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LncRNA MIAT Promotes the Proliferation and Invasion of Colorectal Cancer via Suppressing Apoptosis and Senescence

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Abstract

Background: Myocardial infarction-associated transcript (*MIAT*) is a long non-coding RNA (lncRNA), which functions in a variety of disorders, like myocardial infarction and diabetic retinopathy. Moreover, recent reports have established that *MIAT* is upregulated in several types of malignancies and plays a crucial role in tumorigenesis. Therefore, this research aimed to investigate the expression of *MIAT* in colorectal cancer (CRC) and further evaluate the impact of its knocking-down on the proliferation and migration of the CRC cell.

Method: In this case-control experimental study, we evaluated the expression level of *MIAT* in a series of CRC and marginal tissues using RT-qPCR. Furthermore, the role of *MIAT* was assessed employing RNA interference (RNAi)-mediated suppressing strategy in CRC-derived cells. Subsequently, colony formation, cell cycle analysis, migration, apoptosis, and senescence assays were done to decipher the influence of *MIAT* on initiation and progression of CRC.

Results: Our findings revealed that *MIAT* expression is significantly upregulated in high-grade and vascular invasion tumor tissues. Furthermore, *MIAT* silencing led to G1 arrest in SW116 and SW48 CRC-derived cells. We also found that *MIAT* inhibition contributed to the induction of apoptosis/cellular senescence as well as the limitation of colony formation capability and cell migration in CRC cells. The obtained findings also showed that *MIAT* silencing dysregulated the expression of ATM and CHK2 genes known as DNA damage responsive genes.

Conclusion: The results of the present study demonstrated that lncRNA *MIAT* may control CRC cell proliferation and metastasis through regulating DNA damage-responsive pathway and can be noticed as a potential marker for diagnosis, prognosis, and targeted-therapy of high-grade CRC.

Keywords: *MIAT* long non-coding RNA, Colorectal neoplasms, Cellular senescence, DNA damage

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Introduction

Long non-coding RNAs (lncRNAs) are generally described as the class of RNA molecules longer than 200 nucleotides, which do not have an open reading frame (ORF).[1, 2] Over the recent years, lncRNAs have received a great deal of scientific attention owing to their specific tissue expression patterns and functional significance in various physiological and pathological pathways.[3] On account of their strong cell-type specific and temporal expression, their importance has been verified. lncRNAs have now been identified as critical regulators in multiple biological mechanisms, like gene expression, epigenetic modulation, and chromatin remodeling.[3, 4]

Myocardial infarction-associated transcript (*MIAT*), also known as retina non-coding RNA2 or *Gomafu* is a lncRNA which has been located on human chromosome 12q12.1. LncRNA *MIAT* has a crucial role in different diseases, such as myocardial infarction,[5] microvascular dysfunction,[6] and paranoid schizophrenia.[7] Previous reports have established that its

