

MicroRNA-1179 targets Epiregulin (EREG) regulates the proliferation and metastasis of human multiple myeloma cells

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MicroRNA-1179 (miRNA-1179) is an extensively studied tumor suppressor. However, the significance of miR-1179 in multiple myeloma has not been investigated previously. So, there is a need for research to find out about the significance of miR-1179 in multiple myeloma. However, current investigations have examined the significance of miRNA-1179 in multiple myeloma for the first time by targeting epiregulin (EREG). In this study, 26 multiple myeloma specimens and 16 healthy donor specimens were examined. Multiple myeloma cell lines (U266, RPMI-8226, KMS-11, JLN-3, and IM-9) were used. In this study, expression analysis, cell viability, colony formation assay, and transwell assay were carried out by standard methods. The outcomes revealed the down-regulation of miRNA-1179 in multiple myeloma. Overexpression of miRNA-1179 promotes, while its inhibition suppresses, the survival ability and colony formation of the U266 multiple myeloma cells. Investigation of underlying mechanisms revealed apoptosis to be responsible for the tumour-suppressive effects of miRNA-1179. The proportion of apoptosis in U266 cells rose from 5.32% to 34.86% when miRNA-1179 was overexpressed. Additionally, it was discovered that miRNA-1179 directs its tumor-inhibiting activities toward EREG at the molecular level. While EREG knockdown was found to halt the proliferation of U266 cells, its overexpression could overcome the suppressive effects of miRNA-1179 on the survival ability, mobility, and invasion of the U266 cells. This research proves that miRNA-1179 can be used as a new treatment or drug for multiple myeloma.

Keywords: MicroRNA-1179, epiregulin, myeloma cells, metastasis

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Abbreviations: ATCC, American Type Culture Collection; EREG, Epiregulin; FBS, Foetal bovine serum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; MM, Multiple myeloma; NPCs, normal plasma cells; S.D., Standard deviation

INTRODUCTION

Multiple myeloma is one of the deadliest disorders, with a high fatality rate. Multiple myeloma made up about 2% of all new cases of myeloma and 2.1% of deaths caused by melanoma in the United States (Pinto *et al.*, 2020). Even though research on cancer diagnosis and treatment has come a long way, multiple myeloma is still one of the hardest cancers to cure (Rajkumar & Kumar, 2020). With just over 5 years of median survival, the majority of the patients are administered four or even more different lines of therapy (Boyle *et al.*, 2021).

Researchers across the globe are looking to develop biomarkers for early diagnosis, identify therapeutic targets for efficient treatment, and identify drugs that are effective and safe.

MicroRNAs (miRNAs) have recently gained significant interest as therapeutic approaches to treat human diseases and disorders. miRNAs are non-coding RNA molecules that have the potential to control the expression of protein-coding genes. At the post-transcriptional stage, every miRNA may influence the production of multiple protein-coding genes (Tan *et al.*, 2018). Several studies on miRNAs found that the progression of many human malignancies was often associated with abnormal expression of miRNA (Ramassone *et al.*, 2018; Vannini *et al.*, 2018). For instance, miRNA-1179 has been found to post-transcriptionally suppress the expression of E2F5 to control the formation and spread of pancreatic cancer (Lin *et al.*, 2018). Song and others (Song *et al.*, 2018) observed that miRNA-1179 regulates the progression and metastasis of non-small cell lung carcinoma. Previously, several researchers investigated miR-1179 and found that it regulates chemical sensitivity in ovarian cancer cells (Zhihong *et al.*, 2020), regulates cell cycle progression in glioblastoma cells (Xu *et al.*, 2017) and the vincristine sensitivity of oral cancer cells (Gao *et al.*, 2020). But the significance of miR-1179 in multiple myeloma has not been investigated. So far, there are no reports available on this; therefore, this study intends to find out the significant influence of miR-1179 in multiple myeloma by examining the changes in epiregulin (EREG). The outcomes of this study may help us find out if miR-1179 could be a new way to treat or cure multiple myeloma.

MATERIALS AND METHODS

Study area

The present study was carried out at the Department of Hematology, The Fourth Affiliated Hospital of Traditional Chinese Medicine from March 2015 to November 2018.

