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The Role of miR-143, miR-145, and miR-590 in Expression Levels of CD44 and Vascular Endothelial Cadherin in Oral Squamous Cell Carcinoma

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is the most common head and neck cancer with a continuing rise of incidence in developing countries. Despite the improvement in the clinical outcome of OSCC, the overall 5-year survival rate of patients is still disappointing. MicroRNAs (miRNAs) regulate gene expression in the post-transcriptional stage by targeting mRNAs. Advances in knowledge of the pathogenesis and molecular events lead to the improvement of OSCC treatments.

Materials and Methods: Human oral epidermoid carcinoma cells (KB), HGF1, and HEK 293T cell line were cultured. Lentiviral vectors were constructed and Western Blot analysis was performed. Then, immunocytochemistry staining was performed by using CD44 and vascular endothelial cadherin (VE-Cadherin) antibodies. All data were analyzed using the REST 2009 software. *P* values of ≤0.05 were considered statistically significant.

Results: MiR-143, miR-145, and miR 590 were down-regulated in the oral cancer cell line. Following transfection of Lv miR 143, Lv-miR-145, and Lv-miR-590, the expression of CD44 was markedly decreased in KB cells. In addition, transfection of miR-143 and miR-590 mimics into the oral cancer cell line significantly decreased the expresion level of VE-Cadherin; however, transfection of miR-145 mimic had no significant effect on the expression of VE-Cadherin. Western blot analysis and immunocytochemistry staining confirmed the results.

Conclusion: This study revealed that miR-143, miR-145, and miR-590 negatively regulate CD44 and VE-Cadherin (except miR-145) expression, which might play a crucial role in the induction of cancer stem cells proliferation and angiogenesis in OSCC cells. The knowledge about the involved factors may provide new insights for the clinical use of miR-143, mR-145, and miR-590 in the treatment of patients with OSCC.

Keywords: Cadherin 5, CD44, microRNAs, Mouth, Neoplasms

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Introduction

Histologically, head and neck squamous cell carcinoma (HNSCC) is a heterogeneous tumor composed

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of epithelial cells, stromal cells, and inflammatory cells.[1,2] Oral squamous cell carcinoma (OSCC) is the most common head and neck cancer, with a continuous rise of incidence in developing countries.[2] Despite the improvement in the clinical outcome of OSCC, the overall 5-year survival rate of patients is still disappointing. Metastasis is the main cause of death in OSCC patients.[3] The obtained results reflect the limited advances in the knowledge of the pathogenesis and the molecular events contributing to the OSCC development. Hence, every discovery in the molecular mechanisms of oral cancer results in the developing of advanced therapeutic approaches.[4] MicroRNAs (miRNAs) are endogenous small non-coding RNAs that regulate gene expression in the post-transcriptional stage by interacting with the 3' untranslated region (3' UTR) of the target mRNA. In cancers, multiple miRNAs play essential roles in proliferation, differentiation, apoptosis, survival, motility, invasion, and metastasis via regulating the expression level of their target genes.[3] Previous studies have demonstrated the involvement of miRNAs in the pathogenesis of OSCC.[5]

CD44, a cell-surface glycoprotein, involves cell-cell interactions, cell migration, and adhesion. Therefore, it plays an essential role in tumorigenesis and metastasis.[6,7] Furthermore, CD44 has been described as a cancer stem cells (CSCs) marker in different cancers including HNSCC. CSCs have a great impact on the pathogenesis of HNSCC.[8] The increased expression level of CD44 is associated with more aggressive tumor behavior and poor patient prognosis.[2,7] The tumor suppressor miRNAs target CD44 to inhibit tumor growth and metastasis. For instance, miR-34a, a tumor suppressor miRNA, has been used to target CD44 in renal cancer cells to suppress renal cancer cell growth, tube formation, and metastasis through in vitro and in vivo studies.[9] Moreover, oncomiRs such as miR-221 are positively associated with the CD44 expression level in hepatocellular carcinoma.[10] As another regulator, miR-720 induces epithelial-mesenchymal transition (EMT) phenomenon in renal cell carcinoma through up-regulation of CD44.[11] Therefore, CD44 contributes to EMT in different cancers.[12] EMT is a master switch for cancer metastasis including OSCC.[2,13]

