Original Article

Association of miR-200a and miR-205 Expression with Epithelial-Mesenchymal Transition in Colorectal Cancer Cells

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Abstract

Introduction: MicroRNAs play a critical role in EMT regulation through tightly regulating the transcription factors. EMT (Epithelial-Mesenchymal Transition) in the colorectal cancer cell (CRC) is a highly controlled mechanism, contributing to the development of progressive cancer. Throughout this research, miR-200a/205 was focused as a component implicated throughout regulating the EMT process in colorectal cancer cells. In this sense, the induction of the EMT process was made using colorectal cancer cell lines.

Materials and Methods: The mRNA levels of E-cadherin, Vimentin, β-catenin, Zeb1, and Snail were determined using real time-PCR for characterization of the EMT process. True real time-PCR was conducted for evaluating the alteration amount of microRNAs.

Results: The findings of this study verified the in-vitro EMT model being developed. The in vitro analysis revealed a negative correlation between the Zeb1 and Snail miR-200a and miR205 (P=0.001) (P=0.0001). The results of miR-200a and miR205 are regulated down in vitro.

Conclusion: miR-200a and miR205 may be used as candidates in further research to prevent colorectal cancer's invasive properties via the EMT process.

Keywords: Cancer Stem Cells, Colorectal Cancer, miRNAs, EMT, Transcription Factors

1. Introduction

Colorectal cancer (CRC) is the fourth most common cause of cancer-related death in the world. It is responsible for 10% of cancer-related deaths [1]. Many treatment strategies such as surgery, chemotherapy, and radiotherapy have improved survival rates [2].

However, conventional therapy is not suitable for most CRC patients with metastasis [3, 4]. On the other hand, there is no efficient therapy for advanced CRC with metastasis [5]. Altogether, determining new biomarkers for the diagnosis of CRC and developing novel treatment methods is essential [6]. The development of metastatic cancer involves changes in epithelial structure and function [7]. One of the essential molecular steps of metastasis includes epithelial-to-mesenchymal transition (EMT) [8]. Tumor metastasis includes interlinked steps [9] related to the

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transformation between epithelial and mesenchymal phenotypes [2]. The epithelial-mesenchymal transition (EMT) plays a critical role in developing metastasis in recent years [2]. It disrupts cell-cell adherence, loses apical-basal polarity, causes profound remodeling of the cytoskeleton, and obtain mesenchymal properties [2]. Mesenchymal-epithelial transition (MET), the reversal of EMT, seems to happen after distribution and subsequent formation of metastasis [10]. However, findings about MET needs further investigation [2].

All in all, EMT plays a critical role in human CRC [2]. EMT needs the help of a complex network including molecular signaling ways and regulators: 1- molecules that activate EMT program naming EMT effectors (such as E-cadherin, Vimentin, and β-Catenin); 2- the transcription factors that organize EMT program (such as Snail and Zeb1); 3- some extracellular factors which activate EMT [11]. The process of EMT can be induced by TGF- β resulting in affecting EMT markers of E-cadherin, Vimentin, and β-Catenin and expression, levels of transcription factors of Snails and Zeb1. The transcription factors role in EMT has been determined in different cancers, including CRC [5]. The Snail group activates the EMT program during development, fibrosis, and cancer [12]. It can develop EMT via E-cadherine [5]. Snail help to decrease of E-cadherin in colon cancer [13]. Also, the snail is vital in linking EMT and stem cell characteristics in CRC [5]. Zeb group act as repressors and activators, therefore repress genes of epithelial and activate genes of mesenchyme [5]. This research helps us to understand cancer and metastasis molecular pathways. Therefore, the effort has been put to find novel cancer treatment ways. Decreasing E-cadherin expression indicates lymph node metastases, poor tumor differentiation, and a worse prognosis in patients with CRC [14, 15].

In contrast, increasing Vimentin expression is related to lymph node metastasis [16]. TGF- β is understood to have two different roles. It suppresses tumors in the early stages of tumor development. Yet, on the other hand, it may enhance tumor progression in late stages because it proliferates cells and EMT, and also suppresses immune function in some types of cancers, including breast, prostate, and colon cancers [17]. MicroRNAs, as evolutionarily retained small non-coding RNA molecules, control large programs, including proliferation, apoptosis, and cell division through post-transcription gene expression modification [18]. In EMT, the miR-200 group downregulate, while Zeb group upregulate [18]. In MET, the miR-200 group upregulate, while Zeb group downregulate[19]. Next, it suppresses the expression of the Zeb group and increases the synthesis of E-cadherin that is important for the adhesion of cells [20, 21].

