miR-145-5p suppresses cell proliferation by targeting IGFR1 and NRAS genes in multiple myeloma cells

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Abstract

Objectives: Multiple myeloma (MM) is a common hematological cancer. Hence, it is important to conduct further studies investigating the molecular mechanisms in detail that contributes to myeloma genesis. In addition to genetic changes, epigenetic factors such as miRNAs may influence the expression of myeloma-related genes.

Methods: Our study aimed to detect genes closely related to MM and miRNAs involved in the cancer process by changing the expression of these genes with bioinformatics tools and in vitro methods. Bioinformatics approaches identified hub miRNAs in our study that may have a role in the expression change of genes connected to myeloma. The functional impacts of the chosen miRNA on RPMI8226 and U266 cell lines and the effect of this miRNA on the expression changes of putative target genes were investigated.

Results: The viability of miR-145-5p transfected cells was found to decrease compared to control cells and the expression of IGFR1 and NRAS genes were found to be significantly suppressed in both cell lines at mRNA level. Decreased levels of the IGFR1 and NRAS genes were confirmed in miR-145-5p transfected cells at the protein level as well as compared to control cells. In addition, IGFR1/miR-145-5p interaction was demonstrated via luciferase reporter assay. However, expression levels of EGFR, KLF4, IRS1, CDK4 and CDK6 candidate genes had no statistically significant difference in miR-145-5p transfected cells compared to control cells.

Conclusions: Mir-145-5p was demonstrated to act as a tumor suppressor miRNA and inhibit the proliferation in MM cell lines via targeting IGFR1 and NRAS.

Keywords: IGFR1; miR-145-5p; multiple myeloma; NRAS; RPMI-8226; U266

Introduction

Multiple myeloma (MM), is the 2nd most prevalent hematological malignancy among plasma cell cancers, characterized by the formation and accumulation of differentiated plasma cells in the bone marrow [1, 2]. Serious progress has been made in treating MM in parallel with the recent increase in modern research methods. Although these developments prolong MM patients’ life expectancy, relapse is still a severe problem in most patients [3].
of their target mRNAs. miRNAs can act as tumor suppressors (Ts-miR) or oncogenic miRNAs (oncomiR) in regulating the expression of their targets by binding to the 3' UTR regions [4]. miR-145-5p is a crucial Ts-miR, and it has been shown that miR-145-5p expression is frequently decreased in many cancers, including MM and other cancers such as prostate, breast, glioma, non-small cell lung, bladder, hematological, colorectal, gastric, ovarian and cervical [5, 6]. miR-145-5p is involved in various critical cellular events such as cell proliferation, migration, and apoptosis by targeting many important genes, thus, miR-145-5p is suggested as a potential biomarker in cancer diagnosis and treatment [5, 7]. The importance of miR-145-5p is unclear in myeloma. Bioinformatics tools were used in the current study to define genes involved in the pathogenesis of MM as well as miRNAs that can regulate these genes. In vitro approaches validated the influence of miR-145-5p found in this manner on the MM cancer process, and miR-145-5p-associated genes were identified.

Materials and methods

Bioinformatic analysis to define genes and miRNAs

From the Disgenet database (www.disgenet.org/) (DisGeNET is a database containing one of the largest publicly available collections of numerous genes and variants associated with human diseases), 15 genes had found differential expression levels in MM and associated with MM were selected according to N.PMIDs (total number of PMIDs supporting the association) scores ≥5. The selected genes were confirmed via biochiporal database (a useful and open-access database for exploration of many cancer genomics datasets) (www.biochiportal.org). To define miRNAs related to the 15 MM-associated genes, Enrichr tool (maanyanlab-cloud/Enrichr/) was used. Further confirmation of our findings, GSE38824 (for genes), GSE78865 and GSE16558 (for miRNAs) geodatasets were analysed using GEO2R program (www.ncbi.nlm.nih.gov/geo/geo2r/). The associations between selected MM genes and miRNAs were searched through multiple databases, miRDB database (http://mirdb.org/), Targetscan database (www.targetscan.org), miRabel database (bioinfo.univ-rouen.fr/mirabel/) and miRWalk database (http://mirwalk.umm.uni-heidelberg.de/) were used. Genes recognized as miR-145-5p targets in at least two databases were selected.

