Int J Clin Exp Pathol 2017;10(10):10205-10211
www.ijcep.com /ISSN:1936-2625/IJCEP0046955

Original Article

ErbB3-binding protein 1 inhibits tumor proliferation in esophageal cancer by targeting bcl-2 and p53 gene

Peiliang Zhang[1], Shanlan Yang[1], Shuren Cao[1], Baosheng Li[2]

[1]Institute of Radiotherapy, Linyi Central Hospital, Shandong Province, China; [2]Department of Radiotherapy, Shandong Tumor Hospital, Shandong Province, China

Received December 19, 2016; Accepted February 18, 2017; Epub October 1, 2017; Published October 15, 2017

Abstract: Esophageal cnacer is one of the common malignant tumors with high fatality rate and morbidity, ErbB3-binding protein 1 (EBP1) is the intracellular binding protein of ErbB3. This study aims to investigate the effect of EBP1 on esophageal cancer biological behavior and the underlying mechanism. In our study, esophageal cancer tissues and adjacent non-tumor issues were collected and the relative expression of EBP1 mRNA were examined by real-time PCR. EBP1 mimics is synthesised and transfected onto Eca109 cells. The effects of EBP1 on biological behaviors of Eca109 cells were detected by colony formation assay and flow cytometry, the expression of bcl, p53 proteins were detected by western-blot. The results showed that EBP1 was down-regulation in NSCLC. The number of colony-forming units, and the tumor size and volume in vivo in the Eca109/EBP1 group was obviously lower and the apoptosis rate was significantly higher than the Eca109/NC group and Normal group (*P<0.05*). The expression of p53 protein in Eca109/EBP1 group were significantly lower, and the expression of bcl-2 were dramatically increased compare with Eca109/NC group and Normal group (P<0.05). Therefore, we concluded that that E-cad may inhibit cell proliferation of esophageal cancer in vitro and in vivo by inducing apoptosis. EBP1 may play a anti-oncogene role in esophageal cancer.

Keywords: EBP1, esophageal cnacer, proliferation, apoptosis, tumorigensis

Introduction

Esophageal cancer is one of the common malignant tumors with high fatality rate and morbidity, including esophageal adenocarcinoma and esophageal squamous cell carcinoma (ESCC). According to the statistics of American Cancer Society (ACS) and National Cancer Institute (NCI), the fatality rate and morbidity of esophageal cancer rank the 6th and 8th places in the world, respectively [1]. There are 480000 new cases globally each year, with the 5-year survival rate of only 35.45%. Particularly, the morbidity of ESCC in Iran and East Asia like China always remains high [2]. The pathogenesis of esophageal cancer can be attributed to the comprehensive action of smoking, diet, gastro-esophageal reflux, human papillomavirus and genetic factors [3]. With the continuous development of medical technology and research, diagnostic technology of esophageal cancer has been quite mature. However, how to find out the key effective therapeutic targets or biomarker molecules remains essential to the research, prediction and treatment of esophageal cancer, due to limitation of pathological and genetic complexity of esophageal cancer.

ErbB3-binding protein 1 (EBP1) is the intracellular binding protein of ErbB3, which participates in a series of important physiological processes and signal transduction, and is closely correlated with cell differentiation and proliferation [4-6]. It is indicated in research that abnormal expression of EBP1 can be seen in multiple tumors. For instance, its declined expression can be observed in bladder cancer [7], liver cancer [8], prostate cancer [9, 10], breast cancer [11], salivary adenoid cystic carcinoma [12] and oral squamous cell carcinoma [13]; while abnormally high expression can be seen in glioma cells [14]. However, the role of EBP1 in esophageal cancer remains unclear; therefore, investigating the function of EBP1 in

esophageal cancer may have high research and clinical value. As is suggested in reports, EBP1 expression is closely associated with cancer cell proliferation and metastasis, and over-expression of EBP1 can effectively inhibit cancer cell proliferation [10, 15, 16]. Hence, it is speculated that EBP1 may also exert the function of tumor suppressor in esophageal cancer, serving as the potential biomarker or molecular target.

