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INCREASED MICRORNA-362 LEVEL IN MALIGNANT SKIN MELANOMA

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Abstract – *Objective:* *The malignant skin melanoma is a deadliest skin tumor; however, it could be cured if diagnosed timely. MicroRNAs contribute to the control of cellular physiologic and pathologic procedures and many of them can act as oncogenes and tumor inhibitory factors. The current study aims at comparing the expression level of Mir-362 between the case and the control groups and evaluating its association with the clinicopathological features in patients with melanoma.*

Patients and Methods: *A total of 40 patients with melanoma and 10 healthy subjects in the control groups were enrolled. RNAs were extracted from the paraffinized tissue blocks. The expression of Mir-362 was assessed using the Real-time polymerase chain reaction. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control.*

Results: *Mir-362 expression level was found six-fold higher in the case group, compared to that of the controls ($p<0.05$). No correlation was found between the expression of Mir-362 and other factors assessing in melanoma cases such as the Clark and Breslow indices, mitotic count, as well as demographic characteristics such as age and gender ($p>0.05$).*

Conclusions: *Owing to the up-regulation of Mir-362 expression in the patients with melanoma compared to the healthy subjects, Mir-362 expression could be used as a diagnostic and therapeutic marker for further investigations.*

KEYWORDS: *Melanoma, Mir-362, Up-regulation.*

INTRODUCTION

The malignant skin melanoma, one of the most common types of cancer worldwide, constitutes at least 40% of all types of cancer. Being exposed to sunlight is the main cause of this type of cancer. In addition, exposure to harmful environmental agents, radiotherapy, and even hereditary and genetic features may contribute to the incidence of the disease[1]. There are 3 main types of skin cancer: basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and melanoma; the first 2 types, in addition to the rare types of the skin cancer, are identified as non-melanoma skin cancers (NMSC)[2]. Melanoma is one of the most invasive and life-threatening skin cancers. It is a melanocyte-killing tumor initiated from irregular production of dark pigments such as melanin in melanocytes and is transmitted through the bloodstream to entire the body. Most of the melanoma cells produce melanin; therefore, most of the melanoma tumors are usually brown or black. But, some of them do not produce melanin and emerge in pink or even white colors[3]. MicroRNAs are the regulatory, evolutionally protected, 18-25-nucleotide molecules encoded by eukaryotic DNA. Such protective sequences are encoded by the 30% of the genes in human. MirRNAs control the expression of mRNAs. Such molecules contribute to the regulation of cellular processes such as proliferation, differentiation, and apoptosis as well as intrinsic and acquired immune responses playing critical roles as post-transcriptional regulators in the expression

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of genes. Hence, changes in the expression of MicroRNAs can influence critical bioprocedures such as differentiation and apoptosis[4]. The increased expression of MicroRNA in cancer cells may result from increase and lack of control over the production of a transcription factor or demethylation of GpC island promoters[5]. Some MicroRNAs are the tumor suppressors. Increased expression of MicroRNAs in cancer cells may decrease tumor suppressors in such cells, which is one of the causes of tumor cells emergence. Such MicroRNAs are known as oncomirs[6]. In contrast, some other MicroRNAs target proto-oncogenes. The expression of such MicroRNAs is accordingly reduced in cancer cells, which are followed by the increased expression of proto-oncogenes in the cells[7]. The gene location of Mir-362 is XP11.23. Mir-362 expression is reduced in some cancers, while is increased in some others. For instance, it increases in gastric[8] as well as liver cancers[9], while it is reduced in cervix[10], breast[11], and kidney cancer cells[12]. Mir-362 targets the genes involving in cell cycle progression and proliferation. In addition, the expression of Mir-362, which acts as suppressive agents, is reduced in the cancer cells. Considering the fact that most of the common methods used to screen cancers are incapable of diagnosing the disease in the early stages, the detection of tumor MicroRNAs is a key method for the timely diagnosis of cancer. In addition, MicroRNAs are also used for cancer therapy purposes and they can affect the target cells by making effective changes on such molecules[13]. The main purpose of the present research is to evaluating the expression of Mir-362 in melanoma, since there is no report about the changes in the expression level of Mir-362 in melanoma. Furthermore, the correlation between the expression of Mri-362 and clinicopathological features was investigated in the current study patients.

PATIENTS AND METHODS

Patients

Paraffin-embedded blocks from 40 patients with melanoma and 10 normal samples (as a control group) were analyzed after obtaining their written consent. The samples were referred to the Pathology Laboratory of Razi Hospital (Tehran, Iran) during 2013-2016 and were selected after a confirmed diagnosis by the pathologist. All patients aged between 21-87 years old with no previous use of anti-pregnancy medications, smoking and immune system disorders at least for the past 5 years. Demographic results of the patients were illustrated in Table 1. All Ethical considerations have been considered in this study, which was approved by the Ethics Committee of Islamic Azad University and was performed based on the principles of Helsinki Declaration.

