Down-Regulation of TSLP After EZH2 Silencing in ESCC Cell Line

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Abstract

Background: Esophagus cancer is the sixth most common cause of death all over the world. This type of cancer is divided into two histological subtypes: Esophagus squamous cell carcinoma and Adeno-carcinoma, the first type of this type of cancer approximately include 90% of esophageal cancers in Asian countries. While radio therapy, chemotherapy and surgery are useful in treating, few people are survived. Therefore, it is need to molecular study to predict and prevent this cancer. EZH2 gene is a member of poly comb group proteins, and with other proteins of this group act as epigenetic regulators and have principle role in cellular functions.

Objectives: In this study our aim was to evaluate the impact of EZH2 silencing on TSLP gene.

Materials and Methods: For constantly silencing of EZH2 gene in KYSE-30, we used shRNA in retroviral vector. After viral production in HEK293T cell line, the cells of esophageus squamous cell carcinoma were transduced with viral packages. Silencing of EZH2 gene and the expression of TSLP were evaluated with real-time PCR.

Results: By using retroviral EZH2 shRNA, the expression of EZH2 reduced over 2.5 fold change in KYSE-30. TSLP gene expression reduced over 2 fold change after EZH2 gene silencing in KYSE-30.

Conclusions: Regarding the stablishing of KYSE-30 cell line with EZH2 silenced gene, Our results showed that the decrease of EZH2 expression cause change of downstream genes expression that will be effective on molecular treatment of ESCC.

Keywords: Esophagus Squamous Cell Carcinoma (ESCC), EZH2, TSLP, RNAi

1. Background

Mainly in terms of histology the esophagus carcinoma is classified into two groups: esophageus squamous cell carcinoma and esophagus Adeno carcinoma. Esophagus squamous cell carcinoma (ESCC) is the second type of cancer that has been diagnosed in North of Iran which is often diagnosed in progressed stages (1).

ESCC is the most common form of cancer that include 80% of Esophageus malignancies all over the world and 90% of people suffering from this kind of esophageus carcinoma live in Gonbad-e-Kavus city. Generally esophageus carcinoma is the 8th common cancer in the world (2), and is the 6th causes of death all over the world (3). Neglecting the recent advancements in treating this disease, too many people die all over the world because of this disease, and molecular alterations involved in generating tumor have not been known completely. During three last decades there was little increasing in the prognosis of the disease, therefore, better understanding of molecular alterations in this kind of malignant carcinoma can be effective in promoting the prognosis and treating the disease. While some of molecular alterations have been studied, it can be said with certainty that there are many genes and molecular paths to be known and they need to be detected.

Development direction is one of the molecular paths which has effect both in terms of cells’ normal function and disorders in growth. Different genes play role in this direction, and every day their roles are revealed more and more. One of the genes of this direction is EZH2, which recent studies have shown its role in many carcinomas.

In the present research, the role of EZH2 gene has been studied in the pathogenesis of esophagus carcinoma by applying RNAi and retroviral mechanism. The applying of the results of present research in combination with other genes having role in this kind of carcinoma will grant us a better perception of forming the esophagus carcinoma.

EZH2 is an active member of PRC2. PRC2 is one of the two inhibitor complexes of PCG proteins composing to regulate the epigenetic. EZH2 has a SET domain with the activity of histone methyl-transferase and responds to DNA methyl transferase directly, and regulates the activity of DNMT1 (DNA methyl transferase), DNMT3a, DNMT3b (4).

EZH2 located in 7q35 and encode one member of PCG proteins which regulates the gene expression by epigenetic modification of chromatin structure including methylation and histoneacetylation induction (5). Al-
though the first function of EZH2 is gene silencing by histone H3 lysine 27 trimethylation, the scientific results show that EZH2 acts independently in histone H3 lysine 27 trimethylation in different cancers. The more important point is that EZH2 is concerned more with stem cell characteristics especially cancerous stem cell characteristics and the function of cell at the beginning of becoming cancerous (6).

EZH2 is expressed broadly in embryo’s development and its expression decreases through differentiation and maturity (4).

