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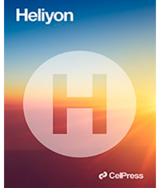

Research article

miR-4486 inhibits colorectal cancer proliferation via targeting MAP2K4 to inhibit the activation of the p38MAPK/JNK signaling

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ABSTRACT

Objective: Since MAP2K4 was reportedly involved in colorectal cancer development and the p38MAPK/JNK signaling transcription, this study aimed to investigate the mechanism by which the microRNA (miR)-4486 acts on colorectal cell proliferation.
Methods: RT-PCR was conducted to measure the expression levels of the MAP2K4 and miR-4486 in NCM460, SW1116, and HCT116 cells. TargetScanHuman site anticipated that MAP2K4 may be a target of miR-4486. The dual-luciferase reporter assay confirmed their relationship. After plasmids of miR-4486 mimic and si-MAP2K4 transfection, MAP2K4 was quantified again, The CCK-8 assay was carried out to assess cell proliferation, while Scratch and Transwell assays were used to evaluate cell migration and invasion. Finally, miR-4486 mimic and SB203580 were applied in HCT116 and SW1116 cells separately or in combination. CCK-8, Scratch and Transwell assay were performed again. In addition, the proteins including c-capase3, Bax, Bcl2, MAP2K4, and the p38MAPK/JNK signaling-related proteins expression levels were quantified by Western blot (WB).
Results: Compared with the NCM460 cells, the expression level of MAP2K4 was elevated, while the expression level of miR-4486 was reduced in SW1116 and HCT116 cells. The results showed that elevated levels of miR-4486 suppressed cell proliferation, migration, and invasion in colorectal cells by downregulating MAP2K4 expression. miR-4486 mimic showed similar effects to SB203580, which promoted colorectal cell apoptosis and inhibited the p38 MAPK/JNK signaling transcription.
Conclusion: miR-4486 may target MAP2K4 to inhibit colorectal cell proliferation by inhibiting the activation of the p38/JNK signaling pathway.

1. Introduction

According to the Global Cancer Report 2020, colorectal cancer (CRC), is one of the malignant tumors that pose a significant threat to human health, with approximately 147,950 people diagnosed and 53,200 deaths reported from CRC [1,2]. CRC pathogenesis is

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highly complex and diverse, and its risk factors include environmental, dietary habits, and familial heredity [3]. It has been reported that surgical resection combined with modern adjuvant systemic therapy has only a 20 % cure rate for CRC [4]. In new CRC diagnoses, 1/5 of patients present with uncontrollable disease, and an additional 1/4 of patients presenting with localized disease later metastasize [5]. Thus, research into more effective treatment modalities is crucial to maintaining the health of CRC patients.

microRNAs (miRNAs) are widely found in eukaryotic cells and are highly well-kept among species, expressed in a time-series and tissue-specific manner. As reported, miRNA treatment is a novel anti-tumor therapy, which can regulate tumor development by regulating the cell cycle and cell proliferation [6]. The latest study demonstrated that although the diagnostic and prognostic applicability of miRNAs in CRC is not well established, miRNAs can be useful to assist in the targeting treatment of drugs [7]. The role of tumor-suppressor microRNAs in colorectal cancer has been studied. For example, miR-455 targets HDAC2 to inhibit CRC cell proliferation [8]. Moreover, miR-30a was reported to be linked with advanced-stage and lymph node metastatic status [9]. miR-4486 is a newly discovered tumor-suppressor miRNA. In 2021, our study proved miR-4486 could improve cisplatin sensitivity in CRC [10], suggesting miR-4486 may be a potential target of CRC treatment.

Mitogen-activated protein kinase kinase 4 (MAP2K4) was predicted as the potential target of miR-4486 via TargetScan v2.0 (http://www.targetscan.org/). Researchers have found that MAP2K4 promotes cancer development and acts as an oncogene. As the upstream member of the mitogen-activated protein kinase (MAPK) signaling pathway, MAP2K4 activates p38 MAPK and c-Jun N-terminal kinase (JNK). Considering this, this study focuses on investigating the relationship between MAP2K4 and miR-4486; based on the p38MAPK/JNK signaling pathway to find the function of miR-4486 on CRC cells.

2. Materials and methods

2.1. Dual-luciferase reporter assay

We amplified the MAP2K4 3′ untranslated region (UTR) sequence by PCR and cloned it into the pSI-Check2 vector (Promega Corporation). Then, Using Lipofectamine 2000 reagent (Invitrogen) we co-transfected 293T cells (Procell Life Science & Technology Inc.) with miR4486 mimic or NC mimic and pSICheck2 MAP2K43′UTRWt or Mut. for 48h. Finally, three independent experiments quantified the Rluc/Fluc value by the Promega dual Luciferase Reporter Assay System.

