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METTL14 regulates M6A methylation-modified primary miR-19a to promote cardiovascular endothelial cell proliferation and invasion

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Abstract. – OBJECTIVE: Increasing evidence indicated that N6-methyl-adenosine (M6A) played a key role in a variety of pathophysiological processes. Methylases could promote the processing of mature mi-RNA in a M6A-dependent manner, thereby participating in the pathological cells' occurrence and development. However, the regulatory mechanism of M6A in atherosclerosis (AS) was still unclear.

PATIENTS AND METHODS: Quantificational Real-time polymerase chain reaction (qRT-PCR) was used to detect the relative expression levels of M6A, methyltransferase, demethylase transferase, miR-19a and other mi-RNA in atherosclerotic vascular endothelial cells (ASVEC). Cell Counting Kit (CCK8) was used to detect cell proliferation, the expression of PCNA was measured by Western Blot (WB) and qRT-PCR. Transwell assays were used to detect the invasion ability of ASVEC. Co-immunoprecipitation (Co-IP) was used to detect the binding of METTL14 to DGCR8. RNA Immunoprecipitation (RIP) was used to detect the binding of METTL14 to miR-19a.

RESULTS: M6A modification levels and METTL14 methylation transferase were significantly overexpressed in ASVEC. Silencing METTL14 inhibited the proliferation and invasion of ASVEC. Low expression of METTL14 suppressed the binding of methylated RNA and RNA splicing related protein DGCR8. Moreover, silencing METTL14 significantly inhibited the expression of miR-19a while promoted the expression of primary pre-miR-19a. However, high expression of METTL14 obviously increased the expression of DGCR8 and methylated m6A. Furthermore, silencing miR-19a inhibited the proliferation and invasion of ASVEC.

CONCLUSIONS: METTL14 increased the M6A modification of pri-miR-19a and promoted the processing of mature miR-19a, thus promoting the proliferation and invasion of ASVEC. These results suggested that METTL14/ M6A/ miR-19a signaling pathway may be a new target for atherosclerosis treatment.

Key Words:
M6A, METTL14, MiR-19a, Atherosclerosis, Proliferation, Invasion.

Introduction

Atherosclerosis (AS) refers to chronic inflammation and fibroproliferative lesions occurring in the lumen of the large and middle arteries[1-3]. AS is the pathological basis of most cerebrovascular diseases, and it has high morbidity, disability and mortality[4-6]. Vascular endothelial cells are closely related to vascular physiology such as vascular regeneration, vasodilation and inflammation[7]. The proliferation and invasion of vascular endothelial cells affect the stability of AS plaques, platelet activation and the occurrence of atherosclerotic complications[8-10].

RNA m6A modification was the most endogenous method of RNA modification and it was widely present in eukaryotic mRNAs and lncRNAs[11,12]. RNA m6A modification was higher in liver, testis and other tissues and highly conserved among multiple species. M6A modification exhibits dynamic and reversible changes in different developmental stages, different tissues, physiological and pathological processes[13]. RNA m6A modifications were involved in the process of disease formation or development[14]. Clancy et al[15] found that FTO was negatively correlated with m6A levels in adipocytes during adipogenesis. Interfering with FTO inhibited the differentiation of adipocytes into adipocytes. Further analysis found that knockdown of FTO promoted m6A levels. Besides, M6A promoted the binding of the splicing factor SRSF2 protein to the adipogenesis regulator RUNXlTl transcript, which

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lead to increased exon retention events during the RUNXlTl splicing process, thereby regulating the differentiation process of adipose primary cells. Zhao et al[16] found that the localization of RNA m6A demethylase ALKBH5 in the nuclear plaque region participated in the metabolic process of RNA and was highly expressed in the testicular tissue of mice. The knockout of ALKBH5 in mice leads to the overall m6A modification level of mRNA elevation, testicular atrophy, and decreased sperm count and viability in mice. Follow-up studies have found that knockout of ALKBH5 resulted in an effect on the p53 pathway, leading to increased apoptosis. Above evidence suggested that ALKBH5 may be involved in spermatogenesis-related processes. Meyer et al[17] found that hypoxic conditions could promote the upregulation of HIFlQ and HIF2a-dependent ALKBH5, thus leading to the down-regulation of m6A levels. The stability of the transcript was promoted and eventually increased the mRNA and protein expression levels. However, the role and function of m6A modification in atherosclerosis remained unclear.

