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MicroRNA-330 Inhibits Growth and Migration of Melanoma A375 Cells: In Vitro study

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Author contribution statement

Nasser Sehati developed the hypotheses for this research.

Behzad Baradaran planned the methodology.

Behzad Baradaran and Nasser Sehati organized, supervised and were responsible for the course of the project and the article.

Behzad Baradaran provided personnel, environmental and financial support, tools, and instruments that were vital for the project.

Behzad Mansoori,, Ali Mohammadi provided biological materials, reagents and referred patients.

Dariush Shanehbandi, Navaz Sadeghie, and Behzad Mansoori were responsible for the execution of the experiments, patient follow-up, data management and reporting.

Behzad Baradaran, and Nasser Sehati were responsible for the interpretation and presentation of the results.

Behzad Baradaran was responsible for overall supervision of this work.

Nasser Sehati was responsible for the construction of the whole or body of the manuscript.

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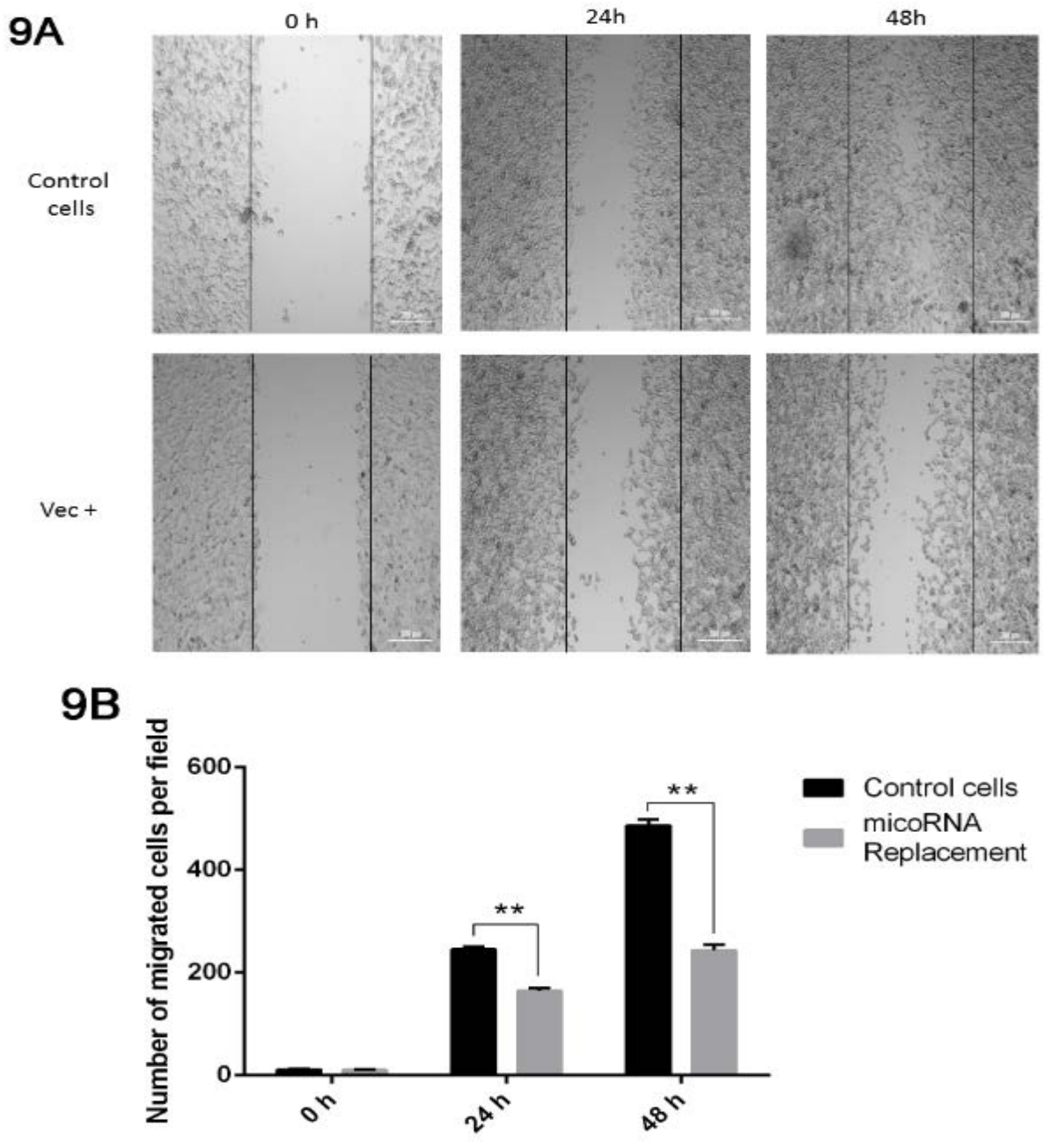
Abstract

Melanoma skin cancer is one of the main causes of male cancer-related deaths worldwide. It has been suggested that miR-330-5p can acts as a tumor suppressor in various types of cancers. So, in this study, we replaced miR-330 in melanoma cancer cells by vector-based miR-330 to evaluate the effects of this miRNA on the growth and migration inhibition of melanoma cancer cells, and to determine the molecular mechanisms underlying its action. By using the MTT assay, the IC50 of Geneticin antibiotic was obtained as 460 µg/ml. The results of the qRT-PCR showed the increased expression level of miR-330 and decreased expression levels of MMP-9, CXCR4, Vimentin, MCAM, AKT1, and E2F1 mRNA in A375 transfected cells. The cytotoxicity assay results demonstrated the inhibition of cancer cells proliferation. Furthermore, the wound healing test results showed a migration reduction of transfected cells with miR-330 compared to non-transfected ones. In addition, DAPI staining revealed the significant nucleus fragmentation in miR-330 replaced cells, which correspond to apoptosis induction in replaced cells. The results showed that

increase in miR-330 expression level could significantly inhibit the tumor cell growth and the migration of melanoma cancer cells.