It is needed to EZH2 for proliferation, migration, invasion, mesenchymal epithelial transferring of cancerous cells. All of these cases are concerned with beginning, progression, and metastasis of cancer. The increasing of EZH2 expression have been observed in different types of cancer such as prostate (7), breast (8), bladder (9), lung (10), liver (11), kidney (12), stomach (13), esophagus (14). In many of these cases, the expression of EZH2 is concerned with more increasing in the number and more invasive behavior of cancerous cells, that this behavior is considered as poor prognoses. Multiple researches have been shown that increase of EZH2 expression increase proliferation, migration, or cell invasion in vitro. The increasing of EZH2 expression in the epithelial cells of mammals in vivo will result in epithelium hyperplasia and eases the beginning of tumor by growth factor 2 receptor (6).

A strong positive relationship was observed in increasing EZH2 expression and increasing in the cellular multiplication in ESCC, these findings suggest that EZH2 has an important potential role in control of the cell proliferation. The activity that can be at least responsible for one part of ESCC disease process, tumorigenesis or ESCC progression, it can also participate in tumor resistance to radiotherapy. Having sensitivity to chemotherapy can be intensified by suppression of the EZH2 expression, this finding suggest a potential effect of EZH2 on the cellular reactions of tumor to cytotoxic drugs and UV ray (15).

As EZH2 acts as an oncogenic factor in many types of cancers frequently, in most cases the targets of EZH2 having been diagnosed in cancer is the suppressor genes of tumor. As clinical reactions of ESCC is not favorable to common treatments such as surgery, chemotherapy and radiotherapy and high probability of returning and metastasis, studying the responsible molecular mechanisms to ESCC disease and detecting a new therapeutic method based on these mechanisms and also the detection of diagnostic marker to diagnosis the disease sooner is felt more than previous time.

TSLP is a member of cytokine interleukin 2 (IL-2) family and a paralog far from interleukin 7 (IL-7) (16), just like IL-7, the TSLP can stimulate thymocytes and promote the production of B lymphocytes. so at first TSLP was studied as a growth factor of B cell (17). For the first time, it was recognized from supernatant on conditioned environment of the cell line of stem cell depending on thymus gland of mouse (18).

TSLP is expressed broadly in vivo in the epithelial cells of lung, skin, intestine, Hassall’s corpuscles, in thymus medulla, lymphoid tissues concerned with mucus and tongue. Also, it is expressed by primary keratinocytes of skin, soft muscular cells and lung fibroblasts (19). Cloning of TSLP has been showed that the heart, liver, spleen, prostate had more TSLP expression in comparison to lung, skeletal-muscular system, kidney, ovary, small intestine and colon (20).

TSLP can impose its biological functions on various cells by its own activities on them. It can activate CD4+T (19) and CD8T cells on the mice and induce proliferation and differentiation of B cells in the human (21). Also, it enhances maturity and proliferation of dendritic and T-naive cells, respectively; and induces releasing of T cells to absorb chemokine from monocytes. In combination with IL-1 and nerosis factor of tumor it can stimulate the production of Th2 cytokines by mast cells of human (22).

2. Objectives

The aim of this study was to stablilize the esophagus cell line in which EZH2 gene has been silenced constantly, and evaluate the expression of TSLP gene subsequently.

3. Materials and Methods

3.1. shRNA and Primer Design

The primer design was performed online from www.ncbi.nlm.nih.gov for EZH2 and TSLP gene, and then it was controlled and approved that primer was Exon junction from Ensemble site, and finally it was analyzed by using oligo7 software for stem loop and hair pain (Table 1).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>EZH2 F</td>
<td>TGGTGGCGGAGGCCTGAAAATCHC</td>
</tr>
<tr>
<td>EZH2 R</td>
<td>TCCCAGTGGCCGGAATGACGC</td>
</tr>
<tr>
<td>TSLP F</td>
<td>CCCAGGGATTCCGGAACCTCAG</td>
</tr>
<tr>
<td>TSLP R</td>
<td>GCACCGATGCCTGTGAAATGTCG</td>
</tr>
</tbody>
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For shRNA, two complement strand of oligonucleotide (up and down strand) were designed, then an digestion site of 5’-BamHI was placed in the ending part of 5’ up
strand and a digestion site of 5’-ECORI was placed at the end of 5’s down strand. These digestion sites allow our two complement oligonucleotides to clone into our target vector directly. A sequence of hairpin having 7-9 nucleotides and terminator sequence of RNA pol III having 5-6 T nucleotides (poly T) were considered too. A place of unique digestion site was considered as well in downstream of terminator sequence for analysis of enzymatic digestion in order to guarantee the cloned piece to be present (Table 2).