Materials and methods

Tissue collection

Esophageal cancer samples were obtained from patients with esophageal cancer that diagnosed in Lin County of Henan Province. 48 pairs of primary esophageal cancer lesions as well as the matched para-carcinoma tissues were collected postoperatively with the patients' consent. Both tumors and the matched para-carcinoma tissues conformed to pathological diagnosis, and specimens were stored in liquid nitrogen immediately after detachment, and then they were transferred to preserve in refrigerator at -80°C. Experiments concerning human tissue samples in this research were approved by the Ethics Committee of the Linyi hospital.

Cell culture and reagents

Eca109 esophageal cancer cells were purchased from ATCC and were cultured in PRMI-1640 medium (Gibco) containing 10% fetal bovine serum (Gibco) in a cell incubator at 5% CO_2 and 37°C. As for the RPMU1640, fetal calf serum and trypsase, they were all bought from Gibco (USA). Lipofectamine™ 2000 and Trizol were bought from Invitrogen (USA), which also supplied the total RNA isolation nut Trizol. Reserve transcription kit M-MLV was purchased from Promega (USA). SYBR premixextaq kit was sourced from Axygen (USA). The target genes along with the upstream and downstream primers of reference genes were supplied by Ribobio (Guangzhou). The thiazolyl blue kit (MTT) was purchased from Dingguo Biochemistry Ltd. (Beijing). The propidiumiodide (PI) was bought from Sigma (USA), whereas RNase A was from Fermentas (Canada). Lastly, the cell apoptosis kit was supplied by eBioscience (USA). All animal experiments were approved by the Ethics Committee of the Linyi hospital.

Construction of Eca109-Ebp1 stable expression cell line

Clone primers of EBP1 gene were designed using Primer Premier 5.0 software, and EBP1 gene was obtained through cloning of cDNA of esophageal cancer tissues. The primer sequences were 5'-CGGAATTCATGTCGGGCGAGGACGAGCA-3' and 5'-CGGGATCCTCAGTCCCCAGCTTCATTTTCTTCT-3', which were loaded into PCDH-CMV-MCS-EF1-puro (Addgene) lentiviral core vector after enzyme digestion. The core plasmids PCDH-puro-EBP1 and PCDH-puro, as well as the packaging plasmids psPAX2 and pMD-2G were imported into 293T cells (Shanghai Cell Bank of Chinese Academy of Sciences) through calcium phosphate method, and the viruses were collected and concentrated 24 h later. Eca109 and KYSE150 cells were infected with viruses for 8 h; 24 h after the virus incubation buffer was removed, puromycin (4 μg/mL, Sangon Bioengineering Company) was used for cell screening for 72 h; monoclone was selected randomly for Western blot identification, and cell line culture was further amplified.

RT-PCR

The cDNA was treated as the template for diluted 20-fold with PBS. 20 μL of the mixture of PCR reaction system (template), 2 × PCR premixed buffer (containing enzymes required by PCR, buffering substance, dNTP and chelating agent, Takara Company) and ddH_2O (at a volume ratio of 3:10:7) was utilized for amplification. Each sample was conducted in triplicate. Real-time PCR machine (Thermo scientific Company) was adopted for detecting amplification. The primers used were shown as follows: Ebp1: 3'-GAAGCCTCACCTCCCAAA-5' and 5'-CCATTATCCAGAATCCCACA-3'; and internal reference GAPDH primers: 5'-ATGACCCCTTCATTGACCTCA-3' and 5'-GAGATGATGACCCTTTTGGCT-3'. The PCR conditions were shown as follows: 40 loops of 95°C for 7 min, 95°C for 10 s, and 60°C for 30 s; followed by at 60°C for 30 s, at 68°C for 2 min and at 20°C for 10 s.

Colony formation assay in vitro

The cells of each groups in the logarithmic growth phase were digested and counted. A total of 1 ml (1 × 10^3 cells/ml) of cells were seeded in 6-well plates. Two weeks later, cells in each well were fixed with 100% methanol for

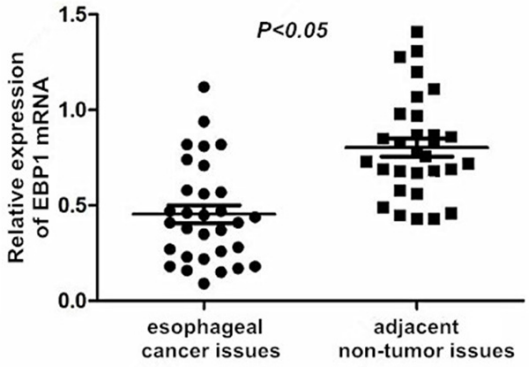

Figure 1. Real-time PCR for the relative expression level of EBP1 mRNA in esophageal cancer issues and adjacent non-tumor issues.

