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Impact of KLF4, SHH, and Hif1a knockdown on miRNA expression in malign melanoma cancer stem cells

KLF4, SHH ve Hif1a susturulmasının malign melanom kanser kök hücrelerinde miRNA ekspresyonuna etkisi

Berrin Ozdil[1] Cıgır Biray Avci[2] Huseyin Aktug[3]

[1] Department of Histology and Embryology, Faculty of Medicine, Suleyman Demirel University, Isparta, Türkiye

[2] Department of Medical Biology, Faculty of Medicine, Ege University, Izmir, Türkiye

[3] Department of Biology, Faculty of Science, Ege University, Izmir, Türkiye

ABSTRACT

Aim: microRNAs (miRNAs) play a pivotal role in gene regulation, influencing various cellular processes such as differentiation, proliferation, and apoptosis. This study investigated the expression of three specific miRNAs (Hsa-miR-21-5p, Hsa-miR-9-5p, and Hsa-miR-200a-5p) in malignant melanoma stem cells (CSCs) and non-stem cells (NSCs).

Materials and Methods: CSCs and NCSCs were sorted from CHL-1 cells based on CD133 marker, a malignant melanoma cell line. CD133+ cells were treated with Hif1a, KLF4, and SHH siRNA and the expression levels of three different miRNAs were compared between groups.

Results: Our findings indicated that Hsa-miR-200a-5p expression was similar in both cell groups. Conversely, Hsa-miR-21-5p and Hsa-miR-9-5p were significantly upregulated in NCSCs. Further analysis showed that the knockdown of KLF4 did not significantly affect the expression levels of these miRNAs. However, silencing SHH resulted in a substantial downregulation of Hsa-miR-21-5p and a significant upregulation of Hsa-miR-9-5p. Additionally, Hif1a knockdown led to the downregulation of both Hsa-miR-21-5p and Hsa-miR-9-5p.

Conclusion: These findings highlight the complex regulatory mechanisms of miRNA expression in different cellular contexts and suggest potential roles for these miRNAs in response to specific gene silencing.

Keywords: Malignant melanoma, cancer stem cell, miRNA, Hif1a, KLF4, SHH.

ÖZ

Amaç: *mikroRNA'lar (miRNA'lar) gen regülasyonunda önemli bir rol oynar ve farklılaşma, proliferasyon ve apoptoz gibi çeşitli hücresel süreçleri etkiler. Bu çalışmada, malign melanom kök hücrelerinde (CSC'ler) ve kök hücre olmayan hücrelerde (NSC'ler) üç spesifik miRNA'nın (Hsa-miR-21-5p, Hsa-miR-9-5p ve Hsa-miR-200a-5p) ekspresyonu araştırılmıştır.*

Gereç ve Yöntem: *Malign melanoma hücre hattı olan CHL-1 hücrelerinden CSC ve NCSC hücreleri CD133 belirteci baz alınarak elde edilmiştir. CD133+ hücreler HIF1a, KLF4, ve SHH siRNA ile muamele edilerek üç farklı miRNA ekspresyon seviyesi gruplar arası karşılaştırılmıştır.*

Bulgular: *Bulgularımız Hsa-miR-200a-5p ekspresyonunun her iki hücre grubunda da benzer olduğunu ortaya koymuştur. Buna karşılık, Hsa-miR-21-5p ve Hsa-miR-9-5p, NCSC hücrelerinde önemli ölçüde yüksek ifade edilmiştir. Daha ileri analizler KLF4'ün susturulmasının bu miRNA'ların ifade düzeylerini önemli ölçüde etkilemediğini göstermiştir.*

Corresponding author: Berrin Ozdil
Department of Histology and Embryology, Faculty of Medicine, Suleyman Demirel University, Isparta, Türkiye
E-mail: *ozdlberrin@gmail.com*
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Bununla birlikte, SHH'nin susturulması Hsa-miR-21-5p'nin önemli ölçüde düşük regülasyonu ve Hsa-miR-9-5p'nin önemli ölçüde yüksek ifadelenmesi ile sonuçlanmıştır. Ek olarak, Hif1a'nın susturulması hem Hsa-miR-21-5p hem de Hsa-miR-9-5p'nin aşağı regülasyonuna yol açmıştır.