Angiogenesis is also a master key in the development of metastasis not only because of supplying the nutrition but also because of providing some pathways for cancer cells to travel to other organs to form new tumors. VE-Cadherin (Cadherin 5) is an adhesive protein expressed by both tumor cells and endothelial cells. Its elevated expression has been shown during neovascularization and tumor growth.[14] Tumor suppressor miRNAs also target VE-cadherin in cancer cells to inhibit tumor cell-mediated vasculogenic mimicry (VM), vascular-like channels lined by tumor cells, instead of endothelial cells. One of these miRNAs is miR-27b that is negatively associated with VE-Cadherin expression in ovarian cancer cells and tumor cell-mediated VM. Forced expression of miR-27b in aggressive ovarian cancer cell line significantly decreases VE-Cadherin expression level.[15] MiR-143 and miR-145 are two miRNAs that are located within the same cluster without sharing any sequence homology.[16] The decreased expression levels of miR-143 and miR-145 have been reported in several cancers such as gastric, ovarian, and pancreatic cancer.[17-20] The reduced expression level of miR-143 is associated with pancreatic cancer aggression and metastasis.[21] According to a published work, miR-143 down-regulates in cancer tissues and cell line. In addition, transfection of miR-143 mimic leads to suppression of non-small cell lung cancer cell proliferation, induction of apoptosis, and inhibition of migration and invasion.[22] A previous study showed that miR-143 inhibits tumor cell invasion in prostate cancer.[23] It has been reported that miR-145 inhibits tumor progression in some cancers such as gliomas,[24] gallbladder cancer,[25] and OSCC.[4,26] A decreased miR-145 level in prostate cancer samples is correlated with advanced clinical stage.[27] In cervical cancer patients, the low miR-145 expression level was

associated with poor prognosis and lymph node metastasis.[28] Different genes and signaling pathways are the potential targets of miR-143 and miR-145. For example, miR-143 inhibits cell growth by targeting ERK5 and MAP3K7 in breast cancer.[29] Also, miR-145 negatively regulates some oncogenes such as Myc, K-Ras, IRS-1, ERK5, and some cell surface molecules like junctional cell adhesion molecule (JAM-A), fascin, and MUC1 to suppress cancer cell motility and invasiveness.[30] The function of miR-590 in cancers is controversial. While it acts as an oncogene in cervical cancer,[31] it functions as a tumor suppressor in colorectal cancer (CRC).[32] Some targets of miR-590 have been identified. For example, in breast cancer, miR-590-5p inhibits CSCs stemness and metastasis through SOX2[33] and in CRC miR-590 inhibits angiogenesis and metastasis via vascular endothelial growth factor-A (VEGF-A).[32] Different genes targeted by the aforementioned miRNAs have been studied. Due to the high mortality rate in OSCC patients, identifying miRNAs target genes provides information for the development of new drugs. Therefore, this study was conducted to investigate the miRNA-143, miRNA-145, and miRNA -590 target genes involved in the development of OSCC.

Materials and Methods

1. Cell culture

Human oral epidermoid carcinoma cells (KB) and human normal gingival fibroblast-like cells (HGF1) were purchased from Pasteur Institute, Tehran, Iran. HEK 293T cell line was obtained from the Stem Cell Technology Research Center Tehran, Iran. KB and HGF1 cell lines were seeded in RPMI 1640 containing 10% fetal bovine serum (FBS) and antibiotics at 37℃ in the 5% CO_2 air atmosphere. HEK 293T cell line was seeded in DMEM containing 10% FBS and antibiotics at 37℃ in a 5% CO_2 air atmosphere.

2. RNA isolation and quantitative real-time PCR analysis

The expression levels of miR-143, miR-145, and miR-590 were assessed in KB cells using qRT-PCR. Briefly, 1 µg of total RNA was converted into specific cDNA derived from mature microRNAs using TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, USA) and quantified in triplicate using the TaqMan microRNA assay. SNORD47 was used as endogenous control. Data were quantified and analyzed using sequence detection system v. 2.3 (Applied Biosystems, USA). The microRNA relative expression in cancer cells was normalized against normal oral gingival cell line. PCR primers used in this study were as follows: miR-143