2.2. HCT116 and SW1116 cells culture and treatment

Human colorectal cancer cell lines, HCT116 cells (cat. no. MXC469, Shanghai Meixuan Biological Technology Co., Ltd) and SW1116 cells (cat. no. MXC368, Shanghai Meixuan Biological Technology Co., Ltd) were cultured with RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.), that added with 10 % fetal calf serum, 100 U/ml penicillin/streptomycin. Cells were incubated at an incubator at 37 °C, 5 % CO2 until they reached the logarithmic growth phase. They were subjected to the following experiments. Experiment 1: miR-4486 mimic and negative control mimic were transfected into cells. Experiment 2: the mixture of NC mimic + si-NC, miR-4486 mimic + si-NC, NC mimic + si-MAP2K4, and miR-4486 mimic + si-MAP2K4 were transfected into cells, respectively. Experiment 3: cells were transfected with miR-4486 mimic, 10 μM SB203580 (S8307, MERCK), or miR-4486 mimic + SB203580, respectively. Notably, SB203580 is an inhibitor of the p38MAPK. After cell culture and treatment, the following assays were performed.

2.3. Quantitative real-time PCR (qRT-PCR)

TRIzol reagent (Invitrogen, USA) was employed to extract the HCT116 and SW1116 cells' total RNA in Experiments 1 and 2. The quantification of miR-4486 and MAP2K4 was employed by SYBR Green assay (Vazyme, China). The 2-ΔΔCt method was used for gene expression quantification. GADPH served as a control. miR-4486 forward primer: 5′- ACA CTC CAG CTG GGG CTG CGC GA-3'; miR-4486 reverse primer: 5' -TGG TGT CGT GGA GTC G-3; MAP2K4 forward primer: 5′- TCC CAA TCC TAC AGG AGT TCA A-3'; MAP2K4 reverse primer: 5′- CCA GTG TTG TTC AGG GGA GA-3'; RT-qPCR was performed at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 10s. The Melting curve analysis was performed for amplification product specificity.

2.4. Cell viability was evaluated by cell Counting kit 8 (CCK8) assay

A CCK-8 kit (Beyotime, China) was applied to assess cell viability in experiment 1. Exponentially growing HCT116 and SW1116 cells (2×10^3 cells/well) were seeded into 96-well plates. The plate was incubated overnight, cells were transfected with plasmids or SB203580 for 72h. Next, Cell viability was assessed at designated time points, and cells with CCK-8 solution at 37 °C for 2 h. Finally, when the fresh medium was set as a control, the OD value was quantified at 450 nm by a PerkinElmer microplate reader (PerkinElmer, Waltham, MA, USA).

2.5. Transwell assay

For the Transwell assays in Experiments 2 and 3, HCT116 and SW1116 cells (1×10^5 cells/well) were seeded in the upper chambers (Corning Costar, NY, USA). The lower chambers were stuffed with DMEM encompassing 10 % FBS. Plasmids or SB203580 were added in the upper chambers, and cells were cultured for 24 h. HCT116 and SW1116 cells penetrated the membrane and were fixed with

methanol, and 0.1 % crystal violet (Sigma–Aldrich) was used for staining. Finally, an inverted microscope (Olympus Corp.) was employed to compute the invaded cells.

2.6. Scratch assay

The scratch assay was performed to determine the cell migration of HCT116 and SW1116 cells in Experiments 2 and 3. A 6-well plate was seeded with HCT116 and SW1116 cells (5×10^5 cells/well) and grown overnight. A sterile pipette tip was used to make a single scrape. After PBS washing of the monolayer, plasmids or SB203580 were used to treat cells for 24h. A fresh medium as the control. The time point of 0 and 24 h of serial photographic images was captured by a Leica TCS 4D microscope (Leica Camera AG, Wetzlar, Germany).

2.7. Western blot

Cell lysates of HCT116 and SW1116 cells in Experiment 3 were prepared by RIPA cell lysis buffer. After the supernatant collection, a BCA kit (Thermo Scientific, Rockford, IL, USA) was employed for total protein measure. 20 μg supernatants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes. TBS-T was used to block the membrane for 2 h at room temperature. Next, after three times of PBS washing, we incubated the immunoblots with primary antibodies overnight at 4 °C. The primary antibodies were against cleave-capase3(1:2000; no. A7684, Abclonal), Bax (1:1000; no. A0207, Abclonal), Bcl2 (1:1000; no. A0208, Abclonal), MAP2K4 (1:1000; no. A14781, Abclonal), p38 MAPK (1:1000; no. A14401, Abclonal), p-p38 MAPK (1:1000; no. AP0526, Abclonal). Sample loading was normalized relative to β-actin (1:100000; no. AC026; Abclonal). Then, the membrane with HRP goat anti-rabbit IgG (H + L) (1:10000; no. AS014, Abclonal) and washed again. Finally, the response was blocked, and ECL was used to detect. Employed Scion Image 4.0 software (Scion Corporation, Frederick, MD, USA) to measure band sizes.

2.8. Statistical analysis

GraphPad 9.0.2 software (GraphPad Software, Inc.) was performed to employ statistical analyses. The results were given as mean ± SD. One-way ANOVA was used to calculate the statistically significant difference. A p-value of ≤ 0.05 was considered to mention statistical significance when each experiment was performed in triplicate at least.