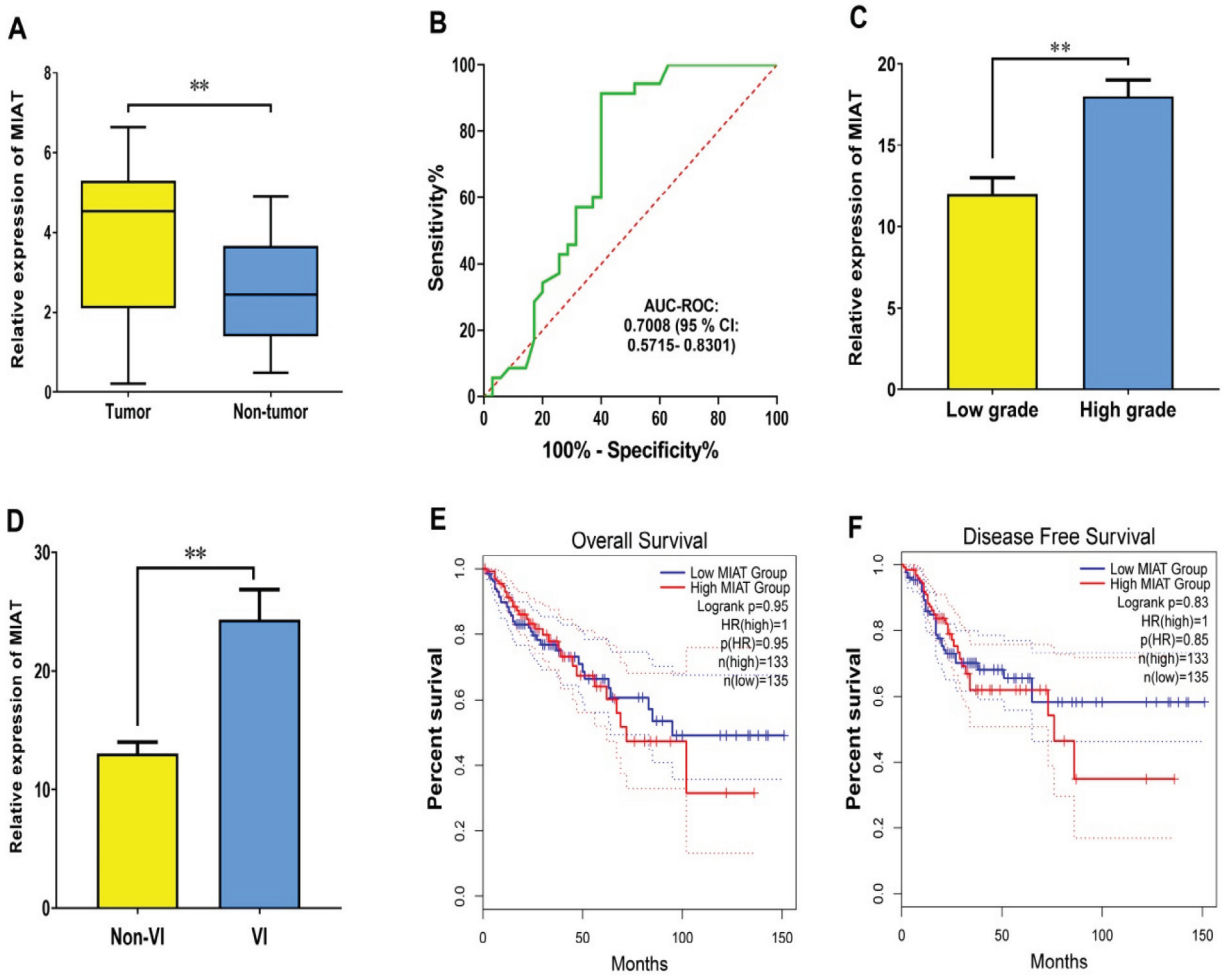

Figure 1. LncRNA *MIAT* is significantly upregulated in CRC tissues. (A) *MIAT* is significantly upregulated in CRC samples compared with that in adjacent normal ones. (B) ROC-curve analysis introduces *MIAT* as a specific and sensitive marker in CRC diagnosis. (C) *MIAT* is significantly upregulated in high-grade tumors in comparison with the low-grade ones. (D) *MIAT* expression is significantly higher in VI (vascular invasion) samples than that in non-VI (non-vascular invasion) ones. (E, F) Kaplan-Meier analysis of *MIAT* expression and overall survival of CRC patients showed that *MIAT* expression is associated with the survival rate in higher stages of CRC. Data are demonstrated as mean ± SD at three independent repeats (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

MIAT: Myocardial infarction associated transcript; CRC: Colorectal cancer; VI: Vascular invasion; SD: Standard deviation; ROC: Receiver operating characterstic

expression is upregulated in different malignancies, such as breast,[8] hepatocellular,[9] cervical,[10] and ovarian cancers.[11] Emerging evidence established that the role of *MIAT* in many cellular processes, including the nuclear bodies' formation and neurogenic commitment.[12] Additionally, a recent report demonstrated that *MIAT* contibuted to the initiation and progression of numerous cancer types.[13] It can induce epithelial to mesenchymal transition (EMT) in different types of cancers, including hepatocellular carcinoma,[14] breast cancer,[15] and melanoma.[16] Numerous miRNAs, like miR-214, miR-22-3p, miR-520d-3p, miR-203a, miR-29a-3p, miR-141, miR-150, miR-302, miR-29, and miR-155-5p, have effective interactions with *MIAT*. Besides these miRs, *MIAT* has interactions with a variety of proteins, like Oct4.[13]

Colorectal cancer (CRC) is considered as one of the most prevalent types of gastrointestinal deficiencies worldwide, and over one million new cases are diagnosed annually.[17] Lately, great body of evidence has reported that lncRNAs play the role of modulators in the growth and metastasis of CRC cells;[18, 19] for example, terminal differentiation-induced non-coding RNA (TINCR) was reported to have a role in proliferation and metastasis of cancer cells via regulating the epithelial growth factor (EGF) signaling pathway through miRNA sponging.[20] Moreover, CRNDE activates the RASnMAPK pathway and interacts with hnRNPUL2 that promotes the proliferation of CRC cells.[21, 22]

It was previously specified that *MIAT* promoted the proliferation, migration, and invasion of CRC cells via sponging the miR-132 and subsequently regulating the expression of Derlin-1.[23] *MIAT* knocking-down in CRC cells led to the limitation of proliferation, apoptosis induction, and suppression of migration/invasion, suggesting that *MIAT* has a tumorigenesis function and could have a substantial performance in the growth and metastasis of CRC.[23]

A previous study reported that *MIAT* has a vital role in tumor initiation, progression, and metastasis.[8] Therefore, the present research aimed to assess the possible dysregulation of lncRNA *MIAT* in a series of colorectal tumor tissues. Furthermore, the present work was conducted to further evaluate the mechanisms underlying *MIAT* contribution to CRC development and progression.