Some miRNAs are associated with CRC [19] and also breast [22], prostate [23], endometrial [24], ovarian [25], and lung [26] cancers. It is displayed that miR-205 decrease in CRC tissues and cell lines [27]. Bioinformatics and cloning studies have estimated that miRNAs may regulate 30% of all human genes, and miRNA can control hundreds of gene targets [28]. MiRNAs are highly supported in sequence between distantly related organisms, indicating their participation in the vital biological process [28]. In this research, the function of miR-200a and miR-205 in inducing the EMT pathway into colorectal cancer cells was examined.

2. Materials and Methods

2.1. Cell Culture

Colorectal cancer (CRC) cell lines were provided from Pasteur Institute of Iran-Tehran and cultured in RPMI-640, completed with 10% fetal bovine serum (GIBCO) and 100U/ml penicillin/ streptomycin (Invitrogen, Carlsbad, CA, USA). Cells of CRC were incubated at 37 °C with atmosphere humidity of 95%,

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5% CO2, and 95% O2.

2.2 TGF-β Treatment

After that, cells had reached about the confluence of 80% in the T-25 flask, and were grown at a seeding density of 2x104/cm in two T-25 flasks. The seeded cells were fed by 0.5% FBS for 24 hours to induce EMT. Next, they were treated by 10 ng/ml TGF-β1 for 48 hours. After inducing TGF-β, cell alteration of morphology was studied using contrast phase microscopy (Olympus IX7071).

2.3 Real-Time PCR

Using AllPrep DNA / RNA / miRNA Universal Kit (Qiagen, Hilden, Germany) to separate colorectal cancer cell lines RNA and miRNA. Nanodrop spectrophotometer (Nanodrop Techniques INC, USA) were used to assess the concentration and purification of extracted RNA. The reverse-transcribed was used on 50ng of RNA. The Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) was used. The cDNA was given to quantify the degree of expression of the genes Zeb1, E-cadherin, Vimentin, β-Catenin, and Snail EMT markers. In EMT array reaction, GAPDH was used as an internal control. The Real-time PCR was performed on the Rotor-Gene 5plex HRM (QIAGEN, Hilden, Germany) in triplicate with SYBR Green Master Mix (Amplicon, Denmark). Table 1 displays the primers used in this analysis.

Table 1. Sequence of the primers used in the real-time PCR experiment

Gene names	Forward	Revers
E-cadherin	TGCTCTTGCTGTTTCTTCGG	CTTCTCCGCCTCCTTCTTC
β-catenin	GGGTAGGGTAAATCAGTAAGAGGT	GCATCGTATCACAGCAGGTT
Vimentin	CCAGGCAAAGCAGGAGTC	CGAAGGTGACGAGCCATT
Snail	CACTATGCCGCGCTCTTTC	TGCTGGAAGGTAAACTCTGGAT
Zeb1	CAAGAGGCGCAAACAAGC	GGTTGGCAATACCGTCATCC
GAPDH	GCTCTCTGCTCCTCCTGTTC	ACGACCAAATCCGTTGACTC
hsa-miR-200a	ACACTATAGCTGGGTAACACTGTCTGGT	TGGTGTCGTGGAGTCGGCGTTCACGTG
hsa-miR-205	ACACTATAGCTGCGTCCTTGACATCATCACC	TGGTGTCGAGTCGGCGCGAAATTCAGTG
U6	ACCTCCCATCTGGGTCGTGCGAGCGTTC	TGGTGTCGTGGAGTCGGCAATTCAGTTG

The variations of the genes listed above have been normalized into the housekeeping gene. Performance compared with the method of 2- ΔΔCT formula.