3. Results

3.1. miR-4486 direct target to MAP2K4 gene

TargetScan was employed to predict the targeting relationship of the MAP2K4 gene and miR-4486. Fig. 1A revealed that miR-4486 may directly target the 3′-UTR of MAP2K4 mRNA and Fig. 1B verified the prediction. These findings suggested that miR-4486 directly targets the MAP2K4 gene. To further determine the effectiveness of miR-4486 and MAP2K4 in CRC, qRT-PCR was conducted to evaluate the miR-4486 and MAP2K4 expression levels in human colorectal cancer cell lines (HCT116 and SW1116) (Fig. 1C). It was found that the miR-4486 expression was downregulated($P < 0.01$), and the MAP2K4 expression was upregulated ($P < 0.05$) in

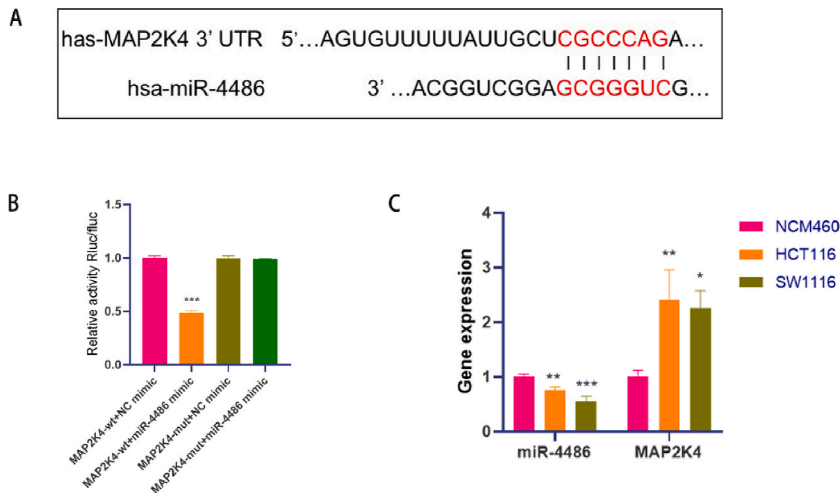

Fig. 1. The targeting relationship between MAP2K4 and miR-4486. A-B: A dual-luciferase reporter assay was performed to display the targeting relationship between MAP2K4 and miR-4486. **C:** qRT-PCR was used to quantify the MAP2K4 expression. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

comparison with the NCM460 cells.

3.2. miR-4486 down-regulates the MAP2K4 expression and inhibits cell proliferation in CRC cells

To further confirm if MAP2K4 expression level was regulated by miR-4486 in CRC cells, HCT116 and SW1116 cells were treated to over-express the miR-4486, respectively. As Fig. 2A has shown, miR-4486 overexpression inhibits the MAP2K4 expression ($P < 0.05$). In addition, HCT116 and SW1116 cells were treated to over-express the miR-4486 or down-express MAP2K4 separately or in combination. Fig. 2B pointed out that si-MAP2K4 and the miR-4486 mimic both downregulated MAP2K4 expression ($P < 0.05$). Importantly, the combination of si-MAP2K4 and the miR-4486 mimic had the greatest effect on MAP2K4 inhibition. Cell viability was determined by CCK8 (Fig. 2C), and revealed that the greater the MAP2K4 inhibition was, the lower the cell viability was ($P < 0.001$).

3.3. Inhibition of MAP2K4 down-regulates the invasive transfer capability of CRC cells

The Scratch and Transwell assays were conducted to explore the MAP2K4 role in invasive transfer capability in HCT116 and SW1116 cells. Fig. 3A showed the scratch healing from 0h to 24h, and Fig. 3C quantified the CRC cell migration. The results revealed that both si-MAP2K4 and miR-4486 mimic treatment restrained the migration of HCT116 and SW1116 cells, and the combination had the greatest inhibitory effect. Fig. 3B, and **D** shows the cell invasion. Importantly, si-MAP2K4, miR-4486 mimic, and the combination

