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Research Article

In vitro Characterization of Mir-203 and Mir-363-3p as Potential Biomarkers in Laryngeal Cancer Cells

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Abstract

Laryngeal cancer (LCa) is the most common and aggressive type of head and neck region cancers. The molecular understanding of Laryngeal cancer has been gaining of interest in recent years. Timely diagnosis is crucial for effective treatment and positive prognosis. The early diagnosis may increase the treatment success at the molecular level. Existing evidence on the association of microRNAs (miRNAs) has provided the chance to further analyze the possibilities of the miRNAs to be applied in early diagnosis and further therapies. Our study aims to show functional analysis of miR-26b, miR-200c-3p, miR-203, miR-363-3p, and miR-1825, which were already identified in our previous microarray studies that could be associated with squamous cell laryngeal cancer. In this study, human epithelial type 2 (Hep-2) cell lines, which are from human laryngeal carcinoma, were transfected with the targeted miRNAs and were throughly observed and analyzed for the alteration in cell proliferation, migration, and invasion using soft agar assays. The result of the experiments detected an inverse association between the decline in miR-203 and miR363-3p and rapid proliferation of Hep-2 cells. The findings indicated that miR-203 and miR-363-3p act as tumor suppressor and slow down proliferation, migration, invasion, and tumorigenesis. Based on the findings on this study, these miRNAs could be considered as promising candidates for being diagnostic biomarkers in laryngeal cancer.

Keywords: Laryngeal Cancer; miR-203; miR363-3p; Hep-2

Introduction

Laryngeal cancer (LCa) is one of the most common head and neck malignancies with the prevalence of 1.1% of all cancer cases. The mortality rate of LCa is 1.1 of all cancer deaths [1]. Histological analysis of LCa has shown approximately 90% of the cancer is squamous cell carcinoma [2,3] which is likely because of lymph node metastasis that causing almost no improvement in the survival rate of the patients [4,5]. Some lifestyles such as smoking and an exorbitant amount of alcohol consumption are some of the risk factors for developing LCa [6,7]. Men are 7-8 times more prone to manifest laryngeal squamous cell carcinoma (LSCC) that accounts for nearly 2% of human malignancies and 90% of all malignant laryngeal tumors [8]. Although various treatment techniques such as partial and total laryngectomy, chemotherapy

and radiation therapy are commonly applied [9,10] there has not been a significant change in mortality rate. This raises question whether genetic factors such as the onset of oncogenes and offset of tumor suppressor genes could be the causative factor for nearly ineffective traditional therapies, since the LCa recurrence after laryngectomy has been observed even after complete removal of the laryngeal [8,9]. Therefore, the identification of genetic factors is essential in designing effective therapy to reduce the recurrence rate of LCa.