Human tissues

In this study, 26 multiple myeloma specimens and 16 healthy donor specimens were collected at the The Fourth Affiliated Hospital of Traditional Chinese Medicine from March 2015 to November 2018. This research was only performed after the patients provided written permission. The research ethics committee (Reg. No. IA-CUC/EREG/2015/02) also authorised the investigation.

Cell lines

Multiple myeloma cell lines (U266, RPMI-8226, KMS-11, JJN-3, and IM-9) were obtained from the American Type Culture Collection (ATCC, USA) and grown in RPMI-1640 media (Gibco, Ireland) with 10% foetal bovine serum (FBS, Gibco, Ireland), streptomycin (100 g/mL) and 1% penicillin (100 U/mL) in ambient temperature at 37°C with 5% CO₂. Normal plasma cells (NPCs) were isolated and grown from a peripheral blood smear as described in earlier methodologies by Adham *et al.* (2020).

Cell Transfection

The miRNA-NC, miRNA-1179 mimics, siRNA-NC, siRNA-EREG, miRNA-1179 inhibitor, and inhibitor NC were constructed by the modified method of Zhihong and others (Zhihong *et al.*, 2019) as per the manufacturer's instructions (RiboBio, Invitrogen, Carlsbad, CA, USA). The appropriate concentrations of miRNA-1179 inhibitor, inhibitor NC, siRNA-NC, and siRNA-EREG were transfected into U266 cells and treated with Lipofectamine 2000 (Thermo Fisher Scientific, China) based on the kit's guidelines.

Expression analysis

Whole RNA extraction from test specimens and cell lines is performed for mRNA expression analysis by utilizing the TRIzol reagent (Invitrogen). The extracted whole RNA was subjected to RNase-free DNase treatment to eliminate the contaminated DNA. Following that, PrimeScript RT Master Mix was used to produce cDNA from the RNA (TaKaRa, Japan). Then miR-1179 expression was compared with snRNA U6, while EREG expression was compared with that of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and actin. Proteins were drawn out of cells using RIPA solution and run on an SDS-polyacrylamide gel for protein expression analysis. Then the proteins were loaded onto a polyvinylidene fluoride membrane and maintained with specific antibodies. The membrane then re-treated at room temperature with secondary antibodies and horseradish peroxidase. The proteins of concern were visualized using an increased chemiluminescence solution (Millipore).

Cell viability

In this study, the cell survival ability of treated U266 cells was examined by the MTT test. The U266 cells/well (5×10^3) were cultured for 0, 12, 24, 48, and 96 h. Thereafter, 20 μ L of MTT was added to each well, and the plates were again incubated at 37°C for 4 h. Formazan crystals were removed by DMSO. Finally, absorbance was measured by a microplate reader to evaluate cell survival ability at 490 nm.

Colony formation assay

The transfected U266 cells were inoculated in 6-well plates and grown for 2 weeks at 37°C. The grown cells were fixed with 100% methanol for 25 min. After that, 0.1% of the colonies were stained with crystal violet and counted under a microscope.

DAPI staining

Transfected U266 cells were grown in 12-well plates for 24 hours at 37°C at a concentration of 1×10^5 cells/well. Subsequently, the cells were harvested by centrifu-

gation, washed with PBS, and fixed with 70% ethanol. Finally, the U266 cells were then stained with DAPI and observed on a fluorescent microscope.

Annexin V/PI Assessment

The Annexin V/PI assay was to be employed to analyze the percentage of apoptotic U266 cells. Transfected U266 cells (1×10^6) were added into six-well plates and maintained to proliferate for 24 h. After that, the cells were harvested, and the percentage of apoptotic U266 cells was evaluated by an Annexin-VFITC apoptosis detection kit (BestBio) and a Cytomics FC500 flow cytometer using CXP software (Beckman Coulter, Fullerton, CA, USA).