Materials and Methods

Tissue samples collection

In this case-control experimental study, a series of cancerous and non-cancerous marginal tissues from 35 patients with CRC were gathered from the Iran National Tumor Bank, which was founded by Cancer Institute of Tehran University of Medical Sciences (Tehran, Iran). The Ethics Committee of Kerman University of Medical Sciences approved the experimental procedures of this study (Ethics code: IR.KMU.REC.1401.329). Written informed consents were taken prior to the experimental procedure. The clinicopathological features of each patient were obtained during tissue gathering by the Iran National Tumor Bank (Table 1). The tissues were kept frozen and stored at -80ºC until the beginning of the RNA extraction experiments.

Cell culture and transfection

In this study, CRC cell lines (SW1116, SW480, HT29, and SW48) were provided by the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran) and grown in RPMI 1640 medium (Gibco, CA, USA), with 10% fetal bovine serum. They were kept at 37ºC in a humidified incubator with 5% CO_2. We further transfected the cancer cells with the SMART pool *MIAT* siRNAs and scrambled siRNA (Dharmacon, Lafayette, CO, USA) using Lipofectamine 2000 (Invitrogen) as described previously.[8]

Gene expression analysis

To extract total RNA, RNAX plus solution (Fermentase, Lithuania) was used based on the manufacturer's protocol. The concentration and purity of the isolated RNA were assessed by measuring 260/280-nm absorbance ratio, and then running it on 1% agarose gel electrophoresis. Afterwards, the isolated RNA was applied for cDNA synthesis using reverse transcriptase enzyme (Fermentase, Lithuania) based on the recommended protocol. Specific primers for amplifying of *MIAT*, β-actin and other studied

genes were designed by Gene Runner software (version 4.0) (Table 2). Quantitative PCR was done employing SYBR Premix Ex TaqTM II (Takara, Japan) on Rotor-Gene 6000 instrument (Corbett Life Science, Sydney, Australia). Gene relative expression levels were computed with the $2^{-\Delta\Delta Ct}$ formula (where $\Delta\Delta Ct$ is $(CT_{target}\text{-}CT_{actin})$ $time_x$ $-(CT_{target}\text{-}C_{Tactin})$ time 0.24 β-actin gene was applied as the housekeeping gene for normalization.

