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ORIGINAL RESEARCH

lncRNA DSCAM-AS1 downregulates miR-216b to promote the migration and invasion of colorectal adenocarcinoma cells

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Background: It has been reported that lncRNA DSCAM-AS1 plays an oncogenic role in breast cancer. In the present study we explored the role of DSCAM-AS1 in colorectal adenocarcinoma (CRA).

Methods: Gene expression was analyzed by qPCR and Western blot. Overexpression experiments were performed to analyze gene interactions. Transwell assays were performed to analyze cell invasion and migration. Methylation-specific PCR (MSP) was performed to analyze DNA methylation.

Results: It was observed that DSCAM-AS1 was upregulated in the primary tumor tissues than in paired non-tumor tissues (within 2 cm around tumors) and was further increased with tumor metastasis. miR-216b was downregulated in primary tumor and further downregulated with tumor metastasis. miR-216b was inversely correlated with DSCAM-AS1 in tumor tissues, but not in non-tumor tissues. In cells of CRA cell lines, DSCAM-AS1 overexpression resulted in the downregulation of miR-216b, while miR-216b overexpression did not significantly affect DSCAM-AS1. DSCAM-AS1 overexpression did not significantly affect cancer cell proliferation but promoted cell migration and invasion. miR-216b inhibited cancer cell migration and invasion and significantly reduced the effects of DSCAM-AS1 overexpression. Methylation-specific PCR showed that DSCAM-AS1 overexpression promoted the methylation of miR-216b gene.

Conclusion: DSCAM-AS1 may downregulate miR-216b to promote the migration and invasion of CRA cells.

Keywords: colorectal adenocarcinoma, lncRNA DSCAM-AS1, miR-216b

Introduction

Colorectal adenocarcinoma (CRA) causes 60,000 deaths and affects more than 1.2 million every year.[1] To data, CRA has been recognized as a common type of malignancy and a leading cause of cancer-related deaths worldwide.[1] Development of CRA is usually accompanied with the occurrence of tumor metastasis.[2,3] Overall survival of CRA patients with non-metastatic tumor within 5 years after initial diagnosis can be up to 90%, but only less than 10% of patients with metastatic CRA can live longer than 5 years.[4,5] Therefore, how to prevent and treat tumor metastasis in CRA patients is a critical problem in the clinical treatment of CRA.

More than 98% of human genome transcripts are noncoding RNAs (ncRNAs), which are RNA transcripts that lack protein-coding ability.[6] Long (>200 nt) ncRNAs, or lncRNAs, were initially characterized as spurious transcriptional

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noise but proved as critical determinants in cancer biology by recent studies.[7,8] More and more studies have shown that regulating the expression of lncRNAs may lead to altered expression of certain oncogenes or tumor suppressors, thereby promoting or inhibiting cancer development.[9,10] However, functions of lncRNAs are only partially understood. DSCAM-AS1 is an lncRNA that promotes breast cancer progression.[11] Our RNA-seq data suggested that DSCAM-AS1 was upregulated in CRA and inversely correlated with miR-216b, which suppress CRA. Our study was therefore carried out to investigate the interaction between DSCAM-AS1 and miR-216b in CRA.

Materials and methods

Patients

Liaoning Cancer Hospital & Institute admitted 144 patients with CRA during the time period from March 2011 to March 2013. Our study selected 70 (39 males and 31 females) of those patients to serve as research subjects according to strict inclusion and exclusion criteria. Inclusion criteria:1) CRA diagnosed by histopathological examinations; 2) no previous malignancy history; 3) first diagnosis and no therapy received before admission. Exclusion criteria: 1) patients with multiple clinical disorders besides CRA; 2) patients who were transferred from other hospitals. Based on the criteria established by AJCC, there were 12 cases of stage I, 18 cases of stage II, 10 cases of stage III and 30 cases of stage IV. Among those patients, distant metastasis occurred in 28 cases. Age of the patients ranged from 29 to 68 years and the mean age was 49.2±6.1 years. All patients signed informed consent.

