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, JNJ-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-inhabiting 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

Received: 01 February, 2023; revised: 13 March, 2023; accepted: 03 April, 2023; available on-line: 17 June, 2023

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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 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 retreated 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 (5×10^4) were cultured for 0, 12, 24, 48, and 96 h. Thereafter, 20 µL 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 centrifugation, 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 (1×10^4) 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 µM 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 µL of DMEM containing 10% FBS. Transfected U266 cells were maintained in 100 µL 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 ± standard deviation (S.D.). The GraphPad Prism 7.0 software tool was used to analyze 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.
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 ($p<0.05$) 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 myeloma-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-
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. Consequently, the downregulation of EREG leads to enhanced cell invasion and migration, indicative of its therapeutic implications.
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.

REFERENCES