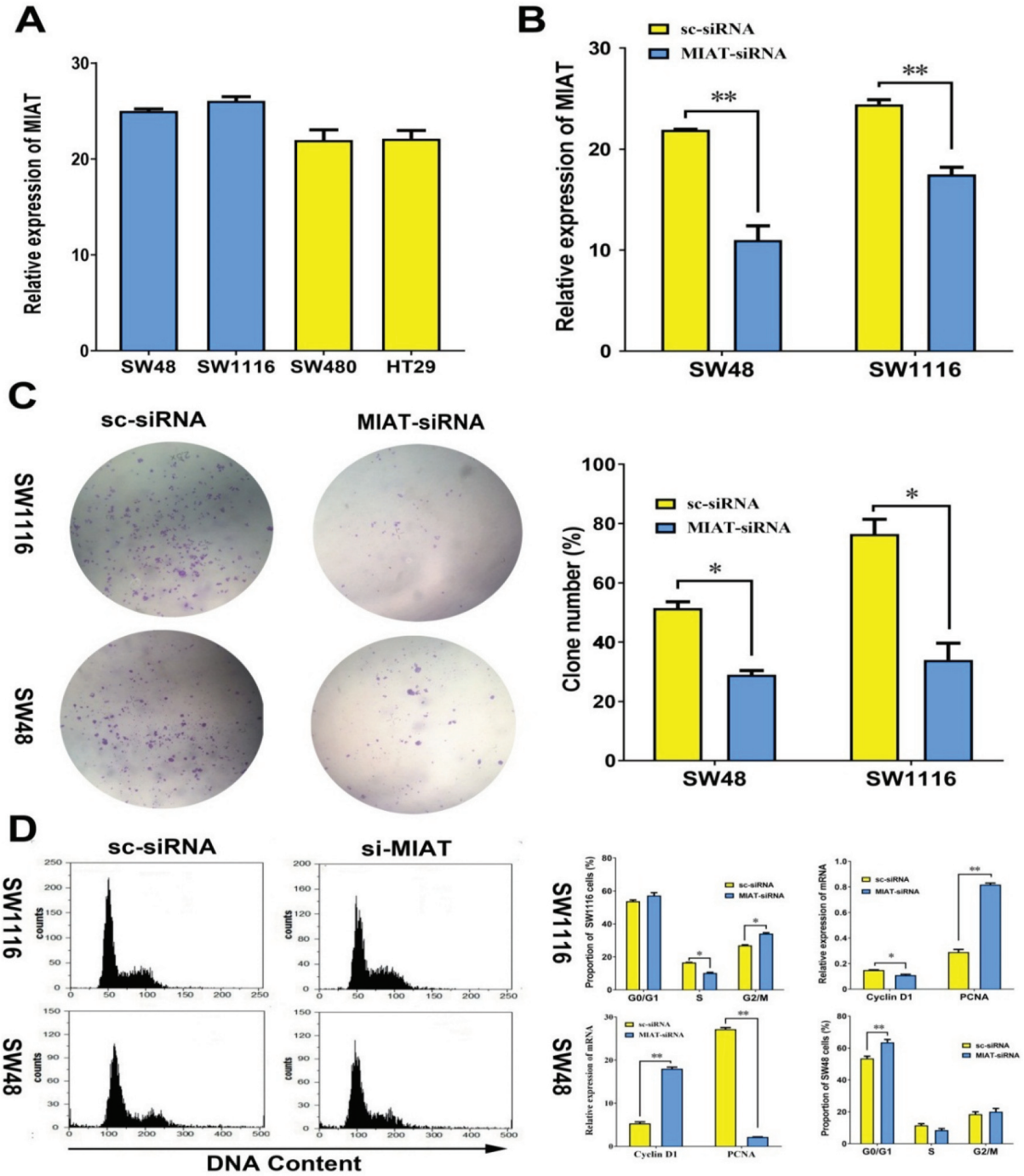

Figure 2. *MIAT* knocking-down causes a significant decrease in proliferation rate of cancer cells and induces G1 arrest. (A) *MIAT* expression in four types of CRC cells shows that its expression is upregulated in SW48 and SW116 cell lines. (B) Downregulation of *MIAT* was confirmed by RT-qPCR following its silencing in CRC cell lines. (C) *MIAT* knocking-down significantly reduces the clonogenic capacity of CRC cells. (D) *MIAT* suppression causes a G1 arrest in CRC cells. Data are demonstrated as mean ± SD at three independent repeats (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ****$P < 0.0001$).

MIAT: Myocardial infarction associated transcript; CRC: Colorectal cancer.

Colony formation experiment

To evaluate the impact of *MIAT* suppression on colony formation ability of CRC cells, wound healing assay was done. Briefly, the limited number of transfected cells were seeded in six-well plates and kept in RPMI 1640 medium with 10% FBS for 2 weeks. Subsequently, the colonies were fixed with 4% paraformaldehyde and then stained with 0.1% crystal violet (Sigma, St. Louis, MO, USA). Finally, the stained colonies were counted.

Flow cytometry analysis

The transfected cancer cells were collected at 48 and 72 hours following *MIAT* suppression and stained with FITC-Annexin V and PI (50 µg/ml propidium iodide, 0.1 % Triton X-100 and 0.1 % sodium citrate). The stained cells were analyzed for cell cycle distribution and apoptosis occurence ratio using a flow cytometer (Partec, Germany). Flowjo 7.6.1 software was utilized for analyzing the obtained data.

SA-β-gal staining

The increase in the activity of lysosomal β-galactosidase was considered to identify senescent cells. To this end, the SA-β-gal method was performed as explained before.[25] Finally, in three randomly selected fields, positive staining cells were quantified via ImageJ software.

Wound healing experiment

The cancer cells were seeded on six-well plates until reaching 90% confluency and were then scratched. After that, the medium was changed to DMEM with 1% FBS, and the cells were incubated for 48 hours. The speed of wound healing was captured and the images were evaluated with ImageJ software at 0 h, 24h, and 48 h.

Western blotting

Radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris, 0.15 M NaCl, 1 mM EDTA, 1 % NP40, 0.25 % SDS, pH 7.4) was used to extract total protein from transfected cells. The proteins were then quantified through Bradford assay, following which the extracted proteins were separated on a 12 % SDS polyacrylamide gel and then transferred to a PVDF membrane (Roche Diagnostics). The blocking procedure was done with 5% BSA in Tris-buffered saline containing 0.1 % Tween-20 (TBS-T). The membrane was incubated in the diluted first antibodies, including ATM (ab17995, Abcam, Cambridge, UK) and CHK2 (ab47433, Abcam, Cambridge, UK) at 4∞C for one night. It was then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 hour. An enhanced chemiluminescence detection kit (Amersham Biosciences, Uppsala, Sweden) was used to view the antibody/protein complexes for the next investigation.