Specimens and cell lines

To detect the in vivo expression, primary tumor tissues and paired non-tumor tissues (within 2 cm around tumors) were obtained from each patient through biopsy. All specimens were confirmed by 3 experienced pathologists.

To perform in vitro experiments, cells of human CRA cell lines WiDr and HT-29 were purchased from ATCC (Manassas, VA, USA) and were cultivated in McCoy's 5a modified medium supplemented with 10% FBS.

Follow-up

Patients were followed-up until their death or for 5 years through outpatient visit or phone call every month. Patients who were lost or died of other causes were excluded (the 70 patients included in this study all completed the follow-up or died of CRA during follow-up).

Real-time quantitative PCR

In order to detect DSCAM-AS1 expression, RNAzol reagent (Sigma-Aldrich, MO, USA) was used to extract total RNA. Reverse transcription was performed using Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit , PCR reaction systems were prepared using Luna® Universal One-Step RT-qPCR Kit (NEB) with 18S rRNA as endogenous control.

In order to detect miR-216b, miRNeasy Kit (Qiagen) was used to extract miRNA. Following reverse transcription performed using Applied Biosystems™ TaqMan™ MicroRNA Reverse Transcription Kit, TaqMan™ Fast Advanced Master Mix (Thermo Fisher Scientific, Schwerte, Germany) was used to prepare PCR reaction systems with U6 as endogenous control.

After PCR reactions, data normalization was performed based on $2^{-\Delta\Delta CT}$ method.

Methylation-specific PCR (MSP)

Conventional methods were used to extract genomic DNA as extracted from WiDr and HT-29. EZ DNA Methylation-GoldTM kit (ZYMO) was used to convert DNA. Taq polymerase was used to perform the following PCR reactions.

Transient transfection

DSCAM-AS1 expression was constructed by inserting DSCAM-AS1 full-length DNA into pcDNA3.1 expression vector (Sangon, Shanghai, China). MISSION® microRNA Mimic hsa-miR-216b and negative control (NC) miRNA were bought from Sigma-Aldrich. Transient transfections were performed using lipofectamine 3000 reagent (Thermo Fisher Scientific) with 10 nM vector and 40 nM miRNA. Cells were harvested at 24 hrs after transfection to perform subsequent experiments. Cells with no transfection were control cells. Cells with empty vector or NCmiRNA transfection were NCcells.

Transwell migration and invasion assay

Cells were harvested at 24 hrs after transfection. Nonserum McCoy's 5a medium modified was used to prepare single-cell suspensions (4×10^4 cells/mL). Cell suspensions were added into the upper chamber with 0.1 mL per well. Matrigel (356,234, Millipore, Bedford, MA, USA) was used to coat the upper chamber membrane before invasion assay. McCoy's 5a medium Modified supplemented with

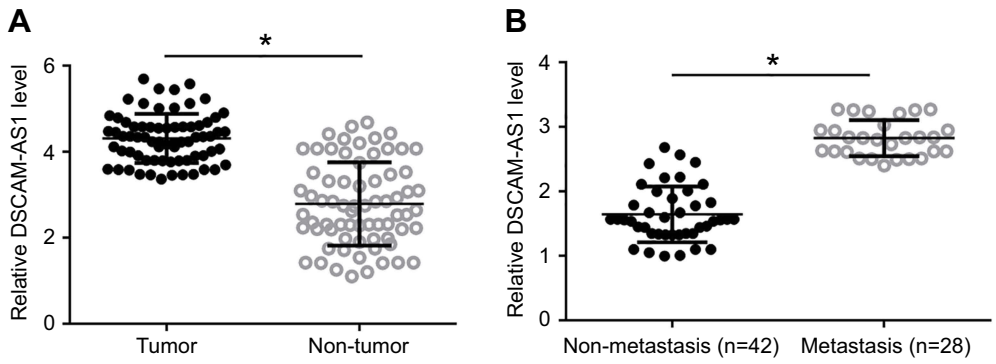

Figure 1 DSCAM-AS1 was upregulated in tumor tissue and affected by distant tumor metastasis. DSCAM-AS1 was significantly upregulated in the primary tumor tissues than in paired non-tumor tissues (**A**). Compared with the non-metastasis group, DSCAM-AS1 was significantly upregulated in the metastasis group (**B**) (*p<0.05).

