MiR-199a mediated the dissemination of human mantle cell lymphoma by interacting with the CCR7/CCL21 pair

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Mantle cell lymphoma (MCL) is a rare but deadly subtype of non-Hodgkin's lymphomas because of it can progress rapidly and has a poor prognosis. MicroRNA-199a (miR-199a) is a potential diagnostic marker and therapeutic target for MCL patients. However, the function and molecular mechanisms of miRNA-199a in MCL cells are still unclear. In this study, we first analyzed the levels of miR-199a and C-C chemokine receptor 7 (CCR7) in the tumor tissues and tumor-adjacent tissues, and found that the level of miRNA-199a was lower, whereas the level of CCR7 was higher in tumor tissues. Moreover, overexpression of miR-199a in MCL cells downregulated the level of CCR7. Then, it was found that chemokine (C-C motif) ligand 21 (CCL21), a ligand of CCR7, promoted Granta-519 and Mino cell growth and migration in a concentration-dependent and time-dependent manner. Otherwise, the CCL21/CCR7 pair elevated the level of phosphorylation of protein kinase B and extracellular regulated protein kinases 1/2, upregulated the level of matrix metalloproteinases-2, matrix metalloproteinases-9, and the markers of the mesenchymal phenotype (N-cadherin and vimentin), as well as decreased the level of E-cadherin. However, the functions of CCL21/CCR7 in the growth, migration, and dissemination of MCL cells were decreased by overexpression of miR-199a. Thus, miR-199a inhibited the dissemination of MCL cells by reversing the function of CCL21/CCR7, providing a theoretical basis for miRNA-199a as a potential novel diagnostic marker and therapeutic target for MCL patients. *Anti-Cancer Drugs* 00:000–000 Copyright © 2018 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Mantle cell lymphoma (MCL), a rare but deadly subtype of non-Hodgkin's lymphomas, has received considerable attention from researchers and doctors because of its aggressive clinical feature and poor prognosis [1]. In terms of morphology, hematologists classify MCL as an aggressive B-cell malignancy. MCL patients are typically diagnosed at 60–65 years of age, and MCL is accompanied by generalized nonbulky lymphadenopathy and frequent extraneous disease burden. Although the median survival of MCL patients has increased with the development of new therapeutic strategies, such as chemoimmunotherapy [2–4], MCL is still considered an incurable cancer. One of the important reasons for this is the rapid dissemination of MCL, which resembles the so-called lymphocyte homing to targeted lymphoid organ to facilitate microenvironment circulation, and thus provides proliferation, survival, and migration signals for tumor cells [5]. Therefore, controlling the dissemination of MCL may provide a new avenue for identifying the novel therapeutic targets for this type of neoplasm.

MicroRNAs (miRNAs) are a group of small (19–25 nucleotides) noncoding RNAs that negatively regulate gene expression through binding to their target mRNAs, which are involved in numerous biological and pathophysiological processes, including development, host defense, aging, and tumorigenesis [6,7]. Currently, accumulating studies suggest that there exists miRNAs aberrant expression in most human tumors [8,9]. More recently, it has been found that overexpression of miRNA-199a contributes toward improving the survival time of aggressive diffuse large B-cell lymphoma patients by modifying drug sensitivity to immune chemotherapy [10]. This result suggests that miRNA-199a may serve as a potential novel therapeutic target for MCL patients. To date, the function and molecular mechanism of miRNA-199a in MCL are still unclear.

C-C chemokine receptor 7 (CCR7) is expressed on naive T and B-cells, some central memory T-cells, and mature dendritic cells. Through interaction with its ligands

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CCL19 or chemokine (C-C motif) ligand 21 (CCL21), which is mainly secreted by lymphatic endothelial cells, CCR7 regulates lymphocyte trafficking and homing to lymph nodes during immune and inflammatory reactions [11]. It was reported that the high level of CCR7 was associated with lymph node metastasis and poor prognosis in squamous cell carcinoma [12], melanoma [13], and nonsmall lung cancer [14]. Stimulation of CCR7 by CCL21 could induce the migration and invasion of CCR7-expressing cancer cells [15,16]. Moreover, some researches indicated that CCR7 played a major role in migration and nodular dissemination of certain lymphoproliferative syndromes including chronic lymphocytic leukemia and MCL [17]. In addition, CCR7 was highly expressed in lymphoma and the tumor of human MCL Granta-519 and Mino cells xenograft, and it was significantly inhibited by anti-CCR7 mAb treatment [18]. Thus, exploring the upstream miRNAs targeting CCR7 might provide hope for MCL patients.

It was reported that overexpression of miR-199a-5p inhibited bladder cancer cell migration and invasion by targeting the 3′-untranslated region of CCR7 to downregulate the level of CCR7 [15]. However, this is not reported in MCL. Therefore, we studied whether and how miRNA-199a mediated cell growth, migration, and dissemination by targeting CCR7 in MCL. As a result, we found lower levels of miRNA-199a and higher levels of CCR7 in MCL neoplasm tissue versus tumor-adjacent tissues. Moreover, overexpression of miRNA-199a reversed the function of the CCL21/CCR7 pair in facilitating the growth, migration, and dissemination of Granta-519 and Mino MCL cells. These results provided a theoretical basis for miRNA-199a as a potential novel diagnostic marker and therapeutic target for MCL patients.

Patients and methods

Patients

Use of patient samples in this study was approved by the Ethical Review Board of the first affiliated hospital of Zhengzhou University. Written informed consent was obtained from each patient before tissue acquisition. A total of 30 MCL specimens were classified according to the World Health Organization Classification of Lymphoid Neoplasms. All collected tissue samples were frozen immediately in liquid nitrogen and stored at −80°C until further use to detect the levels of miR-199a and CCR7.

