

Effect of EZH2 Inhibition on Colorectal Cancer Cells: an *In Vitro* Study

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Abstract

Recently, the epigenetic modifications have been recognized as a regulator of gene expression in various cancers. EZH2 gene is one of the most important components of the PRC2 complex. Overexpression of EZH2 was identified in multiple cancers that considered more attractive the EZH2 role as an oncogene. Some studies report that EZH2 contributes to various aspects of colorectal cancer (CRC). However accurate evaluation of the role of EZH2 in the CRC cancer requires more extensive study. In this study, we treated HCT116 cells with the transduction of EZH2-shRNA. Here we observed that the expression level of EZH2 was lower in the treatment cell groups compared to control cells groups. We also found that inhibiting *ezh2* induced caspase-3 gene expression, while no significant change was observed in the expression of *BAX* gene. Caspase-3 and BAX play a central role in the cell apoptosis. In addition, we found that downregulation of the *Ezh2* gene decreased cell proliferation in CRC cells. Collectively, our results suggest that the reduced expression of *Ezh2* altered the caspase-3 gene expression that could indicate the increase of apoptosis in HCT116 cells, which we suggest to direct effect on decreased cell growth. Meanwhile, suggest that EZH2 can be a useful target for therapy and poor prognosis in cancer.

Key words: EZH2; BAX; shRNA; Colorectal cancer

Introduction

Colorectal cancer (CRC), also known as the large intestine, develops in the colon or the rectum. CRC is the third most commonly diagnosed cancer and the third leading cause of cancer death in both men and women in the US (Society 2014) and very important in Iran (Damandan and Moradpour Hesari, 2016). It is also the third most common cancer in men (746,000 cases, 10.0% of the total) and the second in women (614,000 cases, 9.2% of the total) worldwide (Mir Mohammadrezaei, 2015). Almost 55% of the cases occur in more developed regions (Zhang *et al.*, 2012). The reports indicated that CRC incidence rates

significantly increased between 2000 and 2009 in Iran (Abdifard *et al.*, 2016). According to the reports in 2008, CRC was the fourth most common cancer in males and the third most common cancer in females that the highest incidence rates were found in the central, northern, and western provinces of Iran (Khosravi Shadmani *et al.*, 2017). Hence, our growing understanding of the molecular mechanisms can be helpful in treating or preventing colorectal cancer.

As epigenetic modifiers, the polycomb group (PcG) complexes promote gene repression via particular chromatin modifications and compaction. PcG silencing consists of complex polycomb repressive complex 1 and 2 (PRC1

and PRC2) (Aloia *et al.*, 2013). PRC2 is a conserved multisubunit enzyme that methylates histone H3 on lysine-27 (H3K27me3). PRC2 activity depends upon the SET domain active site of its catalytic subunit (O'Meara and Simon 2012) that includes EZH2, EED, SUZ12, RbAp46/48, and AEBP2 (Tan *et al.*, 2014). Enhancer of zeste homologue 2 (EZH2) is one of the most important components of the PRC2 complex. This gene is located on chromosome 7q35 (Cardoso *et al.*, 2000).

EZH2, a catalytic component of PRC2, trimethylates histone H3K27 and also recruits DNA methyltransferases to silence specific gene expression and contributes to regulation of stem cell properties, differentiation, and regeneration (Chou *et al.*, 2015). The change in expression levels of EZH2 affected by mutations in cancer that replace a single tyrosine in the SET domain of the EZH2 protein (Y641) (Morin *et al.*, 2010). Overexpression of EZH2 has been reported in different human cancers, including prostate (Varambally *et al.*, 2002), colon (Ferraro *et al.*, 2013), glioma (Zhang *et al.*, 2012), lung (Xia *et al.*, 2014), gastric (Cai *et al.*, 2010), and breast cancers (Kleer *et al.*, 2003). Current studies suggest that EZH2 plays a critical role to progress CRC cancer. Furthermore EZH2 is involved in multiple aspects of CRC cell biology like differentiation, regulation of oncogenic EMT (Ferraro *et al.*, 2013), cell-migration (Ferraro *et al.*, 2014) and cell cycle (Fussbroich *et al.*, 2011). The results showed that the depletion of EZH2/PRC2 complex with DZNep mediates and EZH2-shRNA significant reduced colon cancer cell proliferation (Sun *et al.*, 2009; Yao *et al.*, 2016).

