

Original Article

Exploring the role of microRNA-9-5p and microRNA-125b-5p for their therapeutic potential in multiple myeloma

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Article Info

Abstract



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In recent years, microRNA (miRNA) aberrations have gained particular attention in cancer pathogenesis. In our previous study using global miRNA expression microarray, we identified overexpression of miR-9-5p and miR-125b-5p in multiple myeloma (MM) patients. To date, the roles of miR-9-5p and miR-125b-5p are not well understood and require further clarification. This study aimed to investigate the functional role of miR-9-5p and miR-125b-5p in MM by in vitro cell-based assays. Synthetic mimics or inhibitors were transfected into the KMS-28BM MM cell line using nucleofection. The relative miRNA expression level was detected using RT-qPCR. Cell proliferation was measured with MTS assay, while apoptosis was analysed by flow cytometry using the Annexin V-FITC/ PI double staining technique. The study findings revealed that suppression of miR-9-5p with inhibitor decreased cell proliferation significantly, while enforced expression of miR-9-5p by synthetic mimics increased proliferation of these cells compared to the scrambled negative control ($P < 0.05$). Moreover, transfection of the miR-9-5p inhibitor and mimic increased ($P < 0.01$) and decreased ($P < 0.05$) the proportion of early apoptotic cells, respectively. Apart from that, repression of miR-125b-5p significantly increased the number of late apoptotic cells, while overexpression reduced the number of early apoptotic cells compared to the negative control ($P < 0.05$). Inhibition of miR-9-5p and miR-125b-5p exert apoptotic and/or antiproliferative effects in KMS-28BM cells, suggesting their possible role in the treatment of MM.

Keywords: Multiple myeloma, miR-9-5p, miR-125b-5p, Cell proliferation, Apoptosis.

1. Introduction

Multiple myeloma (MM) is a type of blood cancer characterised by clonal expansion of abnormal bone marrow plasma cells [1]. It is the second leading cause of haematological malignancy in adults worldwide [2]. MM causes lytic bone lesions and fractures. Despite recent advances in novel agents such as immunomodulatory imide drugs, proteasome inhibitors, monoclonal antibodies, bispecific T-cell engager, and CAR T-cell therapy, MM remains incurable due to drug resistance and relapse to the current treatment [3].

MM is thought to be caused by genetic lesions among coding genes and epigenetic modifications. Epigenetic regulators generally include non-coding RNA, DNA methylation, chromatin remodelling, and histone modification [4]. Non-coding microRNAs (miRNAs) are small single-stranded RNAs (18–24 nucleotides) that do not encode for known proteins. MiRNAs regulate gene expression at the post-transcriptional level via transcript degradation and/or translational repression [5]. Dysregulation of miRNAs is a frequent event in human malignancies, including MM [6]. They inhibit tumour suppressor genes or activate oncogenes, thereby inducing angiogenesis and tumour cell

proliferative signalling, invasion, and metastasis [7]. miRNAs may function as either oncomirs or tumour suppressor miRNAs, and the type of cellular context plays a critical role in determining their function in cancer [8].

Recent research findings have shown that miR-9-5p and miR-125b-5p play a dual suppressive or promoting role in different cancer cells or cancer subtypes. Overexpression of miR-9-5p was reported in various cancers, including MM [9–14]. Increased expression of miR-9-5p induced progression, metastasis, and invasion of MM cells via activation of the NF κ B signaling pathway [13]. Conversely, the under-expression of miR-9-5p in chronic lymphocytic leukaemia, ovarian, oral, and gastric cancers, suggests its tumour-suppressive function in these cancers [15–18]. Down-regulation of miR-9-5p disrupted cell cycle arrest, apoptosis, and proliferation mechanisms in cancers [17–18].

The miR-125b-5p regulates cell survival, invasion, migration, apoptosis, drug resistance, and the immune system of cancer cells by modulating NF- κ B, p53, PI3K/Akt/mTOR, ErbB2, Wnt/ β -catenin signalling pathways [19–23]. The role of miR-125b-5p in MM pathogenesis remains controversial. It can function as a promoter or

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suppressor in MM depending on the molecular characteristic or classification. Overexpression of miR-125b-5p is thought to induce the progression of MM by inhibiting the activity of various tumour suppressor genes involved in SIRT1/AKT signaling pathways [24-26]. High expression of miR-125b-5p was found to be associated with shorter event-free survival in MM patients [25]. Remarkably, it was shown that increased expression of circulatory miR-125b-5p in extracellular vesicles and plasma was a powerful indicator for relapse in MM after autologous hematopoietic stem cell transplantation [27]. On the contrary, miR-125b-5p demonstrated the opposite role in TC2 or TC3 MM [28]. It has been shown that down-regulation of miR-125b-5p induces cell proliferation and inhibits autophagy and apoptosis via mediating *IRF4* in MM [28].