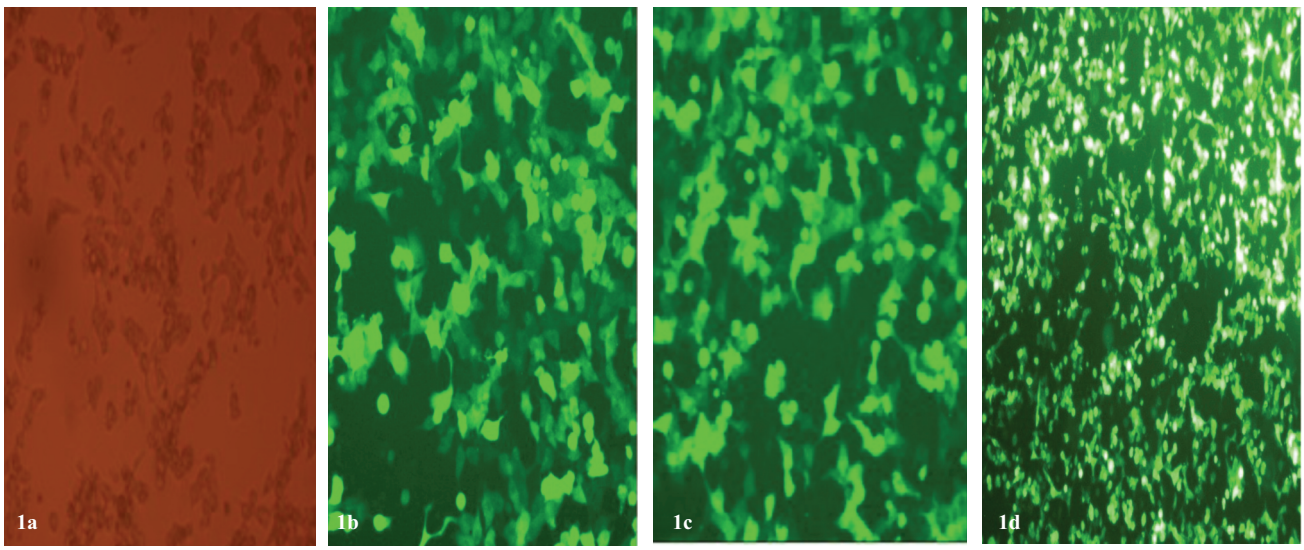

Figure 1. (a-d). GFP expression indicating successful transfection of KB cell line using pLEX.ired recombinant vector resulting in miR-143 (b), miR-145 (c), and miR-590 (d) over-expression compared to non-transfected KB cells (a).

forward: 5′-actgttgagatgaagcactgt-3′; miR-145 forward: 5′-ggtccagttccagga-3′; miR-590 forward: 5'-ggcgagcttattcataaaat-3'; CD44 forward 5′ caacagtcgaagaaggtgtg 3′, reverse 5 ′ tggtctg-gagtttctgacg 3′; VE-Cadherin forward 5′ agccctaccagcccaa 3′, reverse: gccgtgttatcgtga ttatc; and β2-Microglobulin levels, which were used as normalization controls with forward primer: 5′atgcctgccgtgtgaac3′ and reverse primer: 5′atcttcaaacctccatgatg 3′.

3. Lentiviral construction and Lentiviral-mediated overexpression of studied miRNAs

A genomic fragment spanning the miR-143, miR-145, and miR-590 coding regions were amplified by PCR and cloned into the Xho I/Mlu I restriction site of a mammalian expression vector pLEX.jred downstream of the cytomegalovirus (CMV) promoter. For control, the empty vector without any cloned sequence (pLEX-Ctrl) was used. Lentiviral particles were generated by transient calcium phosphate cotransfection of HEK293T cells with lentiviral vector DNA (or pLEX backbone). Lentiviral supernatants were harvested two or three times, every 12 h and concentrated by ultracentrifuge at 47,000×g for 2 h at 4℃. Lentivirus titer was determined by flow cytometry analysis of j.RED positive HCT 293T cells. After 48 hours, lentivirus in the supernatant was transduced into KB cells. All lentiviral vectors expressed increased green fluorescent protein (GFP); hence, they were allowed for measuring the virus titer and their infection efficiency in the infected cells. The number of fluorescent cells in each well was determined by fluorescent microscopy.

4. Western Blot analysis

Cells were lysed using cell lysis buffer (50Mm Tris, pH= 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM EDTA). Later, 70 µg of protein was separated electrophoretically in 12% SDS polyacrylamide gel, transferred into poly vinyl den fluoride membrane, and immersed in 5% non-fat milk powder over 1h

