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Tip60-siRNA Regulates ABCE1 Acetylation and Inhibits the Proliferation, Migration and Invasion of Esophageal Cancer via the Wnt Pathway

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Abstract

Objective: To investigate the effect of Tip60 gene silencing on the ABCE1 acetylation level and cell proliferation, migration and invasion in TE-1 cells of oesophageal cancer. **Methods:** The siRNA sequence of Tip60 was transfected with esophageal cancer TE-1 cells. Transfected siRNA vector cells were used as experimental group (si-T), siRNA no-loaded somatic cells were transfected as control group (si-NC), and untransfected TE-1 cells were used as blank group (Group N). ABCE1 mRNA was detected by qRT-PCR, the expression of ABCE1 protein, proliferation-related protein βcatenin (β-catenin), GSK3β, and c-myc by Western blot, the protein acetylation level by immunoprecipitation, MTT assay for cell viability, scratch healing and Transwell compartment assay for migration and invasion ability. **Results:** After 48 h downregulation of the Tip60 gene, TE-1 cells showed no significant changes in the ABCE1 mRNA and protein expression. The acetylation level of ABCE1 decreased significantly, compared with the control group and the blank group. After Tip60 gene silencing, the expression of β-catenin and c-myc protein decreased, while the expression of GSK-3β protein increased. Cytofunctology experiments showed that the proliferative activity, migration and invasion ability of TE-1 cells in the experimental group were significantly inhibited. **Conclusion:** Down regulation of Tip60 gene can deacetylate ABCE1 protein and inhibit the proliferation activity, migration and invasion ability of esophageal cancer by blocking the conduction of Wnt signaling pathway.

Keywords

Tip60, ABCE1, Acetylation, Wnt Signaling Pathway, Esophageal Cancer

1. Introduction

Esophageal cancer is a common digestive tract tumor with high morbidity and mortality [1]. Although surgery, chemotherapy and radiotherapy have improved the prognosis of some patients with esophageal cancer to a certain extent, the overall survival rate is still unsatisfactory [2]. Modern medical research has found that protein acetylation modification in epigenetics plays a very important role in the occurrence and development of tumors [3]. The acetylation of some key proteins in the body will change their functions, which will lead to the occurrence and development of certain tumors. ATP-binding box transporter E1 (ATP Combined box transporter E1, ABCE1) is involved in the occurrence and development of various cancers as a regulatory factor [4]. Studies have found that ABCE1 is acetylated in lung adenocarcinoma tissue, and its acetylation level is closely related to the proliferation, apoptosis, invasion and metastasis of lung adenocarcinoma [5]. The previous study of the research group found that ABCE1 was highly expressed in esophageal cancer tissue, and it was closely related to the proliferation, apoptosis, invasion and metastasis of esophageal cancer. However, whether ABCE1 can undergo acetylation modification in esophageal cancer tissues and the specific mechanism of promoting the occurrence and development of esophageal cancer is still unclear. The acetylation modification of protein requires the catalysis of acetyltransferase, and the function of the protein after acetylation modification will be changed and strengthened, which is involved in the occurrence and development of tumors [6]. HIV-1 Tat interacting protein (tat-interaction protein, Tip60) is an important member of the acetyltransferase family, which can catalyze the acetylation of various histones and non-histone proteins, and then participate in the occurrence and development of tumors [7]. In this study, we mainly observed the effects of down-regulation of Tip60 on ABCE1 acetylation modification, Wnt signaling pathway, and cell proliferation, migration and invasion through experiments, and provided experimental evidence for the clinical treatment of esophageal cancer.

2. Materials and Methods

2.1. Materials and Instruments

The human esophageal cancer cell line TE-1 was purchased from Biotechnology Development Co., Ltd. (Shanghai, China, Confirm that mycoplasma testing has been done for the cell lines used) and kept in a 37˚C incubator with 5% carbon dioxide. RPMI 1640 and PBS were purchased from Bio-Industry (Israel); fetal bovine serum was purchased from Bio-Company (Shanghai, China); ABCE1 monoclonal antibody and Acetylated-Lysine antibody were purchased from Bio-Co (Abcam, USA) β-actin polyclonal antibody was purchased from Beijing Zhongshan Jinqiao Biotechnology Company; A/G agarose beads were purchased from Biotechnology Company (CST, China; carbon dioxide incubator and Doska FC microplate reader were purchased from Heat (USA); Fascaliber was

purchased from BD Company (USA). Si-RNA interference sequences were designed and synthesized by Biotechnology Company (Guangzhou, China).