Cell culture

Human MCL cell lines Mino, Granta-519, and Z138 were maintained in RIPM-1640 medium (Gibco; Grand Island, New York, USA) supplemented with 10% fetal bovine serum (Gibco), 2 mmol/l L-glutamine (Sigma-Aldrich; St. Louis, Missouri, USA), 100 μg/ml streptomycin (Sigma-Aldrich), and 100 U/ml penicillin (Sigma-Aldrich). These cell lines were cultured at 37°C in an incubator with a humidified atmosphere of 5% CO_2.

Reverse transcription PCR and real-time quantitative PCR

Total RNA was extracted from the cell lines and tissues using Trizol reagent (Invitrogen; Carlsbad, California, USA) according to the manufacturer's instructions. Reverse transcription was performed using the PrimeScript RT (reverse transcription) reagent kit (TaKaRa; Tokyo, Japan). In addition, the changes in the levels of CCR7 and miRNA-199a after the indicated treatment were measured using real-time quantitative PCR with an SYBR Premix Ex Taq Kit (TaKaRa). The primer sequences are as follows: CCR7, 5′-GAG GCT ATT GTC CCC TAA ACC-3′ (forward) and 5′-TGG AGG ACA GTG AAG AAA ACG-3′ (reverse); miR-199a, 5′-GCGCCCAGTGTTCAGA-3′ (forward) and 5′-GT CGTATCCAGTCAG-3′ (reverse); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-GGTCTCCTCT GACTTCAACA-3′ (forward) and 5′-GTGAGGGTCTCT CTCTTCCT-3′ (reverse). The relative expression of each gene was quantified using GAPDH expression as an endogenous control. Dissociation curves were constructed after each experiment to confirm the specificity of product amplification.

MiRNA-199a mimics and transfection

MiRNA-199a mimics were designed and synthesized by Genephama (Shanghai, China). MiRNA-199a mimics sequences were 5′-CGGCAATGAAGAAGATAAA-3′ (siYB-1#1), 5′-TAACCATTATAGACGCTAT-3′ (siYB-1#2). Full-length miR-199a cDNA was amplified from a human lymphoma library using PCR and sub-cloned into a pcDNA3.1 construct (Invitrogen). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

MTT assay

Cells were seeded in a 96-well plate at the concentration of 1.0×10^4 cells per well for 24 h, and transfected with miRNA-199a mimics, followed by treatment with or without CCL21 at 400, 600, 800, 1000, and 1200 ng/ml. After incubation for 24, 48, and 72 h, respectively, the plates were centrifuged, and the supernatants were discarded, followed by a wash with PBS once. A volume of 100 μl serum-free medium containing 0.5 mg/ml MTT was added and incubated for 4 h. The supernatants were discarded carefully and the formazan crystals were dissolved in 100 μl dimethyl sulfoxide. The absorbance of each well was quantified in a microplate reader (Bio-Rad, Hercules, California, USA) at 570 nm with a reference wavelength of 630 nm.

Migration assay

Migration assay was carried out with a chamber containing a polycarbonate membrane (8 μm pore size). After being collected by centrifugation, cells were re-suspended in serum-free RPMI-1640 medium and adjusted to a cellular density of 5×10^5 cells/ml. Then, 100 μl cell suspension was placed in the upper transwell chambers, whereas

500 µl culture fluid containing CCL21 at 400, 600, and 800 ng/ml, respectively, was added in the lower chamber. After incubation at 37°C for 24 h, the cells that migrated to the lower chambers were counted.

Cell apoptosis assay

An apoptosis assay was performed using the Annexin V-FITC Apoptosis Detection Kit (KeyGen Bio TECH; Nanjing, China). Cells were seeded onto a 6-cm dish at a concentration of 3.5×10^5/dish for 24 h and then transfected with miRNA-199a mimics, followed by treatment with or without 800 ng/ml CCL21 for 48 h. Cells were harvested and washed twice with PBS. After re-suspension in 100 µl binding buffer containing 2 µl Annexin V-FITC dye, cells were incubated on ice in the dark for 10 min. Next, 100 µl normal saline with 2 µl propidium iodide dye were added and incubated for 2–3 min at room temperature in the dark. The cells were evaluated immediately by flow cytometry (BD Biosciences; San Jose, California, USA).

Western blotting

Total proteins were extracted by radio-immunoprecipitation assay and 1% phenylmethanesulfonyl fluoride. The protein concentrations were measured using the bicinchoninic acid assay. Equal amounts of protein (80–100 µg) were separated by 12% SDS-PAGE gels and electrotransferred onto polyvinylidene fluoride membranes. After blocking with 5% nonfat milk in TBST buffer [20 mmol/l Tris-HCl, 120 mM NaCl, and 0.1% (v/v) Tween 20] at 37°C for 1–2 h, the membranes were subsequently probed with the primary antibodies at 4°C overnight. After washing with TBST, the membranes were incubated with the secondary antibodies labeled by horseradish peroxidase at room temperature for 40 min. Finally, proteins were detected using an enhanced chemiluminescence detection kit (Thermo Fisher Scientific; Waltham, Massachusetts, USA). The antibodies used in this study included rabbit anti-human GAPDH (Abcam; Cambridge, Massachusetts, USA), rabbit anti-human CCR7 (Abcam), rabbit anti-human matrix metalloproteinases (MMP)-2 (Abcam), rabbit anti-human MMP-9 (Abcam), rabbit anti-human E-cadherin (Abcam), rabbit anti-human N-cadherin (Abcam), rabbit anti-human vimentin (Abcam), rabbit anti-human protein kinase B (also known as AKT), (Abcam), rabbit anti-human phosphorylation of AKT (Abcam), rabbit anti-human extracellular regulated protein kinases 1/2 (ERK1/2) (Abcam), rabbit anti-human phosphorylation of ERK1/2 (Abcam), and goat anti rabbit IgG1 (Abcam). Quantification of visualized bands was performed by densitometry using Image J software (Version 1.8.0; National Institutes of Health, Denver, Colo, USA).