Table 1. Clinicopathological features of the samples

Characteristics	Numbers
Tumor	27
Normal	20
Low-grade	19
High-grade	6
Stage2	5
Stage3	18
Stage4	7
Age	
<50 years	7
≥50 years	18
Sex	
Male	16
Female	11
Survival	
Alive	20
Deceased	4
Lymphatic invasion	
Positive	14
Negative	11
Vascular invasion	
Positive	14
Negative	11
Perineural invasion	
Positive	4
Negative	21

Data analysis

All the experimental procedures were performed in triplicate. To establish the statistical difference between the groups, the PRISM 6.07 software (GraphPad Software Inc., San Diego, CA, USA) was utilized, which analyzed the paired samples t-test, independent samples t-test, and ANOVA Tukey's post hoc test. Moreover, the receiver operating characteristic (ROC) curve was used for finding the sensitivity and specificity of *MIAT* in CRC screening. Survival analysis was also performed using the TCGA database in

GEPIA (http:// http://gepia.cancer-pku.cn/). The statistical significance was set at $P < 0.05$.

Results

MIAT is significantly overexpressed in CRC and its expression is associated with clinical features. *MIAT* expression level was quantified via RT-qPCR in paired cancerous and non-cancerous marginal tissues. As shown in figure 1A, *MIAT* was significantly overexpressed (1.34-fold) in CRC samples compared with the adjacent non-tumor tissues (0.004 ± 0.001 versus 0.001 ± 0.001, $P = 0.003$). Our findings from the ROC curve analysis showed that *MIAT* had an AUC-ROC value of 0.700 (Figure 1B, $P = 0.003$). We then analyzed the association between *MIAT* expression and clinical characteristics of CRC tissues. A high level of *MIAT* expression was significantly associated with tumor grade (1.5 folds) and vascular invasion (1.8 folds) (Figure 1C, D; $P = 0.001$ and 0.001, respectively). There was no association between *MIAT* expression and age, sex, survival, and perineural invasion (Table 1). Moreover, lower *MIAT* expression was found to be associated with more prolonged survival of CRC patients (Figure 1E, F).

MIAT promotes CRC cell proliferation and cell cycle progression from G0/G1 to S phase