RNA Extraction and cDNA Synthesis

First, the tissue samples were deparaffinized and then, the RNA extraction and cDNA synthesis were performed based on the protocol of Rahmatizade et al[14]. The

TABLE 1. The demographic and pathological information of the patients.

		Number	*(%)*
Age	62.84±13.06	40	100
Sex	Male	11	27.5
	Female	29	72.5
Breslow staging	Thin melanoma < 1 mm	25	62.5
	1 mm	2	5
	non thin melanoma > 1 mm	13	32.5
Clark's Anatomic Level	I	5	12.8%
	I-II	2	5.1%
	II	6	15.4%
	II-III	2	5.1%
	III	5	12.8%
	III-IV	3	7.7%
	IV	10	25.6%
	IV-V	3	7.7%
	VI	2	5.1%
Site of invasion	Head	4	10.0%
	Face	15	37.5%
	Upper limb	7	17.5%
	Lower limb	14	35.0%
Mitotic Count	High	4	10.0%
	low	36	90.0%

tissue samples were briefly deparaffinized by xylene (1000 µl) at 37°C for 5 min. Then, micro tubes were centrifuged at 3800 rpm for 5 min, supernatant was removed and then 1000 µl of ethanol were added and inverted for 5 min. Finally, samples were centrifuged at 13000 rpm at 6°C for 5 min and the ethanol and xylene of the micro tubes were entirely removed. Total RNA was extracted using the RNX plus™ kit (Cinnagen, Tehran, Iran) based on the manufacturer recommendations. 100 µl of the tissue sample were homogenized with 500 µl of the RNX-PLUS solution and incubated at room temperature for 5 min. Chloroform (200 µl) was added to the solution and centrifuged at 12000 rpm for 15 min. Supernatant was transferred to another tube and equal volume of isopropanol was added. The mixture was centrifuged at 12000 rpm for 15 min and the resulting pellet was washed in ethanol (70%) and dissolved in DEPC-treated water. The purity and the integrity of the extracted RNA were evaluated by optical density measurements and visual observation of sample electrophoresis on 2% agarose gel using NanoDrop spectrophotometer. The cDNA was synthesized from total RNA by using the commercial kit (Fermentas, Burlington, Ontario, Canada). Each microtube was added with 1 µl of random hexamer (5 µM), 1 µl of oligo (dT) primer (5 µM), 1 µl of deoxynucleotide (dNTP) (10 mM), 5 µl of RNA, 0.5 µl of Moloney murine leukemia virus (MMLV) reverse transcriptase, 2 µl of MMLV buffer, and 9.5 µl of DEPC-treated water. The total volume of the final mixture was expected to reach 20 µl. The samples were incubated at 65°C for 5 min and then placed in ice immediately. Afterward, they were run at 42°C for 60 min.

Real-Time PCR

In the present study, glyceraldehyde phosphate dehydrogenase (GAPDH) was considered as the housekeeping gene because of its permanent expression in most cells and tissues. The sequence of specific primers for Micro RNA and GAPDH was retrieved from the National Center for Biotechnology Information (NCBI) website. The specific primers of these two genes were designed using the Primer Express Software and their specificity was blasted in the NCBI. Table 2 presents the sequence of the primers used in this study. Real-time PCR was used to measure the expression of Micro RNA and GAPDH (as the control at mRNA level). A StepOne Real-time PCR system was used for relative quantification through the measurement of fluorescence increase following the application of SYBR Green. The Real-time PCR reaction was optimized at the final volume of 20 µl. The reactants included 10 µl of SYBR TM (2X) Master Mix (TaKaRa Co., Otsu, Shiga, Japan), 10 µM of the reverse and forward primers (Takapoo Zist Co. Tehran, Iran), 7 µl of deionized water, and 2 µl of the cDNA template. The temperature program of the device was optimized as follows: pre-denaturation at 95°C for 10 s; 30 cycles of denaturation at 95°C for 5 s; annealing and extension at 60°C for 34 s. Each experiment was repeated at least 3 times in order to ensure reproducibility. The melting curve was drawn through measuring the changes in the fluorescence level at different times using the Real-time PCR device. After the amplification reaction using the relative quantitative Real-time PCR, the raw data in the form of ct values were drawn out of the device, calculated through $2^{\Delta\Delta- ct}$, and converted into relative quantity. All experiments were performed at least in triplicate.

Statistical Analysis

The expression level of Mir-362 was analyzed using One-Way Anova (ANOVA). The results are presented as the Mean ± Standard Deviation (Mean ± SD). Statistical analysis was performed with GraphPad Prism (ver. 5.0, La Jolla, CA, USA). Significant differences were considered $p<0.05$.

