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Research Article

EZH2 Gene Silencing Can Affect the Expression of miR-155 and TP53INP1

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Abstract

Many genetic, epigenetic, and cellular studies on cancer are underway today, and the completion of the genetic and epigenetic library of cancer could be the way to treat the disease in the future. In this study, we have investigated the parallel gene expression changes of EZH2 and miR-155. So far no study has examined the role of these two factors simultaneously and the results of this study could be useful for further studies. For this purpose, using specific shRNA, the EZH2 gene of HCT116 cells was downregulated and then the changes in expression of the miR-155 were investigated. For gene expression study, Real-time PCR as a standard quantitative method was used. The findings of this study showed that in HCT116 human colon cancer cells, downregulation of miR-155 using shRNA can reduce EZH2 expression and also can promote a significant increase in the expression of TP53INP1 gene. Based on the results, we can emphasize the interaction between these two genes. Importantly, EZH2 downregulation has been able to decrease the amount of miR-155 that has also increased expression in many types of cancers. It may be of interest in epigenetic treatments of colon cancer, because miR-155 can control a very important tumor suppressor gene, TP53INP1.

***Keywords*:** colorectal cancer, EZH2, miR-155, epigenetic, HCT116 cell line

Introduction

Cancer is the cause of many human deaths in all countries of the world, and most types of cancer have a uniform distribution throughout the world (Farnia Ghafouri Sabzevari, Madjid Momeni-Moghaddam. 2014). In all types of cancer, genetic and epigenetic differences can be detected compared to normal cells of the body. Due to changing dietary habits as well as changes in human lifestyle today, colon cancer is one of the most common cancers worldwide and it kills many people every year (Kuipers et al. 2015). For this reason, it is important undoubtedly to fully identify the molecular and cellular processes of colon cancer, and in fact to identify genetic and epigenetic relationships.

Colorectal cancer (CRC), also known as the large intestine cancer, develops in the colon or the rectum (Ragusa et al. 2015). CRC is the third most commonly diagnosed cancer worldwide and the third leading cause of cancer death in both men and women in the US. Based on reported statistics, colorectal cancer accounts for about 10 percent of all cancer deaths in developed countries (Kuipers et al. 2015).

EZH2 (enhancer of zeste homolog 2) is one of the most important genes in colon cancer and increased expression of this gene in several types of cancer including colon cancer (Z. Chen et al. 2018; Jiang et al. 2019). This gene actually produces a product that is the catalytic subunit of the polycomb repressive complex 2 (J.-F. Chen et al. 2016; Z. Chen et al. 2018). Overexpression of EZH2 has been noticed in many types of cancers, including breast, prostate, colon, gastric, lung, and glioma cancers.

MicroRNA (miRNA) is a type of RNA molecule that does not produce a protein product (non-coding RNA), but by having complementary sequences during a specific process it can inhibit mRNA either through ribosomal inhibition or complete RNA degradation (Teymoori and Momeni-moghaddam 2017). Nowadays, it has been found that any alterations in various types of microRNAs are associated with physiological malfunction or cancers (Shojania et al. 2019; Momeni-Moghaddam, Yossefi, and Oladi 2017). There is some evidence that explain the reduced expression of miR-155 in some types of cancers (Yu et al. 2018; N. Li et al. 2019; Y. Li et al. 2018; Zhang, Zhao, and Deng 2013). Physiological roles of miR-155 are including hematopoiesis by directly targeting SOCS1(Bouamar et al. 2015), a negative regulator for IL-2 signaling (Das et al. 2013), immune system miR-155-5p displays a similar

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responsiveness to pathogen stimuli and triggering immune system responses (Etna et al. 2018), inflammation, and reduction of IgG1(Qiu et al. 2018).

MiR-155 today is known as oncomiR and both overexpression and downregulation are detectable based on cell dynamic, so every change of miR-155 can control the expression of its target tumor protein 53-induced nuclear protein 1 (TP53INP1).

The purpose of this study was to investigate the changes in miR-155 and TP53INP1 expression after the suppression of EZH2 expression in cancer cells.

Materials and Methods

Cell culture

HCT116 cells (Pasteur Institute of Iran) were cultured in Dulbecco's Modified Eagle Medium (DMEM), containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were incubated in a cell culture incubator (Memmert Incubator Oven INB200) with a humidified atmosphere and 5% CO_2 at 37˚C. Culture medium was changed every other day.