Statistical analysis

All experiments were repeated three times. The data were expressed as mean ± SD. All statistical analyses were carried out using the statistics software program SPSS (Version 16; IBM, Armonk, New York, USA). Statistical evaluation was performed using Student's *t*-test (two tailed) between two groups or one-way analysis of variance, followed by the Tukey post-hoc test for multiple comparisons. Results were considered statistically significant at P value less than 0.05.

Results

The MiRNA-199a level was downregulated and the CCR7 level was upregulated in human MCL tissue samples

To determine whether miRNA-199a and CCR7 were involved in the tumorigenesis of human MCL, we evaluated the levels of miRNA-199a and CCR7 in human MCL tissues and negative control (tumor-adjacent tissues) by RT-qPCR. As shown in Fig. 1a, the expression of miRNA-199a was significantly lower ($P < 0.001$) in tumor tissues than that in the negative control. In contrast to miRNA-199a, the expression of CCR7 was obviously higher ($P < 0.001$) in tumor tissues (Fig. 1b). Thus, the differential expressions of miR-199a and CCR7 may be the key reason for the tumorigenesis of human MCL.

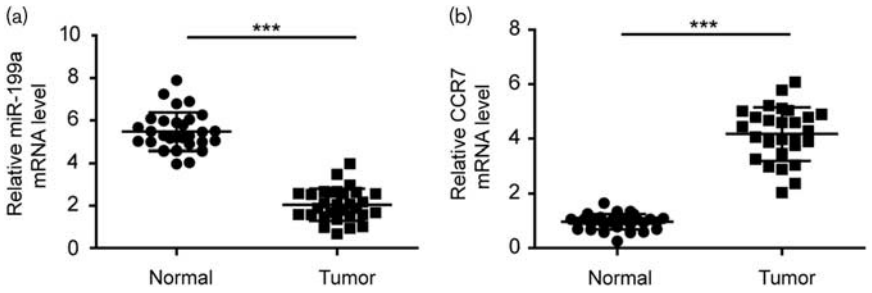
Levels of miRNA-199a and CCR7, and EMT ability in human MCL cell lines

To determine the relationship between miRNA-199a and CCR7, we investigated the levels of miRNA-199a and CCR7 in Mino, Granta-519, and Z138 human MCL cells by RT-qPCR and western blotting. The results suggested that the level of miRNA-199a ranging from the lowest to the highest was in the order of Granta-519, Mino, and Z138, whereas the levels of CCR7 expression in those cells showed the reverse order (Fig. 2a, b and c). After transfection with miRNA-199a mimics, the levels of miR-199a in these MCL cells were upregulated obviously and the levels of CCR7 were downregulated significantly compared with the negative control (Fig. 2d and e). Thus, miR-199a negatively regulated the expression of CCR7. To study the migration ability of these cells, the levels of MMP-9, MMP-2, N-cadherin, vimentin, and E-cadherin in these 3 MCL cells were detected by western blotting. It was found that the levels of MMP-2, MMP-9, N-cadherin, and vimentin were the highest in Granta-519, whereas E-caderin expression was the lowest, indicating that miR-199a may regulate the migration of MCL cells through CCR7 (Fig. 2f). In addition, the migration ability was enhanced in Granta-519 and Mino cells compared with that in Z138 cells, from the aspect of endothelial-mesenchymal transition (EMT) marker levels. Subsequently, Granta-519 and Mino cells were selected for the next experiments.

CCL21 promoted the cell proliferation and altered miR-199a and CCR7 expressions in a concentration-dependent manner

Generally, CCR7 will be activated after binding to its ligands CCL19 or CCL21. First, we analyzed the changes in the growth of Granta-519 and Mino cells after treatment

Fig. 1



The relative mRNA levels of miR-199a and CCR7 in tumor tissues and tumor-adjacent tissues from MCL patients. The tumor tissues and tumor-adjacent tissues from MCL patients were collected and the relative mRNA levels of miR-199a (a) and CCR7 (b) were analyzed by RT-qPCR. CCR, C-C chemokine receptor; MCL, mantle cell lymphoma. ***$P < 0.001$.

with CCL21 at various concentrations for 24, 48, and 72 h, respectively. The results suggested that CCL21 promoted the proliferation of Granta-519 and Mino cells in a concentration-dependent and time-dependent manner (Supplementary Fig. 1a, Supplemental digital content 1, *http://links.lww.com/ACD/A264*). Next, MCL Granta-519 and Mino cells were, respectively, treated with CCL21 at 400, 600, and 800 ng/ml for 48 h. Then, the levels of miRNA-199a and CCR7 were detected by RT-qPCR and western blotting. In terms of miRNA-199a, the results showed that CCL21 inhibited the level of miRNA-199a in a concentration-dependent manner (Supplementary Fig. 1b, Supplemental digital content 1, *http://links.lww.com/ACD/A264*). However, both transcription and translation levels of CCR7 were elevated with increasing concentration of CCL21 (Supplementary Fig. 1c and d, Supplemental digital content 1, *http://links.lww.com/ACD/A264*).

