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Decreased level of miR-1301 promotes colorectal cancer progression via activation of STAT3 pathway

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Abstract: The molecular pathogenesis of colorectal cancer (CRC) has been widely investigated in recent years. Accumulating evidence has indicated that microRNA (miRNA) dysregulation participates in the processes of driving CRC initiation and progression. Aberrant expression of miR-1301 has been found in various tumor types. However, its role in CRC remains to be elucidated. In the present study, we identified miR-1301 was enriched in normal colorectal tissues and significantly down-regulated in CRC. Decreased level of miR-1301 strongly correlated with aggressive pathological characteristics, including advanced stage and metastasis. Bioinformatics and dual luciferase assay demonstrated that STAT3 is a direct target of miR-1301. Gain and loss-of-function assays showed that miR-1301 had no effect on cell proliferation. Overexpression of miR-1301 suppressed cell migration and invasion capacity of pSTAT3-positive LoVo cells, but not pSTAT3-negative SW480 cells, while inhibition of miR-1301 consistently promoted cell migration and invasion in both cell lines. Additionally, miR-1301 inhibition restored the suppressed migration and invasion of STAT3-knockdown LoVo cells. MiR-1301 functioned as a tumor suppressor to modulate the IL6/STAT3 signaling pathway. In summary, this study highlights the significant role of miR-1301/STAT3 axis in CRC metastasis.

Introduction

Colorectal cancer (CRC) is the leading malignant tumors in developed countries with high incidence and mortality. Alteration of driver genes and epigenetic changes contribute to CRC initiation and progression (Chen et al. 2018c). As an essential way of epigenetic regulation, microRNAs are small noncoding RNAs that play essential roles in gene expression regulation by binding to 3'-untranslated region (3'-UTR) of targets (Wang et al. 2019). Aberrant miRNA expression has been extensively reported in CRC using miRNA profiles or independent validation group (Arndt et al. 2009; Chen et al. 2014; Keller et al. 2014; Loo et al. 2015; Lu et al. 2018). The in-depth mechanism studies of dysregulated miRNA network are issues to be solved. The number of direct target of each miRNA ranges from hundreds to thousands. Depending on mRNA targets they regulated, miRNA acts as oncogene or as tumor suppressor gene.

MiR-1301 has been reported mostly as a tumor suppressor in multiple cancer types (Fang et al. 2012; Wang et al. 2017; Wen et al. 2019; Yang et al. 2017; Zhi et al. 2017), but inversely, in prostate cancer, it functioned as an oncogene (Bi et al. 2016; Song et al. 2018). As far as we are concerned, the importance of miR-1301 in CRC is still uncovered. In this study, we utilized public data of 14 tumor types and found the expression of miR-1301 is highest in normal colorectal tissues and significantly down-regulated in CRC. The decreasing trend is only detected in CRC and pancreatic cancer, suggesting miR-1301 is important in digestive tract. Using bioinformatics analysis, we found miR-1301/STAT3 axis may dominate in the network regulated by miR-1301.

STAT3 (Signal transducer and activator of transcription 3), a transcriptional mediator of oncogenic signaling, is constitutively active in ~70% of human cancers (Wei et al. 2019). Distant metastasis causes most of the cancer-related morbidity and mortality after initial treatment (Chang et al. 2017). STAT3 activation in CRC is reported associated with metastasis (Ye et al. 2017). Furthermore, increasing evidences demonstrated

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that knocking down STAT3 expression could suppress the growth of CRC cells *in vitro* and *in vivo*. Here, to further confirm the suppressor role of miR-1301 is mainly via STAT3 pathway, we selected two miR-1301-expressing cell lines, LoVo (pSTAT3-positive) and SW480 (pSTAT3-negative) for function study. Our results may provide a novel therapeutic target for advanced CRC patients.

Results

MiR-1301 is significantly down-regulated in tumor samples and correlated with CRC metastasis

To determine the expression status of miR-1301 in different tissues, we utilized The Cancer Genome Atlas (TCGA, Illumina HiSeq platform) data to analyze miR-1301 expression in 14 types of normal tissues. RNA sequencing data showed miR-1301 is highly expressed in normal colon, rectum, and pancreas (Figure S1A). Notably, as shown in Figure 1A and Figure S1B, down-regulation of miR-1301 is only found in colorectal cancer and pancreatic cancer. This result indicated the expression pattern of miR-1301 is tissue-specific, and decreased level of miR-1301 could be a tumor promoter in gastrointestinal tumors Validation group of our samples

confirmed the down-regulation of miR-1301 in CRC (Figure 1B, $P < 0.0001$, paired t -test).