In this paper, we found that the level of m6A methylation modification and METTL14 were significantly upregulated in atherosclerotic vascular endothelial cells. Silencing METTL14 could inhibit the maturation of primary miR-19a by reducing the level of m6A methylation modification, thereby suppressing the proliferation and invasion of atherosclerotic vascular endothelial cell. Thus, METTLl4/m6A/mi-19a pathway may be a marker molecule and provide potential drug targets for AS treatment.

Patients and Methods

Cell Cultures and Patient Tissues

Human tissue specimens were collected from AS patients with plaques in our hospital from July 2018 to October 2019. We selected a total of 46 cases including 22 males and 24 females aged 50.4-72.4 years old with an average (61.4 ± 11). All patients were diagnosed by angiography. Patients with angina, diabetes and history of myocardial infarction were strictly excluded. In the above patients, plaque tissue was removed during cerebrovascular surgery at the later stage, and part of normal blood vessel tissue was removed as a normal control group. The tissues were labeled immediately after collection and stored in liquid nitrogen. This study was approved by the Ethics Committee of our hospital and all subjects signed the informed consent.

RT-qPCR Assays and Cell Transfection

TRIzol kit (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from ASVEC and tissues. First Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) was applied to synthesize cDNA. Then we used NanoDrop 2000 (Quawell, San Jose, CA, USA) to detect the levels and qualities of RNA. Power SYBRGreen PCR master Mix (Life Technologies; Thermo Fisher Scientific, Waltham, MA, USA) was used to determine quantitative Real-time PCR. All data were calculated by using $2^{-\Delta\Delta Ct}$ methods. GenePharma (Shanghai, China) was used to complete the construction and design of small interfering RNAs (siRNAs) targeting to miR-19a inhibitors or negative control (miR-NC).

CCK-8 Assays

We took cells of ASVEC and control group in the logarithmic growth phase and seed them into a 96-well culture plate and cultured routinely. 10 μL of CCK-8 solution was added to the cells after the cells of each group were cultured for 24, 48, 72 and 96 hours. Next, the cells were incubated for 10 minutes and a microplate reader (9200, Bio-Rad Laboratories, Hercules, CA, USA) was used to measure the absorbance at 405 nm.

Western Blot

ASVEC and control cells from each group were collected 24 hours after transfection. and then the total protein was routinely extracted. BCA kit (Pierce, Rockford, IL, USA) was used to detect the protein concentration. Then, the cells were blocked 5% skimmed milk powder at 37°C for 2 h. PCNA and β-actin (Abcam, Cambridge, UK) primary antibodies were added and incubated overnight at 4°C. We then washed TBST for 3 × 10 min, incubated secondary antibodies for 1 h at 37°C and developed ECL. At last, we used Image J software to analyze the gray value of protein bands. β-actin served as an internal reference to calculate the relative expression.

Transwell Assay

Cell invasion was detected by transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA) with Matrigel. Serum-free medium was placed in the upper chamber and 600 mL of 10% FBS-containing medium was supplemented in the lower chamber. Then we placed Matrigel (BD Biosci-

ences, Franklin Lakes, NJ, USA) in the upper chamber for invasion assays. The number of invasion cells was measured by a microscope.

Co-Immunoprecipitation

Co-immunoprecipitation of proteins was performed in ASVEC using a Pierce Co-Immunoprecipitation Kit (Thermo-Fisher Scientific, Waltham, MA, USA). The rabbit METTLl4 antibody (HPA038002; Sigma-Aldrich, St. Louis, MO, USA) and negative control rabbit IgG antibody were used in ASVEC. The complexes bound after immunoprecipitation were subjected to Western Blot to detect mutual binding between proteins.

RNA M6A Co-Immunoprecipitation

Briefly, the RNAs of ASTEC and its control group cells after transfection of si-METTLl4 and LV-METTLl4 were extracted.