RESULTS

Clinicopathological Features of the Study Participants

In the current study, a total of 40 patients aged 26 to 87 years were enrolled as the case group and 10 healthy subjects were selected as the controls. The clinicopathological features of the study participants are shown in Table 1.

TABLE 2. The sequence of the specific primers of Real-Time polymerase chain reaction (PCR).

Name		*Tm*	*Amplicon Size*
GAPDH F	ATGGAGAAGGCTGGGGCT	62.05	124 bp
GAPDH R	ATCTTGAGGCTGTTGTCATACTTCTC	61.62	
miR-362 F	GTTTAATCCTTGGAACCTAGGT	51.11	80 bp
miR-362 R	GTGCAGGGTCCGAGGT	51.06	

The mean age of the participants in the control and case groups were 64.7±9.93 and 62.84±16.53 years, respectively; the difference between the groups in terms of the mean age was not insignificant (p = 0.92). The mean expression of Mir-362 in the control and case groups was 6.7±1.3 and 1.1±0.19, respectively and the difference between the groups was significant (p = 0.000) (Figure 1). No significant association was observed between the genders in terms of Mir-362 expression (p = 0.42). No significant difference was observed among different age groups regarding Mir-362 expression (p = 0.30). Mir-362 expression in the case group was evaluated based on the Breslow index. The groups were compared based on thin melanoma <1 mm and non-thin melanoma ≥1 mm, and based on the Breslow index, the difference between the groups was insignificant (p = 0.42). The groups were also compared in terms of high-mitotic activity and low-mitotic activity based on Mir-362 expression. No significant association was found between Mir-362 expression and mitotic count (p = 0.43). No significant relationship was observed between the Mir-362 expression and the Clark anatomic level in the study groups (p = 0.39). A correlation was observed between Mir-362 expression and age in the study groups (r = -0.31, p = 0.05). There was no correlation between the expression of Mir-362 and gender in the study groups (r = -0.22, p = 0.16). Also, there was no correlation between Mir-362 and the Breslow index (r = 0.28, p = 0.08) as well as Mir-362 expression and mitotic count (r = -0.16, p = 0.33).

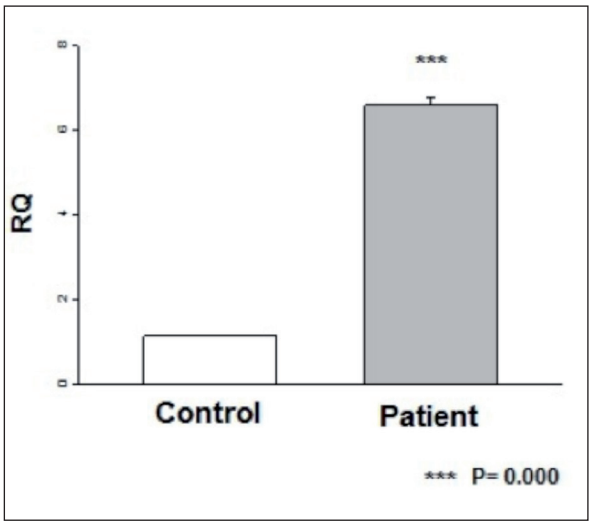

Fig. 1. The Mir-362 mRNA expression in patients and normal samples.

DISCUSSION

Despite advancements in the diagnosis and treatment, skin cancer is one of the leading causes of death worldwide. In addition to genetic and environmental factors, the epigenetic factors also contribute to the etiology of cancer. Here, the most important factor is the timely diagnosis and proper treatment of the cancer, which significantly affects the increased survival of patients[15,16]. There is a close correlation between MicroRNAs and different diseases such as cancer[17].

Adhesines and cell junctions play a significant role in the adhesion and immigration of cells in cancers. Tight cell junctions are the most important ones usually observed in epithelial cells. The tight cell junctions are the solid intracellular junctions with a complex molecular structure, which include adaptor proteins such as zonula occludens (ZO) and other integral membrane binding proteins such as occludin and claudin. These junctions are placed on the interior side of plasma membrane and 2 important types of them are occludin and claudin[18]. Occludin is a 65-kDa, integral membrane protein[19]. Reduced occladin protein in the tumors derived from epithelial cells weakens the tight junctions of epithelium cells and increases the cell movement and risk of metastasis[20,21]. The occludin mRNA is one of the targets of Mir-362; this MicroRNA suppresses the transcription of occludin mRNA and in consequence, reduces its expression. Based on the findings of the current study, the expression of Mir-362 was 6-fold higher in the case group, compared with the controls. There was no significant association between the expressions of Mir-362 and pathological features, such as Clark index and mitotic count, as well as demographic characteristics, such as age and gender. The changes of occludin gene expression in the patients with melanoma were evaluated in the authors' previous study and the results indicated the reduction of occludin gene expression in the patients (data not published). The current study, in line and continuation with previous studies, evaluated Mir-362 expression, which occludin is one of its targeting genes. The current study indicates that the increased expression of Mir-362 by targeting mRNA of occludin gene reduces the expression of occludin, which is an important cell junction protein and affects the progression of melanoma.