In our previous studies using global miRNA expression microarrays, we had identified overexpression of miR-9-5p (FC=8.07; $P < 0.05$) and miR-125b-5p (FC=21.9; $P < 0.05$) in 19 MM patients and 8 MM cell lines compared to 3 normal controls [29]. Although miR-9-5p and miR-125b-5p have shown differential expression in several studies, their biological function and molecular mechanisms in the development and progression of MM are not well understood. As such, the present study aims to explore the functional role of miR-9-5p and miR-125b-5p in MM by *in vitro* cell-based assays.

2. Materials and methods

2.1. Microarray expression datasets

Two miRNA expression profiles (GSE17498 and GSE17306) were obtained from the NCBI GEO public database. GSE17498 (platform GPL8227) contains miRNA profiles of CD138+ plasma cells from 38 MM patients and normal plasma cells (NPCs) from 3 donors. GSE17306 (platform GPL9081) contains miRNA profiles of purified plasma cells obtained from 52 MM and 2 normal donors. Differential expressions of miR-9-5p and miR-125b-5p were analysed using a web-based tool as described previously [30].

2.2. Cell culture

The KMS-28BM and U266 human MM cell lines were purchased from the Japanese Collection of Research Bioresources (JCRB) cell bank. The KMS-28BM is derived from a 77-year-old Japanese female patient. The RPMI 1640 medium (PAN-Biotech, Germany) containing penicillin/streptomycin/amphotericin B (Antibiotic-Antimycotic 100X, Gibco, USA) and 10% of fetal bovine serum (Sigma-Aldrich, Germany) was used for cell culture. The cells were maintained and sub-cultured every 2–3 days at 37°C in a 5% CO₂ incubator. Cells at the logarithmic phase of growth were utilised for all experiments.

2.3. Cell transfection

The synthetic miR-9-5p and miR-125b-5p inhibitor/mimic and scrambled negative control (miR-inhibitor NC/miR-mimic NC) were obtained from GeneCopoeia (USA). The sequences for the inhibitor/mimic are shown in Table 1. Either miR-inhibitor scrambled negative control (miR-inhibitor NC, Catalog no.: CmiR-SN0001-SN, GeneCopoeia, USA) or miR-mimic scrambled negative control (miR-mimic NC, Catalog no.: CmiR-AN0001-SN, GeneCopoeia, USA) was used as a control for all experiments, as appropriate. Generally, 100 µl of 4D-Nucleofector solution (Lonza, USA) was used to resuspend 2×10^6 cells in each transfection. Cells were mixed with 300-500 nM of inhibitor/mimic/NC. Transfection of cells with inhibitor/mimic/NC was performed using program DY-100 in a 4D-Nucleofector system (Lonza, USA). Then, cells in the cuvette were gently transferred to a warm medium in a microplate. The efficiency of transfection was analysed by RT-qPCR as described below.

2.4. RT-qPCR analysis

Total RNAs from the cells were extracted using combined Trizol and spin-column-based RNA isolation methods (Qiagen miRNeasy mini kit, Hilden, Germany). DNA digestion was performed to remove DNA contamination from the sample (Qiagen DNase I, Hilden, Germany). The TaqMan MicroRNA Reverse Transcription Kit was utilised to generate first-strand cDNAs (Applied Biosystems, USA). Amplification of miRNA fragments was performed using the TaqMan MicroRNA assay according to the manufacturer's recommendations (Assay ID: 000449, has-miR-125b, Applied Biosystems, USA; Assay ID: 000583, has-miR-9, Applied Biosystems, USA). RNU6B was used as an internal control miRNA (Assay ID: 001093, RNU6B, Applied Biosystems, USA). Conditions for the RT reaction were as follows: 16°C for 30 mins, 42°C for 30 mins, and 85°C for 5 mins. The TaqMan Fast Advanced Master Mix and StepOnePlus Real-time PCR System (Applied Biosystems, USA) were used for RT-qPCR according to the following conditions: denaturation at 95°C for 20 s, 40 cycles at 95°C for 1 s and at 60°C for 20 s. The differences in miRNA expression between the test and control groups were determined using the formula $2^{-\Delta\Delta Ct}$.