2.2. Experimental Method

2.2.1. Cell Culture

Well-growing esophageal cancer TE-1 cells were selected, and the culture flask was gently shaken several times, suspended with debris floating on the cell surface, and then poured out together with the growth fluid and washed once with Hanks solution. Add 0.25% trypsin solution or trypsin-one EDTA digestion solution for 5 ml from the cell-free face side, and flip the culture flask to immerse the digestion solution in the cells for about 1 min. Flip the culture bottle and place it for 10 min. In order to promote cell digestion, 37˚C of pre-warmed digestive solution can be added, or use the palm of the hand to the outer wall of the bottle with the cell surface, until the cell surface is observed by the naked eye. Pour out the digestive juice, and wash with 4.5 ml of Hanks liquid along the opposite side of the cell layer. When washing, then turn the culture bottle gently, let the liquid flow slowly in the bottle, so as to wash off the digestive juice such as trypsin and digest after pouring off the trypsin. Add a right amount of newly prepared growth solution along the cell surface, wash the cells, and blow them several times to disperse the cells, and pass the culture at 1:2 or 1:3. In 37˚C culture, it can be adherent for about 30 min after inoculation. The growth liquid can be changed for 48 hours. Generally, a monolayer can be formed for 3 - 4 days. The monolayer is formed, and then the maintenance liquid can be changed for testing.

2.2.2. Protein Acetylation Was Detected by Co-Immunoprecipitation Assay

The total protein was extracted and divided into two parts, and one part was used as an internal reference for protein electrophoresis experiments. One part was used for co-immunoprecipitation experiments. For each protein sample, add 5 ul of A/G agarose beads and 5 ul of pure antibody respectively. After mixing, add 2× lysis buffer to the total volume of 450 ul per tube, mix well, and add 400 ul of protein supernatant. The centrifuge tube was fixed on the mixer, 4˚C, 15 r/min, co-immunoprecipitation for 12 hours. Centrifuge at 4˚C and discard the supernatant. A/G agarose beads were washed 3 times with 500 ul of 1× lysis buffer. Centrifuge at 4˚C, 3000 r/min for 3 minutes, and discard the supernatant. After the last wash, discard the supernatant. Mix 35 ul of 1× lysis buffer with an equal volume of 2× SDS loading buffer, and cook at 100˚C for 8 minutes. 3000 r/min, centrifuge for 1 minute. Load the sample onto a PAGE gel, perform SDS-PAGE electrophoresis, electro transfer, and block with nonfat milk powder, then add primary antibodies (Acetylated-Lysine antibody 1:2000, GAPDH 1:1000) and incubate overnight at 4˚C in 3% BSA. After washing 3 times with TBST, secondary antibody (IgG 1:1000) was added and incubated for two hours at room temperature, and then developed by ECL method.

2.2.3. Protein Expression Was Detected by Western Blot

The total protein was extracted, and the protein concentration was determined; the total protein was separated by gel electrophoresis, and the total protein was transferred to PVDF membrane by semi-dry transfer method. After blocking with 5% nonfat milk powder at room temperature for 1 h, the membrane was incubated in the diluted primary antibody for 40 min. After rinsing, the membrane was incubated in the diluted secondary antibody for 30 min. After rinsing, the luminescence reaction was performed in a dark room and exposed to film. Image pro plus software was used to analyze the IOD value of the integrated optical density of the protein bands, and the ratio of the IOD value of the target protein to the IOD value of the internal reference was used to reflect the expression level of the target protein in each group.