In the next phase of our study, *MIAT* expression

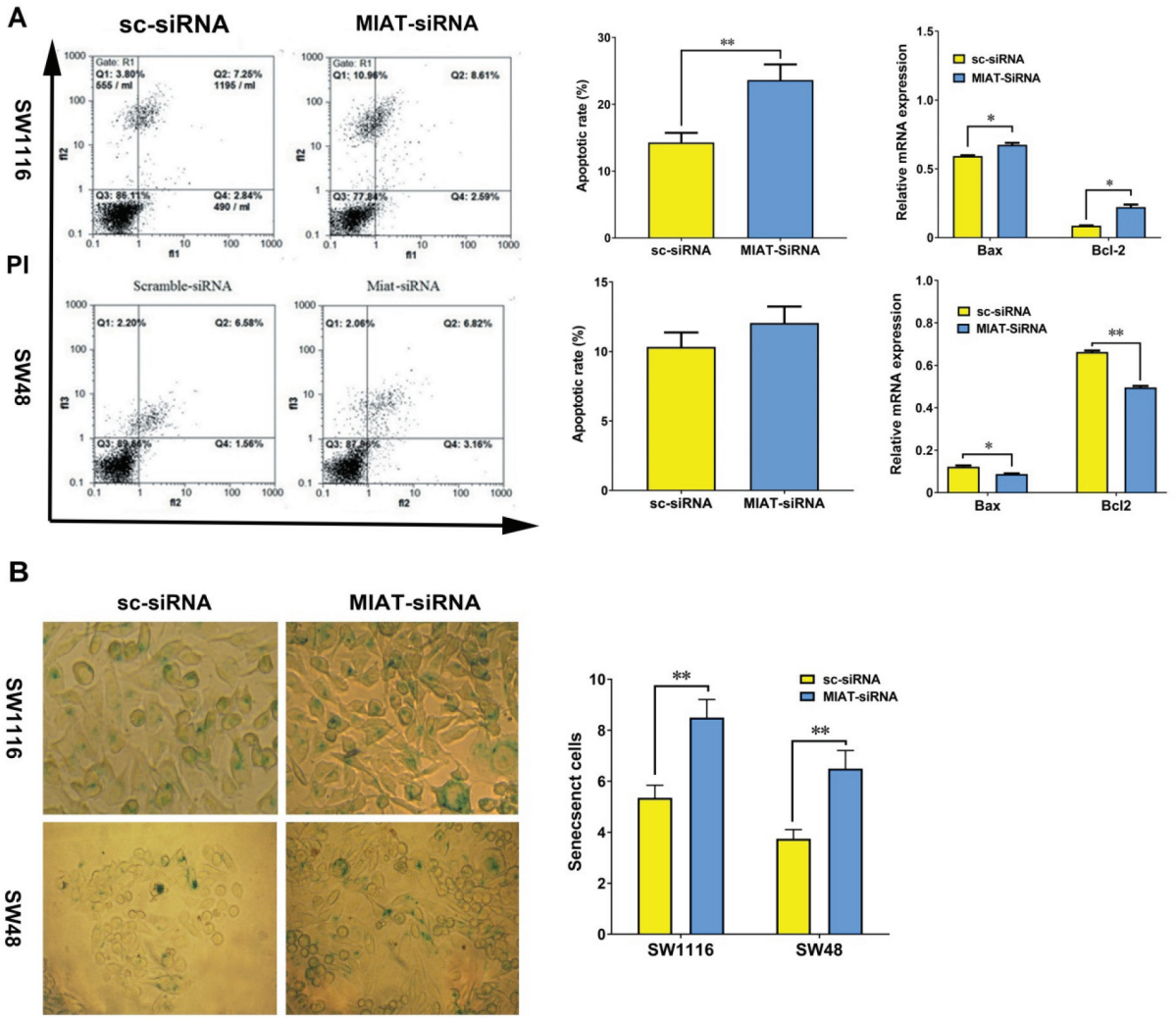

Figure 3. *MIAT* knocking-down causes a noticeable increase in the apoptotic percentage (A) and senescence rate (B) of CRC cells. (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ****$P < 0.0001$).

MIAT: Myocardial infarction associated transcript; CRC: Colorectal cancer

Table 2. Sequences of the primers used in this study

Gene name	Primer (forward) 5′-3′	Primer (reverse) 5′-3′	Amplicon size (bp)
MIAT	CAAAGAGCCCTCTGCACTAG	ACCTTGGTTACCCCTGTGATG	128
β-actin	ACCACCTTCAACTCCATCATG	CTCCTTCTGCATCCTGTCG	120
Bax	GGACGAACTGGACAGTAACATGG	GCAAAGTAGAAAAGGGCGACAAC	150
Bcl-2	CTGCACCTGACGCCCTTCACC	CACATGACCCCACCGAACTCAAAGA	119
Cyclin D1	ACAAACAGATCATCCGCAAACAC	TGTTGGGGCTCCTCAGGTTC	144
PCNA	AGGTGGAGAACTTGGAAATGG	CGTTGAAGAGAGTGGAGTGG	160

MIAT: Myocardial infarction associated transcript; Bax: Bcl-2-like protein; Bcl-2: B-cell lymphoma-2; PCNA: Proliferating cell nuclear antigen

level was evaluated in CRC cell lines (SW48, SW1116, SW480, and HT-29) with RT-qPCR. The expression of *MIAT* was higher in SW1116 and SW48 cell lines compared with that in the other ones (Figure 2A). To obtain a panoramic view about the probable mechanism of *MIAT* in CRC, the *MIAT* expression was knocked down using smart pool si-*MIAT* in SW1116 and SW48 cell lines, which have a high endogenous *MIAT* expression level. Primarily, the *MIAT* knocking-down efficiency was validated with RT-qPCR analysis in SW48 and SW1116 cell lines (Figure 2B, $P < 0.001$). The obtained results demonstrated that the clonogenic ability of the transfected cells significantly decreased following *MIAT* inhibition (Figure 2C; $P < 0.001$).

We further found that downregulation of *MIAT* led to a significant elevation in the ratio of cells in the G0/G1 phase compared with the scrambled siRNA transfected cells in SW1116 ($P = 0.02$) and SW48 ($P < 0.001$). These observations suggested that *MIAT* inhibition contributes to G1 arrest in the studied CRC cell lines. The contribution of *MIAT* in cell cycle arrest was then evaluated via Cyclin D1 and PCNA mRNA levels measurement. Based on the results, their level of expression was respectively down- and up-regulated (Cyclin D1: 3.6-fold; $P = 0.020$; PCNA: 2.6-fold; $P < 0.000$) in the si-*MIAT*-treated cells compared with the scrambled siRNA transfected CRC ones (Figure 2D). These results confirmed the possible role of *MIAT* in tumorigenesis of CRC through cell cycle regulation.