2.4 miRNA Analysis

ALLprep Universal Kit (QIAGEN) isolated Total miRNA from colorectal cancer HT29 cell line. Later, the First Strand cDNA Synthesis Kit (Thermo Scientific, USA) synthesized miR-200a and 205 cDNAs. Particular primers were designed for cDNA synthesis and the miR-200 and 205 screening (Table 1). U6 was applied to standardize miRNA expression levels. Also, Rotorgen Q 5HRM (QIAGEN, Hilden, Germany) assessed the level of miRNA expression based on SYBER green protocol. Tests were evaluated according to RQ analysis.

2.5 Statistical Analysis

The research was conducted via the prism Graph Pad program and Excel and applied each procedure in three consecutive days and duplicate. The findings were defined as mean ± SEM. Student's T-test was used to analyze differences between the two groups, and one-way variance analysis (ANOVA) was used to analyze more than two groups. The data was ultimately statistically significant ($p<0.05$).

3. Results

Alteration of EMT Markers

Phenotype was morphologically changed due to EMT in colorectal cancer cells. Cell morphology was changed into a spindle-like shape, as shown in Fig.1. TGF-β1, as an

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EMT actuating specialist, could build up cancer stem cell-like cells. E-cadherin, β-catenin, and Vimentin are some of the EMT-related markers which were tested in this research. E-cadherin as epithelial cells reduced significantly. On the other hand, β-catenin and Vimentin showed a significant increase after induction of HT29 cell line by TGF-β1. In other word, E-cadherin, significantly, reduced in comparison with the control sample (p=0.001) and contrast, mesenchyme cells markers, β-catenin, and Vimentin, in this research showed a significant increase (β-catenin: 0.01; Vimentin: 0.001) when compared with a control group that is the suggested transformation of epithelial cells to mesenchymal cells (EMT process) (Fig.2).

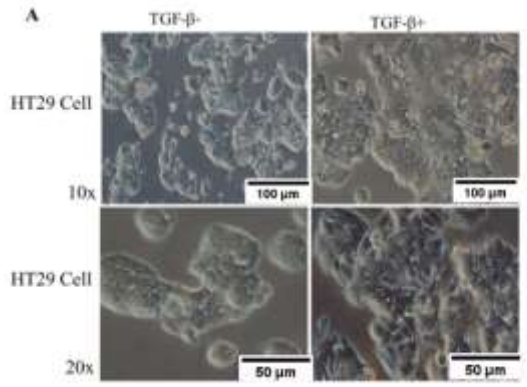

Figure 1. Changes in colorectal cancer cells (HT29) by the transformation from epithelial to mesenchymal. TGF-β transforms the morphology of the cells from epithelial to spindle-like shape. Arrow in the picture shows the change in cell morphology with TGF- β (Scale bar= 100µm , Scale bar= 50µm) in treated and untreated status

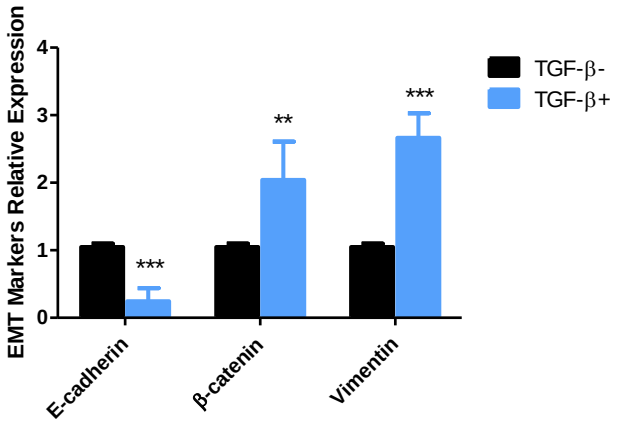

Figure 2. Statistical analysis after EMT induction shows significant changes in the β-catenin, Vimentin, and E-cadherin expression levels. GAPDH normalized the expressions of the EMT markers as a gene for housekeeping. The data were described in triple studies as SEM β-catenin (P=0.01), Vimentin (P=0.001) E-cadherin (P=0.001).