The RNAs were placed in a sonicator and the ultrasound was fragmented for 10 seconds after treatment with DNase I. The RNAs were placed in a sonicator and the ultrasound was fragmented for 10 seconds. Resuspend in 100 ul RIPWash Buffer Magnetic beads were incubated with rabbit m6A anti-(Sysy) and rabbit IgG antibodies for 30 minutes at room temperature to allow the antibodies to bind to the magnetic beads. After being washed with 500 ul RIP Wash Buffer for six times, the beads were incubated with proteinase K Buffer at 42°C for 30 minutes. Phenol: chloroform: isoamyl alcohol was used to purify the RNA, and the amount of pri-miRNA was detected by Real-time quantitative PCR after reverse transcription.

Statistical Analysis

SPSS 15.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to analyze the experimental data. The data was expressed as mean ± standard deviation. $p<0.05$ was considered statistically significant.

Results

METTL14 and M6A Methylation Expression Levels Were Significantly Overexpressed in ASVEC

We compared m6A expression in normal vascular cells and ASVEC. The results showed that the relative expression level of m6A in ASVEC was significantly higher than that of normal vascular cells (Figure 1A). In order to investigate the cause of the high expression of m6A in ASVEC, qRT-PCR was used to detect the relative expression levels of methyltransferase and demethylase. It was found that the expression level of METTL14 in ASVEC was significantly higher than that of METTL3 and WTAP (Figure 1B). However, the demethylases (METTL4, FTO, YTHDF2, KIAA429 and ALKBH5) had no significant difference in expression level in ASVEC (Figure 1C).

Low Expression of METTL14 Inhibited Proliferation and Invasion of ASVEC

To investigate the effect of low expression of METTL14 on the proliferation and invasion of ASVEC, ASVEC were transfected with si-METTL14 and cultured for 3 days. The results showed that METTL14 expression level in ASVEC cells after transfection with si-METTL14 was significantly lower than that in transfected si-NC group (Figure 2A). The proliferation ability of ASCEC

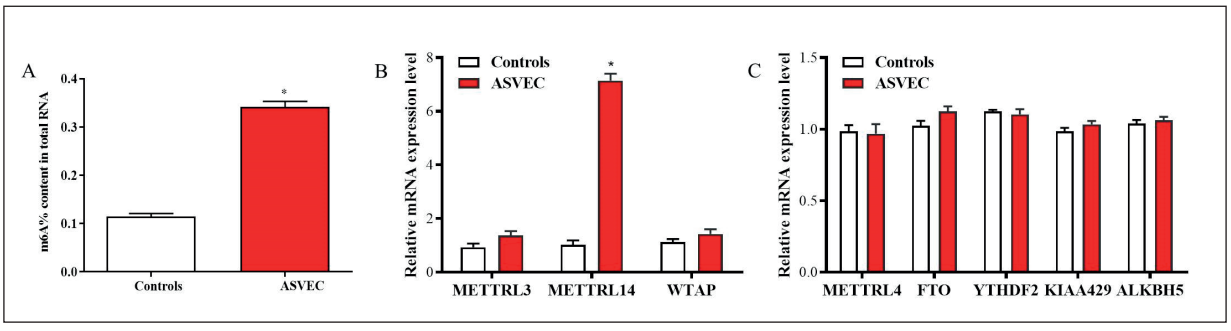

Figure 1. METTL14 and M6A methylation expression levels were significantly overexpressed in ASVEC. **A,** M6A content in total RNA was detected by qRT-PCR in normal vascular cells and ASVEC. **B,** The relative mRNA expression level of METTL3, METTL14 and WTAP was measured by qRT-PCR in ASVEC. **C,** The demethylases (METTL4, FTO, YTHDF2, KIAA429 and ALKBH5) expression level was detected by qRT-PCR in ASVEC. The data were expressed as mean ± SD. *$p<0.05$.

cells after transfection with si-METTL14 was significantly reduced in comparison with si-NC group (Figure 2B). RT-PCR and Western blot were used to detect the expression levels of proliferation-related molecules. The results showed that PCNA in ASVEC after transfection with si-METTL14 was significantly lower than si-NC groups (Figure 2C and 2D). Besides, the invasion ability of ASCEC after transfection with si-METTL14 was significantly lower than si-NC groups (Figure 2E and 2F).