Gene Expression Knockdown

EZH2-specific shRNA was redesigned (origin: pSIREN-retroQ-EZH2, Dr.Yutaka Kondo,AICHI,Japan) by Academic Center for Education, Culture and Research (Mashhad, Iran) and synthesized by Macrogen (South Korea). Co-transfections of shRNA vectors, GP and VSV-G plasmids were performed in HEK293T cell line using calcium phosphate-based protocol, and retroviral particles were enriched using ultracentrifugation (beckman centrifuge) for 120 min at 70,000 g, at 4 °C. The HCT116 cells were plated in six-well plates (Nunc™) at a density of 2 × 105 cells per well and were grown overnight until 50–80% confluency to obtain maximum transfection yield. Then lentivirus was added to the wells. The medium was changed 24h after transfection, and the subsequent experiments were performed 72h after transfection. Puromycin (Invitrogen Corporation, Carlsbad, CA) was applied to select infected cells.

Investigation of gene expression

The specific primers for microRNA used in this study were purchased from Exiqon Company (Exiqon, a leading supplier of flexible solutions for RNA research, is now part of QIAGEN), other primers were designed using provided sequence in NCBI and synthesized by Macrogen Company (South Korea). All primers and sequences are listed in Table 1. RNA extraction (Trizol) as well as cDNA synthesis (Bioneer, south korea) were carried out according to the company's protocols. In this study, Trizol (Thermo Fisher Scientific) reagent used for RNA extraction and Cyber-Green II (Amplicon) was used to perform real-time in a thermal cycler (Corbett Research RG 3000).

Table 1. Primers sequences used for real-time PCR

Primer name	Sequences	NCBI Reference Sequence
TP53INP1F	5’-CCA CGTACAATGACT CTTCT-3’	NM_001135 733.2
TP53INP1R	5’-TTCTTGGTTGGA GGAAGAAC-3’	NM_001135 733.2
EZH2F	5’-TTGTTGGCGGAA GCGTGTAAAAT C-3’	NM_004456. 5
EZH2R	5’-TCCCTAGTCCCG CGCAATGAGC-3’	NM_004456. 5
GAPDHF	5’-GGAAGGTGAAG GTCGGAGTCA-3’	NM_002046. 7
GAPDHR	5’-GTCATTGATGGC AACAATATCCA C-3’T	NM_002046. 7

Results

Downregulation of EZH2 Expression

The expression level of EZH2 gene was examined using real-time PCR method. In Figure 1 it has been shown that the level of EZH2 expression was significantly reduced by shRNA compared to non-treated control group (P<0.05). Moreover, there is a significant difference in gene expression between 24 and 48 hours post transfection.

MTT assay

This colorimetric assay was used to determine the proliferation rate of HCT116 cells after downregulation of EZH2 expression. The results confirmed that the number of viable cells in the

treatment groups were significantly decreased at 24, 48, 72 post transfection ($P < 0.05$, Figure-2). These results suggested that the expression level of EZH2 could regulate the rates of cell proliferation and growth (Figure 2).

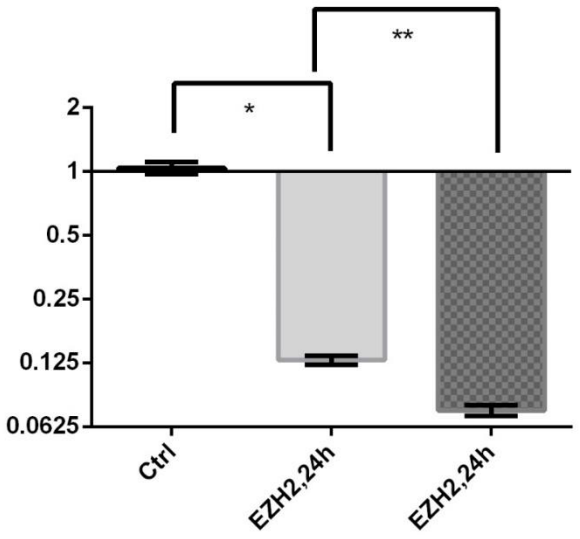

Figure 1. Downregulation of EZH2 expression using its specific shRNA**,** significant decrease of EZH2 expressions have shown after 24 hours and 48 hours compared to control group.