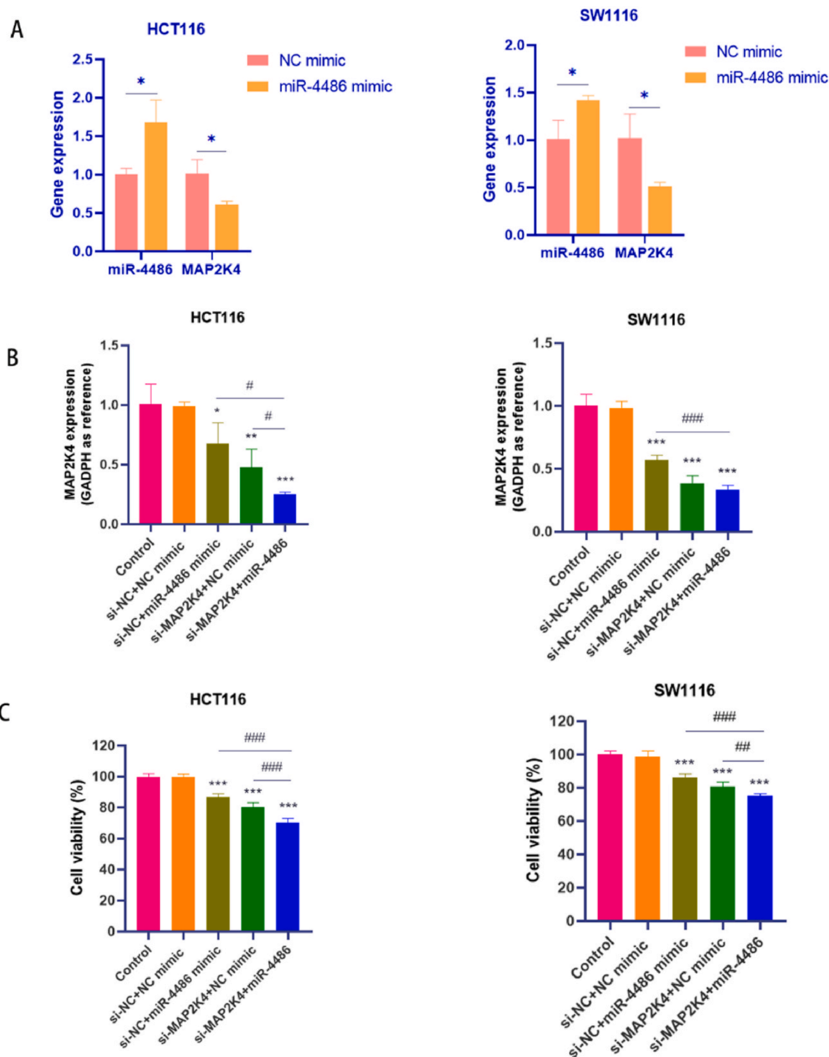

Fig. 2. High expression levels of miR-4486 down-regulate the MAP2K4 in CRC cells. miR-4486 was overexpressed by mimic and MAP2K4 was down-expressed by si-RNA in CRC cells. **A:** qRT-PCR was performed to evaluate miR-4486 and MAP2K4 expression. **B:** MAP2K4 gene expression level. **C:** The cell viability was determined by CCK-8. VS the si-NC + NC mimic group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. VS the si-MAP2K4+ miR-4486 group $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$.

treatment for cell invasion effects showed a similar trend to cell migration (P < 0.001).

3.4. miR-4486 suppress the p38MAPK/JNK signaling activation via inhibiting MAP2K4

Since MAP2K4 is a session of the p38MAPK/JNK signaling, we pondered that miR-4486 may play a key role in its activation. To verify our speculation, the p38MAPK inhibitor (SB203580) was applied in this study. WB was conducted to determine the p38MAPK/JNK signaling-related protein expression level (Fig. 4A). Fig. 4B showed that MAP2K4 protein expression was inhibited by miR-4486 mimic and SB203580 treatment. Furthermore, the phosphorylation of JNK and p38MAPK was analyzed by bar charts. Fig. 4C and D