Sonuç: *Elde edilen bulgular, farklı hücresel bağlamlarda miRNA ifadesinin karmaşık düzenleyici mekanizmalarını vurgulamakta ve spesifik gen susturmaya yanıt olarak bu miRNA'lar için potansiyel roller önermektedir.*

Anahtar Sözcükler: *Malign melanom, kanser kök hücreleri, miRNA, Hif1a, KLF4, SHH.*

INTRODUCTION

Cancer includes a wide range of diseases that is marked by the uncontrolled proliferation of abnormal cells, that can spread to other organs (metastasis), a leading cause of cancer mortality. Malignant melanoma, an aggressive skin cancer arising from melanocytes, exhibits a complex genetic landscape shaped by genetic and environmental influences. Malignant melanoma ranks as the fifth or sixth most prevalent malignancy and has the highest death rate among skin malignancies. It represents 5% and 4% of cancer cases in men and women, respectively (1) and is responsible for 75% of skin cancer-related deaths (2–4).

A particular subset of cells found in tumors with the potential to differentiate into different cell types and self-renewal is known as cancer stem cells (CSCs). These cells are believed to drive tumor initiation, growth, spread, and recurrence due to their stem cell-like characteristics (5). CSCs are marked by specific surface markers. Besides these cells regulate their signaling molecules, that they can be distinguished from the bulk tumor cells (6–8). Their resistance to conventional therapies poses a significant challenge in cancer treatment, as they can survive and regenerate the tumor even after the bulk of the tumor cells have been eradicated (9). They share similar signaling pathways with stem cells and regulate internal signaling through the differential expression of various molecules. The signaling pathways of KLF4, SHH, and Hif1a, which are more active in stem cells, are active in CSCs. However, the literature on the determination of changes related to the silencing of these gene expressions in malignant melanoma stem cells is limited. Another category of molecules that are differentially expressed by CSCs is microRNAs (miRNAs). These short RNAs regulate the expression patterns of targeted genes. miRNA expression is an emerging and increasingly significant area in cancer biology (10–15). Studies on malignant melanoma have shown that certain miRNA expressions are dramatically increased and decreased in malignant melanoma cells compared to melanocytes (16).

miR-21 is an oncogenic miRNA and is frequently upregulated by different subset of cancers, including malignant melanoma. miR-21 is involved in stimulating proliferation, invasion, and avoidance of apoptosis (17). Additionaly, miR-21 has been assocciated with the metastatic behaviour of the melanoma (18). miR-9 has shown in the regulation of cancer progression in addition to neural development. miR-9 modulates gene regulations related with cell adhesion and migration. In different cancer types, miR-9 affects the progression of the cancer differently. In breast cancer, miR-9 is correlated with metastasis, whereas in ovarian, gastric adenocarcinoma and medullablastoma it shows the opposite effect (12). A study on malignant melanoma tissue and cell culture reported miR-9 downregulation in cancerous cells compared to normal cells and miR-9 directly targets NRP1 (12, 19). Another miRNA family is miR-200, known to suppress epithelial-mesenchymal transition (EMT), an important process in cancer metastasis. miR-200 targets ZEB1 and ZEB2 transcription factors that promote EMT (20). The miR-200 family members are dysregulated in cancer tissue (21). Compared to healthy cells, miR-200 is differentially expressed in various cancer types; specifically, in melanoma, cancer cells exhibit lower expression of miR-200 than healthy cells (21, 22).