MicroRNAs, miRNAs, are class of small non-coding RNAs that post-translationally regulate gene expression through binding to 3'UTR region of target mRNA [11-13]. Studies prove the association between miRNAs functions and their occurrence at different cancers [12,13]. However, the roles of miRNAs in the laryngeal carcinogenesis have not been fully explained and therefore, further studies are needed [13,14]. Years of thorough studies in various types of cancers have proven the miRNAs to be the promising biomarkers to detect the malignancies in specific cancer-related tissues. Screening analysis of laryngeal squamous cell carcinoma tissues and adjacent normal tissues revealed the upregulation of miR-21, miR-93, miR-205, miR-708 and downregulation of miR-125b and miR-145 [15] while some can contribute to tumorigenesis of LCa. Lu *et.al* showed the overexpression of miR-9 that caused dysregulation of PTEN, which led to the increased malignancy of LCa [16]. According to another study, miR-10b was shown to effect on invasion and migration through regulation of EMT in LCa cells. Up-regulation of miR-10b was shown to speed up the epithelial-mesenchymal transition (EMT)-mediated invasion and migration in Hep-2 by enhancement of the N-cadherin versus reduction of E-cadherin [17]. Furthermore, the miRNAs are deeply participated in the initiation and progression of several types of cancers. Altered expression of miRNAs can contribute to the malignant phenotype of LCa. For instance, miR-21 that is a well-known onco-miR was revealed as upregulated in LCa. miR-21 down-regulates the B cell translocation gene 2 (BTG2), a cell cycle regulator, that acts as a tumor suppressor. Liu et al. showed that enhanced expression of miR-21 that ultimately results in reduction of BTG2 gene causes elevated proliferation activity of Hep-2 cells and apoptosis arrest. These consequences promotes cancer invasion and metastasis [18,19]. Other miRNA that has a significant role in LCa pathogenesis is miR-205 that is found to be upregulated in laryngeal squoamous cell carcinoma (LSCC). According to a study, miR-205 suppresses the expression of cyclin-dependent kinase 2-associated protein 1 (CDK2AP1). CDK2AP1 is a growth suppressor protein leading to G1/S arrest and cell apoptosis. According to the results of the study, downregulation of CDK2AP1 via miR-205 enhances cell proliferation and invasion through reduction of MMP2/9, c-Myc and Cyclin-D expression [20]. miRNA-93 is another miRNA that take part in dysregulation of cell cycle and acceleration of cancer cell proliferation. MiR-93 over-expression in LCa tissues was shown to increase cancer progression by increasing cancer cell proliferation through suppression of cyclin G2, CCNG2 [21]. In contrast to detrimental impact of miRNAs on cancer progression and high tumorigenesis, presence of miR-203, miR-34a, miR-34b, miR-34c, miR-519b-3p and miR519a functioned as suppressors of LCa [22-24]. miR-203, miR-34a, and miR-34c down-regulate the expression surviving gene whose protein, surviving protein, suppresses cell apoptosis and speeds up cancer cell proliferation [22-24]. In addition, miR-34a and miR-34c control the expression of UDP-N-acetyl-α-dgalactosamine: polypeptide-N-acetylgalactos aminyltransferase7 (GALNT7) which is observed to be one of the causative elements in LCa metastasis [25].

A comprehensive study of the effects of cancer stem cell-specific miRNAs on laryngeal cancer cells and laryngeal cancer stem cells has not yet been conducted as far as we know. The aim of this study is to investigate the effects of miRNAs on cancer cells with oncogenic or tumor suppressor effects in cancer stem cells from squamous cell laryngeal carcinoma. In this study, human laryngeal squamous cancer cell line Hep-2 cells were treated with miR-26b, miR-200c-3p, miR-203, miR-363-3p and miR-1825 mimics to determine tumor cell proliferation, migration and invasion, and functional effects on stem cells.

Materials and Methods

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Cell culture

The Hep-2 cell lines were cultured in RPMI-1640 medium (Gibco-BRL, Bethesda, MA), supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% Penicillin Streptomycin Amphotericin

(PSA), in a 5% CO2 humidified incubator at 37 °C. The cell lines were passaged once they reached 70% confluence.

Transfection of miRNAs into Hep2 cells

miR-26b, miR-200c-3p, miR-203, miR-363-3p and miR-1825 mimics were obtained from SigmaAldrich using a control miRNA from SwitchGear Genomics. miR-203, miR-363-3p, miR-26b, miR-200c-3p and control miRNA were transfected using X-treme GENE siRNA Transfection Reagent in to the Hep-2 cell lines.

q-RT PCR

RNA isolation was performed using Trizol (Invitrogen, San Diego, CA) reagent according to the manufacturer's instructions. The transfected microRNAs were isolated with Trizol, and then their concentrations were measured using NanoDrop ND-2000c (Thermo Fisher Scientific, Inc., Wilmington, DE). miRNA cDNA synthesis was performed according to the manufacturer's instructions using the miRNA RT primers in the loop construct with "Taqman Reverse Transcriptase Kit". qRT-PCR assays were performed by using TaqMan assays. TaqMan miR-26b, miR-200c3p, miR-203, miR-363-3p, miR-1825 and RNU43 probes used as controls were purchased from Applied Biosystems (Foster City, CA). qRT-PCR procedures were performed using the LightCycler480-II real-time PCR device with the TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA) kit followed by the manufacturer's protocol.