2.5. Cellular viability assay

The miRNA inhibitor/mimic/NC was nucleofected into KMS-28BM cells. Approximately 2.0×10^4 cells/well were seeded in a 96-well microtiter plate containing 100 µl of pre-warmed culture medium. Then, 10 µl of MTS reagent (Promega CellTiter 96 AQueous One Solution Reagent, USA) was added and mixed into each sample well at 0 h and after 24 h, 48 h and 72 h post-transfection. The microplate was then placed in a 5% CO₂ incubator at

Table 1. The sequences of synthetic inhibitors and mimics.

miRNA	Sequence	Catalog No.
miR-9-5p inhibitor	UCAUACAGCUAGAUAAACCAAAGA	HmiR-AN0825-SN-5
miR-9-5p mimic	5'-UCUUUGGUUAUCUAGCUGUAUGA-3' 3'-UUAGAAACCAAUAGAUCGACAUA-5'	HmiR-SN0825-SN-3
miR-125b-5p inhibitor	UCACAAGUUAGGGUCUCAGGGA	HmiR-AN0096-SN-5
miR-125b-5p mimic	5'-UCCCUGAGACCCUAACUUGUGA-3' 3'-UUAGGGACUCUGGGAUUGAACA-5'	HmiR-SN0096-SN-3

37°C for 3-4 h. Following incubation, the microplate was measured at absorbances of 490 nm and 630 nm using a plate reader (Synergy HTX, BioTek, USA). The proliferation of cells was determined by subtracting the 630 nm reading from the 490 nm reading.

2.6. Cell apoptosis assay

Synthetic miRNA inhibitor/mimic/NC was transfected into KMS-28BM. Approximately 2.0×10^6 cells were used for each transfection. Cells were harvested after 48 h of incubation. Cold PBS was then added to the cell pellet and washed by centrifugation. The cells were resuspended in Annexin binding buffer at 5.0×10^5 cells/mL. This was followed by staining the cells with Annexin V-FITC and propidium iodide (PI) before apoptosis analysis (Elabscience, USA). The proportion of apoptotic cells was then analysed using a FACS CANTO II flow cytometer (BD BioScience, USA). A total of 10,000 events were recorded for each analysis.

2.7. Statistical analysis

At least two independent experiments were performed for each assay in 3 or 4 technical replicates. Student's *t*-test was used to compare the significance of differences between the means of the two groups. A *p*-value below 0.05 ($P < 0.05$) was considered statistically significant.

3. Results

3.1. Differential expression of miR-9-5p and miR-125b-5p compared with NPCs

Differential expression analysis found that the level of miR-9-5p expression was significantly higher in MM compared to NPCs in the GSE17498 ($P < 0.05$; Fig. 1A) and GSE17306 ($P < 0.01$; Fig. 1B) datasets. The miR-125b-5p was upregulated in MM compared to NPCs in

the GSE17306 dataset, with a low fold change ($P < 0.001$; Fig. 1C).

3.2. Transfection of synthetic miR-9-5p/ miR-125b-5p mimic/ inhibitor into KMS-28BM human MM cells

We first evaluated the knockdown efficiency of miR-9-5p and miR-125b-5p inhibitors in KMS-28BM and U266 MM cells. RT-qPCR findings revealed that the miR-9-5p inhibitor significantly decreased the relative expression of the respective miRNA in KMS-28BM and U266 cells by 31% and 11%, respectively, compared to the inhibitor NC ($P < 0.05$; Fig. 2A). The level of miR-125b-5p was successfully suppressed using synthetic inhibitors up to 92% and 51% in KMS-28BM and U266 cells, respectively, compared to the inhibitor NC ($P < 0.05$; Fig. 2B). As a continuation of our previous microarray study using Asian patients, KMS-28BM cells were selected as host cells in subsequent assays despite the higher knockdown efficiency obtained using this cell line.

When miR-9-5p or miR-125b-5p mimics were transfected into KMS-28BM cells, the synthetic mimics were able to induce the expression level of the respective miRNA by 15 and 27 folds, respectively, compared to the mimic NC ($P < 0.05$; Figs. 2C & D). Our findings showed that synthetic mimics/ inhibitors were successfully delivered into KMS-28BM and modulated the respective miRNA levels in the cells.