2.2.4. mRNA Was Detected by RT-qPCR

RNA was extracted and reverse transcribed with a reverse transcription kit. Fluorescence quantitative kit was used to detect mRNA expression; PCR reaction conditions: pre-denaturation at 94˚C for 4 min, denaturation at 94˚C for 30 s, annealing at 55˚C for 30 s, and extension at 72˚C for 45 s (30 cycles). The melting curve refers to the automatic program of the instrument.

2.2.5. Liposomes Mediate Was Used for Cell Transfection

The cells transfected with siRNA Tip60 were used as the experimental group (si-T), the cells without siRNA transfection were used as the negative control group (si-N), and the normal cells were used as the blank control group (NC). Esophageal cancer cell suspensions were counted, seeded into 6-well plates, and routinely cultured. Dilute 1.25 μl 20 μM siRNA stock solution (V2) with 30 μl 1× riboFECT CP Buffer (V1), mix gently, and incubate at room temperature for 5 min; add 3 μl riboFECT™ CP Reagent (V3), mix well and incubate; The riboFECT CP mixture was added to 465.75 μl of cell culture medium (V4), the total volume reached 500 μl, and the mixture was mixed. After culturing for 48 h, the transfection effect was observed under a fluorescent inverted microscope.

2.2.6. MTT Assay to Detect Cell Viability

Cells were seeded in 96-well plates. Routine culture followed by transfection. 12, 24, 48, and 72 h, take out a plate at each time point, add 30 μl of MTT solution, and continue to incubate for 4 h. Add dimethyl sulfoxide and shake at 80 r/min for 5 - 10 min.

2.2.7. Scratch Test to Detect Cell Migration

The cells were made into a single cell suspension, and 5×10^3 cells per well were seeded into 6-well plates, with 100 μl per well. In conventional culture, when the cells are covered with the bottom of the well, the pipette tip is scratched vertically on the plate surface, and the medium washes off the fallen cells. Add 2 ml of serum-free medium to each well and continue to culture for 48 h, and measure the width of the scratches under an inverted microscope.

2.2.8. Transwell Assay to Detect Cell Invasion Ability

The Matrigel diluent was coated with a filter membrane and polymerized overnight to form a gel. After the chamber was sterilized, the residual glue was removed, and the culture medium was humidified for 1 h. Prepare single cell suspension after transfection. Put the Transwell chamber into a 24-well plate, remove the chamber, and add 600 μl of culture medium to the outside. Add 200 μl of cell suspension to the chamber, and repeat 6 samples for each group of cells. After 48 hours of culture, the chamber was taken out, rinsed with PBS, and the cells in the inner layer of the microporous membrane were wiped off. Crystal violet staining after paraformaldehyde fixation. Count the number of transmembrane cells under the microscope;

2.3. Statistical Analysis

SPSS 26.0 statistical software was used to for data analysis. Data are presented as the mean ± SEM. One way ANOVA was used for statistical analysis. And a value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of Tip60 and ABCE1 Acetylated Proteins in Esophageal Cancer Cell Lines

The results of co-immunoprecipitation and Western blot showed that Tip60 was highly expressed in esophageal cancer TE-1 cells, but low in normal esophageal epithelial HEEC cells. The acetylation level of ABCE1 protein in TE-1 cells was significantly higher than that in normal esophageal epithelial HEEC cells ($p < 0.05$, Figure 1).