Graphical Abstract

The results showed that increasing at miR-330 expression level could repress tumor cell growth and migration of melanoma cancer cells.



Keywords: miR-330; Melanoma cancer; Migration; Proliferation

Introduction

Today, cancer is one of the most serious health issues around the world [Alao, 2007; Helin, 1998]. Among them, the skin cancer is one of the common types of cancers [Newton Bishop, 2010] and the malignant melanoma is the most invasive form of it which some environmental, biochemical, molecular and genetic factors are involved in its development. It originates mainly from the epidermal and dermal layers, the melanocytic pigment cells. This disease develops from the accumulation of melanin seeds and spreads to the outer skin layer [Villanuera and Herlyn, 2009]. Ultraviolet damage is considered to be a major risk factor in this area, with considering the fact that the incidence of melanoma is increasing for populations with a lighter skin as it approaches the equator [Neville et al., 2002]. There is no definitive treatment for melanoma and about 95% of melanoma cases are treated with surgery, but the rate of disease recurrence is very high. Other treatments include chemotherapy, immunotherapy, radiation therapy and a combination of them, all have a weak effect on the cancerous tumor [Burmeister et al., 2002]. Therefore, promoting protective actions in the prevention of skin cancer is essential [Reya et al., 2001].

Micro RNAs are large subgroups of RNAs, which control gene expression after transcription through mRNA translation, inhibit or induce degradation by binding to the 3'-UTR at the end of mRNA [Bader and Lammers, 2011; Mansoori et al., 2014; Mohammadi et al., 2016]. Incorrect Micro-RNA expression or supersession can effectively contribute to tumor formation or tumor progression [Calin and Croce, 2006; Check, 2008; He et al., 2007]. The interaction of microRNAs with target genes determines their role in growth, programmed death, cell differentiation and proliferation, and confirms the direct function of micro-RNAs in cancer. Changing the expression of microRNAs by reducing the expression of the essential genes involved in the proliferation or survival of the cells leads to the formation of a tumor [Schaefer et al., 2010]. On the other hand, Micro RNAs are one of the main types of regulators of the programmed death in the tumorigenic process, and the survival of cancer cells is controlled by manipulating these micro-RNAs [Ruan et al., 2009]. Moreover, these biomolecules regulate the cancer pathways which makes them appropriate targets for cancer treatment. Several strategies are under investigation to manipulate miRNA in vivo. One of them is replacing miRNA in cancer cells [Tufman et al., 2013]. This method is carried out by miRNA replacement in cancer cells through transfecting vectors contains miRNA gene, so the miRNA expression level can be restored to its normal level at cancerous cells and can regulate target gene’s expression naturally [Iio et al., 2010a; Wang et al., 2009]. The miR-330 gene is located on chromosome number 19, which is a fragile region in the genome. The miR-330 is an important regulator of gene expression [Mao et al., 2013] and downregulation of it has been reported in some cancers, including colorectal, esophageal, prostate,. and melanoma cancer [Mueller et al., 2009]. Since the decreased expression of miR-330 in melanoma cancer increases the proliferation and metastasis and reduces apoptosis, it can be used as a therapeutic target for melanoma cancer treatment. In this method, the miRNA expression in cancer cells can be restored to normal levels by replacing the miRNA in cancer cells through miRNA gene transfection by the vector; hence, naturally regulate the expression of target genes [Iio et al., 2010b; Wang et al., 2009].

The aim of this study is to evaluate the miRNA-330 induction in melanoma cancer cell line (A375) and to analyze the effect of this miRNA on growth and migration inhibition of the A375 cells.

Materials and methods

Bacterial culture

E. coli (DH5a) bacteria were obtained from the Genetic Reserves Center and incubated in Luria–Bertani, Miller medium (Himedia, India) at 37 ° C.

Bacterial competent and transformation

Competent of the bacteria was performed prior to the transformation to enhance the permeability of the bacterial membrane. For this purpose, Plasmid containing miR-330 gene (Origene, USA, SKU: SC400344) and glycerol, and 100 mM $CaCl_2$ were mixed within ice bath and incubated for 30 min at 4 °C. Next microtubes were placed at 42 °C in water bath for 60 s to open the gaps of the bacterial wall, allow the plasmid containing miRNA gene to enter into the bacteria easily. Microtubes were incubated in shaker incubator with 150 rpm at 37 °C for 1 h in LB (Broth) medium. The bacterial supernatant was incubated on LB Agar containing Kanamycin antibiotic at 37 °C after centrifuging at 2400 rpm for 5 min. Then, the transformed bacteria were transferred to LB Broth medium.

Plasmid DNA Extraction

Plasmid DNA was extracted by YTA Plasmid DNA Extraction Mini Kit (Yekta Tajhiz, Iran, Cat No: YT9010). Electrophoresis was performed on circular and linear plasmid to ensure the accuracy of plasmid DNA extraction. XHOI restriction enzyme was used to cut circular plasmid to a linear one. After ensuring the correctness extraction was performed on a Maxi scale using YTA Plasmid DNA Extraction Maxi Kit (Yekta Tajhiz, Iran, Cat No: YT9007) protocol.

Cell culture

The melanoma cancer cell line, A375, and skin cell line, HFFF2, were obtained from Pasteur Institute of Iran and incubated in the RPMI 1640 medium (Gibco, USA) enriched with 10% Fetal Bovine Serum (FBS) (Gibco, USA), at 37 °C and humidified air containing 5% CO_2.

MTT test to determine IC_{50}, the appropriate dose of Geneticin antibiotic (G-418)

A375 cells were distributed in 96-well plate with RPMI1640 medium and 10% FBS. Then the wells were incubated at 37 °C with 95% humidity and 5% CO_2 for 24 h. Thereafter, various doses of Geneticin antibiotic (Gibco, USA) were added to the cell culture for 72 h. For determining IC50, the appropriate dose of antibiotic was determined by adding MTT solution and was measured by ELISA Reader (Tecon, Sunrise, Austria) at 570 nm wavelength.