The tumor microenvironment is another factor affecting tumor characteristics; extracellular matrix molecules are responsible for tumor progression. Therefore, it is extremely important to establish the tumor microenvironment to create specific target therapies. To increase the metastatic capacity, melanoma cells manipulate the extracellular matrix and secrete extracellular factors for this purpose (23). Each change or modification in the extracellular matrix affects

cancer cell behavior and has to be examined (24).

Here, we examined the differential roles of specific miRNAs in regulating gene expression in malignant melanoma CSCs and NCSCs, particularly in response to the silencing of key genes such as Hif1a, KLF4, and SHH, thereby contributing to the development of targeted therapies that could potentially disrupt the CSC-driven tumor progression.

MATERIALS AND METHODS

Cell Culture

Non-pigmented human melanoma cell lines, specifically CHL-1 (ATCC® CRL-9446TM), were maintained in EMEM (Eagle's Minimum Essential Medium) (Biowest L0416) with 10% fetal bovine serum (Biowest, S1810). The cells were cultured in a humidified incubator set to 37°C with 5% CO_2. For flow cytometry sorting, cells between passages 6 and 8 were used to preserve experimental reliability and integrity. Regular validation of cell line identity and mycoplasma contamination testing were performed.

Flow Cytometry

Cells were detached from the flask surface using trypsinization, a common enzymatic technique to release adherent cells. Post-detachment, the harvested cells were then resuspended to a concentration of 10^6 cells/ml in 10 ml of cold 1X PBS. Subsequently, the cells were incubated with 10 µL of CD133 phycoerythrin (PE)-conjugated antibody (Miltenyi Biotec Ltd. 130-113-186) and 10 µL of DAPI for 15 minutes at 4°C. After the incubation, the cells were washed with 1X PBS containing 1% dialyzed fetal bovine serum (FBS). Control samples were stained with DAPI alone, without any antibodies. Cell sorting was then carried out using BD FACS Diva 8.0. The sorted cells labeled as CD133+ were identified as CSCs, while CD133- cells were classified as NCSCs. Within the malignant melanoma cell population, the CD133+ subset ranged from 0.1% to 0.4%. CD133+ cells, ranging from passage 2 to passage 4, were used in subsequent experiments. Post-sorting and experimental procedures, cell counting was performed using the Muse® Cell Analyzer, an automated system for cell counting and analysis.

siRNA treatment

To achieve the silencing of Hif1a, KLF4, and SHH genes, CD133+ malignant melanoma CSCs were transfected with varying concentrations (0-200 nM) of siRNA (On-Targetplus Human siRNA, Smartpool, L-005089-00-0005, L-004018-00-0005, L-006036-00-0005 Horizon). The transfection process was carried out to determine the optimal siRNA dosage, which was subsequently validated by RT-PCR for validation. A fold-change cut-off of 2 was established, with changes below this threshold considered negligible. In the experiment, siRNA concentrations ranging from 0-200 nM were utilized. Specifically, the selected doses were 5 nM for Hif1a and KLF4 siRNAs, and 25 nM for SHH siRNA and negative control siRNA (25,26).

RT-PCR

miRNA isolation was performed on cells treated with siRNA and control cells using the miRNeasy Kit 96 kit (Qiagen, 217061). The cells were seeded for 24 hours post-siRNA on Matrigel Basement Membrane Matrix (Corning) at a concentration of 10^6 cells/ml. They were washed with 1X PBS, detached using StemPro™ Accutase™ Cell Dissociation Reagent (Thermo Fisher), and then resuspended in RNA buffer. After that following steps were performed according to the isolation kit (Qiagen, 217061). The quantity and purity of the isolated miRNAs were measured by Nanodrop spectrophotometer (MaestroGen). Ideal RNA purity was indicated by A230/A260 and A260/A280 absorbance ratios of 1.9-2.1. The eluted miRNAs were stored at -80°C. cDNA synthesis was operated using the microScript microRNA cDNA synthesis kit (Norgen, 54410) with miRNAs of suitable quantity and purity (27). The analysis of Hsa-miR-21-5p, Hsa-miR-9-5p, and Hsa-miR-200a-5p with oncogenic or tumor-suppressor roles in malign melanoma stem cells was performed using RT-PCR by LightCycler® 480 SYBR Green I Master (Roche, 4707516001).