Development of mutant EREG

The complementary nucleotides of miRNA-1179 were mutated using the ThermoFisher Scientific mutagenesis kit to construct the mutant EREG (EREG-MUT) by the modified method of Singh and others (Singh *et al.*, 2013)

Target identification

The fee-based online version of TargetScan (<http://www.targetscan.org/version-72/>) was employed to assess the miRNA-1179 targets. In this study, the most suitable sequence of the EREG 3'-UTR was selected for further investigation.

Luciferase assay

The 3'-UTR of EREG (EREG-WT) was cloned into the pGL3-control vector (Promega) downstream of the stop codon of firefly luciferase. Following that, cells transfected with EREG-WT or EREG-MUT were co-transfected with miRNA-1179 or miRNA-NC. The Dual-Luciferase Reporter Assay System (Promega) was employed to conduct the interaction investigation, which included 48 hours of cell culture at 37°C. Renilla luciferase was used for evaluation to normalise the luciferase.

Transwell assay

Transwell inserts with 8 μ m pore sizes that were plain and matrigel-coated were employed for migration and invasion, respectively. In 24-well plates, the transwell insert was placed, and the bottom chamber receives 500 μ L of DMEM containing 10% FBS. Transfected U266 cells were maintained in 100 μ L of DMEM in the top chamber. After 24 hours, the cells on the outer side of the membrane were scrubbed away and maintained at 37°C. The U266 cells that were adhered to the bottom side of the membrane were kept in methanol for 9 min before they were stained by using crystal violet (0.01%). Thereafter, the samples were carefully washed and cells were imaged and counted in at least five random fields under a digital microscope.

Statistical analysis

The trials were repeated in triplicate, and the outcomes were described as mean \pm standard deviation (S.D.). The GraphPad Prism 7.0 software tool was used to analyse the One-way analysis of variance (ANOVA) and Tukey's tests were carried out for multiple group comparisons. A *p*-value <0.05 was considered statistically significant.

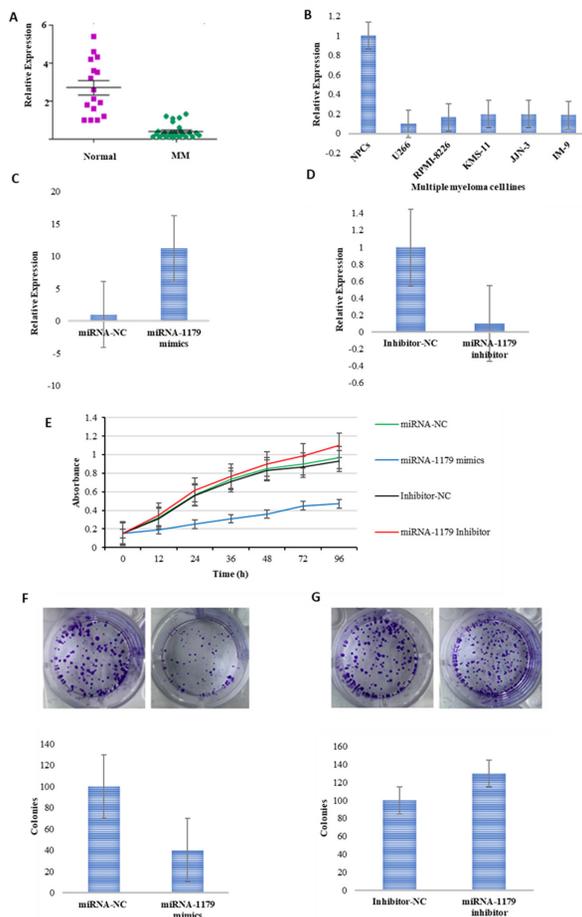


Figure 1. miRNA-1179 expression in multiple myeloma and normal tissues

(A) Relative expression between multiple myeloma and normal tissues; (B) Expression between different types of myeloma cell lines; (C) Relative expression between miRNA-1179 mimics and miRNA-NC; (D) Relative expression between miRNA-1179 inhibitor and inhibitor-NC; (E) Survival ability of the different types of myeloma cell lines; (F) Colony formation of the miRNA-1179 mimics and miRNA-NC cells; (G) Colony formation of the miRNA-1179 inhibitor and inhibitor-NC cells