Cell culture of RPMI-8226 cell line and U266 cell line

AML cells (RPMI-8226 and U266) were incubated in RPMI-1640 medium (EcoTech Biotechnology, Turkey) containing 20 % FBS and 1 % antibiotic (Invitrogen). Cells were cultured at 5 % CO₂ at 37 °C conditions.

miR-145-5p transfection

30 pmol of mimic miR-145-5p miRNA and mimic nontargeting miRNA (nt control) (Thermo Fisher) were transfected to the AML cells via Lipofectamine 2000 Transfection Reagent (Thermo Fisher) according to the protocol of the manufacturer. After culturing for 24 or 48 h, these cells were harvested.

RNA isolation and cDNA synthesis

TRIzol Reagent (MRC Inc.) was used to isolate total RNA from mimicked transfected AML cells. RNA purities and concentrations were evaluated using NanoDrop-2000 (Thermo-Fisher Sci.). cDNA synthesis was conducted with Taqman MicroRNA Reverse Transcriptase Kit (Applied Bio.) (using a total 30 ng RNA) and miRNA RT primers (Thermo Fisher) in accordance with the protocol of the manufacturer for validation of miRNA mimic transcription. cDNA synthesis using 1000 ng RNA was performed with SCRIPT cDNA Synthesis Kit (Jena Bioscience) to determine the expression of putative target genes.

Quantitative real-time (qRT) PCR

qRT-PCR reactions were conducted on a LightCycler480 (Roche) device. Transfection validation was performed with the TaqMan Universal Master Mix kit and TaqMan miRNA probes (Thermo Fisher). RNU43 (control miRNA) (Thermo Fisher) was used for the normalization of miRNA expression. The relative quantitation analysis of IGF1R, NRAS, EGFR, KLFR, IRS1, CDK4, CDK6, and EMT markers (E-cadherin, N-cadherin, and Vimentin) were performed with qPCR SybrMaster (Jena Bioscience). The β-actin housekeeping gene was used to normalize gene expressions at mRNA level. IRS1-F:5'-ACAACACCTTCCTGAGACTCTG-3', IRS1-R:5'-AGTCGCCACAGTGAGACCGT-3' [8], CDK4-F:5'-TGGACCCACGTCAAGGGT-3', CDK4-R:5'-TGCCGAGGCACTGCTGAGC-3' [9], CDKR-F:5'-TGGAGCAGCTTTTGGAAGGC-3', CDKR-R:5'-GACCTCCAGTCAGTGCTTG-3' [10], IGF1R-F:5'-TTTCCCCAGGGCTGGACACCC-3', IGF1R-R:5'-AGCATCTGACGCCTTCC-3' [11], EGFR-F:5'-ATGTCATGCTGGATGAGG-3', EGFR-R:5'-GGAGGAAGGTGGCTGCTATG-3' [12], NRAS-F:5'-GGCCACGGATCCACCCGAC-3', NRAS-R:5'-GGCCACGGATCCACCCGAC-3' [13], KLF4-F:5'-AGCATCTGCCCTCTG-3', KLF4-R:5'-GGCTTCCCTGCTTGGGAGTG-3' [14], β-actin-F:5'-GCCTGCTGCTGGTCCTGGATG-3', β-actin-R:5'-GCCTGCTGCTGGTCCTGGATG-3'. E-Cad-F:5'-TGCCGAGAATAAGGAACGGG-3', E-Cad-R:5'-CTGTAGTGGCAAATGTCCTATG-3' [15], N-Cad-F:5'-CACCCAGCTGACCC-3', N-Cad-R:5'-CCCGAGATGGGGTTGATAAGG-3' [15], Vimentin-F:5'-GAGAACTTGGCGCCTGGAAGGC-3', Vimentin-R:5'-GACTTCCGTGAGTGCCATG-3' [15] qRT-PCR primers were used. All qRT-PCR reactions were applied in of two parallel experiments, and relative quantitation analysis was performed via 2-ddCt method.

