (as an endogenous control), forward primer, 5′-TCACCAGGGCTGCTTTTAAC-3′, reverse primer, 5′-GACAAGCTTCCCGTTCTCAG-3’; mature miR-124, forward primer, 5′- TAAGGCACGCGGTGAATGCC-3′, reverse primer, Universal Primer (QIAGEN, Germany); U6-snRNA, forward primer, RNU6B_2 miScript Primer (QIAGEN, Germany), reverse primer, Universal Primer (QIAGEN, Germany). Real-time

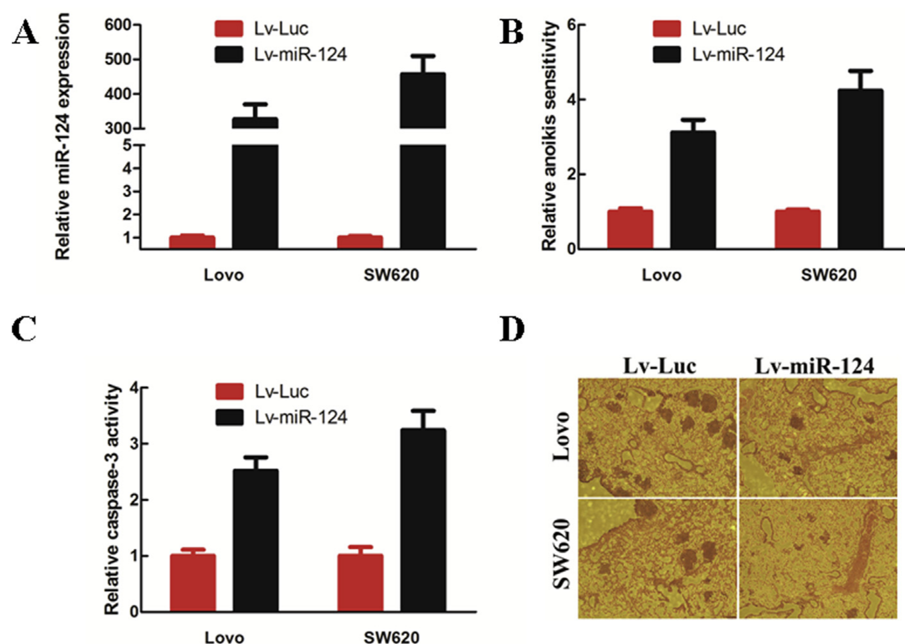

Fig. 1. Overexpression of miR-124 increases anoikis sensitivity of CRC cell *in vitro* and *in vivo*.
(A) qRT-PCR analysis of mature miR-124 levels in CRC cells (**P < 0.01, independent *t*-test).
(B) The anoikis activity of CRC cells stably infected with Lv-Luc or Lv-miR-124 was evaluatedm as entioned in methods and materials.
(C) The anoikis activity of CRC cells stably infected with Lv-Luc or Lv-miR-124 was evaluated by caspase-3 activity assays.
(D) HE staining on the lungs of nude mice injected with CRC cells infected with Lv-Luc or Lv-miR-124 recombinant lentivirus for three weeks.

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