RESULTS

In this investigation, the expression profile of miRNA-1179 was compared between multiple myeloma (MM) and normal cells (Fig. 1A). The relative expression profile of miRNA-1179 in multiple myeloma cells was 1.75-fold, which is considerably lower ($p < 0.05$) than the expression profile in normal tissues (5.5-fold) (Fig. 1A). The expression of miRNA-1179 was assessed in the different types of myeloma cell lines (U266; RPMI-8226; KMS-11; JJN-3; and IM-9) as well as normal plasma cells (NPCs) (Fig. 1B). When compared to NPCs, miRNA-1179 was significantly downregulated ($p < 0.05$) in all myeloma cell lines. NPCs express the highest fold (1.1-fold) compared to different types of myeloma cell lines (below 0.2-fold). Figure 1C shows that miRNA-1179 mimics expressed the highest relative expression (11.5-fold), while miRNA-NC was expressed the least (1-fold). Figure 1D depicts the highest relative expression (1-fold) by inhibitor-NC, and the miRNA-1179 inhibitor exhibits a low expression (0.1-fold). Figure 1E exhibits the cell survival ability of the cell lines. The survival ability of miRNA-1179 mimic cells was highly suppressed

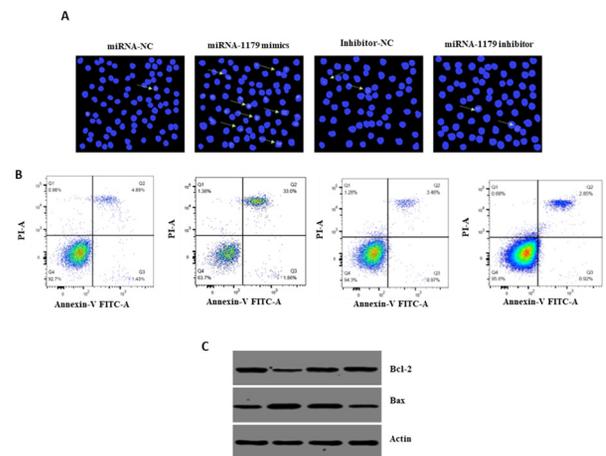


Figure 2. Alteration of the structure of the tested cells

(A) Nucleus structures of the U266 cells; (B) Apoptosis of the U266 cells; (C) Expression of Bcl2, Bax, and Actin

($p < 0.05$) by miRNA-1179 overexpression, whereas the miRNA-1179 inhibitor showed a low level of suppression. Similarly, Fig. 1F expressed the colony formation potential of the miRNA-1179-overexpressing in the miRNA-NC and miRNA-1179 mimics. The miRNA-NC expressed around 100 colonies, while the miRNA-1179 mimics showed around 40 colonies. Figure 1G depicts the colony formation capability of miRNA-1179 overexpression in NC inhibitor and miRNA-1179 inhibitor cells. The inhibitor-NC expresses around 100 colonies, while the miRNA-1179 inhibitor showed around 130 colonies.

DAPI staining revealed that overexpression of miRNA-1179 altered the structure of the nucleus of the miRNA-NC, miRNA-1179 mimics, miRNA-1179 inhibitors, and inhibitor-NC cells (Fig. 2A). The miRNA-1179 inhibitor seems to not affect the nuclear morphology of cells (Fig. 2A), and annexin V/PI staining revealed that the percentage of apoptosis in cells rose from 5.32% to 34.86% when miRNA-1179 was overexpressed. However, inhibition of miRNA-1179 prevented the apoptosis of the tested cells (Fig. 2B). Similarly, the expression of Bax increased while that of Bcl-2 decreased upon miRNA-1179 overexpression. Nonetheless, inhibition of miRNA-1179 in cells exhibited opposite effects (Fig. 2C).