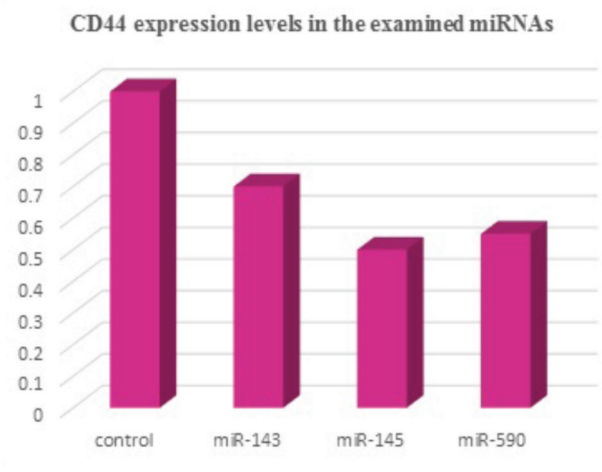

chart 2a

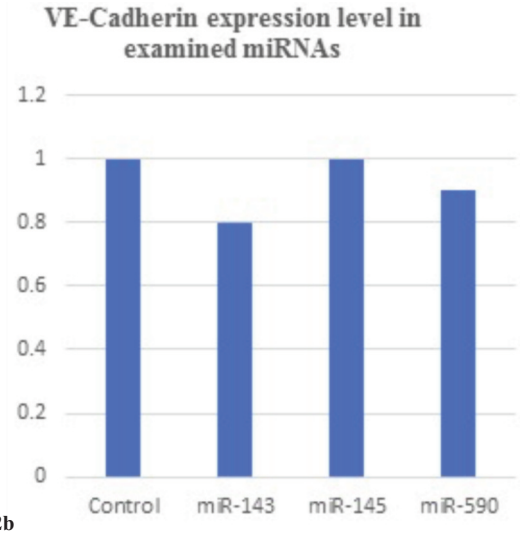

chart 2b

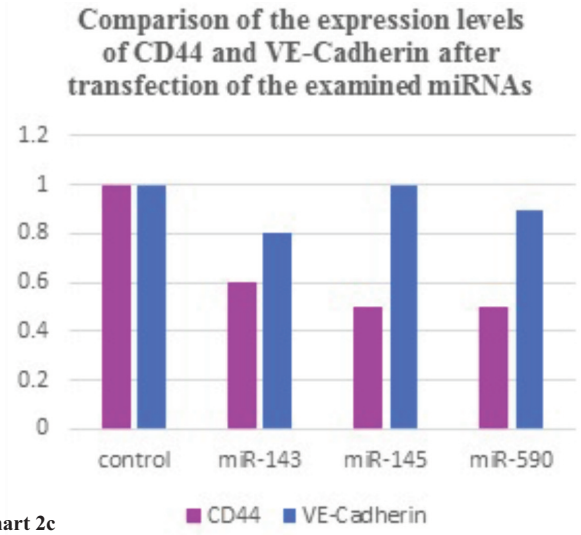

chart 2c

Figure 2. (a-c). RT-qPCR results show the effect of miR-143, miR-145, and miR-590 transfection on CD44 and VE-Cadherin expression levels and the comparison diagram (*$P < 0.05$ vs. Control).

at room temperature. After completion of the transfer, the membranes were incubated with primary antibodies for CD44 (1:200; Thermo Scientific, Std./HCAM Ab 4), Polyclonal anti-rabbit VE-cadherin antibody (1:170; Abcam; 33168) and β-actin (1:170; Abcam; 3257), and horseradish peroxidase-conjugated secondary antibodies (1:1,000, Abcam). Chemiluminescence analysis system was used for antigen-antibody detection.

5. Immunocytochemistry

Cells were seeded on the sterile coverslips in a 6-well tissue culture plates in a sterile tissue culture hood. Then, the cells were fixed by incubating them in 4% (v/v) paraformaldehyde in PBS for 20 minutes at room temperature. After washing three times by PBS, the cells were incubated in 10% goat serum in PBS for 1 hour. Then, the cells were incubated in primary antibodies [CD44 (1:200; Thermo Scientific, Std. /HCAM Ab 4), Polyclonal anti-rabbit VE-cadherin antibody (1:170; Abcam; 33168)], followed by secondary antibodies. In the next step, 3,3′-Diaminobenzidine (DAB) was used to visualize the complex. Then, the cells were counterstained with hematoxylin. Finally, the coverslips were picked up with a forceps and placed on the mounting medium, with the cell-side face down.

Statistical analysis

All experiments were performed at least in triplicate, presented as mean± SD, and analyzed by REST 2009. *P* values of ≤0.05 were considered statistically significant.

Results

Down-regulation of miR-143, miR-145, and miR 590 in the oral cancer cell line

Accumulating evidence indicates that miR-143, miR-145, and miR 590 were down-regulated in several cancers. Therefore, the expression levels of miR-143, miR-145, and miR 590 were examined through the RT qPCR assay. According to the findings of the present study, the expression levels of examined miRNAs were significantly decreased in the oral cancer cell line compared to the normal oral cell line.