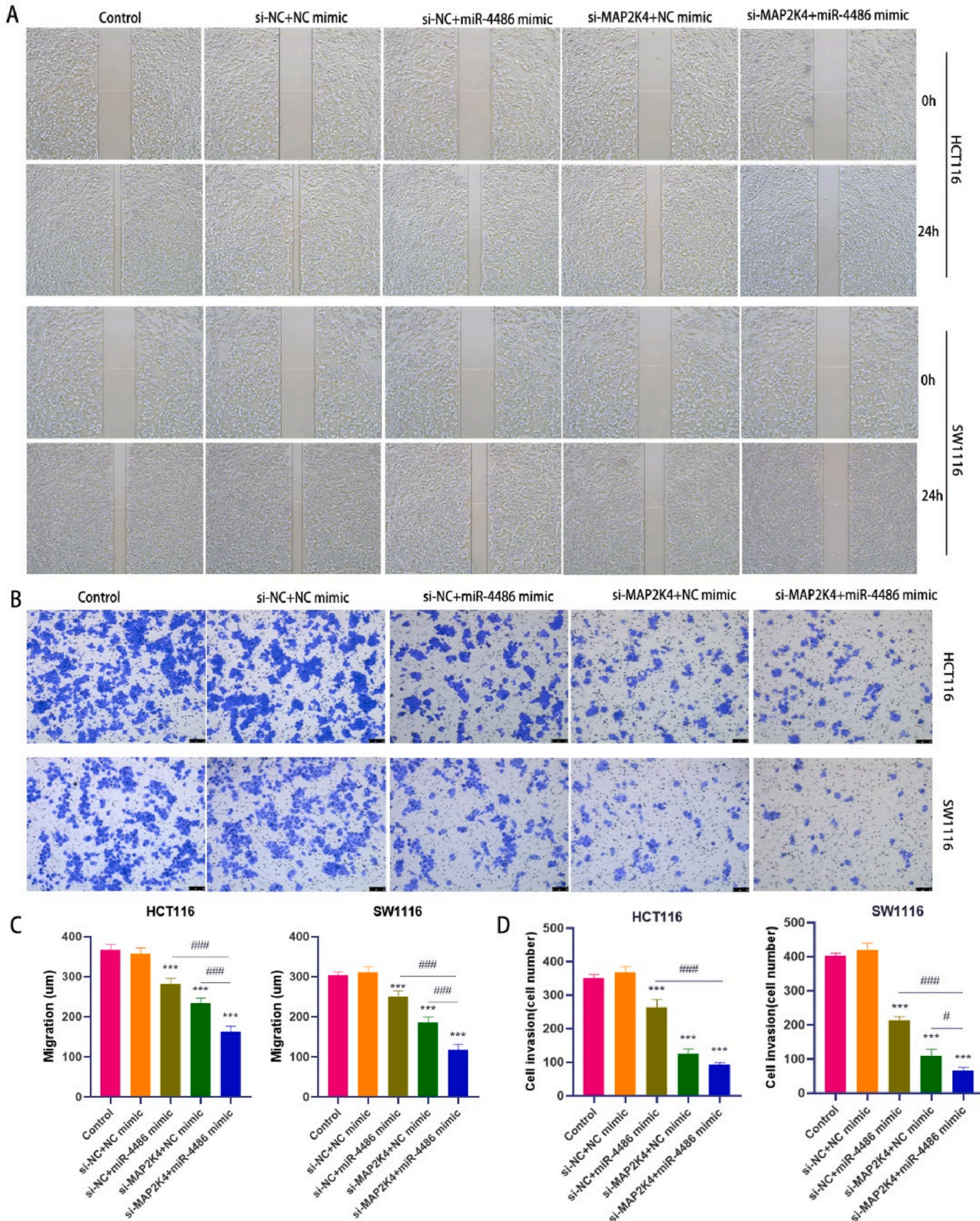

Fig. 3. Inhibition of MAP2K4 down-regulates the migration and invasion of HCT116 and SW1116 cells. miR-4486 was overexpressed by mimic and MAP2K4 was down-expressed by si-RNA in HCT116 and SW1116 cells. **A/C:** Migration of CRC cells was determined by scratch experiments. **B/D:** Invasion of CRC cells was determined by Transwell assay. VS the si-NC + NC mimic group, ***P < 0.001; VS the si-MAP2K4+ miR-4486 group, $^{\#}$P < 0.05, and $^{\#\#\#}$P < 0.001.

showed that the phosphorylation of JNK and p38MAPK both were inhibited by miR-4486 overexpression and SB203580 treatment. It is worth mentioning that the miR-4486 mimic + SB203580 group showed the greatest inhibitory effect on the p38MAPK/JNK signaling activation among the three groups.

3.5. miR-4486 boosts the apoptosis of CRC cells by inhibiting the p38MAPK/JNK signaling

Considering the crucial role of p38MAPK/JNK signaling in cell apoptosis is well established, we determined the cell apoptosis by CCK8 and WB. The cell viability results are displayed in Fig. 5A. Both the miR-4486 mimic and SB203580 treatments inhibited the proliferation of HCT116 and SW1116 cells. Moreover, proteins of Bax, Bcl2, and cleaved-caspase3 were measured by WB (Fig. 5B). Compared with the control group, the Bax expression level was increased (Fig. 5C), and Bcl2 was decreased (Fig. 5D) by miR-4486 mimic and SB203580 treatment significantly. Additionally, the cleaved-caspase3 expression level was decreased by miR-4486 mimic and SB203580 treatment too. Interestingly, the combination of miR-4486 mimic and SB203580 treatment led to the most serious cell apoptosis among the three groups.

3.6. miR-4486 inhibits the invasive transfer capability of CRC cells via inhibiting the p38MAPK/JNK signaling

Finally, Scratch assay and Transwell assay were conducted again under the miR-4486 mimic and SB203580 treatment. Fig. 6A showed the scratch healing from 0h to 24h, and Fig. 6B quantified the cell migration. It was found that both miR-4486 mimic and SB203580 treatment could inhibit the cell migration of HCT116 and SW1116 cells, and the combination showed the most inhibition. Transwell assay results as shown in Fig. 6C and 6D quantified the cell invasion. Importantly, miR-4486 mimics, SB203580, and the combination treatment for cell invasion effects showed a similar trend to cell migration. It suggested that miR-4486 may inhibit HCT116 and SW1116 cell invasive transfer capability by inhibiting the p38MAPK/JNK signaling pathway.