Statistical Analysis

The statistical analysis of expressed miRNAs was conducted in three replicates. miRNA expressions were computed as $\text{relative gene expression} = 2-\Delta\Delta CT$, where $\Delta\Delta CT = [(\text{CT gene} - \text{CT cel} - \text{miR} - \text{u6})\text{treated} - (\text{CT gene} - \text{CT cel} - \text{miR} - \text{u6})]\text{control}$. The relative gene expression values were listed in Table 1. The housekeeping miRNA U6 served as the reference gene in the data analysis. The quantity of the expression was calculated by fold change and fold regulation. Here, we compared CD133+ cell group with other experimental

groups so fold change was calculated as ($Fold\ change = \frac{Expression\ level\ of\ experimental\ group}{Expression\ level\ of\ control\ group}$) where CD133+ cell group is control. Fold regulation was calculated with $d\ Regulation = -\frac{1}{Fold\ change}$. The fold change and fold regulation values of the groups were listed in Table-2.

RESULTS

CD133+ cells were utilized as a CSC group after CHL-1 cells were sorted using the CD133 marker. The CD133+ cells were subjected to Hif1a, KLF4 and SHH separately examining the Hsa-mir-21-5p, Hsa-mir-9-5p and Hsa-mir-200-5p expression. In order to find out relative gene expression $2^{(-Avg.(Delta(Ct))}$ values were calculated (Table-1).

Table-1. Relative expression of the miRNAs calculated by $2^{(-Avg.(Delta(Ct))}$ method.

Symbol	$2^{(-Avg.(Delta(Ct))}$					
	CD133+	CD133-	CD133+/Hif1a-	CD133+/KLF4-	CD133+/SHH-	CD133+/negc
u6	1.000000	1.000000	1.000000	1.000000	1.000000	1.000000
Hsa-mir-21-5p	0.000907	0.002312	0.000350	0.001420	0.000168	0.000833
Hsa-mir-9-5p	0.006287	0.023793	0.001460	0.003016	0.047476	0.003023
Hsa--mir-200a-5p	0.000577	0.000618	0.000547	0.001026	0.000231	0.000410

Table-2. Fold regulation and fold change of the experimental groups' values. As a reference, the fold change and fold regulation values were calculated according to CD133+ group. CD133+ cell group was the control group of siRNA treated and CD133- groups and CD133+/negc group was the negative siRNA treated group.

	CD133+		CD133-		CD133+/Hif1a-		CD133+/KLF4-		CD133+/SHH-		CD133+/negc	
Symbol	Fold Change	Fold Regulation	Fold Change	Fold Regulation	Fold Change	Fold Regulation	Fold Change	Fold Regulation	Fold Change	Fold Regulation	Fold Change	Fold Regulation
u6	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Hsa-mir-21-5p	1.00	1.00	**2.55**	**2.55**	**0.39**	**-2.59**	1.57	1.57	**0.18**	**-5.41**	0.92	-1.09
Hsa-mir-9-5p	1.00	1.00	**3.78**	**3.78**	**0.23**	**-4.31**	**0.48**	**-2.08**	**7.55**	**7.55**	**0.48**	**-2.08**
Hsa--mir-200a-5p	1.00	1.00	1.07	1.07	0.95	-1.05	1.78	1.78	**0.40**	**-2.50**	0.71	-1.41