The prediction of the examined miRNAs targeting genes

Computational algorithms, including TargetScan and microRNA.org, were used to identify potential miRNA target genes. Using

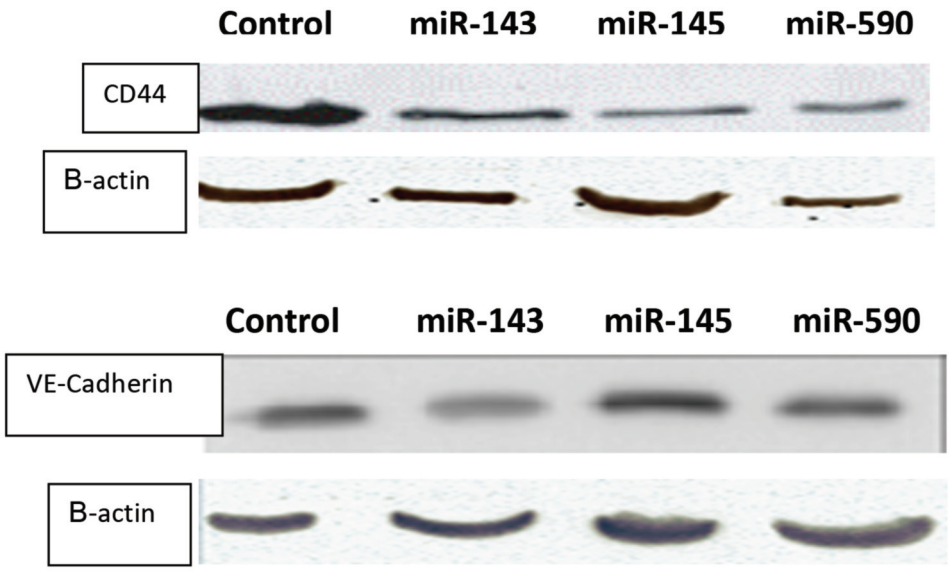

Figure 3. Representative western blot of CD44 and VE-Cadherin in KB cells transfected with miR-143, miR-145 and miR-590; β-actin was used as protein loading control.

these approaches, miR-143, miR-145, and miR-590 are candidate miRNAs for CD44; however, VE-Cadherin is a direct target of miR-143 and miR-590, not miR-145. Therefore, we planned to determine if VE-cadherin is targeted by miR-145.

Overexpression of miR-143, miR-145, and miR-590

Transfection of the pLenti-hsa-miR-143, the pLenti-hsa-miR-145, and the pLenti-hsa-miR-590 expression vectors into the KB cell line showed a minimum of 70% transfection efficiency through GFP expression visualization (Figures 1a-1d). Real-time PCR analysis confirmed the accuracy of transfection and overexpression of three examined miRNAs.

Altered expression of CD44 and VE-Cadherin by Lv miR 143, miR-145, and miR-590

Following transfection of Lv miR 143, Lv-miR-145, and Lv-miR-590, the expression of CD44 was markedly declined in KB cells. In addition, forced expression of miR-143 and miR-590 significantly decreased the expression level of VE-Cadherin; however, the forced expression of miR-145 had no significant effect on the expression level of VE-Cadherin (Figures 2a-2c).

Western Blot analysis

Western blot analysis was performed to verify CD44 as the direct target of miR-143, miR-145 and miR-590 and VE-Cadherin as the direct target of miR-143 and miR-590. Consistent with the results of RT-qPCR analysis, western blotting