20% FBS was added into the lower chamber. Three replicate wells were set for each transfection group. Cells were cultivated for 2 hrs, followed by upper chamber membrane staining using 0.5% crystal violet (Sigma-Aldrich). Staining was performed for 20 mins at room temperature. Stained cells were observed under an optical microscope and counted.

Statistical analysis

Each experiment contained 3 biological repeats. Data were processed using Graphpad Prism 6 software. Paired *t*-test was used to compare tumor and non-tumor tissues. Unpaired *t*-test was used to compare metastasis and non-metastasis groups. Comparisons among multiple-cell treatment groups were performed using ANOVA (one-way) and Tukey test. Linear regression was performed for correlation analysis. Patients were divided into high (n=32) and low (n=38) DSCAM-AS1 (in tumor tissue) groups based on Youden's index. K-M method and log-rank *t*-test were used to plot survival curves and compare survival curves, respectively. $p<0.05$ was the statistically significant cutoff value.

Results

DSCAM-AS1 was upregulated in tumor tissue and affected by distant tumor metastasis

Differential expression of DSCAM-AS1 was detected by RT-qPCR. It was observed that DSCAM-AS1 was significantly upregulated in the primary tumor tissues than in paired non-tumor tissues (Figure 1A, $p<0.05$). Patients were divided into metastasis (n=28) and non-metastasis (n=42) groups. Compared with the non-metastasis group, DSCAM-AS1 was significantly upregulated in the metastasis group (Figure 1B, $p<0.05$).

High expression level of DSCAM-AS1 in tumor tissue predicted poor survival

Patients were divided into high (n=32) and low (n=38) DSCAM-AS1 (in tumor tissue) level groups based on Youden's index. K-M method and log-rank *t*-test were used to plot survival curves and compare survival curves, respectively. It was observed that the overall survival rate of high DSCAM-AS1 level group was significantly lower than that of low DSCAM-AS1 level group (Figure 2).

miR-216b was downregulated in tumor tissue and affected by distant tumor metastasis

Differential expression of miR-216b was also detected by RT-qPCR. It was observed that miR-216b was significantly downregulated in the primary tumor tissues than in paired non-tumor tissues (Figure 3A, $p<0.05$). Compared with the

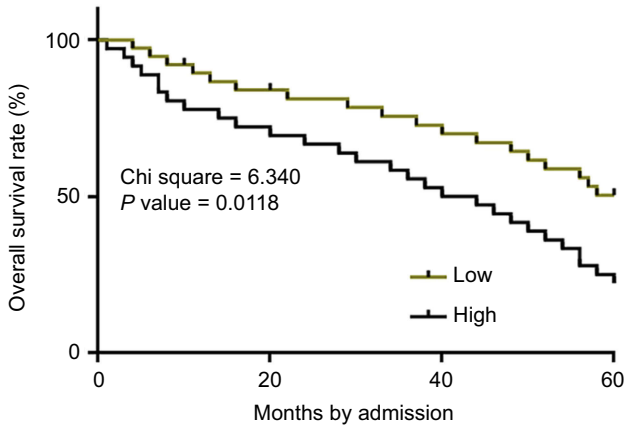

Figure 2 High expression level of DSCAM-AS1 in tumor tissue predicted poor survival. Survival curve analysis showed that the overall survival rate of high DSCAM-AS1 level group was significantly lower than that of low DSCAM-AS1 level group.