To further investigate whether miR-1301 expression is correlated with CRC progression, we analyzed the expression data together with the clinical data of patients. As compared to early stage cases, the level of miR-1301 decreased in patients with advanced pathology (Stage IV) (Figure 1C, $P < 0.05$). Using TNM staging system, we found the expression of miR-1301 is also correlated with metastasis (Figure 1D). The χ^2 test showed the expression of miR-1301 is not correlated with patients' age, gender, tissue site, colon polyps present, and MSI (microsatellite instability) status (Table 1). Therefore, aberrant miR-1301 expression could be associated with CRC metastasis.

STAT3 is a direct target of miR-1301

MiRNA regulation network is very complex, and for a particular miRNA, it may exist hundreds of target genes (Chen et al. 2018a). To systematically analyze the biological process pathway that miR-1301 participates, we performed binding analysis as well as expression correlation analysis. The potential targets ($n = 1888$) of miR-1301 were selected from online software microRNA.org, with mirSVR score < -0.3 . GO analysis revealed that the function of miR-1301 targets mainly correlated with actin cytoskeleton organization, both positive and negative regulation of

Figure 1: Decreased level of miR-1301 is correlated with CRC metastasis.

(A) MiR-1301 expression analysis in normal and tumor (unpaired t -test). (B) Validation of miR-1301 expression in 28 pairs of colorectal adenocarcinomas and adjacent normal tissues (paired t -test). (C) miR-1301 expression in different pathological stage of CRC. (D) Analysis of miR-1301 expression in patients with or without metastasis. Data are showed as mean $\pm$ SD, * $P < 0.05$, **** $P < 0.0001$.

Table 1: Correlation of miR-1301 expression to pathological features of TCGA CRC samples.

Variables	Cases	miR-1301 expression		χ^2	P Value
		High	Low		
Age(years)					
>65	313	146	167	1.830	0.176
≤65	256	134	122		
Gender					
Male	299	144	155	0.277	0.598
Female	270	136	134		
Colon polyps present					
Yes	89	49	40	0.904	0.342
No	204	100	104		
Tissue site					
Colon	416	205	211	0.004	0.952
Rectum	149	73	76		
MSI status					
MSS	391	187	204	1.254	0.263
MSI	166	88	78		

cell proliferation, negative regulation of transcription or gene expression (Figure 2A). We further discovered the genes with annotation in KEGG are mainly involved in cancer related pathways (MAPK, Wnt, and Jak-STAT) (Figure 2B). Particularly, STAT3, a core effector of Jak-STAT pathway, attracted our attention. Though TCGA data showed no correlation between miR-1301 and STAT3 RNA expression, we found in metastasis cases (M1), miR-1301 is negatively correlated with phosphor-Stat3 expression ($P = 0.015$, $r = -0.323$, Figure 2C). Two tightly binding site of STAT3 for miR-1301 were showed in Figure 2D and 2E. To confirm this physical interaction, a dual luciferase assay was performed and the result showed that overexpression of miR-1301 could significantly repress the luciferase activity (wild-type 3'-UTR of STAT3). STAT3 is clearly reported correlated with CRC metastasis, but the study of miR-1301/STAT3 in CRC has not been reported. The similar expression pattern and binding assay confirmed STAT3 is one of direct targets of miR-1301.

Figure 2: STAT3 is a direct target of miR-1301.

(A) GO analysis of 1888 target genes of miR-1301. (B) Pathway analysis of miR-1301 targets using DAVID v6.7 software. (C) Pearson's correlation analysis of miR-1301 and pSTAT3 (Y705) expression in metastasis samples from TCGA CRC cohort. (D–E) Schematic diagrams of wild-type and mutant type 3'-UTR of STAT3. Dual luciferase reporter assay was used to detect the reporter activity in SW480 and LoVo cells. Data were shown as mean $\pm$ SEM, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

Inhibition of miR-1301 promotes CRC cell migration and invasion

MiR-1301 is expressed in both SW480 and LoVo cells, and we performed overexpression and inhibition of miR-1301 in these two cell lines to study its effect on cell phenotype. Transfection efficiency of miR-1301 mimics and inhibitor were determined using QPCR in both cell lines (Figure S2). We firstly investigated cell proliferation using CCK8 assay and observed miR-1301 expression in SW480 cells had no effect on cell proliferation (Figure S3). In LoVo cells, overexpression of miR-1301 could slightly decreased cell proliferation ability. However, test of three independent experiments showed no statistic difference. These

demonstrated the effect of miR-1301 on CRC proliferation is weak and limit.