CCL21 induced Granta-519 and Mino cell migration and endothelial–mesenchymal transition through AKT and the ERK1/2 signaling pathway

Migration is the crucial factor for malignancy and dissemination of MCL. Therefore, we next examined the effects of the CCL21/CCR7 pair on Granta-519 and Mino cell migration. After treatment with CCL21 at 400, 600, and 800 ng/ml, respectively, the migrated cells from upper transwell chambers of Granta-519 and Mino cells increased with increasing CCL21 concentrations (Fig. 3a). Subsequently, we detected the markers of EMT, one of the key factors for tumor malignancy, by western blotting after treatment with 400, 600, and 800 ng/ml CCL21 for 48 h. As shown in Fig. 3b, CCL21 increased MMP-9, MMP-2, N-cadherin, and vimentin expressions in a concentration-dependent manner, whereas the E-cadherin level was obviously decreased in the CCL21 groups of higher concentrations compared with the control. The phosphorylation of ERK1/2 and AKT proteins is usually involved in EMT. Thus, the total and phosphorylation proteins of ERK as well as AKT in Granta-519 and Mino cells were measured by western blotting after treatment with CCL21 at 400, 600, and 800 ng/ml for 48 h, respectively. It was found that the total proteins of ERK1/2 and AKT remained unchanged, whereas the levels of phosphorylation of ERK1/2 and AKT increased with increasing CCL21 concentrations (Fig. 3c). Therefore, CCL21/CCR7 promoted Granta-519 and Mino cell migration by EMT through AKT and the ERK1/2 signaling pathway.

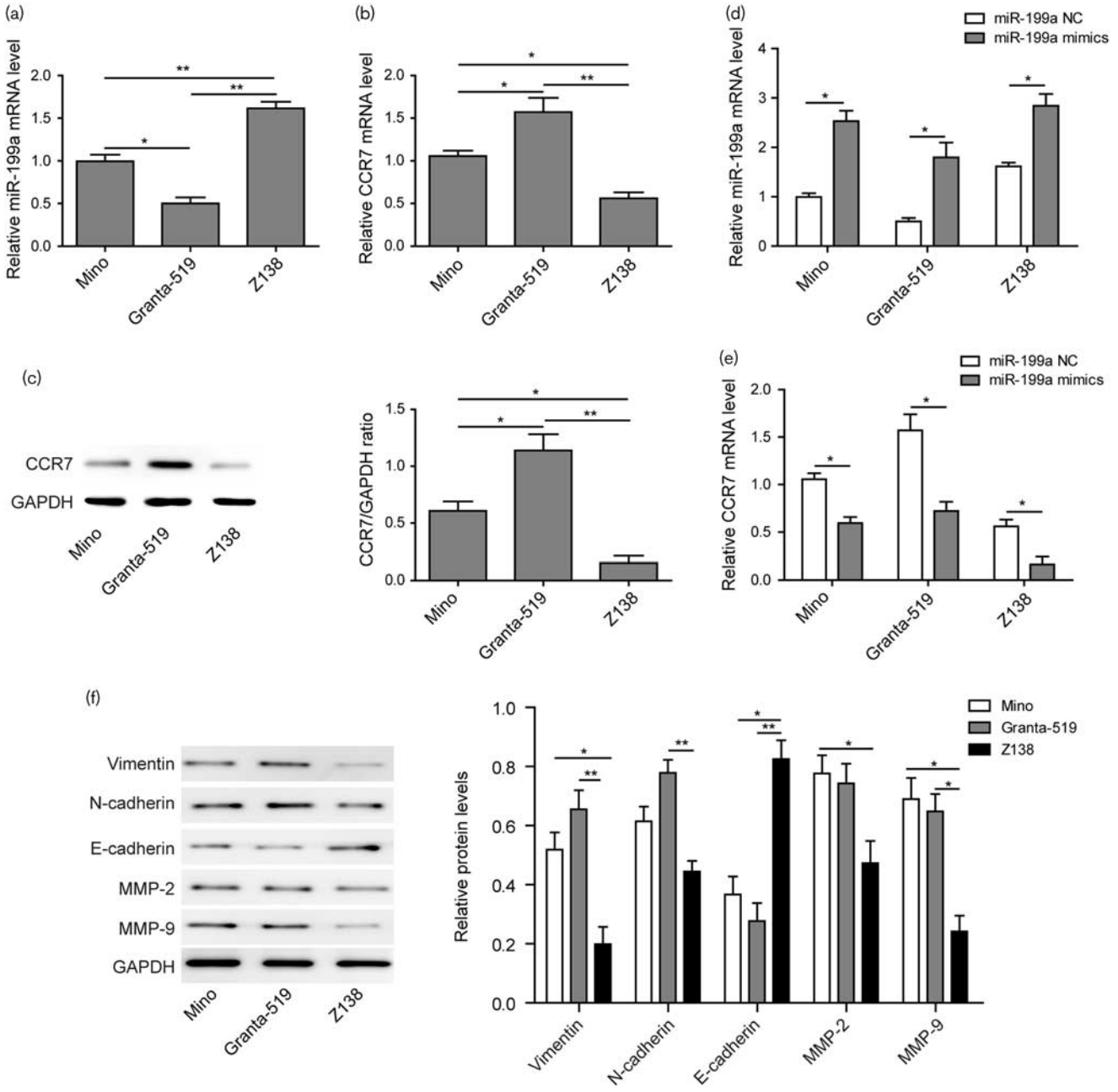
Overexpression of miRNA-199a reversed the effects of CCL21 on CCR7 levels and cell growth