Transfection

The vector carrying miRNA-330 gene, as an expression plasmid for human microRNA-330 and control vector (empty vector without miRNA gene) were obtained from Origene (USA). Transfection was applied with jetPEI® solution (Polyplus, France) based on the protocol description.

Live cell imaging

At 24 hours after transfection, a $3x10^5$ transfected A375 cells were cultured in six-well plates. The cells were washed with PBS and the new RPMI1640 medium was added. Then, wells were checked with Cytation 5 (Biotek, USA) with GFP gene, expressing a fluorescent protein, to verify the correctness of transfection.

Qualitative RT-PCR

Selection of the transfected cells was carried out by Geneticin antibiotic (Gibco, USA). Total RNA was extracted from cells through the RiboEx (GeneAll, Republic of Korea) according to the manufacturer's instructions. The cDNA was synthesized and amplified based on cDNA Synthesis kit (EXIQON, Denmark). The mRNA levels were determined by qRT-PCR using SYBR-Green PCR kit ((EXIQON, Denmark) for miR-330 and the cDNA Synthesis (Thermo Scientific, Rockford, IL, USA) Kit was used for target genes (CXCR4, MMP-9, and Vimentin). The GAPDH gene was used as an internal control (Table 1).

MTT cell proliferation assay

At 48 hours after transfection, $2x10^3$ of both types of A375 cells containing a vector with miR-330 gene and empty vector were cultured in RPMI1640 medium and then added to 96-well plates. MTT solution (2 mg/ml) (Bio Basic, Canada) was added to the cell culture and incubated at absolute darkness at 37 °C for 4 h. Then, the cell proliferation was detected by MTT assay with ELISA reader (Tecon, Sunrise, Austria), at the 570 nm wavelength to detect the cell survival.

Wound Healing Assay

The number of 5×10^5 of each types of A375 cells containing a vector with miR-330 gene and empty vector were cultured in 6-well plate in appropriate conditions for 24 h. Wounds were created by plastic scriber on the cell monolayer. Cells were then washed in 6-well plate and incubated in RPMI 1640 with 10% FBS. The migration activity of cells from the edges was recorded by using an inverted microscope (Optika, XDS-3, Italy) at 0, 24 and 48 h.

DAPI Staining

A number of 2×10^3 A375 cells were cultured in 96-wells for 24 h after transfection. Cells were washed and fixed by PFA (Para-Formaldehyde) and incubated for 1 h. Then, the cells were washed again and Triton-X-100 solution was added and incubated for 10 min. After rewashing with PBS, the cells were incubated with DAPI solution. Analysis was done with citation 5 imaging system (Biotek, USA). The number of fragmented nucleus cells was count and represent as a percentage of the fragmented nucleus in each group.

Results

The electrophoresis of extracted plasmid

After bacterial culture and plasmid extraction in the mini and maxi prep step, electrophoresis was performed on the plasmid without restriction enzyme. Due to the circular plasmid and various spatial forms of plasmid, the result of electrophoresis showed three bands as shown in Fig. 1. At this stage, it was not possible to determine the exact size of the plasmid.

Electrophoresis was performed again after cutting the plasmid with the restriction enzyme, and at this stage, a band was obtained in parallel to about 6.2 kb as shown in Fig. 2.

The appropriate dose of Geneticin for stable selection of miR-330 expressed cells

To obtain IC50 of Geneticin (G-418) antibiotic, MTT test was performed. Cells were treated with 100, 200, 400, 600, 800, 1000 and 1200 µg/ml concentrations of the reagent. The IC50 was obtained as 460 µg /ml for A375 cells. After transfection of vectors, this dose was used to select cells containing the vector. The results are presented in Fig. 3.

miR-330 construct induced expression of this miRNA in the melanoma cells

After transfection and before RNA extraction, cells were photographed with Cytation 5. Transfected cells with vector were observed in green as the GFP gene was expressed (Fig. 4).

The result of the miR-330 expression in a melanoma cell line (A375) showed miR-330 downregulated more than 100 fold compared to the normal skin cell line (HFFF2) (Fig. 5).

The expression of miR-330 gene in A375 cells containing a vector with miR-330 and cells containing the empty vector (plasmid without miR-330) were evaluated by the qRT-PCR assay. The results of the qRT-PCR showed miR-330 was induced after plasmid vector transfection more than 10 folds compared to the negative control (Fig. 6).

miR-330 replacement downregulated the expression of CXCR4, Vimentin, MMP-9, and MCAM genes in A375 cells

The levels of mRNA expression of CXCR4, Vimentin, MMP-9, MCAM, and MELTF genes in A375 cell line was investigated by qRT-PCR after miR-330 induction. The results showed that miR-330 could decrease the expression of CXCR4, MMP9, Vimentin, and MCAM mRNA more than 100 fold, 2 fold, more than 3 fold, and more than 1 fold, respectively. However, the induction of miR-330 has no significant effect on MELTF mRNA expression (Fig. 7).

miR-330 replacement decreased cancer cells proliferation in A375 cells

Cytotoxic effect of the increased miR-330 expression on A375 cells was evaluated by MTT assay. As shown in Fig. 8, MTT assay results showed that the cell proliferation

in miR-330 positive was significantly reduced compared to miR-330 negative vector cells ($P < 0.002$).

miR-330 could inhibit melanoma cell migration

To study the effect of the miR-330 expression on the migration of A375 cells, a Wound healing assay was performed. The results of this assay were evaluated by cell counting which migrated into the scratch area during the period of 0-24-48 hours after scratching time. The migrated cell number in miR-330 replaced cells was decreased compared to the negative control cells (Fig. 9).

miR-330 could alter the nucleus morphology in melanoma cells

In order to elucidate that the miR-330 could fragment the nucleus of cells, apoptosis DAPI staining assay was performed. The result of DAPI staining showed the number of nuclei condensed and fragmented melanoma cells were increased in miR-330 induced cells compared to the negative control (Fig. 10).