To investigate whether miR-1301 affects cell migration capacity, we applied wound scratch assay. In SW480 cells, miR-1301 inhibition enhanced the migration capacity as compared to negative controls, while overexpression of miR-1301 showed no significant effect (Figure 3A). In LoVo cells, gain of miR-1301 inhibited migration, and conversely, loss of miR-1301 promotes the migration capacity (Figure 3B). Furthermore, using a Matrigel invasion assay, we explored the effect of miR-1301 on the invasion capacity of CRC cells. Similarly, the results were consistent with migration assay in SW480 and LoVo cells (Figure 3C).

Figure 3: Inhibition of miR-1301 promotes CRC cell migration and invasion.

(A–B) Wound scratch was performed to detect cell migration ability (A, SW480; B, LoVo). The images were under a microscope at a magnification of 100 $\times$ and photographed at 0 and 48 h. (C) Cell invasion analysis in SW480 and LoVo cells, detected by Transwell chambers with Matrigel. The images were under a microscope at a magnification of 100 $\times$.

MiR-1301 regulates CRC cell migration and invasion through STAT3/MMPs axis

MiR-1301 directly targets STAT3, and we hypothesized the migration and invasion effect of miR-1301 was associated with STAT3 and its downstream targets. We then performed blots of STAT3, pSTAT3, and STAT3 targets MMP2 and MMP9 to confirm our hypothesis (Figure 4A). Both Total STAT3 and pSTAT3 level changed after transfection of miR-1301 in LoVo cells (pSTAT3-positive cells). STAT3 has been confirmed a target for therapy in CRC. Block of STAT3

pathways could depress cell invasion abilities. To further clarify that miR-1301 affects CRC cell invasion by regulation STAT3, LoVo cells were transfected with small-interfering RNA (siRNA) specific for STAT3 to inhibit its expression. Knockdown of STAT3 could inhibit the expression of MMPs (Figure 4B) as well as cell invasion phenotype (Figure 4C). After addition of miR-1301 inhibitor in STAT3-knockdown cells, this effect was attenuated.

To further confirm the role of miR-1301/STAT3 axis in CRC metastasis, we tested whether miR-1301 overexpression could inhibit IL-6-mediated phosphorylation of

Figure 4: MiR-1301 regulates CRC cell migration and invasion through STAT3/MMPs axis.

(A) Western blots of STAT3, pSTAT3, and MMPs after transfection of miR-1301 mimics or inhibitors in LoVo cells. Lane 1: NC. Lane 2: miR-1301 mimics. Lane 3: inhibitor NC. Lane 4: miR-1301 inhibitor. (B) Western blots of STAT3, pSTAT3, and MMPs in LoVo cells after cotransfection of miR-1301 inhibitor/inhibitor NC and STAT3 siRNA/siRNA NC. (C) Cell invasion analysis in LoVo cells. (D–E) Western blots of STAT3, pSTAT3, and MMPs (D: SW480; E: LoVo). (F) Schematic model of miR-1301/STAT3 axis in CRC metastasis.

STAT3 and suppress MMPs expression. As a direct target of miR-1301, total STAT3 and pSTAT3 were down-regulated after overexpression of miR-1301 in pSTAT3-activated cells (Figure 4D and 4E). Phospho-STAT3 is absent in basal SW480 (Figure 4D), in which the suppression role of miR-1301 had no effect. Cell invasion phenotype as well as MMPs blot in SW480 cells confirmed our hypothesis that miR-1301 exerted its suppressor role through STAT3/MMPs axis (Figure 4F).