3.2. The Cloning of shRNA in Retroviral Vector and Plasmid Preparation

In order to clone the shRNA, at first we perform the complement strand of oligonucleotide according to the protocol of company, so that they were ready to ligation reaction. Then we diluted the connected oligonucleotides until density became 0.5 microliter, and then ligation reaction was regulated. A ligation reaction was also performed with scramble shRNA. The cloning of recombinant plasmids were done based on current protocol book.

After overnight culture of bacteria, plasmid was extracted from deposit of bacteria using Zyppy TM plasmid miniprep kit.

3.3. Cell Lines

The KYSE-30 cell line were cultured in RPMI-1640, 10% FBS, 1% Pen-Strep. The HEK293T cell line condition was cultured like KYSE-30 except use DMEM as medium for culture. The transduced cell line was treated with puromycin with 2 microgram per milliliter.

3.4. Viral Production in HEK293T Cell Line and Transduction of Target Cells

It was used from calcium-phosphate method to perform transfection of viral vectors to HEK293T cell line, which the protocol of “Current protocol” book was applied. One milliliter of enrich medium was added which harvested from HEK293T transfectected cells to a well of six wells of KYSE-30 cells. In the next 24 hours we replaced the medium with fresh enrich medium. After 48 hours, we passage this well to a T_{25} flask and in next day we started to select cell by puromycin.

3.5. RNA Extraction and CDNA Synthesis

RNA was extracted from retroviral transduced cells and their control cells, using tri-pure kit (Roche, Nutley, NJ). RNA was treated by DNase I (RNase free, thermo fisher scientific), to avoid DNA contamination. cDNA synthesis was done according company protocol (pars toos).

3.6. Real Time PCR Confirmed the Silencing of EZH2 in KYSE-30

qRT-PCR was used to verify the silencing of EZH2 and the expression of candidate gene based on condition was mention before (23) with the brief description in the following: Using 200 ng RNA in a total volume 20 µL, SYBR green PCR master mix (YTA, Iran), and containing 1/µM from each primers considering GAPDH as reference gene. The temperature condition include initial denaturation for 10 minutes at 95°C, followed by 35 cycles of 15 seconds 94°C, 30 seconds 60°C, and 30 seconds 72°C which performed in BioRAD CFX96 real-time thermocycler.

4. Results

4.1. Virus Production in HEK 293T Cells and Transduction of KYSE-30

To produce virus, the transfection of 3 vectors including psPAX2, pMD2.g and shRNA cloned vector were performed simultaneously in HEK293T cells. The transduction of KYSE-30 cells cheked by GFP markers, and, then puromycine selection was performed.

4.2. TSLP Gene Expression Decrease After the EZH2 Gene Silencing

The expression of TSLP was assessed on EZH2 silenced cell line compared to control cells without shRNA viral transduction. Our data showed the expression of TSLP was significantly reduced in KYSE-30 after EZH2 silencing.

5. Discussion

Genetic and epigenetic changes and subsequently change in different molecular paths have role in the stages
process of esophageal squamous cell carcinoma (24). Differentiation is one of the major characteristics of stem cells. Differentiation process is dependent on sub-type expression of specific genes determining one type of a special cell. The regulation of gene expression contains inhibitory and inductive mechanisms. The inhibiting proteins of polycomb group is an example of important regulatory mechanisms which have role in a stem cell’s ability for producing the differentiated cells. The polycomb group proteins silence the gene expression in cancerous and stem cells. These proteins by creating epigenetic changes inhibit the duplication of special genes.