Discussion

Studies in many countries indicate a high prevalence of skin cancer and the number of cases with this cancer is increasing day by day [Lomas et al., 2012]. Melanoma is treated by surgery in 95% of cases, but the recurrence rate is very high [Saczko et al., 2005]. Although the prevalence of melanoma is increasing throughout the world, however, there is no effective treatment for it yet, therefore, it is necessary to develop new and effective strategies for the treatment of this disease [Villanuera and Herlyn, 2009].

Extensive researches on animal models suggest that restoring the expression of a tumor suppressor micro-RNA in tumor cells can be an appropriate treatment option for cancer, since, a micro-RNA can target and inhibit several oncogenic pathways. On the other hand, conventional therapies such as chemotherapy and radiotherapy have destructive effects on normal cells, but there are still challenges in the field of treatment by the restoring of micro-RNAs that need to be overcome [Calin and Croce, 2006; Check, 2008; He et al., 2007].

Tumor suppressor micro-RNAs in melanoma cancers, like other cancers, involve the micro-RNAs that their expressions have an inverse relationship with the rate and severity of cancer. Achieving effective methods to restore the expression of these tumor suppressor micro-RNAs is very important for the treatment and control of cancer or the side effects of the disease [MacFarlane and R Murphy, 2010; Takamizawa et al., 2004]. Several miRNAs with antitumor properties have been introduced and studied in melanoma cancer, among them, there are miR-148b, miR-181a, miR-23b, miR-503 and miR-330 that their expression levels are reduced in melanoma cancer [Tsao et al., 2012].

The miR-330 is a tumor suppressor microRNA. The reduced expression of it in melanoma cancers increases the proliferation and metastasis and reduces apoptosis. This microRNA can be suggested as a therapeutic target for melanoma cancer, using the new method of treatment by replacing the microRNA by transfection of vectors containing microRNA gene, which can restore the expression of microRNA to normal

levels in cancerous cells with naturally regulating the expression of its target genes [Hodzic et al., 2011; Lee et al., 2009b; Mueller et al., 2009]. As mentioned, the miR-330 expression is declined in melanoma cancer. This microRNA is involved in the processes of proliferation, differentiation, survival, apoptosis, and migration by regulating the expression of various genes, such as Vimentin, CXCR4, and MMP9 [Mueller et al., 2009].

In this study, we studied the expression level of the miR-330 gene in HFFF2 cell line (normal skin cell line), compared to the expression level of the same gene in A375 cell line (melanoma skin cancer cell line). Comparison of the expression of this gene in these two normal and cancerous cell lines indicated a reduction in the expression of this gene in cancerous cells. The results of the qRT-PCR assay showed that the expression of this gene in the HFFF2 cell line is much higher than the A375 cell line, in other words, the gene has been significantly reduced in cancerous cells, which would certainly have a direct effect on the expression of the target genes of miR-330 in A375 cancer cells. Thus, increasing the expression level of miR-330 in these cells and restoring the expression to the normal level can be not only an appropriate target for conducting research but also an appropriate therapeutic approach for the treatment of melanoma cancer.

In this study, initial results of the replacement of the vector containing miR-330 and the miR-330-free vector were shown by the Live Cell Imaging device, indicated the success of the vector transferring using the jetPEI reagent.

The presence of GFP in the vector structure helped us to ensure the transfection and insertion of the vector before RNA extraction. Then, we were able to detect cells with stable expression in the cells transfected with the vector that was seen in green on the Cytation 5 device and could be distinguished from other cells. After ensuring the stability of the cells containing the microRNA, we continued experimentation.

Because of having Geneticin antibiotic resistance gene in the vector, we used an appropriate dose of this antibiotic in cell culture according to the IC50, therefore it can be claimed that only cells received the vector were able to survive in the culture medium containing Geneticin (G-418) and the remaining cells were mostly eliminated.

The results obtained from qRT-PCR showed a significant increase in the expression of the miR-330 after replacement by miR-330 vector in A375 cancer cells compared with the empty vector received cells indicated that the transfection was correct and had a acceptable performance to enhance the expression of tumor suppressor miR-330. The ability to express the gene after transferring into the genome is important in this regard, which indicates the correct incorporation of the gene into the cell's DNA and its functionality.

A study by Mueller et al. showed that miR-330 was significantly reduced in melanoma carcinoma; they reported a link between melanoma cancer and decreased expression of miR-330 in various cell types of melanoma, indicating the tumor suppressor role of miR-330 in melanoma carcinoma [Mueller et al., 2009].

In addition, in 2015, Meng et al. showed miR-330 function as an oncogene in human esophageal cancer by targeting programmed cell death [Meng et al., 2015].

In 2012, studies by Peter Dynoodt et al. on malignant melanoma cell lines revealed that the transfer of miR-145 mimic into these cancer cells prevented migration of cancer cells [Dynoodt et al., 2013]. This study is consistent with our research in proving the role of Micro-RNAs in inhibiting cellular migration at melanoma cancer.

Furthermore, our study showed that miR-330 could induce nucleus fragmentation in melanoma cells, this result proposed miR-330 might induce apoptosis in melanoma cells. Our results also showed a significant reduction in AKT1 and E2F1 mRNA expression after inducing miR-330. Along with our study, Lee et al. showed that miR-330 could induce apoptosis via regulation of E2F1 and suppression of Akt phosphorylation [Lee et al., 2009a].

In addition, by using the Wound Healing Assay we concluded that increased expression of miR-330 reduced the migration and growth in the cells containing miR-330 vector compared to control cells (containing miR-330-free vector) in A375 melanoma cancer cell line. Therefore, it can be concluded that miR-330 has an inhibitory role in the growth and migration of melanoma cancer.