In the study, the target of miR-1179 was analysed and identified by online TargetScan analysis. In this EREG was selected for analysis for its potential oncogenic role in multiple myeloma, and it has not been studied as the target of miRNA-1179. TargetScan analysis showing EREG 3'-UTR sequence as the target of miR1179 (Fig. 3A). The interaction between miRNA-NC and miRNA-1179 mimics was further analyzed by the luciferase assay (Fig. 3B). The luciferase assay of miRNA-NC is high in EREG-WT, while the luciferase assay of miRNA-1179 mimics is very low. In EREG-MUT, there is little difference in luciferase activity between miRNA-NC and miRNA-1179 mimics. Moreover, significant upregulation ($p < 0.05$) of miRNA-1179 was seen in both multiple myeloma tissues compared to normal tissues (Figs 3C and 3D). Multiple myeloma tissues were expressed up to 7-fold, while normal tissues were expressed below that up to 2-fold. However, the expression of EREG was considerably suppressed in miRNA-1179 mimics upon miRNA-1179 overexpres-

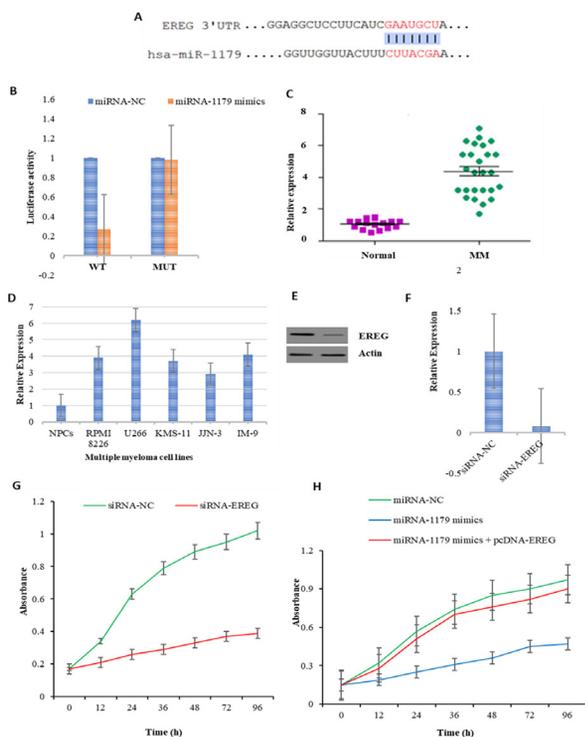


Figure 3. Expression of miRNA-1179

(A) TargetScan analysis showing EREG 3'-UTR sequence as the target of miR1179; (B) Luciferase assay between miRNA-NC and miRNA-1179 mimic; (C) Relative expression in between normal cells and multiple myeloma cells; (D) Relative expression of the different types of myeloma cell lines; (E) Expression of EREG and Actin; (F) Relative expression of the siRNA-NC and siRNA-EREG; (G) Cell Survival ability of the siRNA-NC and siRNA-EREG; (H) Cell Survival ability of the siRNA-NC, miRNA-1179 mimic and miRNA-1179 mimic + pcDNA-EREG

sion (Fig. 3E). Additionally, the knockdown of EREG inhibited the U266 cell survival ability (Fig. 3F). In this assay, siRNA-NC expressed the highest fold compared to siRNA-EREG. The survival ability of the siRNA-NC and siRNA-EREG cells was expressed in Fig. 3G. The survival ability of siRNA-EREG cells was highly suppressed ($p < 0.05$) by miRNA-1179 overexpression, whereas siRNA-NC had a low level of suppression. The survival abilities of miRNA-NC, miRNA-1179 mimics, and miRNA-1179 mimics + pcDNA-EREG were shown in Fig. 3H. This study showed that EREG overexpression eliminated the inhibitory activity of miRNA-1179 on the survival of cells (Fig. 3H).

The impact of miRNA-1179 expression on miRNA-NC, miRNA-1179 mimics, and miRNA-1179 mimics + pcDNA-EREG cell motility and invasion was examined. This study's outcomes revealed that the migration of miRNA-NC, miRNA-1179 mimics, and miRNA-1179 mimics + pcDNA-EREG were inhibited by 69%. However, EREG overexpression restored the motility potential of miRNA-1179 overexpression in the tested cells (Fig. 4A). Additionally, invasion of the tested cells was decreased by 76% compared to the control. Nevertheless, EREG inhibited the inhibitory activity of miRNA-1179 on tested cell invasion (Fig. 4B).