4. Discussion

This study has demonstrated that miR-4486 could target MAP2K4. Compared with NCM460 cells, HCT116 and SW1116 cells

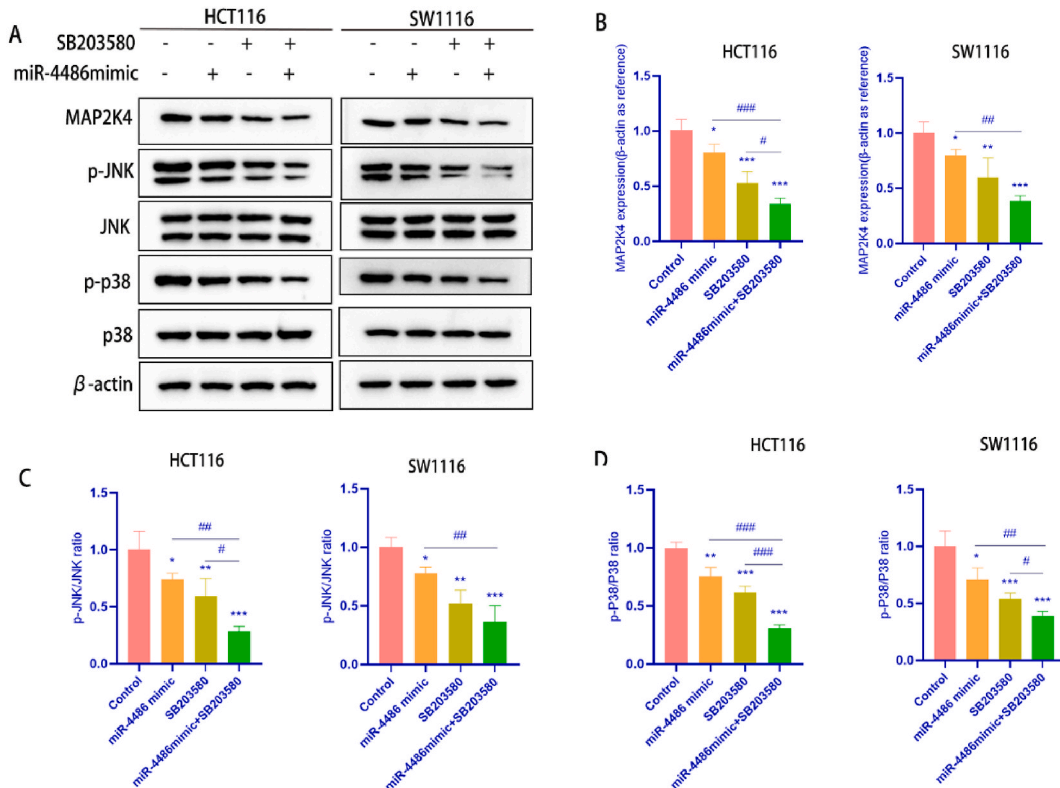

Fig. 4. miR-4486 suppresses the p38MAPK/JNK signaling activation by inhibiting MAP2K4. miR-4486 mimic and SB203580 were applied in HCT116 and SW1116 cells separately or in combination. **A:** WB was performed to determine MAP2K4, p-JNK, JNK, p38MAPK, and p-p38MAPK protein expression (the original image is provided in the supplementary file). Bar charts were used to analyze **(B)** MAP2K4 expression, **(C)** the p-JNK/JNK ratio, and **(D)** the p-p38MAPK/p38MAPK ratio. VS the control group, *P < 0.05, **P < 0.01, and ***P < 0.001. VS the miR-4486 mimic + SB203580 group, $^{\#}$P < 0.05, $^{\#\#}$P < 0.01, and $^{\#\#\#}$P < 0.001.