To further explore and find the molecules led to the reduction in migration of cells transfected by miR-330 in the wound healing assay test, our results showed a significant reduction in mRNA levels of CXCR4, Vimentin, MMP-9 and MCAM genes after miR-330 transfection. Besides, the results did not show the significant change in MELTF mRNA level. Excessive expression of CXCR4 has an effect on the growth of tumors, angiogenesis, metastasis, and resistance to treatment [Chatterjee et al., 2014]. Scala et al. showed that the CXCR4 expression level was increased in human melanoma [Scala et al., 2006]. As a result of miR-330 replacement in melanoma cancer cell line, a significant reduction was observed in the expression of the CXCR4 gene in cells transfected with miR-330, which that reduction reflected the important role of this gene in melanoma cancer.

As like, the MMP-9 is increased in expression level in the process of metastasis and cell migration [Zheng et al., 2010]. Nikkola et al. showed that MMP-9 plays an important role in metastasis of melanoma cancer and its rate is increased in people with melanoma cancer [Nikkola et al., 2005]. As a result of miR-330 replacement in the melanoma cancer cell line, we observed that the expression of MMP-9 was decreased; it could be concluded that miR-330 replacement has a positive effect on the MMP-9 gene in melanoma, and this can inhibit metastases in melanoma cancer.

As the same way, Vimentin is a member of intermediate filament proteins and a common marker of epithelial-mesenchymal transition (EMT) that is effective in the development of tumor, metastasis, and invasion in melanoma cancer [Ye et al., 2016]. In a study conducted byMan Li et al. it was concluded that the high expression of Vimentin was observed in patients with melanoma cancer [Li et al., 2010].

The melanoma cell adhesion molecule (MCAM) involves in cell tocell junction and expressed highly in advanced-stage of melanoma. MCAM expression significantly increased migration and invasion of melanoma cell [Watson-Hurst and Becker, 2006].

Because of miR-330 replacement in the melanoma cell line, we observed a reduced expression level of Vimentin, so it can be concluded that Vimentin has an important effect on the miR-330 replacement in melanoma cells.

Therefore, miR-330 could induce apoptosis by down-regulation of E2F1 and AKT1. Besides miR-330 replacement reduced CXCR4, Vimentin, MMP-9, MCAM mRNA related to the migration of melanoma cancer. (Fig. 11).

Conclusion

In brief, our studies show that miR-330, as a tumor suppressor micro-RNA, is a good choice for micro-RNA replacement technique, and the possibility of using it in target therapy of cancer is suggested. Furthermore, the miR-330 can inhibit the migration of melanoma cancer in vitro by reducing the expression of invasive factors such as CXCR4, Vimentin, MMP-9, and MCAM that play an important role in the treatment of melanoma cancer. In addition, it can regulate apoptosis by reducing E2F1 and AKT1 levels. Our findings suggest that miR-330 can be used as a therapeutic target in the treatment of melanoma, however, further studies on miR-330 in melanoma cancer and its possible pathways are required to understand more about miR-330 tumor suppressor role.

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Conflict of Interest

All the authors declare no conflict of interest.

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Figures

Fig. 1. The electrophoresis of plasmid extracted without restriction enzyme

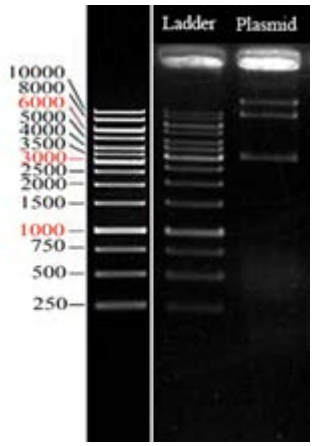


Fig. 2. The electrophoresis of plasmid extracted after cutting with the restriction enzyme

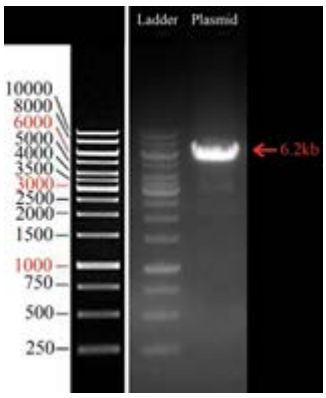


Fig. 3: The effect of Geneticin (G418) antibiotic on the A375 cell line at different doses (Reduced viability of the cells was shown with P <0.05)

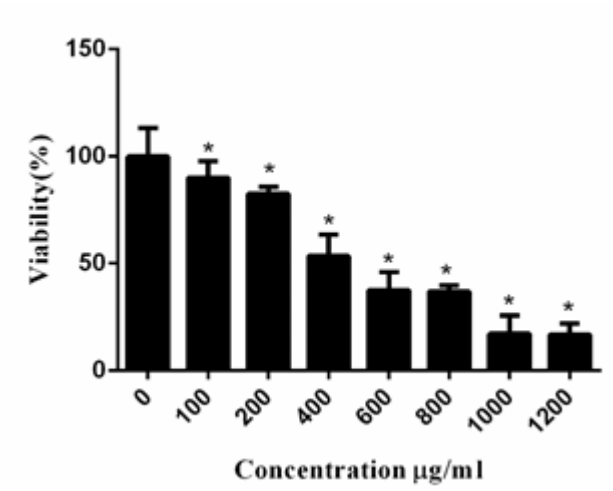


Fig. 4: Images of cells transfected with miR-330 in A375 cells. PCMV-miR-330 vector could induce GFP protein expression in the cells, which emitted green fluorescent showing the stable miR-330 expressed cells.