Discussion

Substantial evidence has shown that aberrant miRNA expression is common and involved in CRC initiation and progression (De Robertis et al. 2018; Grassi et al. 2018; Liu et al. 2018). The miRNA-based targeted therapy has become a promising approach for anti-tumor therapy (Sun et al. 2018). Therefore, identifying novel markers and systematic investigation of the mechanisms underlying CRC initiation and development may help to identify effective therapeutic targets for CRC therapy. In the present study, of the 14 organs tested, we identified the expression of miR-1301 is enriched in colorectum and pancreas and significantly down-regulated in tumor patients ($P < 0.05$ for pancreatic cancer and $P < 0.0001$ for CRC). Decreased level of miR-1301 is correlated with CRC progression, including advanced stage and metastasis. Our function assays indicated that inhibition of miR-1301 promoted cell migration and invasion in both LoVo and SW480 cells. Further in-depth mechanism uncovered miR-1301 regulating network in CRC metastasis. Notably, STAT3 was validated as an essential target of miR-1301. We confirmed miR-1301-mediated inhibition is mainly via STAT3 pathway, indicating that miR-1301/STAT3 may be a promising therapeutic target for CRC.

Previous studies have reported that miR-1301 is differentially expressed and mostly presented as a tumor suppressor in several types of human cancers. The biological functions of miR-1301 depend on different target it binds to. Yang et al. reported that miR-1301 directly targets BCL9 and inhibits Wnt/ β -catenin signaling thus to hepatocellular carcinoma (HCC) invasion and angiogenesis (Yang et al. 2017). Controversially, another study reported miR-1301 was highly expressed in HCC cell lines as well as tumor samples and specifically targeted the tumor suppressive KLF6-FL. Functionally, enhancement of miR-1301 promoted cell migration and angiogenesis (Liang et al. 2014). In glioma cells, miR-1301 inhibited glioma cell proliferation and blocked the cell cycle to G1 by negatively regulating N-Ras and its downstream targets, MEK-ERK1/2

(Zhi et al. 2017). However, in our proliferation test, miR-1301 showed limit effect on CRC cells. A better explanation could be found in our GO and pathway analysis. Though miR-1301 targets BCLs or N-Ras which could positively regulate cell proliferation, several targets unreported like CDKN1 and BAX exerting inhibitory roles in cell proliferation may be an offset. Recently, other research teams have also discovered the novel miR-1301/STAT3 axis in cancer progression. LncRNA ABHD11-AS1 could promote tumor progression in papillary thyroid carcinoma and act as a competitive endogenous RNA to enhance STAT3 expression by sponging miR-1301 (Wen et al. 2019). Similarly in hepatocellular carcinoma, LINC01433 promotes cancer metastasis and progression through modulating the miR-1301/STAT3 axis (Huang et al. 2019).

STAT3, a member of the STAT protein family, is typically triggered by the binding of cytokines and growth factors to their related receptors. Numerous studies have indicated that activated IL-6/STAT3 signaling is one of the crucial pathways involving in CRC. Hyperactivation of STAT3 pathway promotes the expression of genes that involved in cell cycle arrest (e.g., Cyclin D1), survival (e.g., Bcl2, survivin), and metastasis (e.g., vimentin, MMPs) that contribute to CRC initiation and progression. Targeting STAT3 by RNAi or miRNA is an appealing anti-cancer strategy. Full understanding of miRNA-STAT3 regulation would be helpful for CRC therapy. MiR-124 and miR-874 have been reported directly targeted STAT3 and inhibited CRC proliferation (Zhang et al. 2013; Zhao and Dong 2016). In the present study, we found miR-1301 directly binds to STAT3 and is negatively correlated with pSTAT3 (Y705) expression in metastasis patients. To confirm the tumor suppressor role of miR-1301 is mainly via STAT3 pathway, we selected two CRC cell lines, LoVo (pSTAT3-positive, high metastatic capacity) and SW480 (pSTAT3-negative, low metastatic capacity). In LoVo cells, transfection of miR-1301 mimics inhibited total STAT3 and pSTAT3, and suppressed cell migration and invasion capacity. Due to the absence of pSTAT3 in SW480 cells, miR-1301 overexpression did not change pSTAT3 expression and invasion capacity. These result indicated the inhibitory effect of miR-1301 on CRC migration and invasion is STAT3-dependent.

Conclusions

In conclusion, our research revealed that miR-1301 is enriched in normal colorectal tissues and significantly down-regulated in CRC. Decreased level of miR-1301 can potentially promote CRC cell migration and metastasis via

activation of STAT3 pathways. These results suggest that miR-1301 is a promising therapeutic target for advanced CRC patients.

Materials and methods

TCGA data access

Clinical data and gene expression data of 14 tumor types were downloaded from UCSC Xena.

Tissue samples

CRC tissues and adjacent normal tissues from 28 patients were kindly endowed from the Fourth Military Medical University.