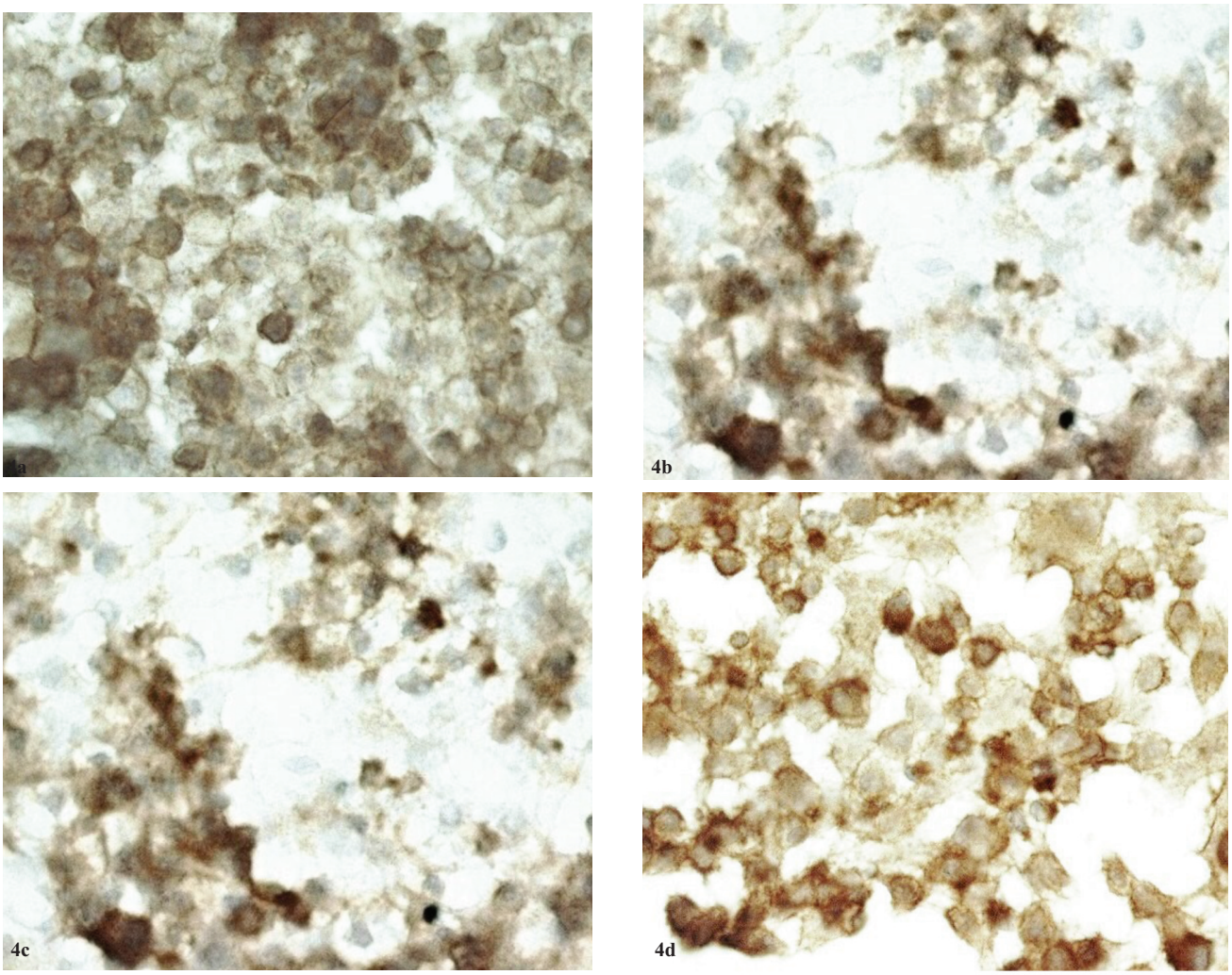

Figure 4. Immunostaining of the cells shows CD44 cell membrane staining in KB cells, miR-143, miR-145, and miR-590 transfected cells. Notice the strong staining in KB cells compared to weak staining in the miRNAs transfected cells. The number of stained cells was remarkably decreased in the transfected cells.

data further confirmed that the examined miRNAs negatively regulate the expression of CD44 while miR-143 and miR-590 negatively regulate the expression level of VE-Cadherin by targeting their 3′ untranslated region (UTR) (Figure 3).

Immunocytochemistry analysis

Cell membrane staining for both CD44 and VE-cadherin antibodies confirmed the results of qRT-PCR and Western blot analysis. Non-transfected KB cells showed a strong membrane staining compared to miRNA transfected cells, except miRNA-145 transfected cells stained with VE-Cadherin antibody that showed nearly the same staining level as non-transfected KB cells (Figures 4 and 5).

Discussion

In the present study, the effect of miR-143, miR-145, and miR-590 on CD44 and VE-Cadherin expression levels was investigated. Decreased expression levels of three examined miRNAs were found in the current study. Similar to our results, a declined expression level of miR-143 has been reported in colon cancer and prostate cancer.[34,35] In addition, down-regulation of miR-145 has been noticed in some cancer cell lines and cancers such as head and neck cancer,[36] breast cancer,[37] and lung carcinoma.[38] Down-regulation of miR-143 and miR-145 was indicated in a previous study on OSCC cell line.[39] Reduced level of miR-590 was also identified in colorectal cancer.[32] All these findings may prove the role of