Decreasing microRNAs Expression Genes (miR-200a and miR-205) after Inducing TGF-β1

miR-200a and miR-205 were selected in the current research since they both play an essential role in EMT process. The relative expression levels of miR-200a and miR-205 were studied after actuating TGF-β1. The relationship between expression levels of miR-200a and miR-205 with TGF-β1, using real-time PCR was evaluated. This study suggested the association of TGF-β1 induction with the expression of microRNAs. As shown in the following figure, investigations showed that both miR-200a and miR-205 were down-regulated significantly after induction of EMT. (Fig.3).

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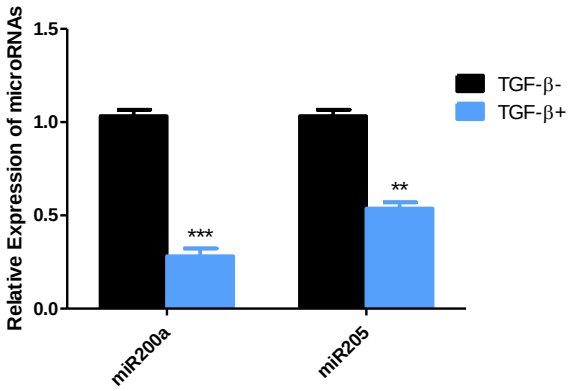

Figure 3. microRNA-200a/205 expression standard in a colorectal cancer cell line (HT29). HT29 was induced within two days with or without 10ng / ml of TGF-β1. miR-200a and miR205 showed significant down regulation .The expression of microRNAs as a housekeeping gene was as compared to U6. The data were described in triple studies as SEM (P=0.001) (P=0.01).

Correlation between Transcription Factors of Zeb1 1 and Snail Expression Associated with miR-200a and miR-205

Real-time PCR identified Zeb-1 and Snail as transcription factors are both targets of miR-200a and miR-205 genes. The miR-200a and miR-205 expression levels were correlated with Zeb1 and Snail as transcription factors involved in EMT. According to the data analysis, low expression of miR-200a/205 has been correlated with significant up-regulation of Zeb1 and Snail. Zeb1 and snail were significantly increased after miR-200a and miR-205 suppression (zeb1: P=0.01; snail: P=0.001) (fig.4).

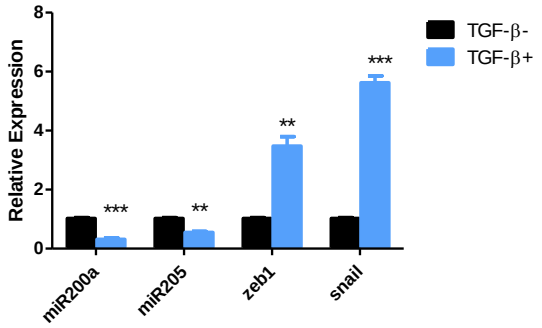

Figure 4. Expression of microRNAs in correlation with the transcription factors. The expression miR-200a/205 has reversed concerning other transcription factors. Snail and Zeb1 are up-controlled with the down-control miR-200a and miR205. GAPDH normalized the expressions of transcription factors as a housekeeping gene, and the level of expression of microRNAs compared to U6. The data were described in triple studies as SEM (snail: P=0.001, zeb1: P=0.01)

4. Discussion

Metastasis cancer is a significant cause of death of patients suffering from a solid tumor. Colorectal cancer is the fourth cause of death worldwide related to cancer [1]. In recent years, molecular genetic studies have been conducted to search for gene-related processes, and it has revealed that some miRNAs have a direct role in tumorigenesis. Moreover, miRNAs expression correlates with some biomolecules. Therefore, a survey on this process has a critical role because of its pathogenic effect. In this research, the usage of TGF-β1 triggered the EMT process in CRC. Zeb1 and Snail transcription factors regulated the EMT process, and the decrease in the expression of these two variables contributed to a shift in the expression of the EMT process. The epithelial cell showed mesenchymal stem cell properties by reducing the E-cadherin

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volume and growing the sum of β-catenin and Vimentin. The results of this study showed that HT29 epithelial cells exhibit morphologically more fibroblastic form in the presence of TGF-β1, which is one of the characteristics of the EMT process. On the other side, these molecular-level experiments showed that the properties of the stem cells in these cells were followed by a decrease in the level of E-cadherin and a rise in the rates of β-catenin and Vimentin. Epithelial to mesenchymal transition (EMT) is a process in which cells lose some characteristics, for instance, cell polarity and cell-cell contact, and cause some mesenchymal properties, and finally may increase mortality [5].