DISCUSSION

Multiple myeloma is one of the most devastating and incurable human malignancies and causes a large number

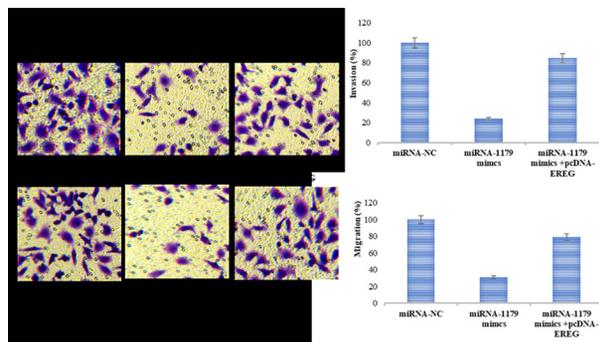


Figure 4. Motility and invasion of U266 cells by miRNA-1179
(A) Activity of miRNA-1179 on U266 cell invasion; (B) Motility potential of miRNA-1179

of human deaths (Wong *et al.*, 2020). The current work intends to explain the significance of miRNA-1179 in multiple myeloma and develop it as a therapeutic target for the disease's management. Prior research has shown that miRNA-1179 acts as a tumor inhibitor in various human malignancies. Li and Qin (Li & Qin, 2019) have shown that HMGB1 is the target of miRNA-1179, which slows down the growth of gastrointestinal cancer. Similarly, miRNA-1179 has been revealed to inhibit the formation of breast cancer cells *via* the notch signalling cascade (Li *et al.*, 2018). Consistently, the present study revealed tumour-suppressive effects of miRNA-1179 in human multiple myeloma cells and suggested that its downregulation in multiple myeloma cells might be facilitating the genesis and progression of multiple myeloma.

Cancer involves a diversity of genetic alterations resulting in the conversion of a normal cell into a malignant cell (Li *et al.*, 2018). Apoptosis eliminates the malignant cells and controls tumor progression. The suppression of apoptosis is essential in the development of human malignancies (Wanner *et al.*, 2021). The current study also found that apoptosis is responsible for the tumor-suppressing effects of miRNA-1179. This means that miRNA-1179 could be used to treat different types of myelomas. Several studies showed that miRNA-1179 can control the development of cancer by targeting a large number of genes. For example, it has been shown to target E2F5 in pancreatic cancer (Lin *et al.*, 2018), PTEN in ovarian cancer (Zhihong *et al.*, 2020), HMGB1 in gastric cancer (Li & Qin, 2019), and ZEB2 in hepatocellular carcinoma (Gao *et al.*, 2019). However, this is the first investigation to identify EREG as a target of miRNA-1179. Previously, EREG has been shown to be elevated in gastric cancer and acts as a predictor of poor prognosis (Xia *et al.*, 2019). Similarly, EREG has already been demonstrated to enhance colorectal cancer proliferation (Vychitilova-Faltejskova *et al.*, 2017). In another study, it was found that EREG makes salivary adenoid cystic carcinoma spread to the lungs (Yang *et al.*, 2017). The expression of EREG was observed to be enhanced in the current investigation. However, miRNA-1179 overexpression inhibits its expression post-transcriptionally to exert its tumor-suppressive effects in multiple myeloma, indicative of its therapeutic implications.

CONCLUSION

The current study found that miRNA-1179 was suppressed in multiple myeloma. Its overexpression suppresses the development and proliferation of human myeloma cells by inhibiting the expression of EREG.

These findings imply that miRNA-1179 might be used as a potential therapeutic agent for the therapy of multiple myeloma. Therefore, more in vivo investigation is necessary for this research area.

Declarations

Acknowledgement. The authors acknowledge the facilities provided by the superiors.

Authors Contribution. Xiao Liu and Wei Li conducted research activities and were involved in article writing. Lan Qin supervised the research activities and helped with article writing. Fei Fei helped proofread the manuscript. All authors read and approved the final version of the manuscript.

Conflicts of interest. There are no conflicts of interest.

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