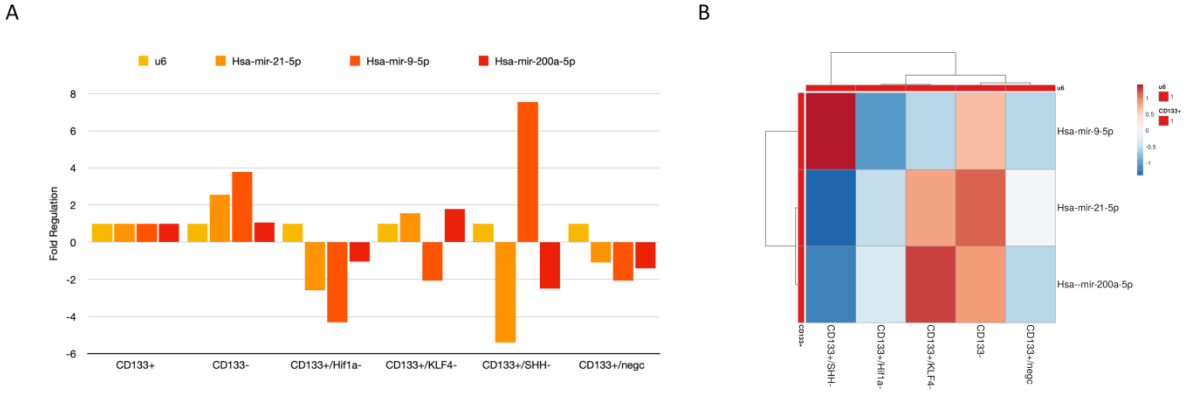

Figure-1. Fold regulation values of the experimental groups were shown in A) bar graph and B) heatmap generated by Clustvis.

The expression of four miRNAs, one of which was control miRNA (u6), was examined for expression analysis. These miRNAs were Hsa-miR-21-5p, Hsa-miR-9-5p, and Hsa-miR-200a-5p. The expression of Hsa-miR-200a-5p was similar in CD133+ and CD133- cell groups (1.07x), whereas Hsa-miR-21-5p and Hsa-miR-9-5p expression were upregulated in CD133- cells (2.55x and 3.78x, respectively) (Table-2). The expression of all three miRNAs did not significantly change in the KLF4 siRNA treated group. While Hsa-miR-21-5p is negatively regulated (-5.41x) in SHH siRNA application, Hsa-miR-9-5p is positively regulated (7.55x). Hsa-miR-21-5p and Hsa-miR-9-5p were both negatively regulated (-2.59x and -4.31x, respectively) in the Hif1a siRNA group. Fold regulation graph and heatmap were illustrated in Figure-1. The heatmap was generated by Clustvis (28).

DISCUSSION

Malignant melanoma is an aggressive and lethal type of skin cancer that originated from melanocytes. Melanoma accounts for fewer than 5% of all skin cancer cases, but due to its high metastatic potential and resistance to conventional treatments, it is the primary cause of skin cancer-related mortality. A promising area of research for addressing malignant melanoma from an epigenetic perspective focuses on miRNAs. These small non-coding RNA molecules control gene expression post-transcriptionally, influencing epigenetic modifications that can affect cancer progression or repression (29–31). Altered expression of miRNAs can contribute to various hallmarks of cancer, including uncontrolled cell proliferation, evasion of growth suppressors, resistance to apoptosis, and enhanced metastatic capability (32). In a review article, it was reported that the characteristics of initiating primary tumors are found only in melanoma cell subgroups characterized by CD133 expression, while CD133- melanoma cells do not possess tumorigenic properties (33). Another study showed that both CD133+ and CD133- cell groups have potential to form colonies, but CD133+ group more potent besies both could initiates tumor formation in mice (34). Additionally, a meta-analysis study demonstrated that both CD133+ and CD133- groups can form tumors; however, CD133- cells derived from patient samples did not metastasize (34). Even, the CD133 marker is utilized for the precise isolation and characterization of malignant melanoma stem cells, distinguishing them from non-stem cancer cell populations, there should be additional markers for better characterization and tracing the cell lineage.