METTL14 Bound to DGCR8 and Reduced Methylated RNA Which Bound to DGCR8

Previous studies have reported that M6A modification was involved in the process of mRNA splicing. Recent research showed that M6A modification participated in the process of pri-miRNA[18]. Alarcón et al[19] found that m6A could label pri-miRNAs. The main principle was that METTL3 could combine with DGCR8 molecule, which was the key molecule involved in RNA maturation. METTL3 recognized DGCR8 to participate in the maturation of pri-miRNA by relying on m6A modification. Considering that METTLl4 and METTL3 had similar synergies, we imagined whether METTLl4 could participate in the pri-miRNA maturation process in a similar manner. Co-immunoprecipitation was used to detect the relationship between METTL14 and DGCR8 by using METTL14 antibody. We found that METTL14 could also bind DGCR8 in ASCEC. Moreover, the binding capacity of METTL14 and DGCR8 was significantly weakened after further treatment with RNase. This indicated that METTL14 was involved in the maturation of pri-miRNA and RNA partially mediated the binding of METTL14 to DGCR8 (Figure 3A). Then we conducted RIP experiments with DGCR8 antibody in ASCEC and compared the effect of knocking down METTL14 on the binding level of METTL14 and DGCR8 and the expression level of m6A modification. When ASCEC was transfected with si-METTL14, the expression level of METTL14 binding to DGCR8 and its M6A expression level was significantly lower than si-NC group. This suggested that silencing METTL14 reduced the expression level of binding to DGCR8 and M6A methylation modifications (Figure 3B).

Silencing METTL14 Inhibited miR-19a Expression while Promoted pre-miR-19a Expression

We further investigated which miRNAs were regulated by METTL14 to participate in the occurrence and development of ASCEC. We

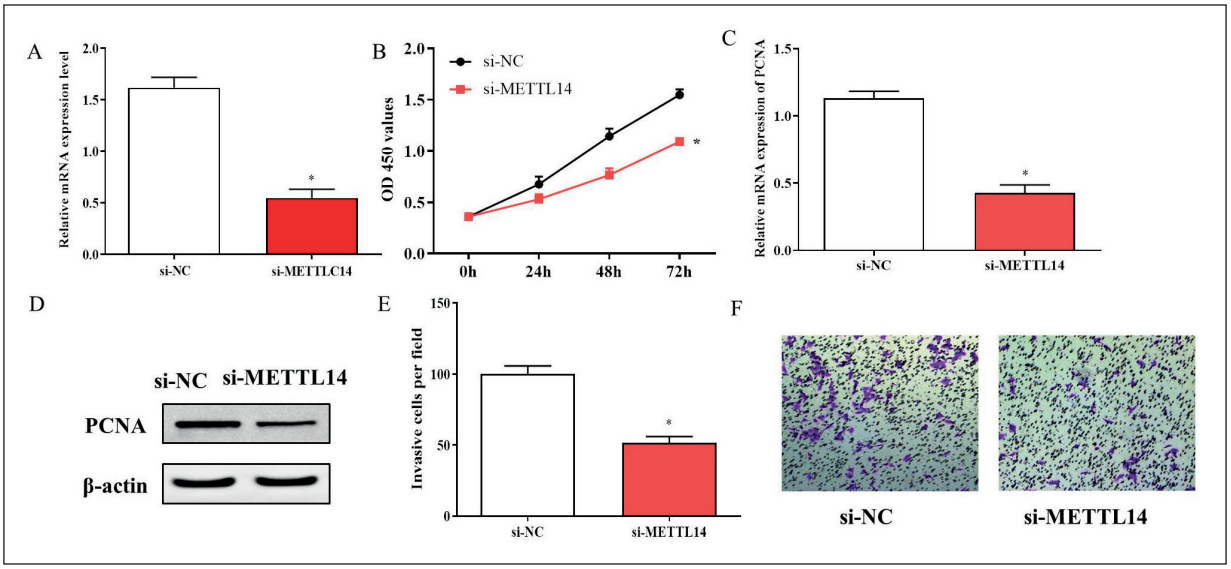

Figure 2. Low expression of METTL14 inhibited proliferation and invasion of ASVEC. **A,** The relative mRNA expression level of METTL14 was measured by qRT-PCR after transfection of si-METTL14 in ASVEC. **B,** CCK-8 assay was used to detect the proliferation of ASVEC after transfection of si-METTL14. **C-D,** QRT-PCR and Western blot were used to measure PCNA relative expression level. **E-F,** Transwell assays were used to cell invasion capacity of ASVEC. The data were expressed as mean ± SD. * $p<0.05$.