Regulation of cell growth and cell death are two important processes in developing cancer. In the present study, we investigated the association between EZH2 and cell proliferation, caspase-3 and BAX genes.

Materials and methods

Cell culture

HCT116 cell line was purchased from Pasture Institute of Iran. The cell line was maintained in DMEM mediums, containing, 1X Penicillin-

Streptomycin and 10% fetal bovine serum (FBS). The Cell line was incubated at 37 °C in a humidified atmosphere with 5% CO₂.

EZH2 gene expression knockdown

EZH2-specific short hairpin RNA (shRNA) sequences were synthesized by university of medical sciences (Mashhad, Iran). Cotransfection of shRNA vector, VSV-G, and GP plasmids in the HEK293T cell line was performed according to the calcium phosphate-based Trono lab protocol (Barde, Salmon *et al.* 2010), and produced retroviral particles were enriched using ultracentrifugation (40-mL culture medium per 50-mL Beckman tube, ultracentrifugation for 120 min at 70,000×g, at 4 °C). shRNA was used to downregulate EZH2 in HCT116 cells. The enriched viral particles were used to transduction of HCT116 cells. The cells were plated in six-well plates at a density of 2 × 10⁵ cells per well and grown overnight until they were 50–80% confluent to obtain maximum transfection efficiency. Then we added lentivirus to the wells. We changed the medium 24 h after transfection, and the subsequent experiments were performed 72 h later. Puromycin (Invitrogen Corporation, Carlsbad, CA) was applied to select infected cells.

RNA extraction and qRT-PCR

RNA was extracted from transduced and control cells of HCT116 using RNX-plus solution (Cinnagen, Tehran, Iran). To avoid DNA contamination, extracted RNA was treated with DNaseI (RNase free, Thermo Fisher Scientific), followed by heating and using EDTA to inactivate enzyme. After cDNA synthesis, specific primer sets (Table 1) were used to perform quantitative real-time PCR (qRT-PCR). qRT-PCR was performed using 200 ng RNA in a total volume 20 µL, SYBR green PCR master mix (Parstous, Mashhad, Iran), and containing 1 µM from each primers considering GAPDH as the reference gene. The temperature condition includes initial denaturation for 10 min at 94 °C, followed by 35 cycles of 15 s 94 °C, 30 s 60 °C, and 30 s 72 °C which were performed in a BioRad CFX96 real-time thermocycler.

Table 1. Specific primer sets used in qRT-PCR

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
EZH2	TTGTTGGCGGAAGCGTGAAAATC	TCCCTAGTCCCGCGCAATGAGC
GAPDH	GGAAGGTGAAGGTCGGAGTCA	GTCATTGATGGCAACAATATCCACT
BAX	CGCCCTTTCTACTTTGCCA	GTGAGGAGGCTTGAGGAGTC
Caspase-3	ACTGGACTGTGGCATTGAGA	GCACAAAGCGACTGGATGAA

Cell proliferation (MTT) assay

After the cell counting (2×10^3 cells/well) were seeded into 96-well plate, cultured for 24 h, 48h, 72h and 96h and every plate separately tested for cell population changes using MTT assay standard protocol. Cell proliferation was measured the optical density (OD) using test wave length for 490 nm at room temperature.

Statistical analysis

The Student's t-tests was performed to determine the relationship between EZH2, Caspase-3 and BAX expressions and to compare cell viability among the treatment and non-treatment groups. Significant p value were evaluated by excel 2010 and the MTT assay statistical analysis was performed with GraphPad Prism 6. Values of $P < 0.05$ were considered to be statistically significant.

Results

Influence of EZH2 downregulation on gene expression and cell proliferation

The expression level of EZH2 mRNA was examined using RT-PCR in HCT116 cells. As shown in Fig.1, the level of EZH2 expression was significantly reduced by shRNA-EZH2 compared to non-targeting shRNA as a control cell line ($P < 0.05$). Subsequently, downregulation of EZH2 induced upregulation of caspase-3 nearly 4-fold and no significant change was observed in the expression of BAX ($P > 0.05$, Fig. 1).