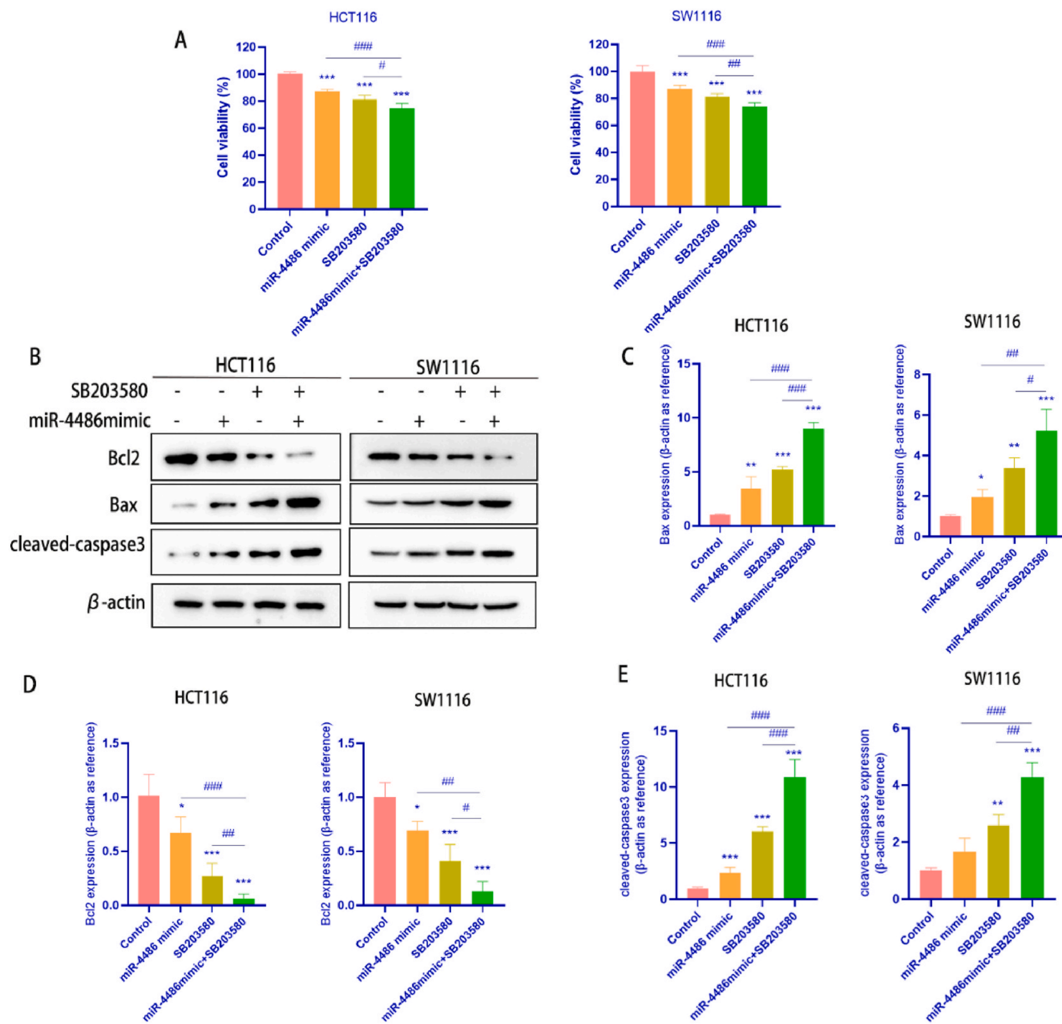

Fig. 5. miR-4486 boosts the apoptosis of CRC cells by inhibiting the p38MAPK/JNK signaling. miR-4486 mimic and SB203580 were applied in HCT116 and SW1116 cells separately or in combination. **A:** Cell viability. **B:** WB was conducted to quantify Bcl2, Bax, and cleaved-caspase3 protein expression. Bar charts were used to analyze the **(C)** Bax, **(D)** Bcl2, and **(E)** cleaved-caspase3 expression (the original image is provided in the supplementary file). Vs the control group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Vs the miR-4486 mimic + SB203580 group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$.

expressed significantly more MAP2K4 expression and miR-4486 expression levels decreased. In addition, the overexpression of miR-4486 significantly downregulated the MAP2K4 expression, while the invasive transfer capability of CRC cells was reduced. Considering the crucial role of MAP2K4 in the p38MAPK/JNK signaling pathway, and the signaling pathway is correlated with cancer cell proliferation, migration, and metastasis [11], SB203580 was employed to find the miR-4486 effects for CRC cell proliferation. It was found that miR-4486 may suppress the p38MAPK/JNK signaling pathway activation by inhibiting MAP2K4, leading to downregulation of the CRC cell proliferation ability and virulence. This study may provide a reference for miRNA adjuvant therapy in CRC.

Previously, Liu, J et al. found that miR-4486 could inhibit the proliferation of glioma cells by interrupting the lncRNA small nucleolar RNA host gene 20 (SNHG20) expression [12]. Liu, J et al. last study further reported that the miR-4486 expression level was increased in the endometrium of polycystic ovary syndrome (PCOS) rats [13]. In the present study, we demonstrated that miR-4486 could boost the apoptosis of CRC cells. These findings suggested that miR-4486 may play different roles in various human diseases, either through positive or negative influences.

JNK, p38MAPK MAPK, and ERK are members of the MAPK family and have some upstream activators, like ASK1 (Apoptosis Signaling Kinase 1), BRAF, and MAP2K4 [14,15]. Among them, MAP2K4 was reported to be an unfavorable factor for the prognosis of cancer patients. For example, in 2014, MAP2K4 inhibition was demonstrated to be beneficial for suppressing the migration of pancreatic cancer cells [16]. In the same year, MAP2K4 was also reported to promote the metastasis of human prostate cancer [17]. However, other research has demonstrated that MAP2K4 is a tumor suppressor gene that inhibits tumor development. Previous studies have found that a high MAP2K4 level could inhibit osteosarcoma cell invasive transfer capability [18], moreover, the MAP2K4