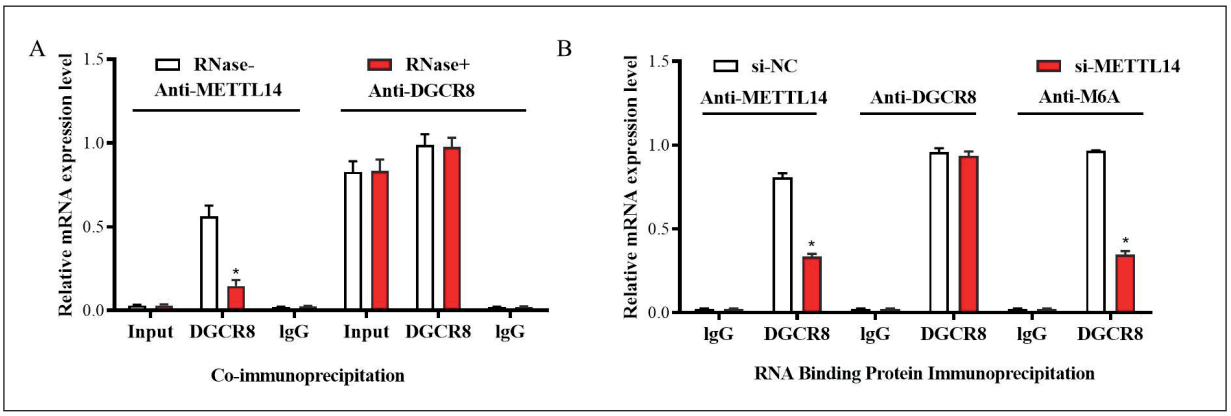

Figure 3. METTL14 bound to DGCR8 and reduced methylated RNA which bound to DGCR8. **A,** Co-immunoprecipitation reagent was used to measure the relationship between METTL14 and DGCR8. QRT-PCR and Western blot were used to measure mRNA expression level. **B,** The relationship between METTL14 and m6A was detected by Co-immunoprecipitation reagent after transfection of si-METTL14 and si-NC, respectively. QRT-PCR and Western blot were used to measure mRNA expression level. The data were expressed as mean ± SD. *$p<0.05$.

found that 10 pri-RNAs of miRNA contained M6A markers by mining M6A-seq nuclear RNA and miRNA for atherosclerosis data (Figure 4A). RNA containing these markers may be regulated by METTL14. We also found that mature miR-19a was down-regulated mostly in ASCEC after transfection of METTL14. Moreover, silencing METTL14 decreased the expression level of mature miR-19a while overexpression of METTL14 increased the expression level of mature miR-19a (Figure 4B). Accordingly, pri-miR-19a expression increased