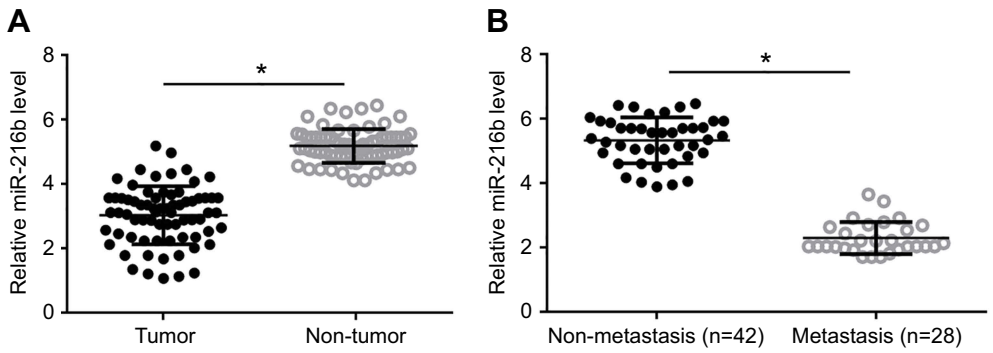

Figure 3 miR-216b was downregulated in tumor tissue and affected by distant tumor metastasis. miR-216b was significantly downregulated in the primary tumor tissues than in paired non-tumor tissues (**A**). Compared with the non-metastasis group, miR-216b was significantly downregulated in the metastasis group (**B**) (*p<0.05).