Proliferation assay

In this study, we aimed to investigate the role of miR-203, miR-363-3p, miR-26b, miR-1825, and miR-200c-3p in Hep-2 cell proliferation. To assess their effects, a cell proliferation test was conducted using the Hep-2 cell line. The utilization of miRNAs allowed for a more effective and efficient evaluation of their impact on cell proliferation, employing the xCELLIGENCE System device. Additionally, the migration-invasion phenotype of Hep-2 cells was analyzed using the CIM plate wells of the xCELLIGENCE System, with the invasion test conducted using a Matrigel Matrix-Tris HCl coating buffer mixture. The obtained data were analyzed using the xCELLIGENCE software.

Migration and invasion assay

In order to assess the impact of miRNAs on the migration and invasion phenotype of Hep-2 cells, a migration-invasion assay was conducted using CIM plate wells of the xCELLIGENCE system. For the invasion test, Matrigel Matrix (Corning, NY, USA) in combination with Tris HCl coating buffer was utilized to create a coated surface. This coating mixture facilitates the assessment of the invasive behavior of the cells, simulating a physiological extracellular matrix environment. The xCELLIGENCE system allows for real-time monitoring and quantification of cell migration and invasion, providing valuable insights into the effects of miRNAs on these cellular processes.

Soft agar colony assay

Hep-2 cells transfected with miR-203 and miR-363-3p were harvested 24 hours post-transfection and subsequently plated onto soft agar plates, following the manual protocol. The preparation of the soft agar plates involved the utilization of 2X RPMI medium, FBS, Phosphate-Buffered Saline Agar (PSA agar), and H_2O. Afterward, the cells were fixed and stained using a solution comprising 0.01% crystal violet and 10% ethanol dissolved in distilled water. To eliminate background staining, the plates were washed three times with distilled water. Finally, the colonies were enumerated.

Statistical analysis

The log-transformed data underwent rigorous statistical analysis using the "two-sided Student's t-test". A P-value threshold of less than 0.05 was employed to establish statistical significance. To visually represent the variability, error bars were meticulously plotted by incorporating the ± standard error values.

Results

miR-203, miR-363-3p, miR-26b, miR-1825 and miR-200c-3p transfection into Hep2 cells

miR-203, miR-363-3p, miR-26b, miR-1825 and miR-200c-3p were successfully transfected into Hep2 cells. qRT-PCR were used to confirm the transfection of miRNAs (miR-203, miR-363-3p, miR-26b, miR-1825 and miR-200c-3p) into Hep2 cells (Figure 1).

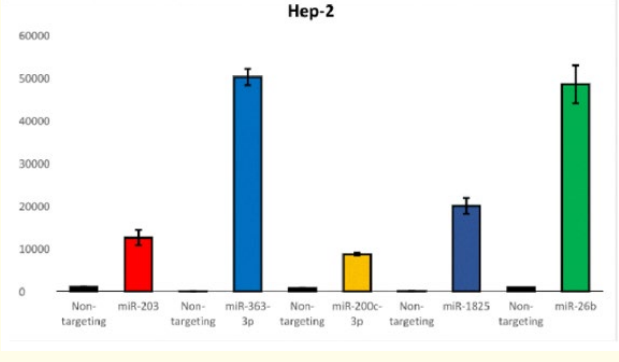

Figure 1: Confirmation of Transfection Success Rate for miRNAs using qRT-PCR. miR-203, miR-363-3p, miR-26b, miR-1825, and miR-200c-3p were successfully expressed in Hep-2 cells.

miR-203 and mir363-3p proliferation of Hep2 cells

After successful transfection, proliferation assay was performed in xCELLIGENCE. proliferation levels of the transfected miR-203 and miR-363-3p cells were checked by the use of control miRNA. As expected, the miRNAs acted as a tumor suppressor. The results showed a statistically significant p values of 0.0007 and 0.001 for miR-203 and miR-363-3p, respectively. No significant change in the proliferation capacities of miR-26b, miR-200c-3p and miR-1825 was observed. The p-values of these microRNAs were found to be 0.2, 0.09, and 0.1, respectively. Those remaining below the non-target graph indicate slower proliferation, while the remaining ones indicate an increase in proliferation. Accordingly, miR-1825 has increased proliferation, while miR-203 and miR-363-3p decreased proliferation (Figures 2, 3).