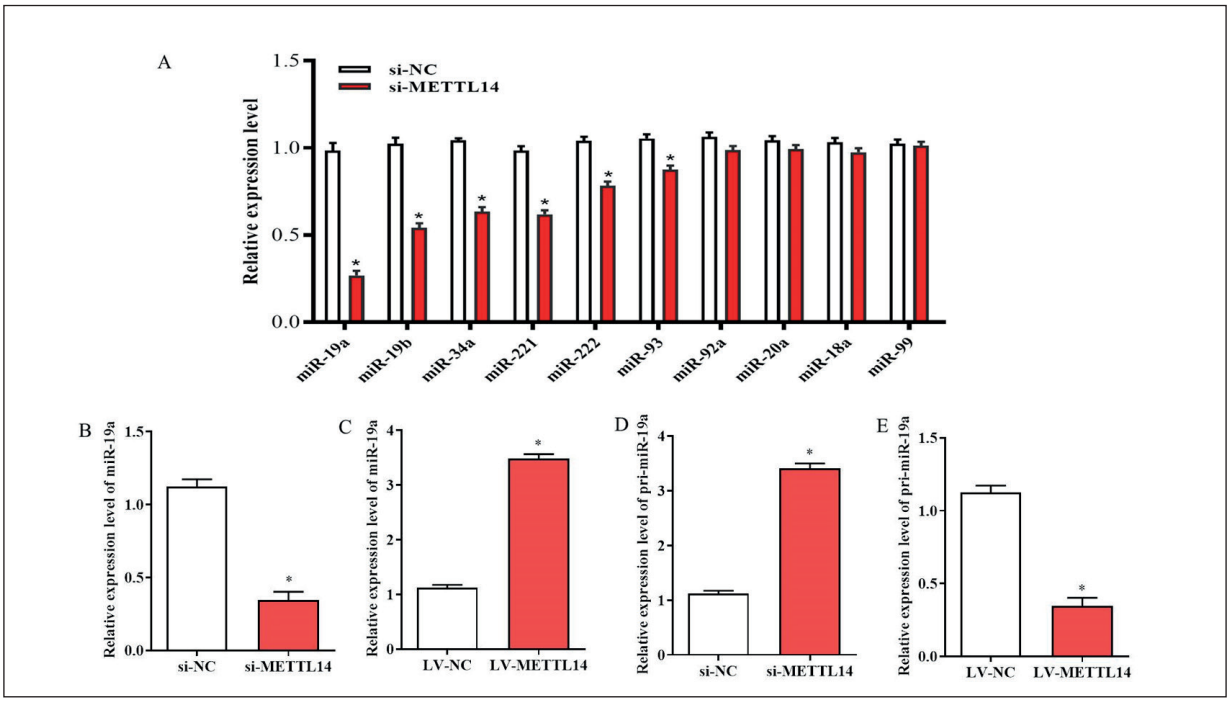

Figure 4. Silencing METTL14 inhibited miR-19a expression while promoted pre-miR-19a expression. **A,** QRT-PCR was used to measure the relative expression level of tagged mi-RNA in ASVEC. **B-C,** The relative expression level of miR-19a was measured by qRT-PCR in ASVEC after transfection of si-METTL14 and LV- METTL14 respectively. **D-E,** QRT-PCR was used to relative expression level of pri-miR-19a in ASVEC after transfection of si-METTL14 and LV-METTL14, respectively.

in METTL14-inhibited cells while pri-miR-19a expression reduced in METTL14-overexpressed cells (Figure 4C).

METTL14/ M6A Methylation Modification Mediated pri-miR-19a Processing by Binding to DGCR8

We further aimed at investigating that the METTL14/ M6A methylation modification mediated pri-miR-19a processing by binding to DGCR8 and promoting the formation of mature miR-19a. The results of RIP experiments showed that overexpression of METTL14 promoted pre-miR-19a binding to DGCR8 in comparison with LV-NC group (Figure 5A). Further RIP experiments revealed that METTL14 overexpression could promote M6A methylation modification of pre-miR-19a in comparison with LV-NC group (Figure 5B). These results suggested that METTL14/M6A methylation modification mediated pri-miR-19a processing and the formation of mature miR-19a via binding to DGCR8.

Low Expression of miR-19a Inhibited Proliferation and Migration of ASCEC

To analyze the effect of miR-19a on the occurrence and development of atherosclerosis, ASCEC was transfected with miR-19a inhibitors and miR-NC, respectively, and cultured for three days. The results showed that miR-19a inhibitors could significantly reduce the expression of miR-19a in ASCEC (Figure 6A). Silencing miR-19a significantly suppressed ASCEC proliferation (Figure 6B). PCNA expression levels were also significantly decreased in proliferation-related molecules (Figure 6C and Figure 6D). Similarly, low expression of miR-19a could also reduce the invasive ability of ASCEC (Figure 6E and Figure 6F).