3.3. MiR-9-5p repression inhibited cell growth and induced apoptosis in KMS-28BM human MM cells

Interestingly, the *in vitro* functional study demonstrated

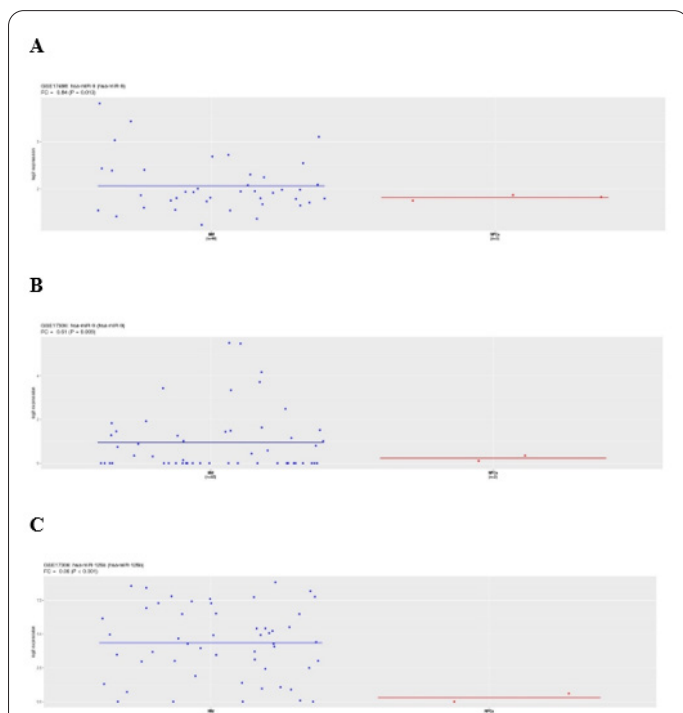


Fig. 1. Differential miRNA expression analysis of GEO datasets, GSE17498 and GSE17306. The miR-9-5p was significantly overexpressed in the **A**: GSE17498 dataset ($P=0.013$). **B**: GSE17306 dataset ($P=0.009$). **C**: The miR-125b-5p was significantly overexpressed in the GSE17306 dataset ($P < 0.001$).

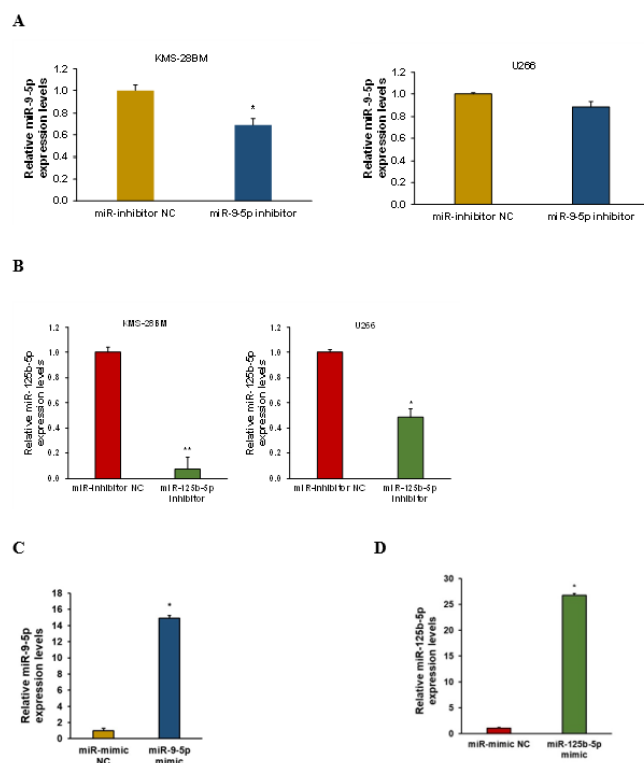


Fig. 2. Synthetic inhibitor/mimic modulated the expression of miR-9-5p/miR-125b-5p in MM cells. The relative miRNA expression level in KMS-28BM and U266 cells transfected with **A**: miR-9-5p inhibitor or inhibitor NC. **B**: miR-125b-5p inhibitor or inhibitor NC. The relative miRNA expression level in KMS-28BM cells transfected with **C**: miRNA-9-5p mimic or mimic NC. **D**: miR-125b-5p mimic or mimic NC. * $P < 0.05$, ** $P < 0.01$

that transfection of the miR-9-5p inhibitor reduced the proliferation of KMS-28BM cells while enforcing expression using the synthetic mimic of the miR-9-5p induced proliferation of MM cells compared to the control ($P < 0.05$; Figs. 3A & B). Apart from that, the percentage of early apoptosis cells was increased in cells transfected with the miR-9-5p inhibitor at 48 h post-transfection as analysed by flow cytometry ($P < 0.01$; Fig. 3C). Conversely, there was a significant decrease in the number of early apoptosis cells when the cells were transfected with miRNA mimic ($P < 0.05$; Fig. 3D).