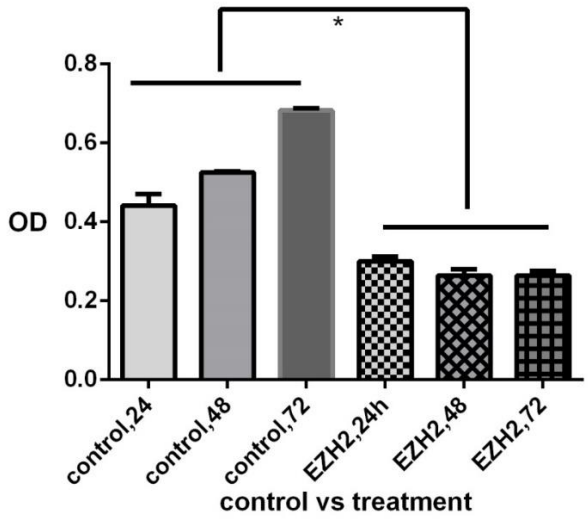

Figure 2. Downregulation of EZH2 expression inhibits the proliferation of HCT116 cells (P <0.05). Curves show the growth of HCT116 cells at 24, 48 and 72 h in control and treatment groups by MTT assay. As shown in the figure, inhibition of EZH2 considerably reduced cell growth.

Molecular findings

RNA extraction was performed for the control and treatment groups and expression of EZH2, miR-155 and TP53INP1 were assessed. As shown in figure-3, a decrease in expression of EZH2 and miR-155 and an increase in expression of TP53INP1 were observed in treatment group (Figure 3).

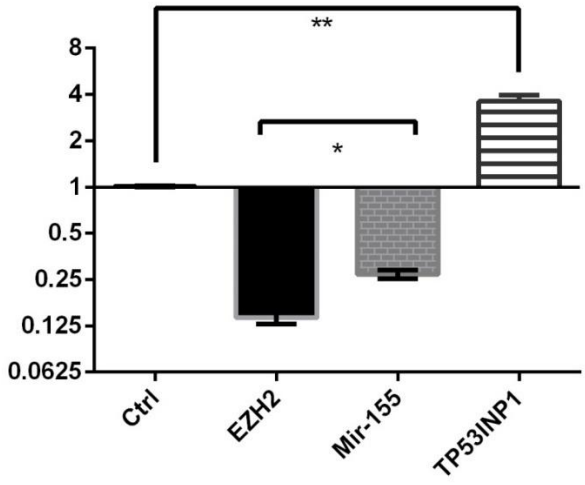

Figure 3. miR-155, EZH2 and TP53INP1 expression in treated and control groups. Level of miR-155 and EZH2 were significantly decreased after 24 h in comparison with control group, but the expression of TP53INP1 compared to control group was significantly increased (P<0.05).

Discussion

In this study, first the EZH2 was downregulated using specific shRNA and then the expression changes of miR-155 and also TP53INP1 were investigated. Although many scientific papers have investigated the role of EZH2 and miR-155 in cancer (Lui et al. 2018; N. Li et al. 2019; Lin et al. 2016; J.-F. Chen et al. 2016), so far, the relationship between these two genes has not been investigated. Hence, finding new information in this area can be important.

The main question was, could we find a synergistic effect between the EZH2 and the miR-155? Did this mean that if we can artificially reduce the EZH2 expression by molecular methods using specific shRNA, will there be a decrease in the second gene?

Given the effect of the EZH2 on the process of cell proliferation, it is clear that any decrease in this gene can lead to a decrease in cell proliferation, which was also tested in this study and confirmed by the MTT assay method. Some articles have clearly mentioned the role of EZH2 on inhibition of apoptosis (Liu et al. 2017).

Researchers have found that any decrease or increase in the expression of miR-155 can have a

completely adverse effect on TP53INP1, indicating that TP53INP1 is regulated by miR-155. In molecular experiments by real-time PCR, these findings were fully confirmed (Seillier et al. 2012; Zhou et al. 2016).

TP53INP1 is a known tumor suppressor, which its expression is downregulated in many types of cancers (Seillier et al. 2012). So, EZH2 not only is a well-known factor in cancer, it can also affect other factors such as TP53INP1 by mediating factors like miR-155.

In conclusion, given that the highly sensitive role of EZH2 and its reduction can lead to decreased gene expression of miR-155 and increased gene expression of tumor suppressor TP53INP1, it could serve as a basis for designing and developing an epigenetic drug to control colon cancer and even other cancers.

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