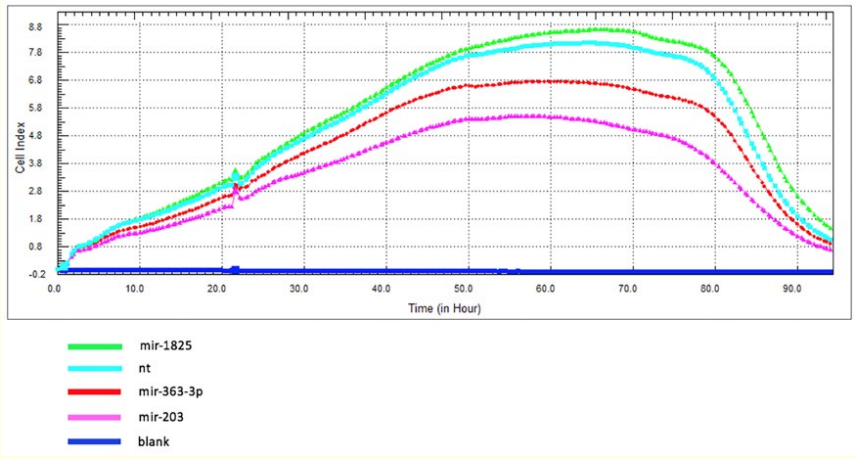

Figure 2: Functional Analysis of Selected miRNAs (miR-26b, miR-200c-3p, miR-203, miR-363-3p, and miR-1825) in Human Laryngeal Squamous Cell Carcinoma. Proliferation graphic shows the mir-1825, mir-363-3p and mir-203 on Hep-2 cells.

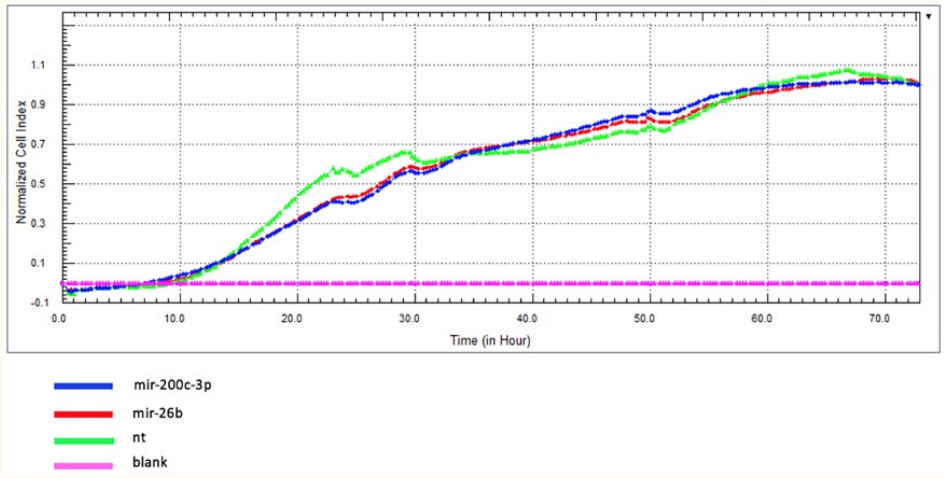

Figure 3: Functional Analysis of Selected miRNAs (miR-26b, miR-200c-3p, miR-203, miR-363-3p, and miR-1825) in Human Laryngeal Squamous Cell Carcinoma. Poliferation graphic shows the mir-26b, and mir200c -3p on Hep2 Cells.

mir-203 and mir-363-3p inhibited migration of Hep2 cells

Migration and invasion assays of miR-203 and miR363-3p, resulted with a significant proliferation assay. Invasion and migration changes in miR-203 and miR-363-3p treated cells, which were found to be significantly altered in accordance with the proliferation results, were assessed using the CIM plate wells of the xCELLigence system. Upon transfection with miR-203 and miR-363-3p, Hep-2 cells lost their migration capacity significantly shown in Figure 4. According to the findings, these miRNAs acted as tumor suppressors.