Discussion

Atherosclerosis is closely related to the occurrence of cardiovascular and cerebrovascular diseases such as coronary heart disease and hypertension[20,21]. Atherosclerosis process begins with vascular endothelial cell dysfunction. Most cardiovascular disease mediators could activate endothelial cells and increased expression of chemokines, cytokines, adhesion molecules and other pro-inflammatory factors[22]. Vascular endothelial cell injury and endothelial dysfunction are the initial links of AS and they are involved in the initiation and progression of AS. Endothelial dysfunction and morphological damage cause leukocyte endothelial cell adhesion, vasoconstriction, platelet aggregation, oxidative stress, smooth muscle proliferation and thrombosis[23]. Therefore, the occurrence and development of vascular endothelial cells have an important effect on atherosclerosis. RNA m6A modification was one of the most typical RNA modification methods[13]. Similar to DNA methylation or histone methylation, RNA m6A modification was also catalyzed by methyltransferase and demethylase systems. Important molecules in the methyltransferase system included METTL3, METTLl4, WTAP, KIAAl429 etc.[24-27]. Important components of the demethylase enzyme system included FTO, ALKBH5 etc.[28,29]. Dynamic changes in the expression levels of methyltransferases and demethylases jointly

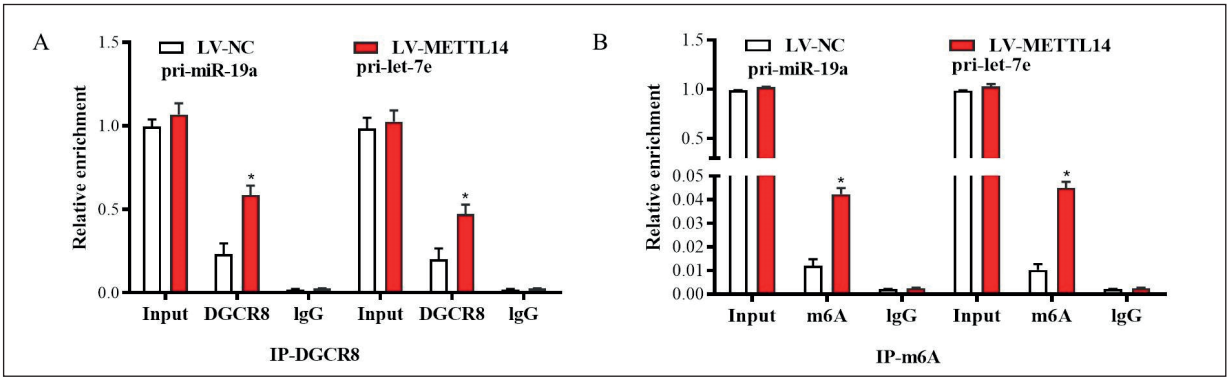

Figure 5. METTL14/ M6A methylation modification mediated pri-miR-19a processing by binding to DGCR8. **A,** RIP experiment was used to detect the content of primary mi-RNA bound to DGCR8 in ASVEC with METTL14 overexpression. **B,** RIP experiment was used to detect the content of M6A-modified primary mi-RNA in atherosclerotic cells with METTL14 overexpression.