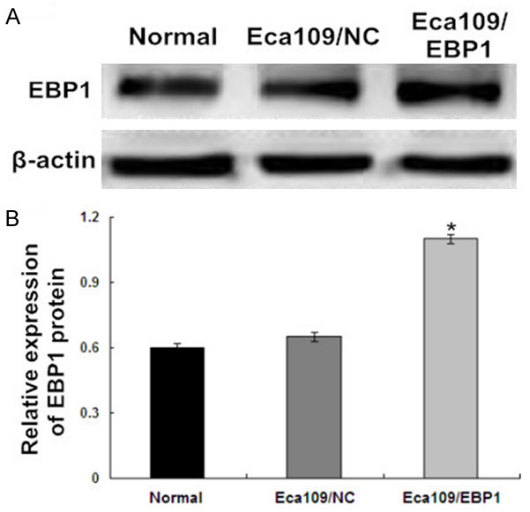

Figure 2. The expression of EBP1 were detected by Western-blot. A. Protein blotting stripe. B. The relative expression of EBP1 protein. *, $p<0.05$ versus the Eca109/NC group and Normal group.

30 min and stained with 0.1% crystal violet for 30 min. Cell colony-forming units were counted. Three repeats were tested.

Subcutaneous tumor formation in cells in vivo

Eca109-EBP1 or KYSE150-EBP1 cells were divided into two groups for injection, 100 μL (containing 1 × 10^6 cells) cell suspension was mixed with 100 μL matrigel (BD), and four 5-week-old male nude mice (SPF level BALB/c nude mice, which were bought from Shanghai SiLaiKe Laboratory Animal Co., Ltd) were assigned in each group for subcutaneous cell inoculation. Cell suspension in experiment group was injected into posterior left leg of mice, while that in control group was injected into posterior right leg of mice, cell were grown for about 4 weeks, tumors were obtained for weighing, and photos were taken.

The cell apoptosis were analyzed by flow cytometry

The cells of each groups in the logarithmic growth phase vaccinated in 60 mm^2 culture dishes with a density of 1 × 10^6 cells per dish. After 48 hours of the drug processing, collect the cells, take 4 groups of 105 cells for washing twice with pre-cooled PBS, and re-suspend them in 400 μL of 1 × Binding buffer with the addition of 10 μL Annexin V-FITC, blend the mixture gently till it becomes even, after that, start the reaction avoiding light at 4°C for 15 to 20 minutes; subsequently, 5 μL propidium iodide were added and mixed. At last, the cell apoptosis rate were detected by flow cytometer. Three repeats were tested.

Western blot

Cell lysis was conducted with RIPA lysis buffer containing protease inhibitor and phospholipase inhibitor (Sigma), and cells were placed on ice for 20 to 30 min of incubation, followed by centrifugation at 14000 rpm for 15 min and determination of protein concentration. Lysis buffer with equivalent protein content was taken for SDS-PAGE electrophoresis, then proteins were transferred onto polyvinylidene fluoride membrane (PVDF), blocked with TBST containing 3% BSA for 30 to 60 min at room temperature, and incubated with anti-EBP1 primary antibody (1:1000, Abcam), anti-P53 primary antibody, anti-bcl primary antibody (1:1000, Cell Signaling Technology), and anti-β-actin (1:5000, Sigma) at 4°C overnight. On the following day, cells were washed with TBST buffer for 5 min for three times, followed by incubation with rabbit or mouse secondary antibody (1:2000, Santa Cruz) at room temperature for 1 h, and washing with TBST buffer for 5 min for three times. Cells were reacted with substrate and exposed. β-actin and Tubulin were treated as the internal reference strips of experiment, respectively.