Cell viability assay

Proliferative potentials of RPMI-8226 and U266 cells transfected with miR-145-5p mimic and nt-control were evaluated with Cell Proliferation Detection Kit-8 (CVDK-8; EcoTech Biotechnology, Turkey). Briefly, cells were seeded at 5 × 10⁵ cells per well on 96-well plates (SPL Life Sci.) Cell viability assay was performed in two parallel experiments. 24 h later, transfection was performed, and cell’s viability was measured at 24 h, 48 h, and 72 h. CVDK-8 reagent diluted in 1/10 in basal cell culture medium was used for color development, and measurements were recorded at 450 nm with Varioskan Flash Microplate reader (Thermo Fisher).
Western blot analysis

RPMI-8226 and U266 cells were seeded in 6-well plates at $4 \times 10^5$ cell density and were transfected with miRNA mimics after 24 h. 48 h later, harvested cells were subjected to western blotting to determine the effect of miR-145-5p on the protein level of the IGF1R and NRAS genes. Western blot analysis was performed as described in our previous study [16]. Western blot images were quantified using Image J.

Luciferase reporter assay

To determine whether IGF1R is a target of miR-145-5p, the luciferase reporter assay was conducted using MM cells. Forward primer including a NheI restriction site, 5'-CTAGCTAGCTAGCGTCATCAAGGCGTACC-3' and reverse primer including XhoI restriction site, 5'-CCGCTCGAGGCGGTGCAAACCTGATCAACAGT-3' was used to clone 498 bp long IGF1R 3'UTR including the estimated miR-145-5p binding site. Bold sequences include restriction digestion sites along with flanking sequences. Amplified 3'UTR region was inserted into the LightSwitch™ 3'UTR Reporter GoClone plasmid (SwitchGear Genomics). RPMI8226 cells were seeded on a 6-well plate at $8 \times 10^4$ cells per well in triplicates. Co-transfection of IGF1R 3'UTR luciferase reporter plasmid and miR-145-5p mimic or non-targeting miRNA mimic (nt control) was performed using Lipofectamine 2000 reagent (Thermo Fisher Scientific). 48 h after transfection, cells were lysed, and luciferase activity was measured using LightSwitch assay reagent (SwitchGear Genomics) according to the manufacturer’s instructions. Relative luciferase activity was normalized to protein concentrations. All experiments were performed in triplicates.

Statistical analysis

Statistical analysis was performed using Student’s t-test, and data are presented as mean ± standard error. Data with a p-value less than 0.05 were considered as statistically significant. GraphPad Prism 9 and SPSS 28 programs were used to visualize data.