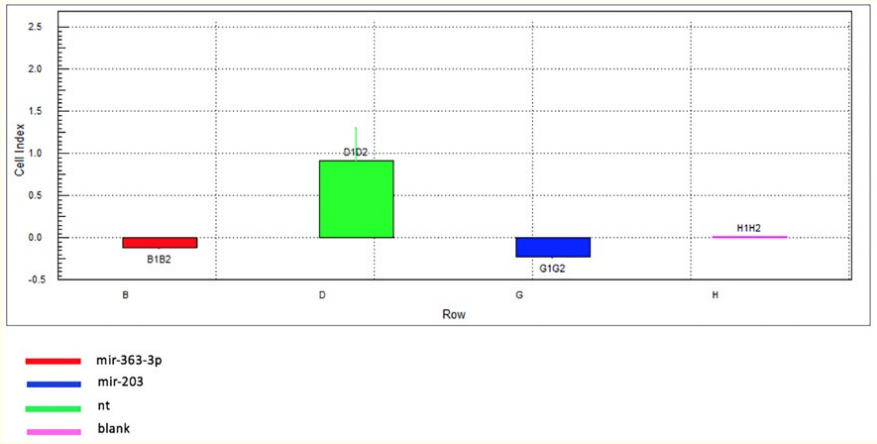

Figure 4: Migration Assay Confirming the Role of miR-203 and miR-363-3p in Tumor Progression in Hep-2 Laryngeal Cancer Cells.

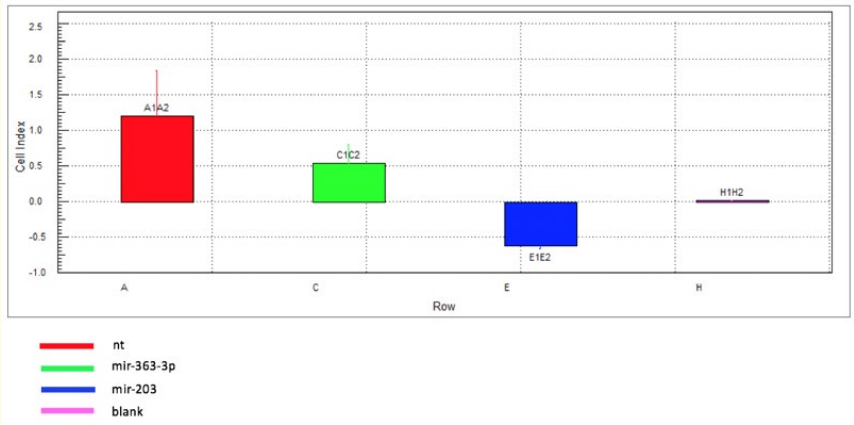

Figure 5: Invasion Assays Confirming the Role of miR-203 and miR-363-3p in Tumor Progression in Hep-2 Laryngeal Cancer Cells.

mir-203 and mir-363-3p restricted colony forming ability of Hep2 cells

Hep-2 cells were transfected with miR-203 and miR-363-3p whether to see there is a change in forming colonies. As a result, Hep-2 cells were transfected with miR-203 and miR-363-3p lost the ability to form colonies when compared to the un-transfected control. It was determined that the ability of transfected cells to compare with the control group resulted in a reduction in forming colonies. This significant reduction shows that miR-363-3p and miR-203 behaving as tumor suppressors. In these analyses, we

observed the effect of miRNAs on Hep-2 laryngeal cancer cells compared to normal cells (Figure 6). As a result, we found that mir-363-3p and mir-203 were highly significant.