After transfection with miRNA-199a mimics for 24 h, Granta-519 and Mino cells were treated with or without 800 ng/ml CCL21 for 48 h, and the levels of miR-199a and CCR7 were measured by RT-qPCR and western blotting. As shown in Fig. 4a, overexpression of miRNA-199a reversed the inhibition of CCL21 on the miR-199a mRNA level. Meanwhile, the upregulation of CCR7 in transcribed and translated levels induced by CCL21 was inhibited by miRNA-199a mimics (Fig. 4b and c). Next, the cell proliferation and apoptosis were also detected. The results suggested that overexpression of miRNA-199a decreased the effects of CCL21 on cell proliferation (Fig. 4d). In the Granta-519 cells, the apoptotic cells in the combined treatment group (~13.31%) were lower than those in the miR-199a mimic group (~21.21%), but the live cells in the combined treatment group were slightly higher than those in the miR-199a mimic group. Thus, this indicated that CCL21-mediated CCR7 activation only blocked miR-199a-induced toxicity partially.

Overexpression of miRNA-199a decrease the effects of the CCR7/CCL21 pair on migration, EMT markers, AKT, and the ERK1/2 signaling pathway in Granta-519 and Mino cells

Subsequently, we examined changes in migration, EMT markers, AKT, and ERK1/2 signaling pathways in Granta-519 and Mino cells after transfection with miRNA-199a mimics for 24 h, followed by treatment with or without 800 ng/ml CCL21 for 48 h. It was found that the migration of Granta-519 and Mino cells triggered by CCL21 was