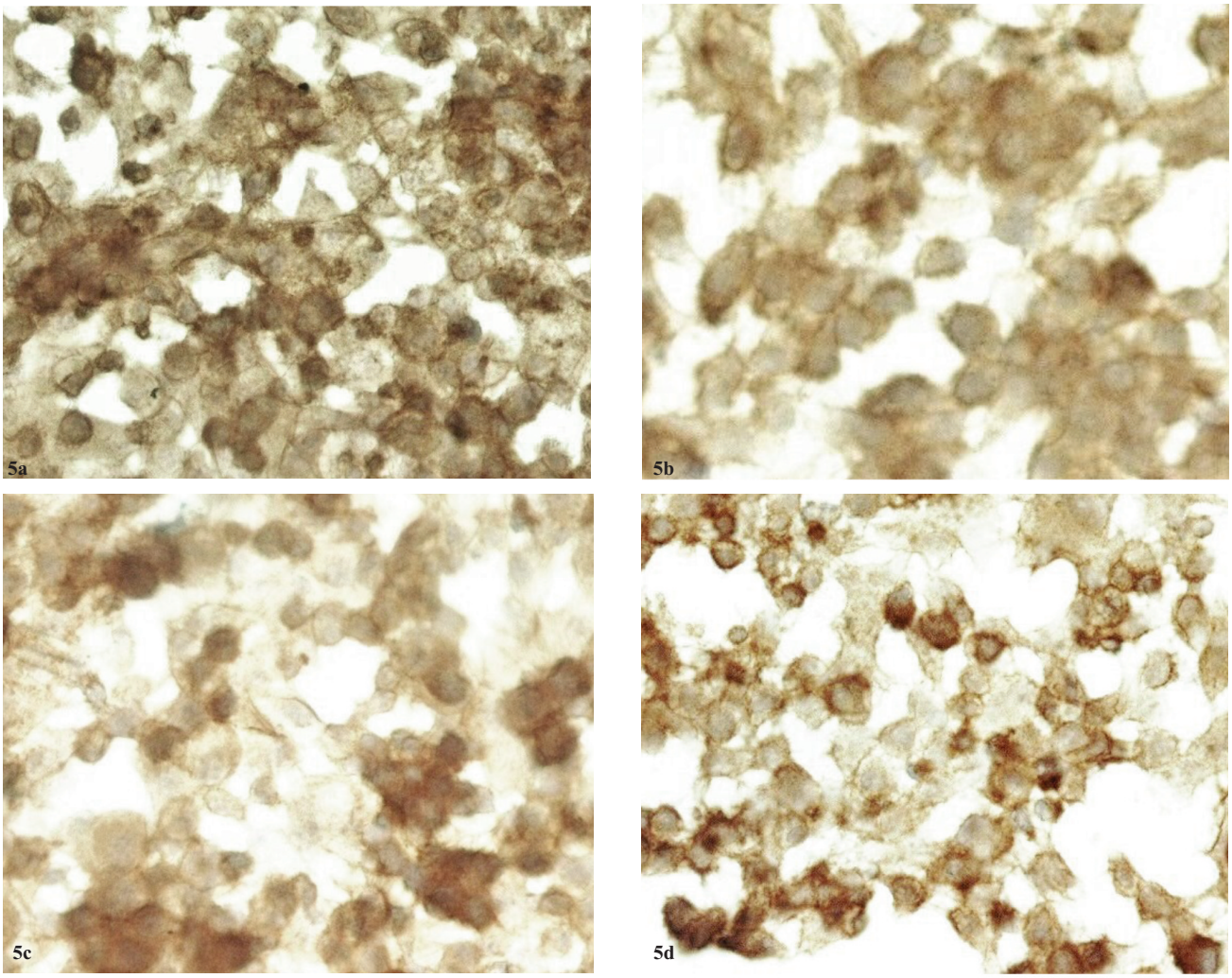

Figure 5. Immunostaining of the cells indicates VE-Cadherin cell membrane staining in KB cells, miR-143, miR-145, and miR-590 transfected cells. The strong staining is visualized in KB cells and miR-145 transfected cells. The miR-143 and miR-590 transfected stained cells show a weaker staining. Also, the number of stained cells was significantly decreased in miR-143 and miR-590 transfected stained cells.

the examined miRNAs as the tumor suppressors in different cancers. In breast cancer, low miR-143 expression was negatively correlated with tumor size and lymph node metastasis.[40] In melanoma, ectopic expression of miR-143 inhibited cancer cell proliferation and invasion by targeting cyclooxygenase-2 (COX-2).[17] Also, miR-143 inhibited angiogenesis in lung cancer cells by targeting VEGF.[18] VEGF is the most studied angiogenic growth factor and is the main target of different genes and miRNAs. In prostate cancer samples, the decreased expression of miR-145 was correlated with advanced clinical stage.[27] In cervical cancer patients, low expression of miR-145 was associated with poor prognosis and lymph node metastasis.[28] The lower expression level of miR-590-5p in colon cancer was associated with larger tumor size or lymph node metastasis.[41] In a published work on hepatocellular carcinoma, decreased expression of miR-590-5p inhibited cancer cell growth through Wnt pathway;[42] however, another study on hepatocellular carcinoma demonstrated that over-expression of miR-590-5p promoted cancer cell proliferation and invasion by targeting TGF-β RII.[43]