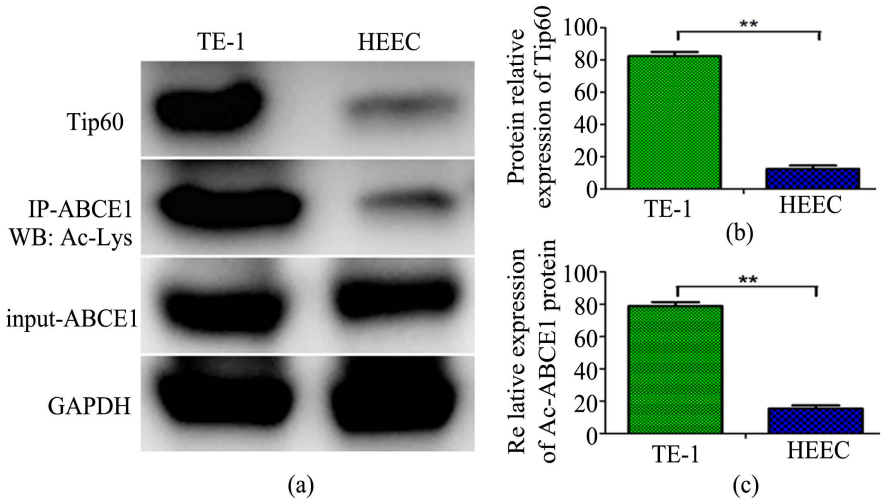

Figure 1. Tip60 regulates ABCE1 acetylation in HEEC and TE-1 cells. (a) Western blotting and co-immunoprecipitation assays were used to determine Tip60 expression and ABCE1 acetylation. The protein expression of (b) Tip60 and (c) acetylated ABCE1 were quantified. Compared with the TE-1 cells, Tip60 expression and ABCE1 acetylation were downregulated in the HEEC cells.

3.2. Effect of Tip60 mRNA and Protein Expression and ABCE1 Acetylation Level after RNA Interference on Tip60

The synthesized siRNA-Tip60 contains a green fluorescent label. After successful transfection of TE-1 cells, green fluorescent expression can be seen in the cells (Figure 2). The results of qRT-PCR detection of Tip60 mRNA and protein expression in three groups of cells showed that the expression of Tip60 mRNA and protein in si-T group was significantly lower than that in si-N group and N group ($p < 0.05$, Figures 3(a)-(c)), indicating that the design and synthesis of RNA interference sequences down-regulation Tip60 was effective. The acetylation level of ABCE1 protein in si-T group decreased significantly ($p < 0.05$, Figure 3(a) and Figure 3(d)). There was no difference in ABCE1 protein acetylation between si-N group and NC group ($p > 0.05$).

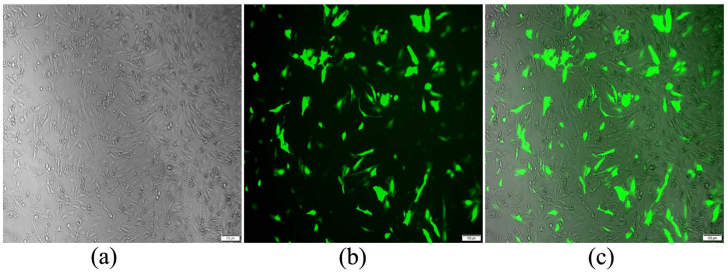

Figure 2. Fluorescent transfection effect of RNA interference with Tip60 transfected TE-1 cells ((a) Bright field; (b) fluorescence field; (c) superposition of (a) and (b)).