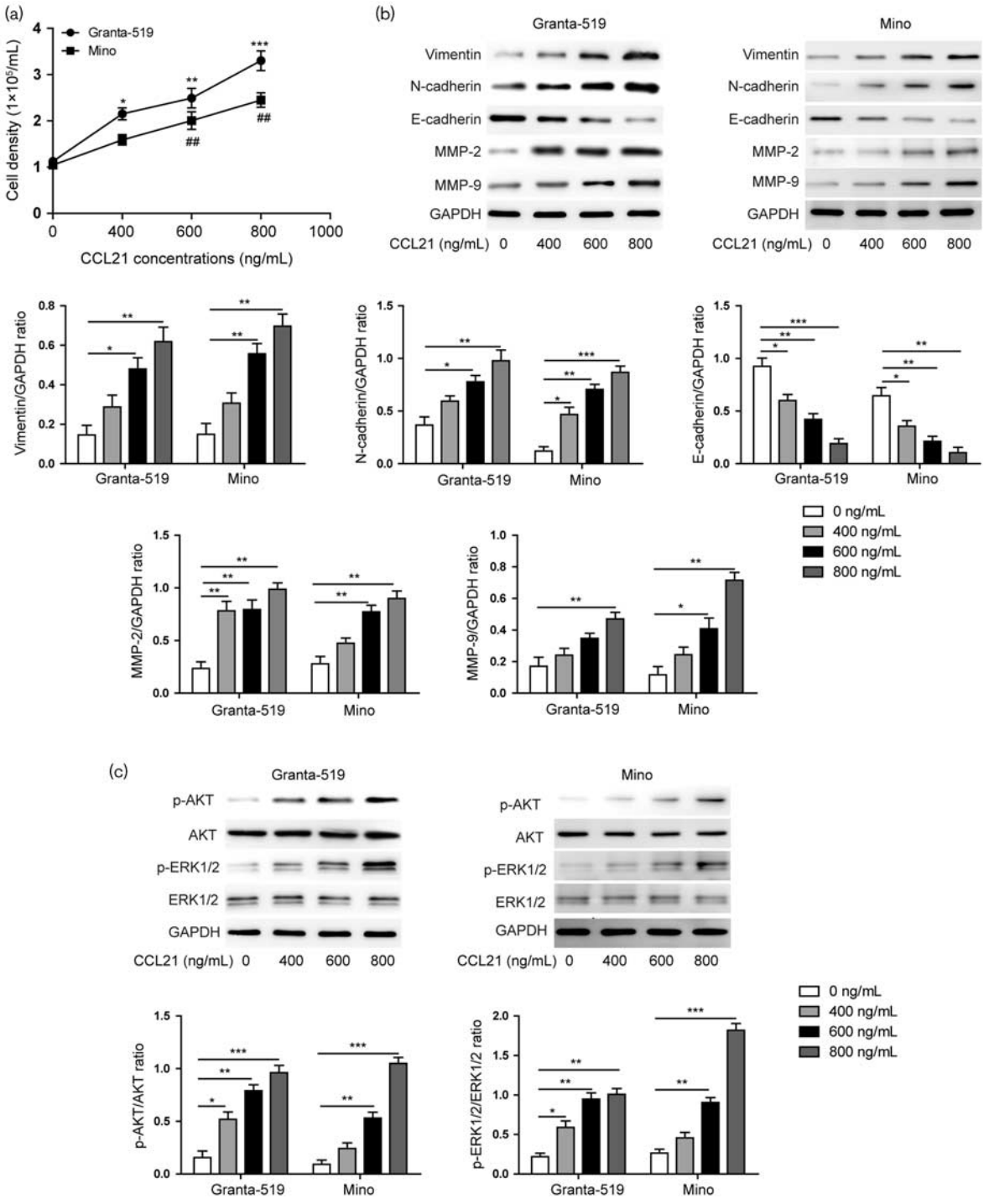
Fig. 2



The levels of miRNA-199a, CCR7, and EMT ability in human MCL cell lines Mino, Granta-519, and Z138. The levels of miRNA-199a (a) and CCR7 mRNA (b) were detected by RT-qPCR. The protein level of CCR7 was measured by western blotting and analyzed using Gel Analyzer software (c). GAPDH was used as an internal control. After transfection with miRNA-199a mimics, the mRNA levels of miRNA-199a (d) and CCR7 (e) were determined by RT-qPCR. The levels of MMP-2, MMP-9, N-cadherin, vimentin, and E-cadherin in all 3 MCL cells were detected by western blotting and analyzed using Gel Analyzer software (f). CCR, C-C chemokine receptor; EMT, endothelial–mesenchymal transition; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MCL, mantle cell lymphoma; MMP, matrix metalloproteinases. $^{*}P < 0.05$; $^{**}P < 0.01$.

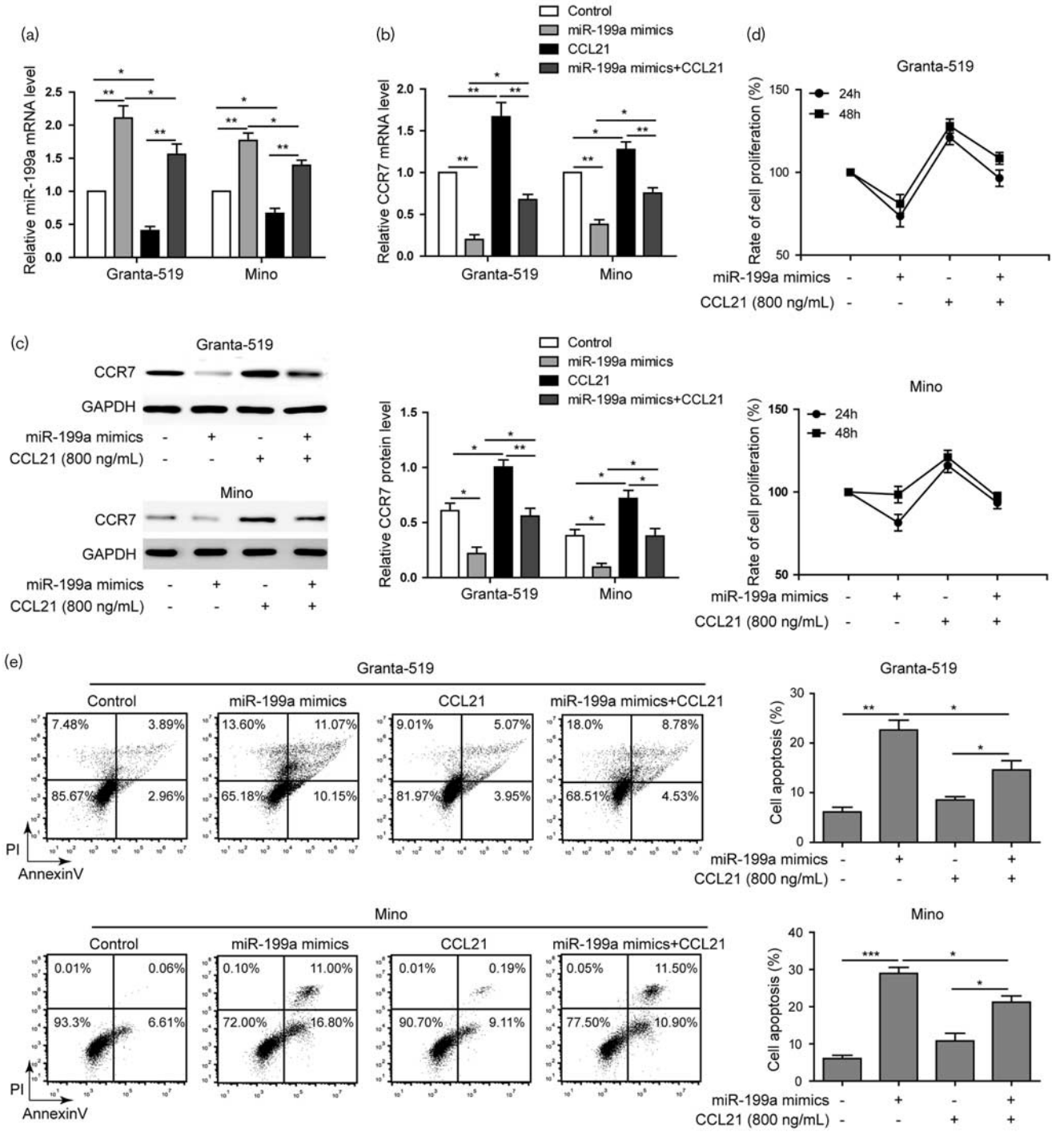
weakened by overexpression of miRNA-199a (Fig. 5a). Meanwhile, the effects of CCL21 on the levels of EMT markers including MMP-9, MMP-2, N-cadherin, vimentin, and E-cadherin were almost abolished when Granta-519 and Mino cells were pre-transfected with miRNA-199a mimics (Fig. 5b). Furthermore, CCL21-triggered upregulation of phosphorylation of AKT and ERK1/2 proteins was also blocked by overexpression of miRNA-199a (Fig. 5c). Thus, miRNA-199a decreased the effects of the CCR7/CCL21 pair on the migration, EMT markers, AKT, and ERK1/2 signaling pathways in Granta-519 and Mino cells.