RNA extraction and quantitative real time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, USA). The miRNA was then reverse transcribed to cDNA according to the manufacture of microRNA First Strand cDNA Synthesis (Poly A Tailing) Kit (Sangon, China). Quantitative real-time PCR amplifications were performed with the SYBR Premix Ex Taq™ II kit (TAKARA, Japan) and specific primers for miR-1301 or U6 (Sangon, China, Table 2). The real time PCR was detected by CFX96™ real-time PCR system. Relative expression was normalized according to formulas $2^{-\Delta\Delta Ct}$, and U6 was set as an internal control.

GO and pathway analysis

Online software DAVID v6.7 was used for Gene Ontology (GO) and pathway analysis of miR-1301 targets.

Cell lines

Colorectal adenocarcinoma cells SW480 and LoVo were purchased from ATCC (American Type Culture Collection) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco USA). Cells were maintained at 37 °C in atmosphere of 5% CO₂.

Transfection

MiR-1301 mimics (Forward: UUGCAGCUGCCUGGGAGUGACUUC; Reverse: AGUCACUCCAGGCAGCUGCAAUU), miR-1301 inhibitor (Sequence: GAAGUCACUCCAGGCAGCUGCAA), and STAT3 siRNA were synthesized and purified (GenePharma, China). Target sequence

of STAT3 siRNA was: UCCAGUUUCUAAUUUGUUGACGGGUC. SW480 and LoVo Cells were reverse transfected with HiPerFect transfection reagent (Qiagen, USA) according to the manuals. For mimics and siRNA, the final concentration was 10 nM, while the concentration for inhibitor was 20 nM.

Luciferase reporter assay

The sequence of wild type or mutant type of STAT3-3'UTR (300 bp) were synthesized and constructed into the pmir-GLO™ luciferase reporter plasmid (Promega, USA). CRC cells were cotransfected with 500 ng of pmir-STAT3-WT or pmir-STAT3-MUT and 10 nM miR-1301 mimics or negative controls in 24-well plates. After transfection of 24 h, the luciferase activity was measured with a dual luciferase reporter assay system (Promega, USA).

Cell proliferation assay

Cell Counting Kit-8 (Dojindo Laboratories, Japan) was performed to detect cell viability according to a previously reported protocol (Wang et al. 2019). Briefly, the transfected cells were seeded into 96-well plate at a density of 2000/well with five replicates. At indicated time, 10 µl CCK-8 solution was added into each well. After incubation for 2–3 h, the viable cells were measured at a wavelength of 450 nm.

Cell migration and invasion assay

Wound scratch assay was performed to evaluate migration capacity, and detail method was described as previously (Chen et al. 2018b). For cell invasion assay, the Transwell chambers (Corning, USA) with Matrigel (BD Biosciences, USA) were applied. Transfected cells were treated with Mitomycin C (10 µg/ml) before plating to inhibit cell proliferation. 2×10^4 cells in a total volume of 100 µl serum-free medium were then plated in the upper chamber. The cells were incubated at 37 °C for 24 h (LoVo) or 48 h (SW480). The invaded cells on the lower chamber were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and washed with PBS. The invasion capacities were analyzed by counting the number of invaded cells in five randomly picked fields as observed under a microscope at a magnification of 100×.

Western blotting

Protein extraction and quantification were carried out according to a previously described protocol (Chen et al. 2018c). The antibodies used in this study were as follows: mouse anti-Stat3 (#9139, 1:1000; Cell Signaling Technology), rabbit anti-Phospho-Stat3 (Tyr705) (#9145, 1:1000; Cell Signaling Technology), rabbit anti-MMP9 (YT5357, 1:1000; ImmunoWay), rabbit anti-MMP2 (10373-2-AP, 1:2000; Proteintech), and mouse anti-GAPDH (YM3029, 1:5000; ImmunoWay).

Statistical analysis

GraphPad Prism (GraphPad Software, Inc.) was used for statistical analysis in this study. All experiments were repeated at least three

Table 2: Primer sequences for qRT-PCR.

Gene	Primer sequence (5'-3')
miR-1301	F: CGAGCTGCCTGGGAGTGAC
U6	F: CTCGCTTCGGCAGCAC

times and the results were presented as the mean $\pm$ SD. A P value < 0.05 was considered statistically significant.

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Research involving human participants: The work has been performed in accordance with Declaration of Helsinki, and all patients involved in this study had given written informed consent.

Conflict of interest statement: All authors declare that there are no conflicts of interest.

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