3.4. MiR-125b-5p repression promoted apoptosis in KMS-28BM human MM cells

The MTS results showed that there were no significant changes in the cell proliferation rate in cells transfected with the miR-125b-5p synthetic inhibitor nor mimic compared to the negative control (Figs. 4A & B). Remarkably, the proportion of late apoptotic cells was increased when the cells were repressed with the miR-125b-5p inhibitor compared to the control ($P < 0.05$; Fig. 4C). On the contrary, there was a significant decrease in the percentage of early apoptotic cells when enforced with miR-125b-5p mimics ($P < 0.05$; Fig. 4D).

4. Discussion

MiRNAs exhibit their potential for developing novel prognosis markers and therapeutic targets due to their crucial role in disease prognosis and progression [31]. For example, miR-34a, miR-221/222, and miR-29b have shown their potential as therapeutic targets in MM [32-34]. Thus, evaluation of the impact of miRNA expression modulation becomes a promising tool used to identify more efficacious prognostic and therapeutic targets in MM.

The role of miRNAs in cancer pathogenesis, including multiple myeloma (MM), has been extensively studied, highlighting their potential as therapeutic targets and biomarkers. For instance, miRNAs like miR-9-5p and miR-125b-5p have been implicated in modulating cell proliferation and apoptosis in MM, suggesting their therapeutic potential. Similarly, studies on other cancers, such as esophageal squamous-cell carcinomas (ESCC), have shown how long non-coding RNAs (lncRNAs) like U731166 can influence tumour metastasis by sponging specific miRNAs, such as miR-3607-3p, as demonstrated by Wang et al. (2024) [35]. Additionally, the complex interaction networks involving miRNAs, such as the PBX1/miR-141-miR-200a/EGR2/SOCS3 axis, have been explored in the context of Th17 cell differentiation, illustrating the intricate regulatory mechanisms mediated by miRNAs [36]. Furthermore, the therapeutic and biomarker properties of miRNAs have been reviewed, emphasizing their potential in cancer treatment [37]. A comprehensive analysis of miRNAs in cancer cells highlights their diverse roles and potential applications in oncology [38].

Consistent with our previous microarray findings using MM patient samples and cell lines, differential expression analysis of GEO datasets, GSE17498 and GSE17306, demonstrated that miR-9-5p is significantly overexpressed in MM in these datasets, while miR-125b-5p is overexpressed in the GSE17306 dataset compared to NPCs [29]. Furthermore, *in vitro* studies revealed that miR-9-5p and miR-125b-5p inhibit apoptosis and/or