Fig. 3



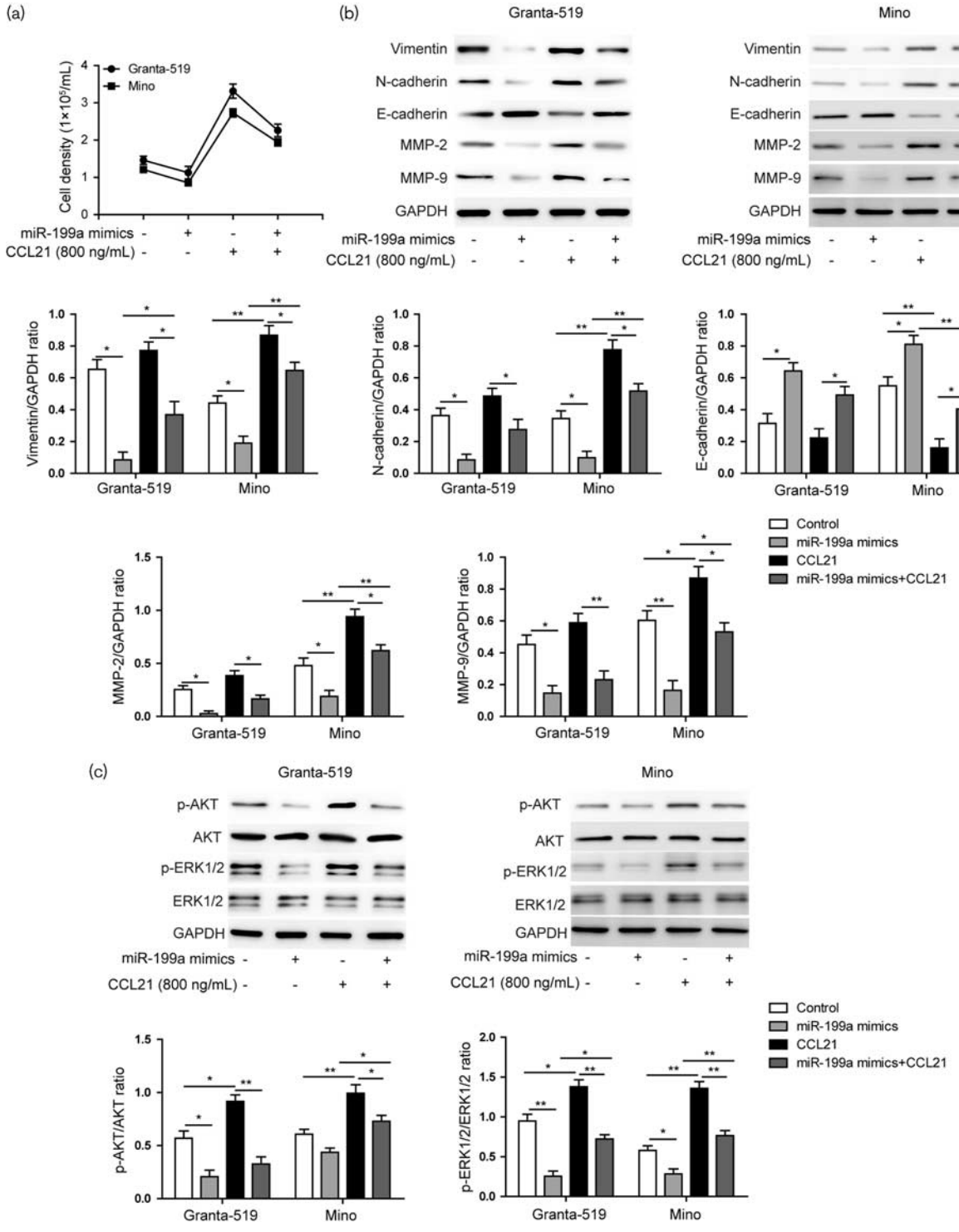
The effects of CCL21 on Granta-519 and Mino cell migration and related signaling pathway. After treatment with CCL21 at 400, 600, and 800 ng/ml for 24 h, the migration cell numbers were counted (a). After treatment with CCL21 at 400, 600, and 800 ng/ml for 48 h, the levels of EMT makers (b), as well as phosphorylation and total AKT and ERK1/2 proteins (c) were measured by western blotting and analyzed using Gel Analyzer software. GAPDH was used as an internal control. CCR, C-C chemokine receptor; EMT, endothelial–mesenchymal transition; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\#\#}P < 0.01$.

Fig. 4



The effects of miRNA-199a on the levels of CCR7 and proliferation and apoptosis of Granta-519 and Mino cells triggered by CCL21. After transfection with miRNA-199a mimics, the cells were treated with or without CCL21. The levels of miRNA-199a (a) and CCR7 mRNA (b) were detected by RT-qPCR. The protein level of CCR7 was measured by western blotting and analyzed using Gel Analyzer software (c). GAPDH was used as an internal control. Subsequently, the cell proliferation was determined using the MTT assay (d). Next, the apoptotic cells were analyzed by flow cytometry after staining by Annexin V-FITC and PI dyes (e). CCR, C-C chemokine receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Fig. 5



The effects of miRNA-199a on Granta-519 and Mino cells' migration and related signaling pathway induced by the CCL21/CCR7 pair. After transfection with miRNA-199a mimics, the lymphoma cells were treated with or without CCL21. The migration cell numbers were counted (a). The levels of EMT makers (b), as well as phosphorylation and total of AKT and ERK1/2 proteins (c) were measured by western blotting and analyzed using Gel Analyzer software. GAPDH was used as an internal control. CCR, C-C chemokine receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; $^{*}P < 0.05$; $^{**}P < 0.01$.