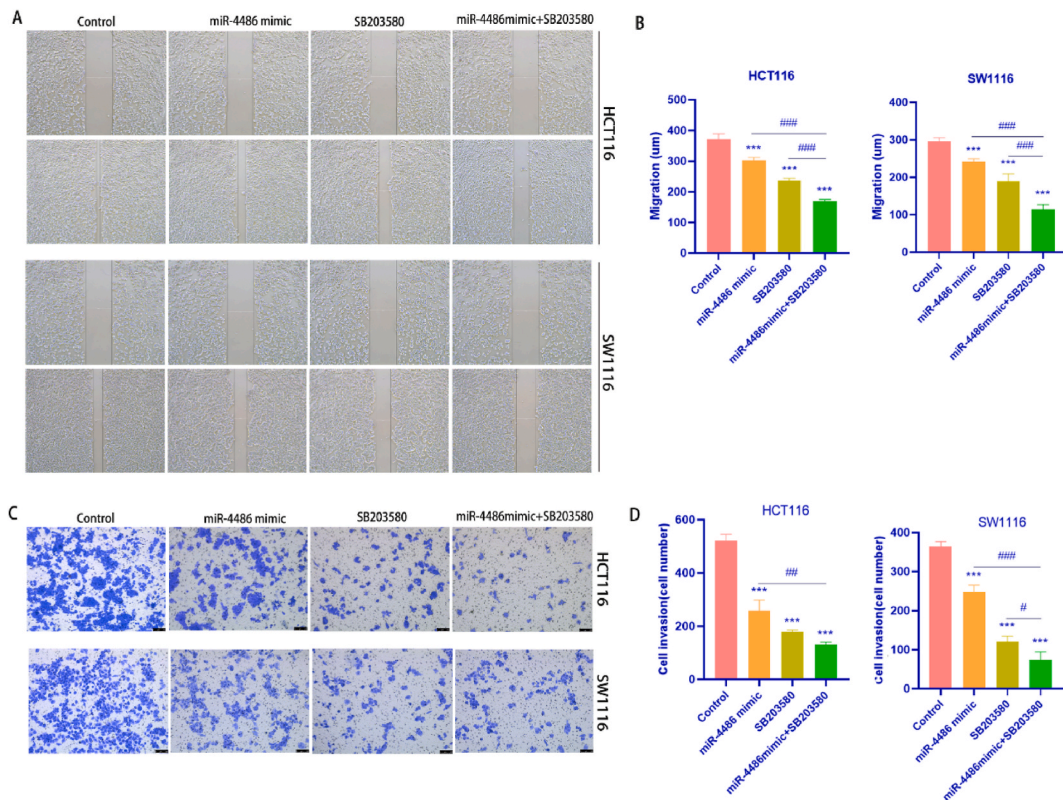

Fig. 6. miR-4486 inhibits cell invasive transfer capability of CRC cells via inhibiting the p38MAPK/JNK signaling. miR-4486 mimic and SB203580 were applied in HCT116 and SW1116 cells separately or in combination. **A/B:** The cell migration was determined by scratch experiments. **C/D:** The cell invasion was determined by the Transwell assay. Vs the control group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Vs the miR-4486 mimic + SB203580 group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$.

overexpression could target to increase the E-cadherin expression level to inhibit the EMT of ovarian cancer [19]. As can be seen, the mechanism of MAP2K4's role in various cancers is elusive. Our results revealed that MAP2K4 was over-expressed in the CRC cells. When the MAP2K4 gene was interrupted, the proliferation of CRC cells was inhibited. Our results verified that MAP2K4 is an oncogene in CRC.

The p38MAPK/JNK signaling pathway is associated with cell differentiation and apoptosis. As reported, *Yongzhi Guo* et al. declared that the inhibition of the p38MAPK/JNK signaling upholds the osteoblast differentiation [20]. JNK and p38MAPK both are proapoptotic factors, their phosphorylation was induced by different factors, such as proinflammatory cytokines and ROS [21]. In addition, the activation of p38 MAPK/JNK pathways could induce VEGF secretion from malignant glioma cells, and VEGF could induce angiogenesis [22]. As is well known, angiogenesis plays a key role in the malignant progression of the tumor. Thus, we speculated that the p38MAPK/JNK signaling pathway may play an important role in CRC development. Our study revealed that when the p38MAPK/JNK signaling was blocked by miR-4486, CRC cell apoptosis was boosted. Specifically, it showed an expression decrease of Bcl2 and an increase of Bax and cleaved-caspase3.

Clinically, miR-4486 can target MAP2K4 to influence the proliferation and the invasive transfer capability of CRC cells. The introduction of miR-4486 expression can inhibit the growth of CRC tumors by suppressing the activation of the p38MAPK/JNK signaling pathway. miR-4486 is expected to be used for targeted therapy of CRC. However, the stability and specificity of miRNA in vivo remain major challenges, necessitating the development of effective delivery systems. This study has some limitations. On the one hand, the p38MAPK inhibitor, SB203580, is applied to explore the effects of the p38 MAPK signaling pathway on the development of various kinds of tumors as usual. p38MAPK includes p38α, p38β, p38γ and p38δ [23]. However, although SB203580 targets p38α and p38β, it is less selective for other p38 isozymes [24]. Thus, the inhibition of SB203580 was limited. On the other hand, this finding needs to be studied in vivo, to allow for the possibility of clinical translation. In conclusion, this finding is of great significance to the basic research of CRC treatment.

Data availability statement

The data sets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Foundation

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CRediT authorship contribution statement

Weiwei Wang: Writing – original draft, Methodology, Data curation, Conceptualization. **Linxia Chen:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Feipeng Xu:** Project administration, Methodology, Investigation. **Rihong Chen:** Validation, Software, Formal analysis. **Qidong Li:** Writing – review & editing. **Lirui Zou:** Validation, Investigation. **Honghui Hu:** Visualization, Software. **Wenjing Zhu:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

none declared.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38926.

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