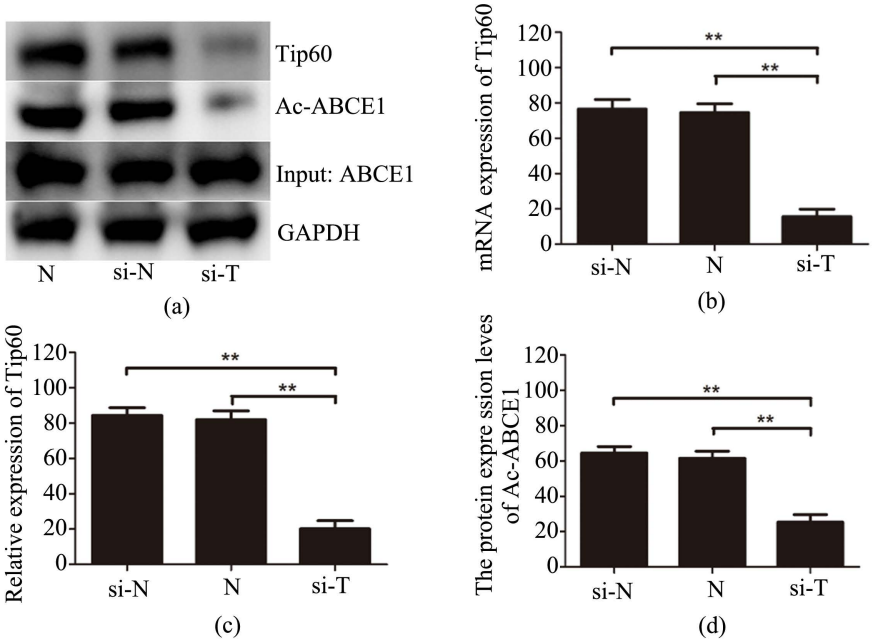

Figure 3. Tip60 regulates ABCE1 acetylation in HBE and TE-1 cells. (a) Tip60 expression and ABCE1 acetylation were significantly reduced in cells transfected with siRNA. (b) Tip60 mRNA relative expressions were significantly reduced in cells transfected with siRNA. (b) The protein expression of (c) Tip60 and (d) acetylated ABCE1 in the transfected cells was quantified. **$p < 0.01$ as indicated.

3.3. Tip60-siRNA Suppresses TE-1 Cell Proliferation, Invasion and Migration

In order to assess the effect of Tip60 on esophageal cancer cell proliferation, we performed an MTT assay, an MTT assay was performed. The cells were transfected with an siRNA directed against Tip60 and a negative control. The proliferative ability of the si-T-transfected cells was lower compared with that of the si-N-transfected cells at 48 and 72 h. The MTT assay demonstrated that TE-1 cell proliferation was markedly suppressed by si-1 siRNA at 72 h ($p < 0.05$; **Figure 4(a)**). Wound healing and Transwell assays were performed to evaluate the suppressive effect of Tip60-siRNA in esophageal cancer cells. As expected, there was a marked reduction in the wound healing, migration and invasion of the TE-1 cells transfected with si-T siRNA when compared with the control cells ($p < 0.01$; **Figures 4(b)-(d)**). Downregulation of Tip60 expression and acetylation of ABCE1 inhibits tumor growth.

3.4. Tip60-siRNA Activates the Wnt Signal Pathway

Western blotting was used to investigate the genes involved in the Wnt signal pathway. Downregulating the expression of Tip60 in the TE-1 cells markedly inhibited c-myc and β-catenin expression compared with the control cells (**Figure 5**). Furthermore, downregulation of Tip60 increased the expression levels of the