CSC and EMT are two concepts addressing tumorigenesis, growth, and metastasis and EMT (Epithelial-mesenchymal transition) is an essential process in the induction of tumor metastasis. TGF- β guides epithelial cells to mesenchymal ones. Several transcription factors, such as snail and Zeb1 have been implicated in the control of EMT [29]. In the present study, the EMT model was first developed using TGF-β1- in HT29 cells, and then, it was confirmed by examining EMT markers in terms of phenotypic and molecular levels.

Snail is another transcription factor that behaves as a regulator that leads to EMT and metastasis to many different types of tumors. Increasing snail may cause CSC. Analysis of snail in human CRC in a prior study determined that about 78% of the tumor samples overexpressed this protein. The other studies found snail in primary colon cancer epithelium and liver metastasis, but snail was undetectable in normal mucus [29]. CSC cells are commonly assumed to play a significant role in tolerance to malignant tumors and postoperative recurrence [30].

In the previous study miRNA family was determined as center stage of cancer molecular biology and its dysregulation is an important stage of many cancers including Colorectal cancer [31].

Moreover, the other researchers observed that miR-205 was decreased in CRC tissues and cell line. MiR-205 was found to be decreased in many cancer types including prostates, oral cancer and breast cancer and its overexpression was able to inhibit proliferation of breast cancer. Therefore, its determined miR-205 is able to play an important therapeutic strategy for the treatment of CRC [32].

Some studies have also shown that miR-200a play an important role in the EMT process in pancreatic cancer cells that alter the expression of EMT markers, as well as invasion and migration [33].

Mirzaei et al. have revealed that the down-regulation of miR-200a could involve in Epithelial mesenchymal transition process control [34].

Also, miR-200c plays a vital role in metastasis in the colon, and it may be a diagnostic marker and therapeutic target for patients with CRC. Researchers in the other study has also shown the importance of increasing miR-200 family in colorectal cancer metastasis. Moreover, it demonstrates that miR-200 family expression correlates with the upregulation of E-cadherin and downregulation of Vimentin expression. In the current study, the expression level changes of miR-200a and miR-205 were examined and identified in the development of invasive properties in colorectal cancer cells by the epithelial-mesenchymal transition. It has been observed in the present study that miR-200a and miR-205 showed a substantial reduction after induction of EMT. Thus miR-200a and miR-205 tend to have an essential role in the recruitment of stem cell-like cells in colorectal cancer. Determining miR-200a/205 role in epithelial-mesenchymal transition molecular mechanism and metastatic colorectal cancer can be an essential area for further research to help develop new therapies to control, screen and treat colorectal cancer It is determined that increasing expression of miR-200 family cause negative regulation of its targets

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ZEB1 which regulate E-cadherin and Vimentin expression to trigger an EMT alteration in CRC cells [8]. All of these studies indicate that EMT is orchestrated by complex processes involving regulators of different signaling pathways [5]. Thus, miR-200a and miR205 are becoming increasingly crucial for the development of colorectal cancer. The current study indicated that with the fall of miR-200a and miR-205, EMT markers of β-catenin and Vimentin rise significantly. On the other side, following a decrease in miR-200a, miR-205, E-cadherin demonstrated down-regulation. Further research into the miR-200 family may open up new opportunities for drug development and other therapeutic strategies for treating colorectal cancer.

5. Conclusion

This study suggest that miR-200a and miR-205 are a significant regulator of EMT by targeting Zeb1 and Snail mRNAs. Thus, miR-200 /205 expression may be a good candidate for further study and could be used to prevent EMT treatment in patients with colorectal cancer. To sum up, the authors induced the EMT model on the colorectal cancer cell line (HT29) and showed that miR-200a /205 expression was significantly reduced in this process. Therefore, it can be concluded that miR-200a/205 reduction plays an influential role in inducing the EMT process and creating an invasive and metastatic condition in colorectal cancer. Thus, with further research on miR-200a/205, the induction of EMT reversibility to MET may be investigated to control and prevent the onset of metastatic colorectal cancer.

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Conflict of interest

The authors declare no conflict of interest.

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