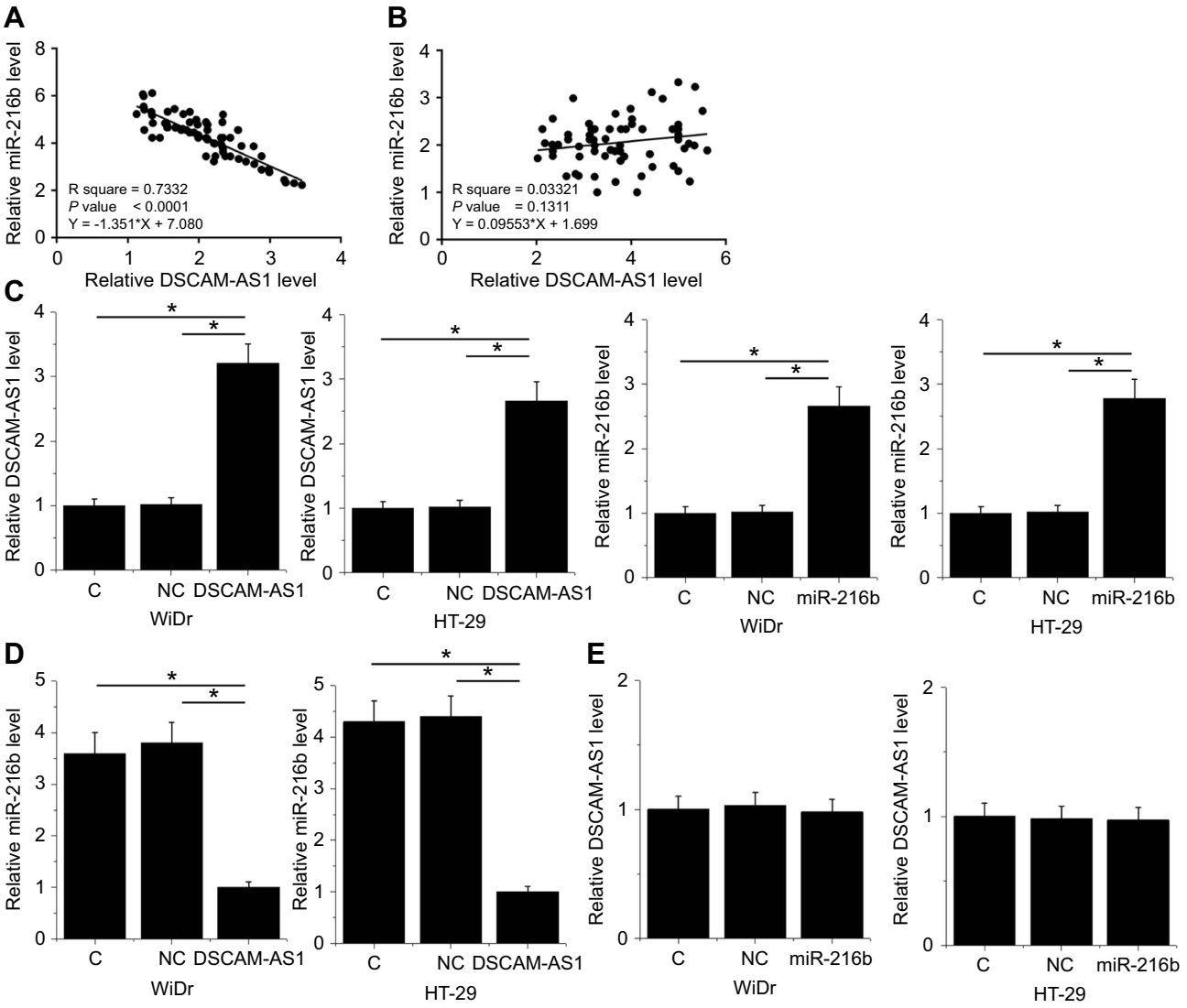

Figure 4 DSCAM-AS1 downregulated miR-216b in CRA cells. Linear regression showed that miR-216b and DSCAM-AS1 were significantly and inversely correlated in tumor tissues (**A**) but not in non-tumor tissues (**B**). After miR-216b and DSCAM-AS1 overexpression in cells of WiDr and HT-29 cell lines (**C**) SCAM-AS1 overexpression resulted in the downregulation of miR-216b (**D**), while miR-216b overexpression did not significantly affect DSCAM-AS1 (**E**) (*p<0.05).

non-metastasis group, miR-216b was significantly downregulated in the metastasis group (Figure 3B, $p<0.05$).

DSCAM-AS1 downregulated miR-216b in CRA cells

Linear regression was performed for correlation analysis between expression levels of miR-216b and DSCAM-AS1. It was observed that miR-216b and DSCAM-AS1 were significantly and inversely correlated in tumor tissues (Figure 4A), but not in non-tumor tissues (Figure 4B). To further analyze the interaction between miR-216b and DSCAM-AS1, miR-216b and DSCAM-AS1 were overexpressed in cells of WiDr and HT-29 cell lines (Figure 4C, $p<0.05$). Compared with control (C) and NC groups, cells with DSCAM-AS1 overexpression showed downregulated miR-216b (Figure 4D, $p<0.05$). However, cells with miR-216b overexpression show no significantly altered expression of DSCAM-AS1 (Figure 4E, $p<0.05$).

DSCAM-AS1 regulated cancer cell migration and invasion, but not proliferation through miR-216b

Compared with C and NC groups, DSCAM-AS1 overexpression did not significantly affect cancer cell proliferation (data not shown) but promoted CRA cell migration (Figure 5A, $p<0.05$) and invasion (Figure 5B, $p<0.05$). miR-216b inhibited cancer cell migration and invasion and significantly reduced the effects of DSCAM-AS1 overexpression.

DSCAM-AS1 overexpression promoted the methylation of miR-216b gene

MSP was performed to analyze the effects of DSCAM-AS1 overexpression on the methylation of miR-216b gene. Compared to the transfection of pcDNA3.1 vector, transfection of DSCAM-AS1 expression vector led to obvious increased methylation rate of miR-216b gene (Figure 6).