Results

15 MM-associated genes were selected according to N.PMIDs (Total number of PMIDs supporting the association) scores ≥ 5 from Disgenet database (Figure 1). These genes are also related to MM in the cbioportal database. The hub miRNAs that can control the expression of the selected MM associated 15 genes are shown in Figure 2. All but one of these miRNAs were human miRNAs. Figure 3 shows the top five human miRNAs (miR-145-5p, miR-206, miR-29a-3p, let-7a-5p, miR-98-5p) and their associations with a wide number of genes, including 15 chosen genes (mirnet.ca). Among these miRNAs, miR-145-5p appears to be the most significant miRNA targeting MM-related genes (Supplementary Figure 1) (mirnet.ca). According to the results of GSE36824 dataset analysis, it was determined that the expressions of IGF1R, KLF4, CCND2 and CDC6 genes were higher in the cytogenetically high-risk group compared to the low-risk group in MM patients (logFC: 1.87; 2.23; 2.5; 1.98 respectively). Based on the findings of the GSE78865 dataset analysis, the expression of the hub miRNAs miR-145-5p and miR-98-5p in exosome samples obtained from MM patients was considerably lower than in the control group (logFC: 2.84 and 1.2 respectively). Of the hub miRNAs, only miR-145-5p (logFC: 1.3) was shown to be considerably lower in patients with various MM-related cytogenetic abnormalities in GSE16558 compared to individuals with normal FISH results. As a result of qRT-PCR performed to determine whether the transfection process was successful or not, it was determined that the amount of miR-145-5p increased sufficiently in both cell lines (RPMI-8226 and U266) in which the transfection was performed compared to the control group and the transfection was successful (Supplementary Figure 2A). After determining that the miRNA mimic level used was sufficient, the effect of miR-145-5p on the viability of RPMI-8226 and U266 cells was examined. We found that cell viability in both cell lines decreased at a statistically significant level at 48 h and 72 h following miR-145-5p transfection (Supplementary Figure 2B). As a result of our literature review and in silico analysis, IGF1R, NRAS, EGFR, KLF4, IRS1, CDK4, and CDK6 genes, which are predicted to be potential targets of miR-145-5p, were chosen for further analysis using qRT-PCR (Supplementary Figure 3) (miRTargetLink2). We found that the expression of IGF1R and NRAS were significantly suppressed in miR-145-5p transfected RPMI-8226 and U266 cells compared to the corresponding control groups. No statistically significant change was detected in the expression levels of IRS1, CDK4, CDK6, EGFR, and KLF4 genes in RPMI-8226 and U266 cells respectively (Supplementary Figure 4A). Epithelial-mesenchymal transition (EMT) has an important role in the invasion and metastasis of cancer cells [17]. Therefore, in RPMI-8226 and U266 cells, to understand whether miR-145-5p has a role in the EMT process, E-Cad, N-Cad, Vimentin expressions, which are important EMT-specific marker genes, were determined by qRT-PCR. E-Cad expression increased significantly in miR-145-5p transfected cells compared to control cells and N-Cad expression decreased significantly but Vimentin expression did not change significantly in RPMI-8226 and U266 cells (Supplementary Figure 4B). Western blotting was performed to investigate the expression of IGF1R and NRAS at the protein levels, whose expression was significantly reduced at the mRNA levels in miR-145-5p transfected cells in both cell lines compared to the control groups. As a result, a significant decrease in the level of IGF1R and NRAS protein was detected in the cells transfected with miR-145-5p in both cell lines compared to the control cells (Supplementary Figure 4C). Luciferase reporter assay was performed for miRNA-target validation via transfecting RPMI-8226 cells.
with miR-145-5p mimic and luciferase reporter clone containing IGF1R 3′UTR. As a result of the IGF1R gene-specific luciferase activity assay, we found that miR-145-5p targets the IGF1R 3′UTR region to regulate its expression (Supplementary Figure 4D).

### Discussions

Alterations in the expression of various genes linked to a number of diseases, including MM, could be caused by a wide range of genetic and/or epigenetic factors. MiRNAs, for
example, can play a crucial role in the development and progression of many diseases, particularly cancers, by altering the expression of many target genes. Therefore, determining the relationship between miRNAs and their target genes is very important in elucidating the nature of cancer. However, a miRNA can target numerous genes and can regulate their expressions. On the other hand a gene’s expression can be altered by more than one miRNA [18]. Furthermore, the link between miRNAs and target genes may differ based on the kind of cancer and even the subtype of the same malignancy. The current study first investigated the relationship between MM-related genes and miRNAs using bioinformatics tools. Transfecting miR-145-5p, shown to be more strongly related with selected genes, into MM cell lines was used to investigate the effect of miR-145-5p on cancer cell proliferation. The expression levels of selected genes IGF1R, NRAS, EGFR, KLF4, IRS1, CDK4, and CDK6 were then examined in mimic miR-145-5p transfected MM cell lines. The EGFR, KLF4, IRS1, CDK4, and CDK6 genes have been connected to a variety of malignancies, including MM. Various studies have shown that miR-145-5p directly affects the contribution of the cancer process in various cancers by disrupting the expressions of these genes. For instance, miR-145-5p has been demonstrated to have a direct influence on cell proliferation and migration by targeting IRS1 in AML cells. According to the study of Wang Y et al., miR-145-5p inhibits hepatocellular carcinoma cells via targeting IRS1/AKT axis [19]. Similarly, miR-145-5p has been demonstrated to inhibit cell proliferation on lung adenocarcinoma by binding to the 3′UTR region of EGFR [20], and it has also been associated with resistance to cisplatin in non-small-cell lung carcinoma through regulating the expressions of CDK4 and CDK6 [21]. Even though EGFR, KLF4, IRS1, CDK4 and CDK6 are strong candidate genes, in our current study we found that miR-145-5p overexpression had no statistically significant influence on the expression levels of these candidate genes in myeloma cells. This suggests that rather than miR-145-5p dysregulation in MM, distinct genetic and epigenetic mechanisms regulate the expression of associated genes. As a result, *in silico* identification of miRNA-target gene axis and *in vitro*-*in vivo* investigations of these connections could be critical for understanding cancer processes.