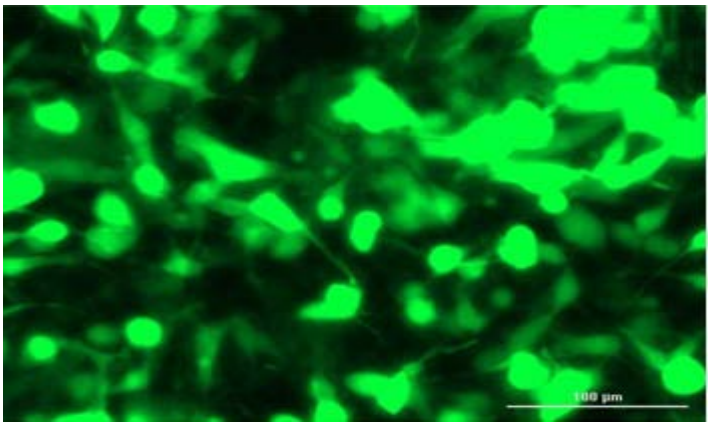


Fig. 5: MicroRNA expression in A375 and HFFF2 cells (**** Low expression of microRNA was shown in the melanoma cells with P <0.0001)

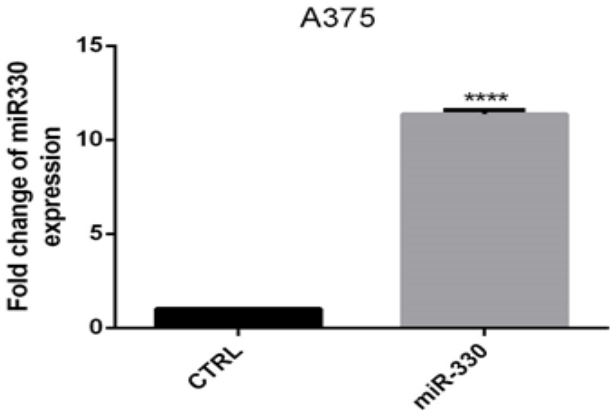


Fig. 6: The effects of microRNA replacement on miR-330 gene expression in A375 cells transfected with a vector containing miR-300 gene compared to control cells (cells transfected with empty vectors). **** Increased expression of microRNA was shown with P <0.0001

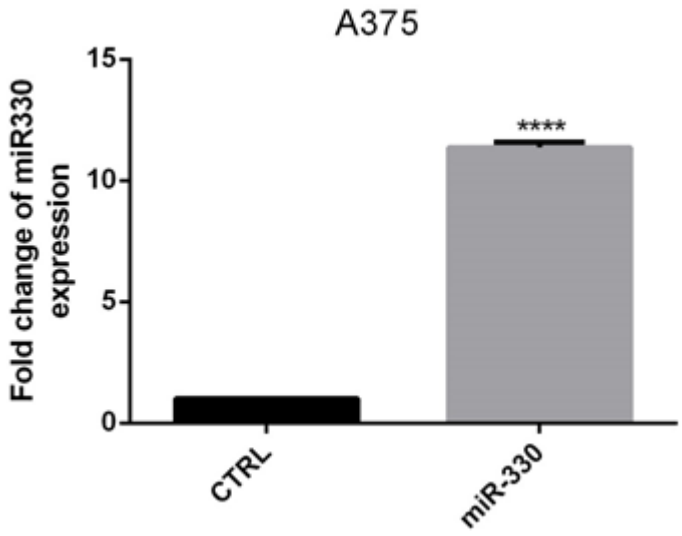


Fig. 7: The effect of miR-330 replacement on CXCR4, Vimentin, MMP-9, MCAM, and MELTF in Melanoma A375 cell line. The levels of CXCR4, Vimentin, MMP-9, and MCAM mRNA expression were decreased in miR-330 induced cells (miRNA replacement) compared with control cells (7A, B, C, D). Besides there is no significant change in MELTF mRNA expression (****P<0.0001, **P<0.01 and * P<0.05).

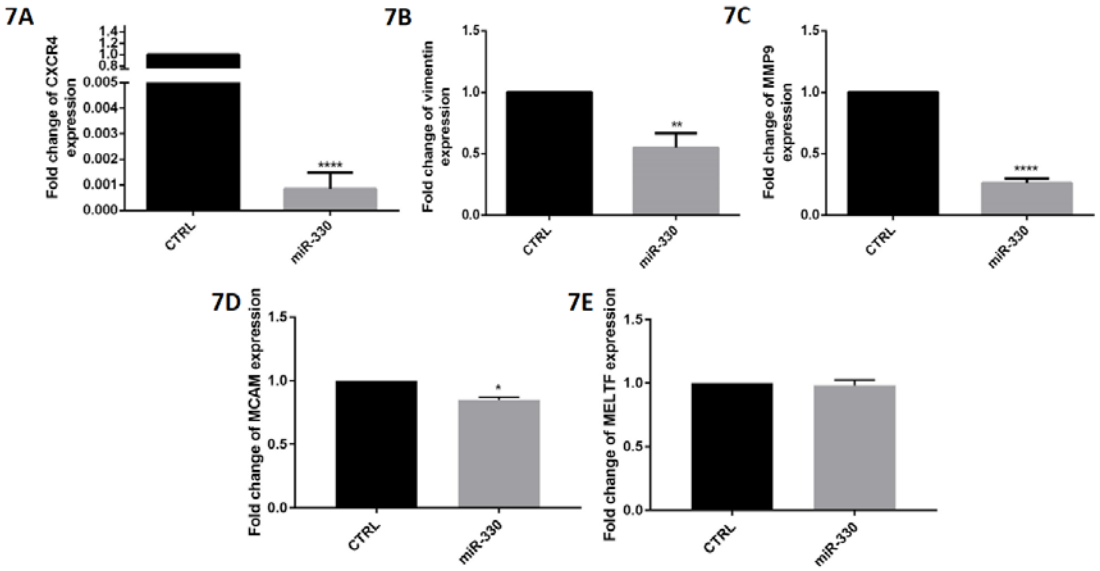


Fig. 8: Cytotoxic effect of increased miR-330 expression on A375 cell line (** P <0.01).