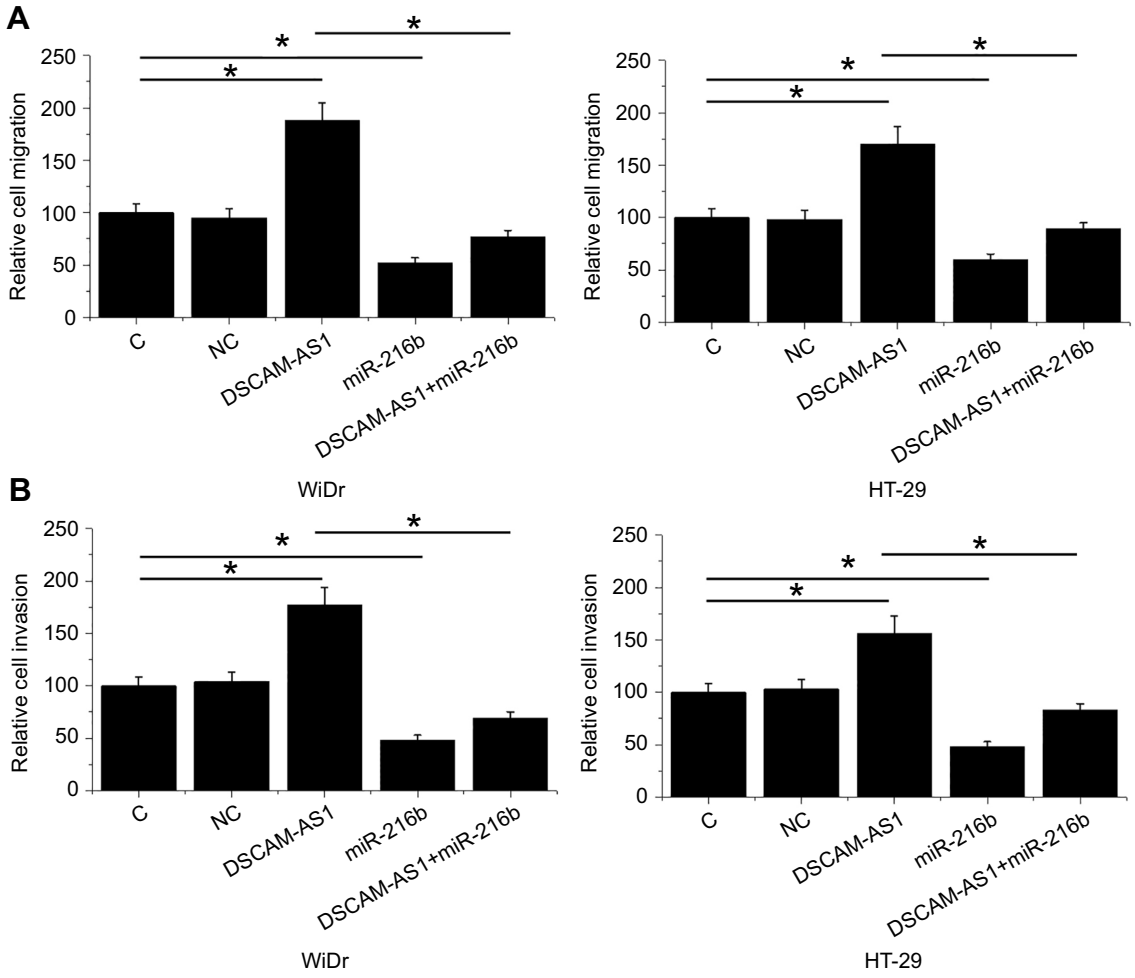

Figure 5 DSCAM-AS1 regulated cancer cell migration and invasion, but not proliferation through miR-216b. DSCAM-AS1 overexpression promoted CRA cell migration (**A**) and invasion (**B**) miR-216b inhibited cancer cell migration and invasion and significantly reduced the effects of DSCAM-AS1 overexpression (*$p<0.05$).