MTT assay was used to determine the effects of the downregulation of EZH2 expression on the proliferation of CRC cells. The results showed

that the number of viable cells in the shEZH2 group was significantly decreased at 24, 48, 72 and 96 h after the transduction ($P < 0.05$, Fig. 2). These results suggested that overexpression of EZH2 could regulate the rates of cell proliferation and growth. As shown in Fig. 2, inhibition EZH2 considerably reduced Cell growth.

Discussion

EZH2, a catalytic component of PRC2, trimethylates histone H3K27 and also recruits DNA methyltransferases to silence specific gene expressions and contributes to regulation of stem cell properties, differentiation, and regeneration (Chou *et al.*, 2015). Previous study demonstrated that compared to normal tissues, colorectal cancer tissues expressed higher levels of EZH2 (Yao *et al.*, 2016).

Apoptosis is one of the most important pathways in tumor suppression. Caspase-3 is a frequently activated protease in mammalian cell apoptosis (Porter and Janicke 1999). In the present study, we have demonstrated up-regulated expression of EZH2 in CRC cell line compared with the paired non-shRNA cell line. We also observed that downregulation of EZH2 induces caspase-3 expression in CRC cells (4.5 folds). These results are consistent with the previous findings that downregulation of EZH2 induced the apoptosis of glioma cells (Zhang *et al.*, 2012) and is essential for the induction of apoptosis in CRC cells (Yao *et al.*, 2016). In contrast, EZH2 silencing did not have clear impact on the BAX expression (1.5 fold, Fig. 1). The results showed, that EZH2 mRNA was highly expressed in CRC cell lines and the proliferation and migration ability of RKO and HCT116 cells was inhibited obviously after downregulation of EZH2.

Consistent with that previous results on CRC cell lines (He *et al.*, 2015), we found that the reduced proliferation is caused by shRNA-EZH2 transduction. After the cells were transduced with shRNA for 4 days, HCT116 cells transduced with grew significantly slower than the cells control with $P < 0.05$ (Fig. 2).

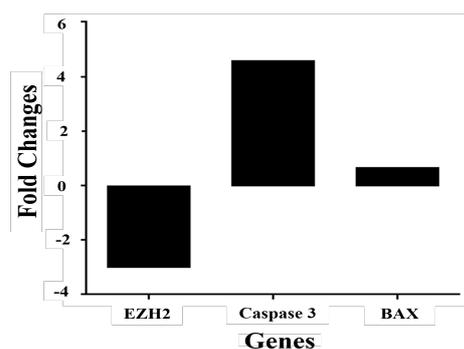


Fig. 1. Downregulation of Ezh2 expression shRNA-EZH2 mediate and increase caspase-3 (4.5 Fold) and little change in BAX mRNA (1.5 Fold) and levels cause inhibition EZH2 in the HCT116 cell line.

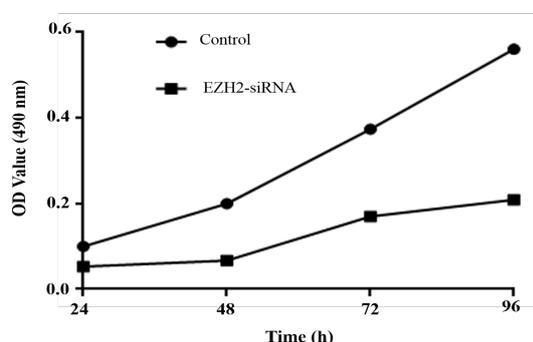


Fig. 2. Downregulation of EZH2 expression inhibits the proliferation of CRC cell line ($P < 0.05$). Curves of HCT116 cells growth were showed at 24, 48 and 72 h after EZH2-shRNA transduction by MTT assay.

These results indicate that the EZH2 gene is essential for cellular proliferation. In this study, statistical analysis revealed a significant inverse correlation between increase caspase-3 expression and decrease cell proliferation. We suggest that decreased cell growth is linked with increased cell death through increased expression of apoptosis.

Collectively, we demonstrated that the EZH2 gene is highly expressed in CRC cell line, and the downregulation of EZH2 expression induces the level of caspase-3 expression and inhibition cell proliferation in HCT116 cells. The other hand, there is no statistically significant difference of BAX expression between treatment and non-treatment groups. However, to further validate the role of EZH2 in colorectal cancer larger studies are needed.

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