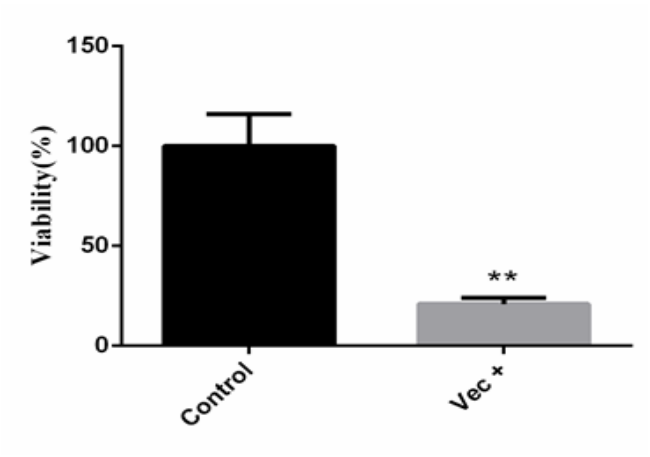


Fig. 9: Migration rate of vector-containing cells with miR-330 gene was reduced compared to cells containing the miR-330-free vector at 0, 24 and 48 hours ***(9A)*** Migration of cells transfected with vectors containing miR-330 were compared to cells containing the miR-330-free vector at 0, 24 and 48 hours (9B) (** P <0.01).

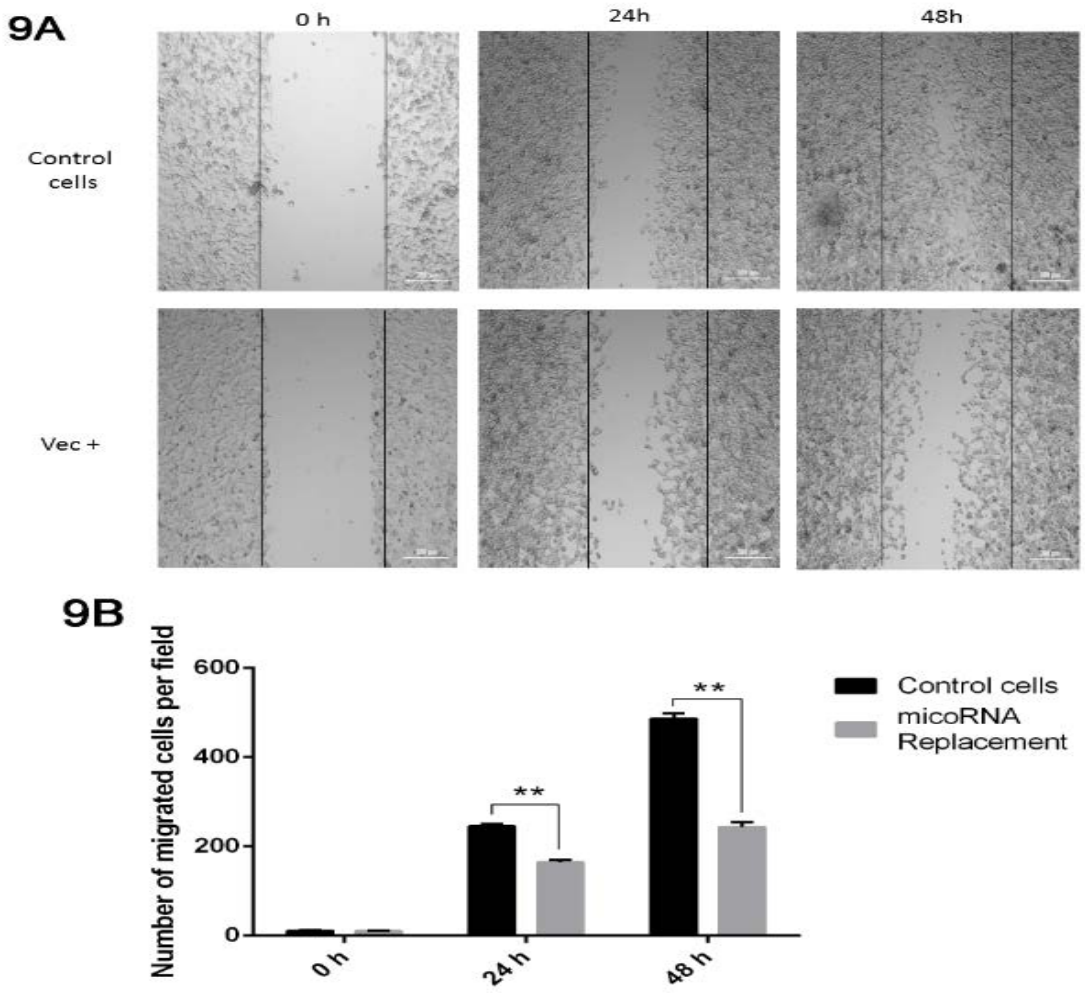


Fig. 10. miR-330 could fragment the nucleus of melanoma cell. Chromatin fragmentation and their percentage were evaluated using DAPI staining in A375 (10A, B). Relative AKT1 (10C) and E2F1 (10D) mRNA expression showed a reduction in their mRNA levels after induction of miR-330. (* P<0. 05).