MIAT restricts apoptosis and cellular senescence in CRC cells

To determine the effects of *MIAT* on the apoptosis of CRC cells, SW48 and SW1116 cells were stained with Annexin-V/propidium iodide and then analyzed through flow-cytometry. Flow-cytometry data revealed a significant increase in apoptosis in *MIAT* silencing CRC cells in comparison with the scrambled siRNA transfected ones ($P = 0.200$). In line with the results of flow-cytometry, dysregulation of Bax ($P = 0.01$) and Bcl-2 ($P < 0.001$) expression, known as apoptosis markers, was observed following *MIAT* suppression (Figure 3A). Furthermore, the number of senescent cells was significantly elevated following *MIAT* restriction (Figure 3B; $P = 0.003$). The findings herein confirmed the role of *MIAT* as an antiapoptotic and senescence inhibitor factor in CRC progression.

MIAT induces cell migration in CRC cells

In the next phase, we analyzed the effect of *MIAT* restriction on cell migration through the scratch test. Wound healing assay demonstrated that *MIAT* suppression caused a significant decrease in cell motility in CRC cells (Figure 4A; $P < 0.001$).

MIAT contributes to regulation of ATM/CHK2 expression in CRC cells

DNA repair mechanisms have a crucial role in DNA-damaging cytotoxic treatment resistance, besides radiation. Therefore, we evaluated the effects of *MIAT* suppression on the expression of ATM/CHK2, well-known DNA repair factors, in SW1116 and SW48 cells. Evaluating the expression level of ATM/CHK2 proteins showed that their expression was significantly reduced when *MIAT* was silenced in CRC cells (Figure 4B; $P < 0.001$).

Discussion

Based on the results of the present study, there is a mechanism through which lncRNA *MIAT* regulates CRC progression. In the current research,

we found that *MIAT* is upregulated in CRC tissues compared with normal ones. Based on *MIAT* expression analysis in CRC tissues, it may function as an appropriate diagnostic marker, with an AUCROC value of 0.700. Moreover, our expression-related data in CRC tissues demonstrated that *MIAT* expression significantly increased in high-grade CRC tissues in comparison with the low-grade ones. The above-mentioned results are in accordance with our previous report