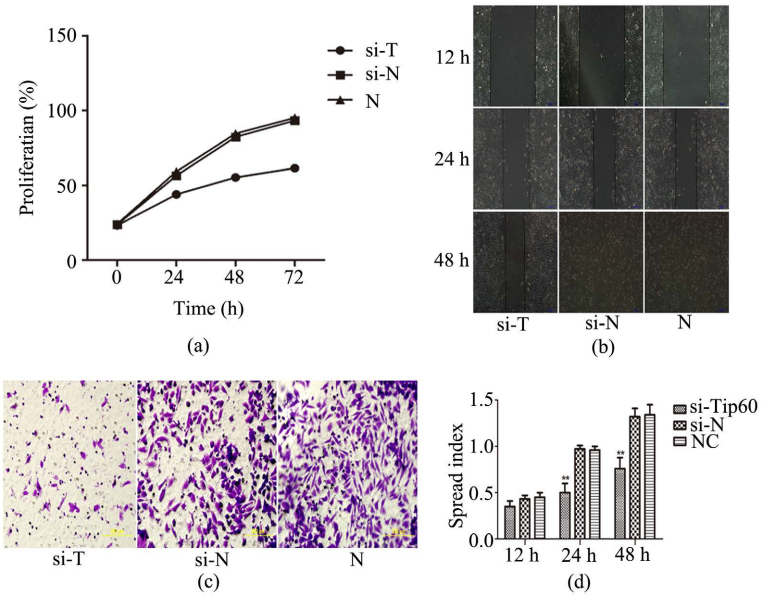

Figure 4. Tip60-siRNA suppresses TE-1 cell proliferation, migration and invasion. (a) The proliferation rate of the si-T, si-N and N groups of cells was investigated using an MTT assay. *$p < 0.05$ vs. the si-N and N group. (b) Tip60 downregulation inhibited the wound healing ability of the TE-1 cells. (c) Downregulation of Tip60 expression reduced cell and invasion, as observed via a Transwell assay with and with Matrigel. (d) The spread index of the wound healing assay was quantified. Experiments were performed in triplicate. **$p < 0.01$ vs. the Si-N and N group. Tip60, Tat interactive protein 60 kDa; si-T, cells transfected with Tip60-siRNA; si-N, cells transfected with negative control siRNA; N, untransfected cells; siRNA, small interfering RNA.

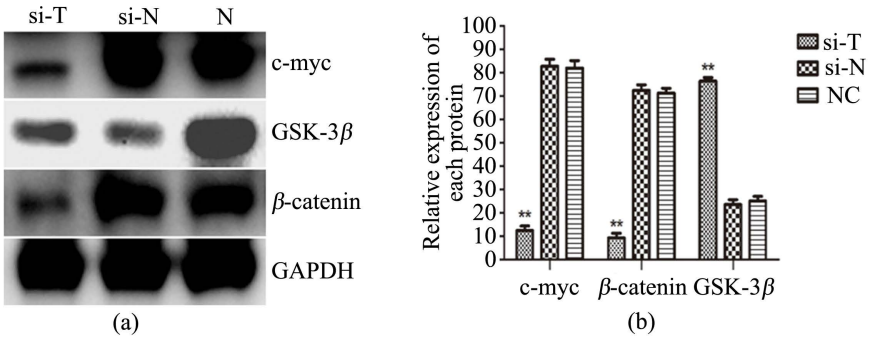

Figure 5. Tip60-siRNA activates Wnt signal pathway. Western blot analysis of Tip60, c-myc, β-catenin, GSK-3β and GAPDH in the TE-1 cells following transfection with si-T or si-N.

apoptosis-associated protein GSK-3β in the TE-1 cells (**Figure 5**). Collectively, the results indicate that downregulation of Tip60 expression directly activates the Wnt signal pathway to suppress tumor cell proliferation, invasion and migration.

4. Discussion

The incidence of esophageal cancer is relatively insidious, and most patients are already in the middle and late stages when diagnosed, and are accompanied by lymph node or distant metastasis, so the overall prognosis of patients with esophageal cancer is not ideal [8]. Conventional treatments have low remission rates for advanced cancer patients and often lead to negative side effects, including anemia and multiple organ dysfunction syndrome [9]. With the in-depth study of human oncogenes in modern medicine, targeted therapy provides better options for patients with advanced esophageal cancer. Therefore, it is particularly important to improve the early diagnosis rate of esophageal cancer, and to screen and identify esophageal cancer tumor-related factors to provide new clinical targets for diagnosis and treatment.

ABCE1 is highly expressed in a variety of malignancies, and is considered as an oncogene, and it is closely related to the occurrence and development of a variety of malignancies [10]. New studies have found that ABCE1 expression is higher in lung cancer tissues than in normal lung tissue, and is closely related to the proliferation, invasion and metastasis of non-small cell lung cancer [11] [12]. ABCE1 expression was observed in gastric cancer tissues and significantly increased, participating in the new regulatory axis of FOXO3a-Parkin-ABCE1, and further regulating the proliferation, migration, and apoptosis of gastric cancer cells [13]. Studies have shown that acetylation can lead to the function of protein, induce tumor and promote tumor development. At present, there are no relevant reports on whether ABCE1 protein is acetylated in esophageal cancer and what effect it will have on esophageal cancer after acetylation.

The acetylation modification of proteins requires the catalysis of acetyltransferase, and the protein function of acetylation modification is changed and

strengthened, participating in the occurrence and development of tumors [14] Studies have confirmed that the inhibition of acetyltransferase expression changes the acetylation state of acetylated proteins in tumor cells [15]. In the mechanism of silencing or down-regulation of acetyltransferase-promoting deacetylation of related proteins, silencing of Tip60 has the potential to exert higher deacetylation and inhibit tumor cells. Experiments have confirmed that after the inhibition of acetyltransferase expression, the effect of inhibiting tumor cells in different tumor cells by deacetylation pathway is not the same as [16] [17] [18]. Acetylyltransferase Tip60 is an important member of the acetyltransferase family, which can catalyze the acetylation of various histones and non-histones.