The expressions of IGF1R and NRAS genes at both the mRNA and protein levels were shown to be reduced in mimic miR-145-5p transfected MM cells compared to the control group. *IGF1R* overexpression has been associated with poor
prognosis in MM [22], its inhibition has been demonstrated to overcome Bortezomib resistance in MM cells [23]. The preclinical data supporting the significance of the insulin-like growth factor (IGF) system in cancer is compelling. Despite outcomes from preclinical research and early clinical trials were quite hopeful, most major randomized phase II and III trials in most malignancies, including MM, have been mainly unsatisfactory [24]. The relationship between miR-145-5p and IGFI R has been shown in various cancers in the literature. For instance, in our previous study, we demonstrated that CASC11 increases the aggressiveness of cancer cells via the miR-145/IGFI R axis in prostate cancer [16]. In another study it has been shown that miR-145-5p inhibits cell proliferation via IGFI R in colorectal cancer, and also it has been reported that miR-145-5p/IGFI R axis may regulate apoptosis and proliferation of retinoblastoma cells [25, 26].

NRAS, another gene whose expression level was found to be dramatically affected by miR-145-5p in MM cells in our study, is one of the most frequently mutated genes in MM. In the study of Ryland et al., for example, the NRAS mutation was found to be 15.1% in a mutation analysis of 86 individuals [27]. According to our observations, NRAS, which plays a significant role in the pathogenesis of MM, can be disrupted by genetic mutations and dysregulated miR-145-5p expression. NRAS-miR-145-5p relation has been shown in some cancers and other diseases. For example, miR-145-5p has been shown to increase the susceptibility of acquired Gefitinib-resistant cells to Gefitinib by suppressing the expression of the NRAS and MEST genes in non-small cell lung cancer [28]. Loss of miR-143 and miR-145 has increased cellular proliferation in Condyloma acuminatum via targeting NRAS [29]. Li X et al. showed that in Osteosarcoma cells, circ0000073 overexpresses NRAS by sponging miR-145-5p and miR-151a-3p, resulting in methotrexate resistance proliferation, migration, and invasion [30]. Based on the literature findings and our study results, we believe that the link between miR-145-5p and IGFI R/NRAS in MM may be important in the disease pathogenesis.

In addition, in our study, three crucial EMT marker genes (E-Cad, N-Cad and Vimentin) were compared in terms of expression levels. The increase in N-Cad and Vimentin expression level and decrease in E-Cad expression level in the EMT process are accepted as hallmarks in cancer [31, 32]. Similar to the literature, we showed increased E-Cad expression and decreased N-cad expression in miR-145 mimics transfected MM cells compared to controls in our study. This suggested that miR-145 may potentially contribute to microenvironment invasion and metastasis of cancer cells. However, the fact that the data obtained in the study is at the mRNA level and requires confirmation at the protein expression level can be considered as a limitation.

Conclusions

Further elucidation of the molecular mechanisms involved in IGFI R and NRAS may contribute to future targeted therapy development. Therefore, the findings of our work, which explain the interaction between miR-145-5p and IGFI R/NRAS in MM cells, might be useful. The absence of in vivo studies restricts the generalizability of our findings. This aspect should be investigated more in future studies. In conclusion, our study revealed that miR-145-5p might reduce MM progression by controlling cell proliferation by targeting IGFI R and NRAS.

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References


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