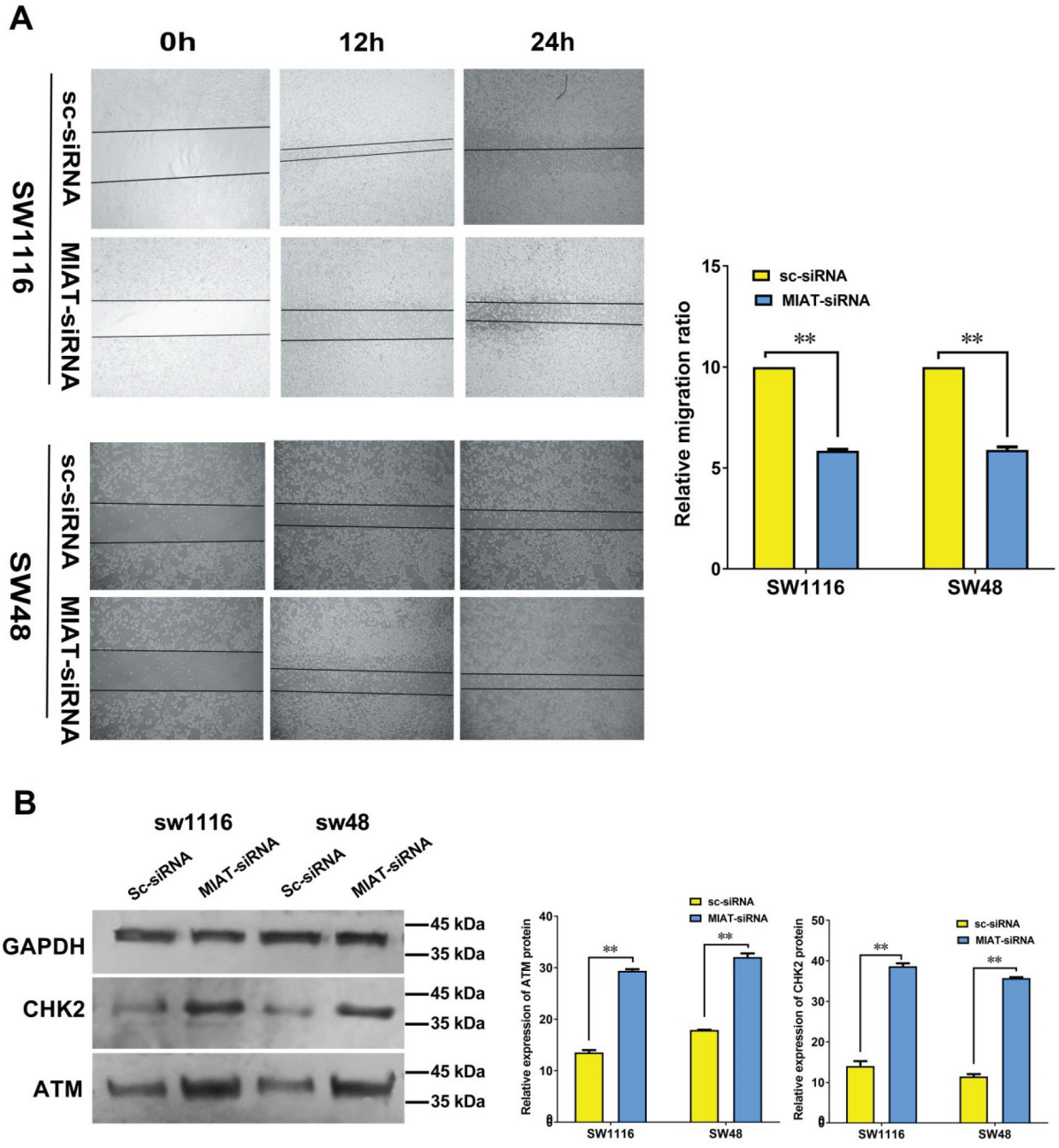

Figure 4. (A) *MIAT* inhibition decreases the migration ability of CRC cells, significantly. (B) *MIAT* suppression led to a significant elevation of ATM/CHK2 expression at the protein level in CRC cells. (*$P < 0.05$, ** $P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).
MIAT: Myocardial infarction associated transcript; CRC: Colorectal cancer

showing that *MIAT* is upregulated in high-grade breast cancer tissues. Recent studies have reported that non-coding RNAs regulate functional cellular processes, including proliferation, migration, differentiation, genomic stability, and metastasis.[26-28] A recent report established that lncRNA *MIAT*, a well-specified disease-related lncRNA, contributed to various cellular functions, such as proliferation, invasion, metastasis, and apoptosis.[8] The mechanisms of *MIAT* regulation are highly complex and involve numerous steps.[29] In line with our findings, *MIAT* has been upregulated in various malignancies, such as prostate, gastric, thyroid, ovarian cancer.

To evaluate the possible role of *MIAT* in CRC, we suppressed its expression employing siRNA-mediated silencing in two CRC cell lines, SW1116, and SW48, which have high endogenous expression of *MIAT*. We then investigated its suppression effect on proliferation and apoptosis of the CRC cells. Previously, its contribution to proliferation, migration, and invasion of CRC cells, by inhibiting miR-132 and regulating Derlin-1 expression, was specified.[23] In line with previous research, we found that *MIAT* may contribute to initiation and progression of CRC via inducing cancer cell proliferation/cell cycle, and restricting apoptosis/senescence.

Alternations in cell phenotypes, specified as EMT, have been reported to have a role in the tumorigenic process.[30] EMT capacitates the cancer cells to gain invasive characteristics and metastatic growth features. A previous study demonstrated that *MIAT* could increase EMT status in osteosarcoma cells via regulating the miR 150 5p/ZEB1 pathway.[31] In this regard, the role of *MIAT* in the wound healing process was investigated in this study. The obtained results confirmed the vital role of *MIAT* in the migration of CRC cells. Overall, our findings suggested that *MIAT* may have a significant function in the migration and metastasis of CRC.

Our findings also demonstrated that the expression level of ATM/CHK2 proteins decreased following *MIAT* suppression. The ataxia-telangiectasia mutated serine/threonine kinase (ATM)/checkpoint kinase 2 (CHEK2, best known as CHK2) cascade is one of the two significant signaling pathways motivating the DNA damage response (DDR), a series of procedures vital regarding the maintenance of genomic stability, acting as a barrier toward tumorigenesis and tumor progression.[32] ATM is involved in cell metabolism, oxidative stress, chromatin remodeling, reaction to uncapped telomeres, and the spindle assembly checkpoint.[33] In contrast, CHK2 is involved in mitosis and is necessary for chromosomal stability maintenance.[33, 34] Previous studies have reported that the ATM-CHK2 pathway has an onco-suppressive effect.[35, 36] Moreover, a number of human malignancies have been discovered to have elevated ATM and/or CHEK2, including breast cancer,[37] and colon cancer.[38] The current study confirmed the aforementioned findings regarding the role of ATM/CHK2 pathway in cancer progression and initiation.

Conclusion

In conclusion, high expression of *MIAT* is involved in CRC progression. Moreover, *MIAT* has substantial roles in proliferation, apoptosis, cell cycle, and metastasis of CRC cells. *MIAT* could be also involved in the ATM/CHK2 pathway, resulting in CRC progression and initiation. However, to some extent, the mechanisms and regulatory pathways are elucidated, necessitating further in vivo studies to clarify them extensively.

Acknowledgement

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Conflict of Interest

None declared.

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