Discussion

New diagnostic makers and therapeutic targets are required for MCL patients because of MCL properties including rapid aggression, poor prognosis, and short median survival [19,20]. miRNA has been a novel hot point in the field of cancer treatment because of its function of negatively regulating genes associated with tumorigenesis and metastasis by binding to the 3′-untranslation region [21,22]. It was reported that a higher level of miRNA-199a was linked to better overall survival of diffuse large B-cell lymphoma patients [10], and CCR7 mediated lymphoid dissemination of many cancers including lymphomas [23]. Therefore, we focused on whether and how miRNA-199a influenced MCL cells' dissemination through interaction with CCR7 in this research.

In this study, we first analyzed the levels of miRNA-199a and CCR7 in tumor tissues, and found that miR-199a was downregulated, whereas CCR7 was upregulated in tumor tissues compared with the negative control (Fig. 1). Consistently, the levels of miRNA-199a and CCR7 in three kinds of human MCL cell lines Mino, Granta-519 and Z138 were contrary (Fig. 2a, b and c). Intriguingly, the expression of CCR7 in all 3 MCL cells was inhibited after transfection with miRNA-199a mimics (Fig. 2d and e). Furthermore, the levels of MMP-2, MMP-9, N-cadherin, and vimentin were the highest, whereas the E-caderin level was the lowest in Granta-519 cells (Fig. 2f). Meanwhile, the lowest levels of miR-199a and the highest level of CCR7 were also found in Granta-519. Thus, we suspected that miRNA-199a might influence the growth, migration, and dissemination of MCL cells by interaction with CCR7. In terms of the EMT marker levels, the migration ability was elevated in Granta-519 and Mino cells, compared with that in Z138 cells (Fig. 2f). Thus, the expression levels of CCR7 in MCL cells may influence the ability of migration and dissemination. Otherwise, the levels of CCR7 in various MCL cells are different, but may always higher than normal cells, which were verified in clinical results (Fig. 1).

The next aim was to determine whether and how CCR7 influenced the growth, migration, and dissemination of MCL cells. Yang *et al.* [23] first reported that CCR7 facilitated the migration and invasion of non-Hodgkin's lymphomas cell Hut78 after the activation of its ligand CCL21. To confirm the function of CCL21 in cell growth and the expressions of miR-199a and CCR7 in MCL cells, we performed cell proliferation, RT-qPCR, and western blotting assays in Granta-519 and Mino cells. These results showed that the growth was stimulated significantly by CCL21 in a concentration-dependent and time-dependent manner Supplementary Fig. 1a, Supplemental digital content 1, *http:///links.lww.com/ACD/A264*). Meanwhile, the mRNA and protein levels of CCR7 were increased and the level of miR-199a was obviously decreased (Supplementary Fig. 1b, c and d, Supplemental digital content 1, *http:///links.lww.com/ACD/A264*). These results implied an important role of the CCL21/CCR7 axis in tumor progression.

EMT is the key biological process for the ability of migration of neoplastic cells [24,25]. EMT was characterized by the properties of loss of epithelial cell polarity and generation of mesenchymal features. Along with the process of EMT, a series of cellular markers showed changes, including decreased E-cadherin that is the symbolic marker of epithelial cells [26], as well as the increased vimentin and N-cadherin, which are markers of the mesenchymal phenotype [27,28]. Otherwise, MMP such as MMP-2 and MMP-9 were upregulated to promote the EMT progression [29,30]. Thus, we detected the levels of these proteins after treatment with CCL21, and found that the alternations in these proteins in Granta-519 and Mino cells were similar to those mentioned above (Fig. 3b). Thus, the CCL21/CCR7 pair induced the migration and dissemination of Granta-519 and Mino cells. Several studies have proven that ERK and the AKT pathway are involved in tumor growth and dissemination by upregulating the expression of MMP-2 and MMP-9 [30–32]. The activities of ERK1/2 and AKT proteins are triggered through phosphorylation. Thus, we examined the levels of phosphorylation of ERK1/2 and AKT after the administration of CCL21, and found that the levels of phosphorylation of ERK1/2 and AKT were markedly increased at higher concentrations of CCL21 (Fig. 3c). Therefore, ERK1/2 and AKT signaling pathways might be correlated with migration and dissemination induced by CCL21/CCR7.

Whether overexpression of miRNA-199a inhibited the growth, migration, and dissemination of Granta-519 and Mino cells through downregulating CCR7 expression was further determined. Upregulation of CCR7 and increase in the cell proliferation rate induced by CCL21 were constrained after pre-transfection with miRNA-199a mimics (Fig. 4b, c and d). Furthermore, overexpression of miRNA-199a counteracted the effects of CCL21 on the migration of Granta-519 and Mino cells (Fig. 5a). Otherwise, the activated effects of CCL21 on the EMT marker and related ERK1/2 and AKT signaling pathways were decreased by overexpression of miR-199a (Fig. 5b and c). Thus, miRNA-199a influenced the dissemination of Granta-519 by downregulating CCR7.

In conclusion, we first analyzed the levels of miR-199a and CCR7 in the tumor tissues, and found that the level of miRNA-199a was lower, whereas the level of CCR7 was higher in tumor tissues. Moreover, we proved that overexpression of miRNA-199a reversed the function of the CCL21/CCR7 pair in facilitating the growth, migration, and dissemination of Granta-519 and Mino cells. These results provided evidence for the potential usefulness of miRNA-199a as a new diagnostic marker and therapeutic target for MCL patients.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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