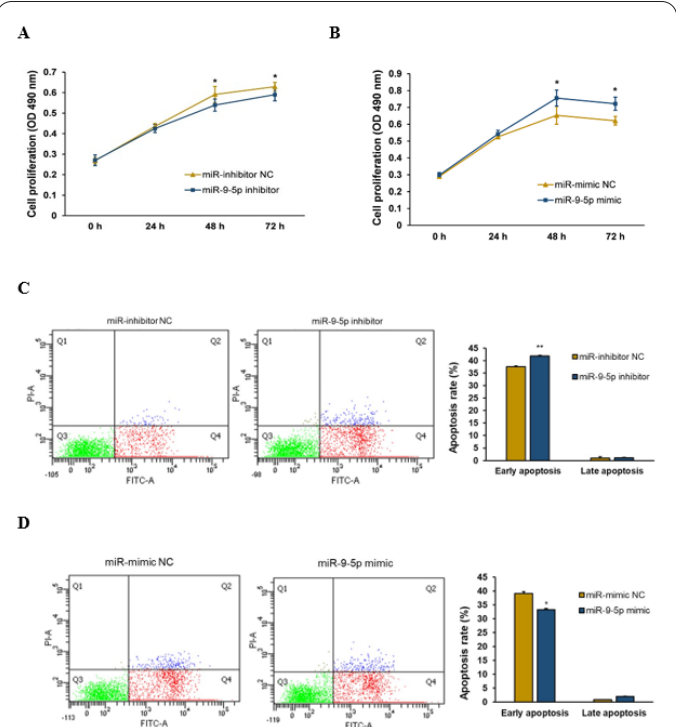


Fig. 3. miR-9-5p repression inhibited proliferation and induced apoptosis in KMS-28BM MM cells. Cell proliferation of KMS-28BM cells transfected with **A:** Inhibitor or inhibitor NC. **B:** Mimic or mimic NC. Flow cytometry scatter plots of the number of apoptotic cells in KMS-28BM cells transfected with **C:** Inhibitor or inhibitor NC. **D:** Mimic or mimic NC. NC = negative control * $P < 0.05$, ** $P < 0.01$

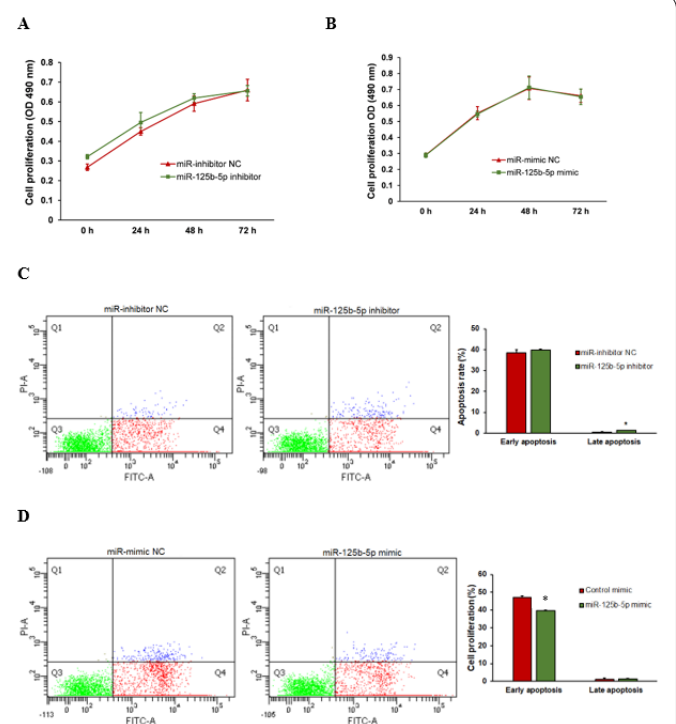


Fig. 4. miR-125b repression induced apoptosis in KMS-28BM MM cells. Cell proliferation of KMS-28BM cells transfected with **A:** Inhibitor or inhibitor NC. **B:** Mimic or mimic NC. Flow cytometry scatter plots of the number of apoptotic cells in KMS-28BM cells transfected with **C:** Inhibitor or inhibitor NC. **D:** Mimic or mimic NC. NC, negative control * $P < 0.05$

promote cell growth in KMS-28BM. In contrast, inhibition of miR-9-5p and miR-125b-5p reverses the protective effects against those cells. This scenario implies that miR-

9-5p and miR-125b-5p play a crucial role, at least in part, in MM progression by regulating the proliferation and apoptotic mechanism of MM cells.

As far as our concern, functional studies of miR-9-5p and miR-125b-5p in MM are very limited, and none were conducted using the MM cell line of Asian origin. For the first time, we revealed that inhibition of miR-9-5p and miR-125b-5p exerts apoptotic and/or anti-proliferative effects in KMS-28BM cells. Thus, this study supports the function of miR-9-5p and miR-125b-5p as oncomirs, in the tumorigenesis of MM.

Collectively, our results indicated that miR-9-5p and miR-125b-5p promote tumorigenesis in the KMS-28BM MM cell line and may be potential therapeutic targets for MM. This study delivers greater insight into the functional role of miR-9-5p and miR-125b-5p in MM.

Conflict of interest

The authors declare that they have no conflict of interest.

Consent for publication

All authors have stated their consent for publication.

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Availability of data and material

The authors declare that all data have been embedded in the manuscript.

Author contributions

All authors contributed to the study conception and design. Experiment, data collection, and analysis were performed by Bong IPN, Mohd Dali NS and Othman N. The first draft of the manuscript was written by Bong IPN. The manuscript was reviewed and edited by Bong IPN and Mohd YA. All authors read and approved the final manuscript.

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