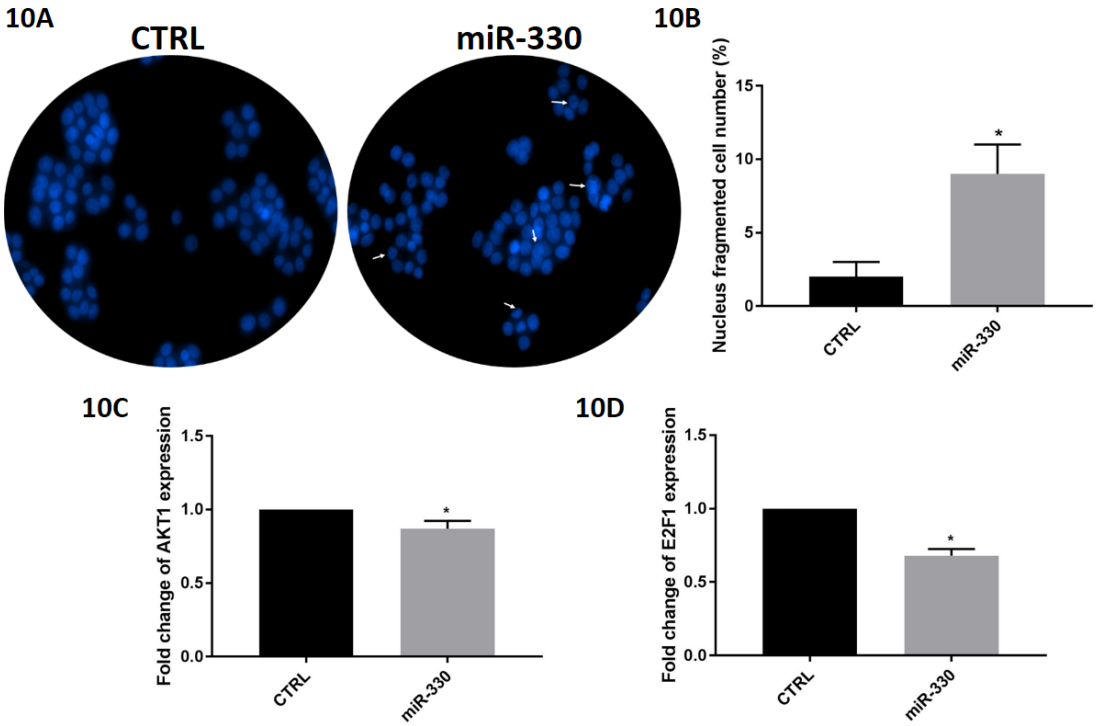
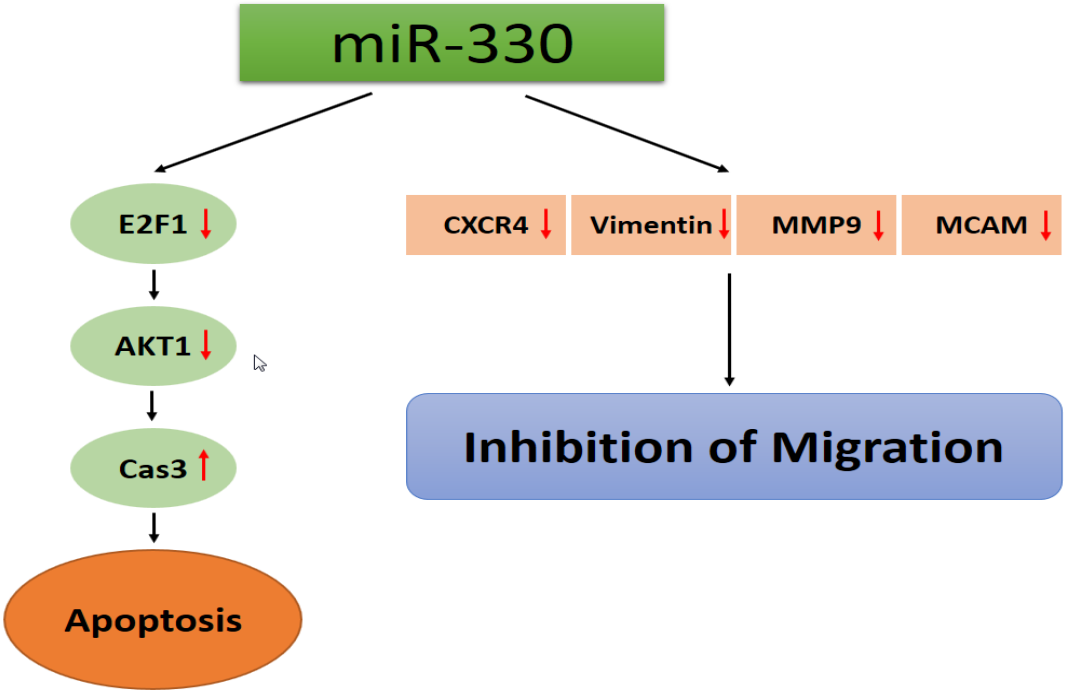


Fig. 11. A schematics representing the effect of miR-330 in apoptosis and migration pathways.



Tables

Table 1: Primer sequences of employed target genes

Name	Product length	Forward and Reverse	Sequences
GAPDH	166	F	5’- CAAGATCATCACCAATGCCT -3’
		R	5’- CCCATCACGCCACAGTTTCC-3’
MMP-9	170	F	5’-GCGCACAAATCCCTTCTACC-3’
		R	5’-ATCCGTGTAGCACATTCTGTCC-3’
CXCR4	145	F	5’- GGAGGGGATCAGTATATACA -3’
		R	5’- GAAGATGATGGAGTAGATGG -3’
Vimentin	125	F	5’- CTCTGGCACGTCTTGACCTT -3’
		R	5’- TTGGACATGCTGTTCCTGAA -3’
MCAM	144	F	5’-GACTCCAACACAACCACTGGC-3’
		R	5’-CAGGACCAGGATGCACACAATC-3’
MELTF	164	F	5’-TCGTCAGGCACACAACCGTC-3’
		R	5’-GGCGTGGGGTGGTATCTGT-3’
AKT1	169	F	5’-GCTGCACAAACGAGGGGAG-3’
		R	5’-CCGCTCCGTCTTCATCAGCT-3’
E2F1	243	F	5’-CCACCCTCCAATCTGCACTT-3’
		R	5’-GACCAAAACAGCGAGGAAGC-3’