In the current study, we also confirmed an inverse correlation between the expressions of three examined miRNAs and CD44 in the KB cell line. These findings may suggest that miR-143, miR-145, and miR-590 play a critical role in 'stemness' of oral cancer cells and EMT phenomenon which enhance tumor growth and metastasis. Metastasis is the main reason for cancer mortality.[3] Accumulating evidence indicates that CD44 promotes the angiogenesis and invasion of tumor cells.[16] A previously published work indicated that miR-143 and miR-145 inhibit the expression of CSC markers and stemness factors including CD44 in prostate cancer cells.[44] Another published investigation has shown that miR-145 regulates the expression of CD44 in gastric cancer, which is correlated to cancer cell self-renewal properties and improvement of chemo-sensitivity.[45] Moreover, in oral cancer, miR-145 controls the stemness of cancer cells via 5-aminolevulinic acid.[32] A published study on gastric cancer revealed that miR-143 and miR-145 inhibit EMT by suppressing MYO6.[46] In addition, miR-590-5p inhibits stemness and metastasis in breast cancer by targeting SOX2.[33] Some other miRNAs are also involved in CSCs characteristics and metastasis via different signaling pathways. For example, miR-1 can repress CSCs properties in breast cancer cells by targeting the Wnt/β-catenin pathway.[47] According to a previous study on bladder cancer, decreased expression of microRNA-34a functions as an anti-metastatic microRNA and suppresses angiogenesis by targeting CD44.[16] The role of miR-590-3p has been shown in regulating the EMT phenomenon through increasing E-cadherin expression and decreasing N-cadherin and Vimentin expression levels in glioma and glioblastoma multiform.[48] In addition, miR-590-3p also suppresses cancer cells migration, invasion, and EMT in glioblastoma multiforme by targeting ZEB1/2.[49] The current investigation may suggest CD44 as another target to promote EMT by miR-590. Altogether, these findings suggest that CD44 may be a promising therapeutic target in cancers.

In addition, in the present study, an inverse correlation was found between miR-143 and miR-590 mimic transfection and VE-Cadherin expression level. The altered expression level of VE-Cadherin by miR-143 and miR-590 might suggest the critical role of the aforementioned miRNAs in tumor angiogenesis and metastasis. MiR-143 also suppresses angiogenesis in CRC by targeting VEGF and HIF-1-α.[50] Although our study did not find any change in the expression level of VE-Cadherin after transfection of miR-145 mimic a previous study on osteosarcoma cell line has revealed that miR-145 inhibits tumor cell invasion and metastasis via targeting VEGF, a master key of angiogenesis.[51] The miR-145 also has an inhibitory role in breast cancer by targeting N-RAS and VEGF-A.[52] A published study on lung cancer demonstrated that stromal miR-143/145 expression promotes neoangiogenesis.[53] Additionally, miR-590 inhibits angiogenesis and metastasis in CRC by targeting nuclear factor 90

(NF90)/vascular endothelial growth factor A (VEGF-A) axis.[41] Besides, miR-590-5p inhibits angiogenesis in CRC by targeting CD31.[41] Previous work on hepatocellular carcinoma indicated the VM formation due to the down-regulation of E-cadherin and up-regulation of VE-Cadherin. Furthermore, this study demonstrated that ectopic expression of miR-27a-3p suppressed VE-Cadherin expression in HCC cells. In addition, a previous study on breast cancer revealed that VE-Cadherin enhances cancer cell proliferation and invasion via TGF-β signaling.[54] Taken together, our results along with previous findings propose that miR-143, miR-145, and miR-590 inhibit CSCs, EMT, angiogenesis, and metastasis in OSCC by targeting VE-Cadherin and CD44.

Conclusion

miRNAs are the major regulators of gene expression in cancers, which are involved in controlling of different steps of tumor progression. This study revealed that miR-143, miR-145, and miR-590 negatively regulate CD44 and VE-Cadherin expression (except miR-145), which may alter the tumorigenic characteristics of CSCs and induce angiogenesis in OSCC cells. The knowledge about the involved factors may provide new insights into the clinical use of miR-143, mR-145, and miR-590 in patients with OSCC. Moreover, the altered expression levels of examined miRNAs can be used as diagnostic biomarkers for OSCC. Several factors and genes are involved in the development of oral cancer.[70-72] Identification of genes and molecules associated with oral cancer progression and metastasis provides our understanding of biology of cancer.[73] Gaining new knowledge improves the early detection, diagnosis, treatment, and survival rate of oral cancer.[74]

Acknowledgment

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Conflict of Interest

None declared.

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