Results of the current study show the increased expression of Mir-362 in patients with melanoma, compared with the healthy controls. There were also different reports about the changes in Mir-362 expression. Some studies explained the increased expression of Mir-362; for example, Xia et al[8] showed that the increased expression of Mir-362 was indicated in gastric cancer cases. Based on their findings, Mir-362 inhibits the expression of CYLD tumor suppressor. In gastric cancer, Mir-362 induces cell proliferation and resistance to apoptosis by activa-

tion of NF-kB signaling pathway. Increased activity of NF-kB and its regulatory genes causes P65 translocation through an unknown mechanism. The increased activity of NF-kB induces cell proliferation and resistance to apoptosis. Mir-362 directly targets CYLD tumor suppressor which causes cell proliferation and resistance to cell apoptosis[8]. In another study, the up-regulation of Mir-362 was reported in the cases with gastric cancer; based on the findings of this study, the increased expression of Mir-362 was significantly higher in gastric cancer cells compared to that of the healthy gastric mucosal cells, and the expression of Mir-362 has inverse association with the expression of CD82 mRNA. There was also an association among the expression of Mir-362-3P, E-cadherin, N-cadherin, and vimentin in gastric cancer cells. Cadherins in the epithelial cells cause junctions in epithelial cells, consequently keeping such cells together. Therefore, the invasion and metastasis are increased following the expression of Mir-362 and reduction in cadherins[22]. The increased expression of Mir-362-3P was also reported in liver cancer cells. Inhibition of Mir-362 expression reduces cell proliferation and migration, as well as invasion and metastasis. CYLD tumor suppressor is one of the main targets of Mir-362 causing tumor growth, metastasis, and even cancer progression[9]. The reduced Mir-362 expression was also pointed out in some studies; for instance, the expression of Mir-362 was investigated in cervix cancer cases and the results showed its reduced expression. Lower expression of Mir-362 has direct correlation with lymph node metastasis and vascular invasion. Mir-362 is a negative regulatory; SLX (sineoculis homebox homolog) 1 is its target gene and plays a key role in DNA repair[10]. The reduced expression of Mir-362-3P was also observed in breast cancer cases. Mir-362-3P and Mir-329 act as tumor suppressors and their expression plays negative regulatory roles in breast cancer. Both the mentioned Mir inhibit cell proliferation, migration, and invasion through reducing the expression of p130Cas. Regulation of p130Cas expression is of great importance for the survival and movement of different cells, and regulates the progression of cancer through the stimulation of tyrosine kinase receptor signal. Reduced expression of Mir-362-3p and Mir-329 is performed by the methylation of genes. DNA methylation is one of the regulatory mechanisms of gene transcription. Improper DNA methylation in cancers usually results in silencing the tumor suppressing genes[11]. Reduced expression of Mir-362-3p was also found in kidney cancer cells. Mir-362-3p acts as tumor suppressor gene in kidney cancer. Mir-362-3p inhibits proliferation, immigration, and regulation of cancer cells and induces cell apoptosis and cessation of cell cycle at G1 phase. The nemo-like kinase is the target gene for Mir-362-3p, and high expression of Mir-362-3p reduces the NLK expression. NLK belongs to MAP kinases family. MAP kinases are the ones that can play a role in cell cycle and cell differentiation[12]. In the current study, MicroRNA was introduced as a factor influencing the cancer development. Also, the Mir-362 expression was 6 times higher than the normal samples. Tumor is a heterogeneous tissue. Although the increased expression of Mir-362 was observed in all the patients, can be considered as one of the most cancerogenic agents, which can be used as a biomarker to diagnose and treat the disease.

CONCLUSIONS

Pathological and physiological features vary based on cancers and the molecular pathways contribute to their different progression. In addition, since the target genes of Mir-362 vary in different cancers, changes in the expression of Mir-362 are associated with the type of cancer and signaling pathways of the target genes; for instance, in some cancers, the increased expression and in some others, the decreased expression, were reported. Differences in the expression of microRNAs in different cancers can be attributed to differences in the origin of cancer cells and their surrounding stroma tissue. We firstly evaluated the expression of such microRNAs in melanoma. Based on our findings, the expression of Mir-362 was higher in melanoma samples, compared with the healthy ones, and this increase had no correlation with demographic characteristics as well as clinicopathological features using the Breslow and Clark indices and mitotic count. Thus, Mir-362 could be used as a marker to diagnose and treat melanoma using additional testing.

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Ethical Approval:
There is none to be disclosed.

Conflict of Interest:
The Authors declare that they have no conflict of interests.

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