Here, we targeted three molecules for silencing. The selection of KLF4, Hif1a, and SHH for this study is based on their significant roles in cancer biology and their potential impact on malignant melanoma stem cells. KLF4 is a critical factor in development, cellular reprogramming, and cancer. This molecule can play role as an oncogene or a tumor suppressor (35). It is involved in various signalling pathways. KLF4 expression was shown to be reduced in gastric cancer, hepatocellular carcinoma, and lung cancer, where it serves as a positive prognostic marker. On the other hand, elevated KLF4 levels are linked to poor outcomes in breast cancer, prostate cancer, colorectal cancer, and skin squamous cell carcinoma, indicating a potential oncogenic role (36). Hif1a upregulation has been implicated in the aggressive phenotypes and worse survival rates observed in various cancers. While a higher level of Hif1a is linked with elevated tumor suppressive signs such as apoptosis and anti-tumor inflammation, it shows a stronger connection with immune-response signs, highlighting its dual role in cancer development (37). The SHH signaling pathway is crucial in the progression of various cancers, including malignant melanoma. SHH signaling has been implicated in promoting cell proliferation, survival, and metastasis by modulating the tumor microenvironment and enhancing EMT. The hedgehog signaling pathway is modulated by miRNAs. Specific miRNAs, such as miR-200 and miR-21, have been shown to influence SHH signaling in melanoma cells. For instance, miR-21 is known to enhance the expression of SHH pathway components, thereby promoting melanoma cell survival and metastasis (38).

In our study, we selected three miRNAs (mir9, mir21, and mir200) from among oncogenic and protooncogenic miRNAs. We then assessed the variations in their expression across the experimental groups. u6 was used as a control for miRNA expression assessment. While no alterations were observed in the expression of the chosen miRNAs upon KLF4 silencing, a

noteworthy outcome emerged, revealing a significant increase in mir9 expression upon the silencing of SHH. miR9 features a conserved sequence spanning from insects to humans. Investigations into neurogenesis have unveiled mir9 expression during various stages of the developmental process (39), as SHH signalling active in neurogenesis. While mir9 expression was high in gastric and neural cancers, it was found to be low in ovarian cancer (39). In addition, mir21 expression decreased with SHH silencing by 5.41x fold. High expression of SHH was associated with GBM and it was determined that the increase of its ligand, PTCH protein, created a resistance to temozolomide in GBM treatment. The GBM study additionally revealed an association between mir9 and PTCH, impacting the SHH pathway. Notably, this association was observed to be independent of the SHH expression level (40). In a study conducted in GBM CSCs, it was stated that mir9 is expressed at high levels in CSCs (41). mir200 has been indicated as a tumor suppressor in various types of cancer (21). The mir 200 family can be classified as mir200a, mir200b, mir200c. Studies comparing healthy tissue and melanoma have shown that the expression of these three mir200s is lower than in healthy tissue (21). In here only with SHH silencing CSCs lowered expression mir-200a-5p, while there was no change in Hif1a and KLF4 silencing.

CONCLUSION

This study demonstrates the differential expression of miRNAs Hsa-mir-21-5p, Hsa-mir-9-5p, and Hsa-mir-200a-5p in CD133+ and CD133- cell populations. Hsa-mir-21-5p and Hsa-mir-9-5p were significantly upregulated in CD133- cells, suggesting their potential roles in the differentiation state of these cells. The knockdown of KLF4 did not affect miRNA expression significantly, indicating that KLF4 might not be a major regulator of these miRNAs under the studied conditions. SHH siRNA treatment showed contrasting effects on Hsa-mir-21-5p and Hsa-mir-9-5p, with the former being downregulated and the latter upregulated, indicating a complex regulatory mechanism between miRNA expression. The modulation of SHH signaling affects these miRNAs in opposite directions of regulation. Hif1a silencing led to the downregulation of both Hsa-mir-21-5p and Hsa-mir-9-5p, highlighting its role in the regulation of these miRNAs. These findings provide perspective on the regulatory networks of miRNAs in various cell types especially cancer stem cells and their possible implications for cellular differentiation and gene silencing.

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