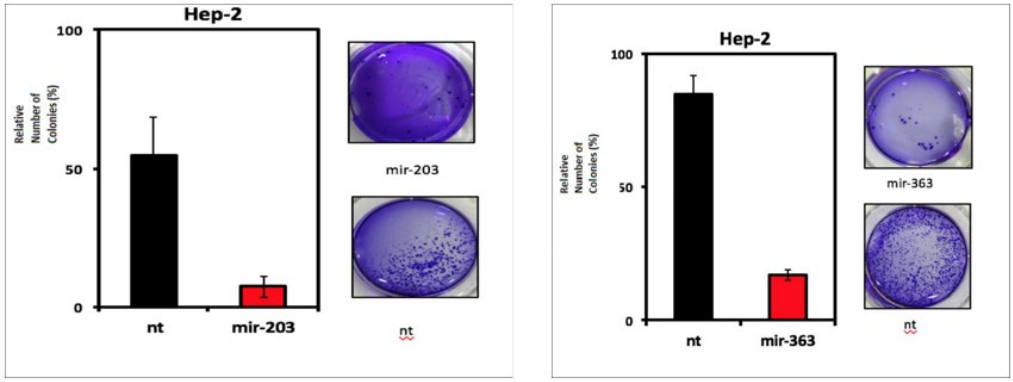

Figure 6: Effect of miR-203 and miR-363-3p on Tumor Cell Colonization Ability.

Discussion

Laryngeal cancer is recognized as one of the most prevalent forms of cancer within the head and neck region, and it exhibits the highest mortality rate in the field of otolaryngology [26]. The majority of laryngeal malignancies consist of squamous cell carcinomas originating from the epithelial cells, accounting for approximately 90-95% of cases [27]. The early stage of LCa can be effectively treated with either surgical intervention or radiotherapy as standalone therapies, allowing for the preservation of laryngeal function. However, the existing treatment options for advanced stages of LCa do not adequately prioritize the preservation of laryngeal function and often result in poorer outcomes [28]. In the context of cancer progression, recurrence, and therapy resistance, miRNA-based approaches have emerged as promising therapeutics. These approaches aim to reverse the alterations in miRNA expression that contribute to these processes. In line with this perspective, the objective of the current study was to explore potential miRNA biomarkers associated with early-stage and non-metastatic squamous cell laryngeal cancer. The identification of miRNA alterations associated with LCa in its early stages holds promise for inhibiting tumor progression to the metastatic stage and preventing tumor relapse following laryngectomy. To this end, we conducted additional functional analyses on specific miRNAs that were detected in microarray studies previously conducted by our colleagues. These analyses aimed to gain further insights into the functional roles of these miRNAs and their potential as therapeutic targets in LCa [29].

MicroRNAs (miRNAs) have emerged as crucial regulators in various cellular processes, including cancer initiation, progression, and metastasis [30-32]. Recent studies have demonstrated the significant involvement of miRNAs in laryngeal squamous cell carcinoma and other cancer types [33,34]. miRNAs have been shown to play piv-otal roles in cell growth, proliferation, and apoptosis, as well as in the regulation of almost every cellular process, depending on their location and time-varying expression [31,32,35]. The dysregulation of miRNAs has been increasingly linked to cancer pathogenesis, including laryngeal carcinogenesis [33,34]. Altered miRNA expression profiles have been observed in solid tumors compared to healthy tissues, suggesting their potential as biomarkers for diagnosis, prediction of therapy response, and treatment outcome [31]. Numerous studies have identified cancer-associated miRNAs, some of which are classified as oncogenic, while others act as tumor suppressors [31,34].

In the context of laryngeal squamous cell carcinoma, comprehensive expression profiling of miRNAs has revealed their significant involvement in the development and progression of this malignancy [33]. Moreover, miRNAs have been found to

play critical roles in various physiological processes, including immunity, proliferation, and stem cell maintenance, which are all closely associated with cancer [36]. miRN-As released into the bloodstream have been found to be highly stable and deranged in cancer patients compared to healthy individuals, suggesting their potential as diagnostic and prognostic biomarkers [36].