The mechanism used by PCG proteins to regulate epigenetically contains the composing of two PCG regulatory complexes, under the title of PCR1 and PCR2. PCR1 is composed from EED, EZH1, EZH2, SUZ1 proteins. This complex has methyl transferase activity and targets lysine 27 of histone 3. As abnormal regulation of differentiation can result in cancer, the ability of PCG proteins in suppression of the stem cells differentiation process indicates their oncogenic activity. Numerous PCG proteins relate to oncogenic process. By reviewing papers on this matter we found out that the expression of EZH2 increases in numerous cancers such as lymphoma, tumors of prostate, breast, bladder, ovary, endometrium, stomach, intestine, head and neck, skin melanoma, hepatocellular carcinoma, esophageal squamous cell carcinoma (15).

In Yamada et al. (14) study on 136 samples of esophageal squamous cell carcinoma the high expression of both EZH2 and Bmi1 with each other not singularly was a bad independent prognostic factor in esophage squamous cell
carcinoma which support the suppression of tumor’s suppressor gene by Bmi 1 in a dependent method on EZH₂. This result indicates that both EZH₂ and Bmi 1 together and not singularly can be suggested as potential candidates for new therapeutic target in ESCC (19).

In another study it was used from Immunohistochemistry (IHC) and Fluorescence In-situ Hybridization (FISH) to determine the distribution and abundance of protein expression and EZH₂ number in the ESCC sick being treated by chemotherapy. High gene expression of EZH₂ was observed in 54.1% of ESCC patients, but in none of the normal samples of esophagus was not observed. Also, it was observed that there is a multilateral positive relationship between high expression of EZH₂ and increasing of cellular extension in ESCC. These results propound the potential important role of EZH₂ in controlling the extension of cell which at last can be the responsible of generating tumor or ESCC progression (25).

In another study the expression of H₃K₂₇me₃ (lysine 27 of trimethylatedhistone3) that EZH₂ can cause this kind of changing specially was considered by IHC in 98% of the patients in the first stages of the ESCC disease and 30 healthy individuals with normal of esophagus. The increasing of H₃K₂₇me₃ expression was observed in 45.9% of the ESCC patients and just in 20% of cases the normal tissue of esophagus was observed. In this research high expression of EZH₂ in 54.1% of the sick with ESCC was observed, so the relationship between the expression of H₃K₂₇me₃ and EZH₂ was evaluated and it was found out that there is a positive relationship between them.

Recent studies have showed that histone changes which are important for growth of normal cell and also important aspect in the biology of cancer. Such changes, histone 3 lysine 27 trimethylation (H₃K₂₇me₃), are needed to the suppressor set of polycomb 2 which intervenes suppression a great number of necessary genes for cell progression, tumor’s differentiation and extension (26). In present study, we revealed the EZH2 expression has impact on downstream gene such as TSLP, therefore it can enhance the cancer progression by regulation this gene.

Another study has reported that TSLP is the most probabilistic responsible candidate gene for the pathogenesis of eosinophilic esophagus inflammation (18). Also some studies showed the involvement of TSLPR gene in leukemia (27). A group of recent studies have showed that TSLP play role in the growth and metastasis of pancreas and breast cancer, especially those ones which show the increased infiltration of TH₂ cells (28). In the examination of cells in breast and pancreas cancers and related fibroblasts to cancer it was showed that TSLP is produced in response to tumor-derived inflammatory cytokines and possibly other unidentified stimulators.

In B-all the activation of downstream of TSLP signaling paths promote the growth of malignant cells directly, so that in pancreas and breast cancer TSLP cooperate in various components of tumor surrounding which influences secured escape, metastasis and growth.

There were numerous reports that TSLP/SLPR can be a useful prognostic marker and can be a new target for therapeutic intervention in cancer. In line of these studies, we showed silencing of EZH2 cause downregulation of TSLP that suggest the effect of TSLP on ESCC. Also, we suggest it needs to consider to EZH2 downstream genes if this gene select as marker for therapy.

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Footnotes

Authors’ Contribution: Gholamreza Karami Madani: prepare the proposal and manuscript, performing the experiments; Abolfazl Rad: design the project, troubleshooting the experiments; MMF: had a critical revision on the manuscript.

Conflict of interest: The authors declare that they do not have any conflict of interest.

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