Malignant transformation of tumors is closely associated with the aberrant expression of the Wnt signaling pathway. When the Wnt signaling pathway is abnormally activated, multiple factors can interact with the β-catenin, making the β-catenin transfer from the cytoplasm to the nucleus, thus inducing cell carcinogenesis [19]. Qin [20] *et al.* showed that the related factors of Wnt signaling pathway are changed after β-catenin downregulation. It is speculated that silencing the key factors in Wnt signaling pathway in esophageal cancer will inhibit the epithelial stromal transition and then hinder the proliferation of esophageal cancer cells, and further inhibit the invasion and metastasis of esophageal cancer cells. Gene molecules that play key roles in the Wnt pathway process mainly include GSK-3 β, β-catenin, and c-myc, and are also important molecular markers to predict tumor invasion and metastasis.

This experimental study confirmed by coimmunoprecipitation experiments that ABCE1 protein was modified in esophageal cancer TE-1 cells, and its acetylation level was significantly higher than that expressed in HEEC of normal esophageal epithelial cells. The present study confirmed that Tip60 is highly expressed in esophageal cancer TE-1 cells, and its high expression was synchronized with the hyperacetylation status of ABCE1 protein, indicating that Tip60 and ABCE are also involved in the occurrence and development of esophageal cancer. However, whether Tip60 acts as an acetyltransferase can catalyze the acetylation modification of ABCE1 and then regulate the occurrence and development of esophageal cancer needs further verification. Further studies found that the intracellular ABCE1 acetylation level was significantly reduced in TE-1 cells after RNA interference occurred to silence Tip60, suggesting that the acetylation modification of ABCE1 is regulated by the acetyltransferase Tip60, or Tip60, the upstream molecule of the acetylation of ABCE1 protein. The results of cell proliferation activity measured by MTT showed that the proliferative activity of oesophageal cancer TE-1 cells was significantly inhibited, suggesting that the downregulation of Tip60 to reduce the ABCE1 acetylation level could inhibit the proliferative ability of oesophageal cancer cells. The results of scratch healing and Transwell invasion experiments showed that the healing migration distance of oesophageal cancer cells was shortened, and the number of cells crossing the filter membrane was significantly reduced. This suggested that after the downregulation of Tip60 reduced the ABCE1 acetylation level, the healing

and migration ability of esophageal cancer cells was delayed, and its invasion ability was significantly inhibited. In this study, the expression of three Wnt pathway-related proteins was confirmed by Western blot testing that β-catenin and c-myc proteins decreased significantly after down-regulation and GSK-3 β expression decreased ABCE1 acetylation levels in esophageal cancer cells. This indicates that after the downregulation of Tip60 reduced the ABCE1 acetylation levels, it inhibited the Wnt signaling pathway, which then inhibited the proliferation, migration and invasion of TE-1 cells in esophageal cancer.

In conclusion, the downregulation of the acetyltransferase Tip60 expression by exogenous RNA interference was able to reduce the ABCE1 acetylation levels and inhibit the Wnt signaling pathway, which subsequently inhibited the proliferation, invasion, and migration of esophageal cancer cells. Tip60 as an acetyltransferase may be an important upstream molecular event to regulate the acetylation of ABCE1 and then alter the biological function of esophageal cancer, providing new ideas and molecular targets for the clinical treatment of esophageal cancer.

The limitations and shortcomings of this study: this study confirmed the effect of Tip60 on esophageal cancer cells through ABCE1 acetylation only at the cellular level, but the validation of its tumor tissue and in vivo function and other specific regulatory mechanisms need further investigation. This is of great significance to provide molecular biological indicators for the early diagnosis and prognosis of esophageal cancer.

Conflicts of Interest

The authors declare that they have no competing interests.

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