In this study, miRNAs related to human laryngeal squamous cell carcinoma was investigated and 5 different miRNAs were selected from miRNAs, which was found significant in earlier studies. These miRNAs are miR-26b, miR200c-3p, miR-203, miR363-3p, and miR-1825. According to the functional analysis findings, miR26b, miR200c-3p and miR-1825 did not show a significant change. However, miR-203 and miR-363-3p has a noticeable reduction in the proliferation abilities of Hep-2 cells was determined (Figure 2). These findings show that miR-26b, miR-200c-3p and miR1825 have no effect on the tumorigenic process, but miR-203 and miR-363-3p may have a significant effect. The result of this meaningful outcome continued at the advanced functional stages.

The results obtained from migration and invasion assays confirmed that miR-203 and miR-363-3p assist tumor progression on Hep2 LCa cells. miR-203 and miR-363-3p transfected Hep2 cells have reduced migration-invasion capacity compared to non-transfected Hep2 cells (Figure 3). These results led to the finding that miR203 and miR363-3p are acting as tumor suppressors (Figure 4, 5). Several studies presented the evidence of anti-tumorigenic impact of of miR-203 in different cancers, including lung [37], prostate [38] and breast cancers [19]. miR-203 expression was elevated in advanced ovarian cancer and the altered expression was conducted with poor prognosis [37]. It is also reported that miR-203 inhibits epithelial-mesenchymal transition via directly binding through 3 UTR region of Snai2 mRNA in prostate, breast and ovarian cancers [39,40].

Although the mechanism of tumor suppression and proliferation differs in each evidently because of the miR-203 target site and all results showed causin anti- invasion and -proliferation of cancer cells that leads to inhibition of metastasis. Similar to previous studies, our investigation on laryngeal cancer showed that significant reduction that caused proliferation of Hep-2 cells in LCa. Based on the migration and invasion assay that was conducted within our investigation, the remarkable drop in the amount of miR-203 was directly correlated with elevation of Hep-2 cell lines' invasion and proliferation. The findings on miR-203 show similar results with the previous studies that were conducted on miRNAs' impact on laryngeal cancer's progress [29,30,35] which is likely to shed light in utilizing miR-203 for LCa diagnosis and possible therapeutic techniques. Parallel to miR-203 findings, analysis of other miRNAs on LCa detected remarkable decline in level of miR-363-3p in head and neck cancers [41] which is consistent with the our findings. To look the role of miR-363-39 in other cancers, tumor-suppresive role of miR-363-3p is detected in colorectal cancer as well. Researchers reported that ectopic overexpression of miR-363-39 in colorectal cancer cells suppresses cell proliferation and metastasis while its inhibition promotes cell proliferation and restrain apoptosis [42,43].

We also investigated the effect of miRNAs on the ability of tumor cells to colonize. According to the results, it was determined that in the miR-203 and miR-363-3p transfected cells, the control group significantly decreased their ability to form colonies as compared to Hep-2 cells. Quantitative statistical analyzes also supported found results (Figure 6).

Conclusion

Dysregulation miR-203 and miR-363-3p was significantly reported and linked to their tumor-suppressive potential for LCa. Our studies showed that overexpression of these miRNAs reduced cell proliferation, migration-invasion and colony formation ability of LCa, Hep-2 cells. We found positive effects on squamous cell laryngeal carcinoma detected in miR-203 and miR-363-3p among five different miRNAs. We have determined that miR1825, miR-200c-3p and miR-26b have no effect on squamous cell laryngeal cancer. It is understood that miR-203 and miR-363-3p can easily a candidate for diagnostic markers according to the data obtained in this study. This study will lead the work to be done in pharmaceutical fields, which will provide a step for the recovered of the disease in the diagnosis of treatment. Achieved results in this study will lead to other projects. Based on the results of the study, miR-363-3p and miR-203 might have a potential of being prognostic biomarker in LCa.

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