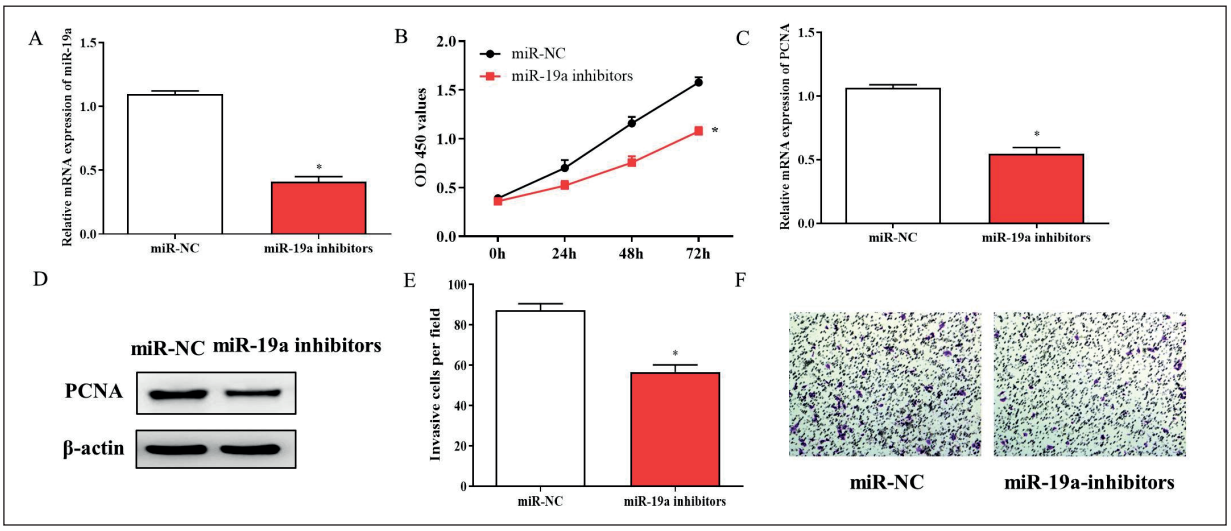

Figure 6. Low expression of miR-19a inhibited proliferation and migration of ASCEC. **A,** The relative mRNA expression level of miR-19a was measured by qRT-PCR after transfection of miR-19a inhibitors in ASVEC. **B,** CCK-8 assay was used to detect the proliferation of ASVEC after transfection of miR-19a inhibitors. **C-D,** QRT-PCR and Western blot were used to measure PCNA relative expression level after transfection of miR-19a inhibitors in ASVEC. **E-F,** Cell invasion capacity of ASVEC was measured by transwell assays after transfection of miR-19a inhibitors in ASVEC. The data were expressed as mean ± SD. *p<0.05.

regulated the level of RNA m6A modification and thus participated in the regulatory process of various diseases[30-32]. In our study, we found that the level of M6A methylation modification was up-regulated in ASVEC. Then we detected the expression levels of methyltransferase and demethylase transferase in ASVEC and found that METTLl4 had the highest expression level in ASVEC. However, there is no significant difference in other enzyme expressions. This indicated that METTLl4 was involved in the expression of M6A methylation modification in ASVEC. When ASVEC was transfected with si-METTLl4, the expression level of METTLl4 decreased significantly. Besides, low expression of METTLl4 could inhibit the proliferation and invasion of ASCEC, which suggested that METTLl4 was involved in the proliferation and invasion of ASCEC. However, its mechanism needs to be further explored.

MiR-19a was a typical microRNA. MiR-19a was demonstrated to be involved in the development and development of many tumor cells[33-35]. For instance, Chen et al[36] found that miR-19a was significantly up-regulated in glioma tissues and cells, and that low expression of miR-19a significantly inhibited the proliferation and invasion of glioma cells. Research by Niu et al[37] showed that miR-19a was significantly increased in clear cell renal cell carcinoma tissues and overexpression of miR-19a could significantly increase the proliferation and invasion of clear cell renal cell carcinoma cells. It has been reported in the literature that m6A modification could mark pri-miRNAs molecules and recognize DGCR8 molecules through a METTL3/ m6A-dependent manner, thereby participating in the maturation process of pri-miRNAs[38]. In addition, studies have found that METTL3 and METTLl4 synergistically could form a stable heterodimer structure at a ratio of 1:1. It maintained the stability of each other's protein level and obtained higher m6A methylation transferability than METTL3 or METTLl4 alone[26,29]. In our study, we found that METTL14 could bind to DGCR8 and mediated RNA binding of METTL14 to DGCR8 in ASVEC. Silencing METTL14 reduced M6A levels bound to DGCR8. When ASVEC was transfected with si-METTL14, miR-19a expression levels were proved to decrease the most in ASVEC. Moreover, the low expression of METTL14 inhibited miR-19a but promoted the expression level of pri-miR-19a. Further experiments found that over-expressing METTL14 could promote the binding of pri-miR-19a to DGCR8 and facilitate the M6A methylation modification of pri-miR-19a in ASVEC. The above results suggested that METTL14/ M6A promoted DGCR8-mediated processing of pri-

miR-19a and the formation of mature miR-19a in ASVEC.

Conclusions

We firstly discovered the effect of METTL14/ m6A/miR-19a signal path on proliferation and invasion of atherosclerotic vascular endothelial cells. METTL14 and M6A relative expression levels were highly expressed in ASVEC. METTL14 mediated the processing of primary miR-19a to mature miR-19a by promoting M6A modification, thereby promoting the proliferation and invasion of ASVEC. Therefore, this study could provide a theoretical basis for the treatment of AS with METTL14/ M6A/ miR-19a signal path.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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