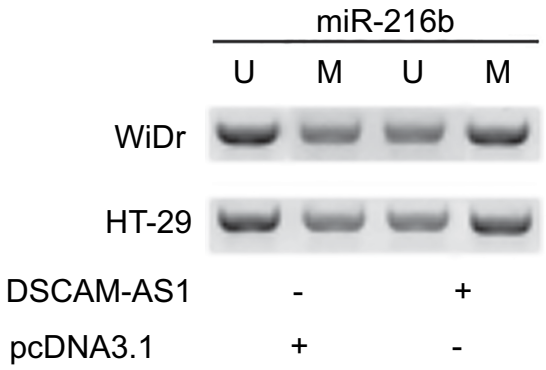

Figure 6 DSCAM-AS1 overexpression promoted the methylation of miR-216b gene. Methylation-specific PCR (MSP) was performed to analyze the effects of DSCAM-AS1 overexpression on the methylation of miR-216b gene. It was observed that DSCAM-AS1 expression vector led to obvious increased methylation rate of miR-216b gene.
Abbreviations: U, unmethylated; M, methylated.

Discussion

DSCAM-AS1 has been characterized as an oncogenic lncRNA in breast cancer,[12] while its involvement in other types of malignancies is unknown. Our study first reported the oncogenic role of DSCAM-AS1 in CRA and proved that the actions of DSCAM-AS1 in CRA are likely mediated by the downregulated expression of miR-216b.

Although miR-216b has been characterized as a tumor suppressor in many types of cancer,[13,14] the function of miR-216b in CRA is still controversial. Zou et al reported that miR-216b promoted cell growth in colon cancer, indicating its role as an oncogene.[15] In contrast, Niknafs et al characterized miR-216b as a tumor suppressor in colon cancer and proved that it targeted HMGB1-mediated JAK2/STAT3 signaling way to inhibit cancer development.[11] Our study observed the downregulated expression of miR-216b in tumor tissues of CRA patients, and the overexpression of miR-216b mediated the inhibited migration and invasion of CRA cells. Therefore, our study supported the role of miR-216b as a tumor suppressor in CRA.

Our study first reported the upregulation of DSCAM-AS1 in CRA tissues and proved that DSCAM-AS1 expression was upregulated with tumor metastasis, suggesting the involvement of DSCAM-AS1 in metastatic CRA. Tumor metastasis is a major cause of deaths among CRA patients.[16] Our study also proved that the high expression level of DSCAM-AS1 in CRA tissues is closely correlated with poor survival of CRA patients. Therefore, DSCAM-AS1 may serve as a prognostic indicator for CRA.

The interactions between lncRNAs and miRNAs have been widely investigated in cancer biology.[17] lncRNAs may serve a sponge of miRNAs to inhibit the function of miRNAs in cancer biology.[18] Our study proved the role of DSCAM-AS1 as an upstream inhibitor of miR-216b, while no promising target of miR-216b on DSCAM-AS1 was observed. DSCAM-AS1 and miR-216b were not significantly correlated in non-tumor tissues. We showed that DSCAM-AS1 is likely involved in the methylation of miR-216b gene to downregulate its expression. However, the detailed mechanism remains to be further investigated.

In conclusion, DSCAM-AS1 may participate in the metastasis of CRA by downregulating miR-216b.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Liaoning Cancer Hospital & Institute. The research has been carried out in accordance with the World Medical Association Declaration of Helsinki. All patients and healthy volunteers provided written informed consent prior to their inclusion within the study.

Author contributions

Fei Liu designed the study. Fei Liu, Jianhui Jia, Liping Sun, Qingrui Yu, Hongyan Duan, Dexin Jiao, Zhiqiang Gong, Shendong Zhu, Kexin Jiang, Yijiang He, Liping Chen, Yanni Zhang and Han Sun carried out the study. Fei Liu, Jianhui Jia, Liping Sun, Qingrui Yu, Hongyan Duan, Dexin Jiao and Zhiqiang Gong participated in the experiments and statistical analysis. Fei Liu wrote the manuscript. Fei Liu and Jianhui Jia revised the manuscript. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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