Statistical analysis

All statistical analyses were performed with Student's t-Test and represented as mean ± standard deviation (SD) unless noted other-

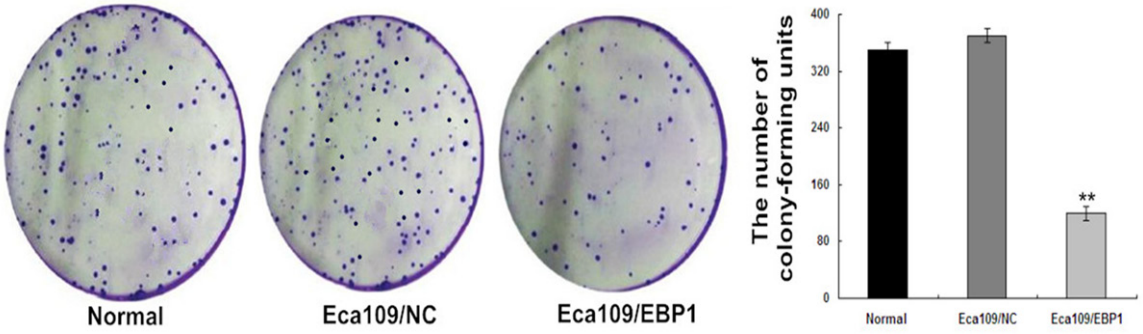

Figure 3. The effects of EBP1 on cell proliferation were detected by colony formation assay. **, p<0.01 versus the Eca109/NC group and Normal group.

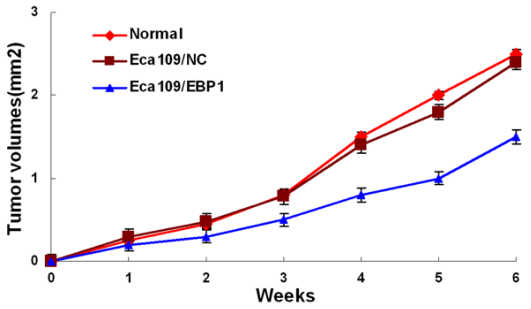

Figure 4. Animal experiment in vivo, Eca109/EBP1 group were significantly smaller in size and volume compared with the Eca109/NC group and Normal group.

wise. No animal or sample was excluded from the analysis. The *p* values were designated as: **, p<0.05; **, p<0.01*.

Results

Low expression of E-cad in esophageal cancer

The RT-PCR results demonstrated that the EBP1 mRNA expression in esophageal cancer issues was obviously lower than adjacent tissue,the results were validated by the non-parametric sum of ranks of paired samples, and the difference was significant (P<0.05, **Figure 1**). It was suggested that EBP1 in esophageal cancer is low expression and plays a tumor inhibitory effect possibly.

E-cad were successfully transfected

The Western blot results showed that compared with the Eca109/NC group and Normal group, the expression of EBP1 protein in Eca109/EBP1 group elevated markedly (P< 0.05, **Figure 2**).

EBP1 inhibit cell proliferation in vitro

The number of colony-forming units in the EC109/EBP1 group was obviously lower than the EC109/NC group and Normal group (P<0.05, **Figure 3**). It was suggested that the EBP1 can inhibite cell colony formation in vitro.

EBP1 inhibit tumor growth in vivo

Esophageal cancer nude mouse subcutaneous planting tumor model were successfully established, and the animals were closely calculated for tumor growth and tumor-formation rate for 6 weeks. The results showed that Eca109/EBP1 group tumors were significantly smaller in size and volume than Eca109/NC group and Normal group (P<0.05, **Figure 4**). It was suggested that the EBP1 could inhibit tumor growth in vivo.

EBP1 induced cell apoptosis

The Flow Cytometry results demonstrated that the apoptosis rate of cells in the EC109/EBP1 group was significantly higher than the EC109/NC group and Normal group (P<0.05, **Figure 5**). It was suggested that the EBP1 could promote cells apoptosis.

The expression of p53 and bcl-2 were detected by Western-blot

The Western-blot results showed that the expression of p53 protein in Eca109/EBP1 group were significantly lower than Eca109/NC group and Normal group, whereas the expression of bcl-2 were dramatically increased compare with Eca109/NC group and Normal group (P<0.05, **Figure 6**).

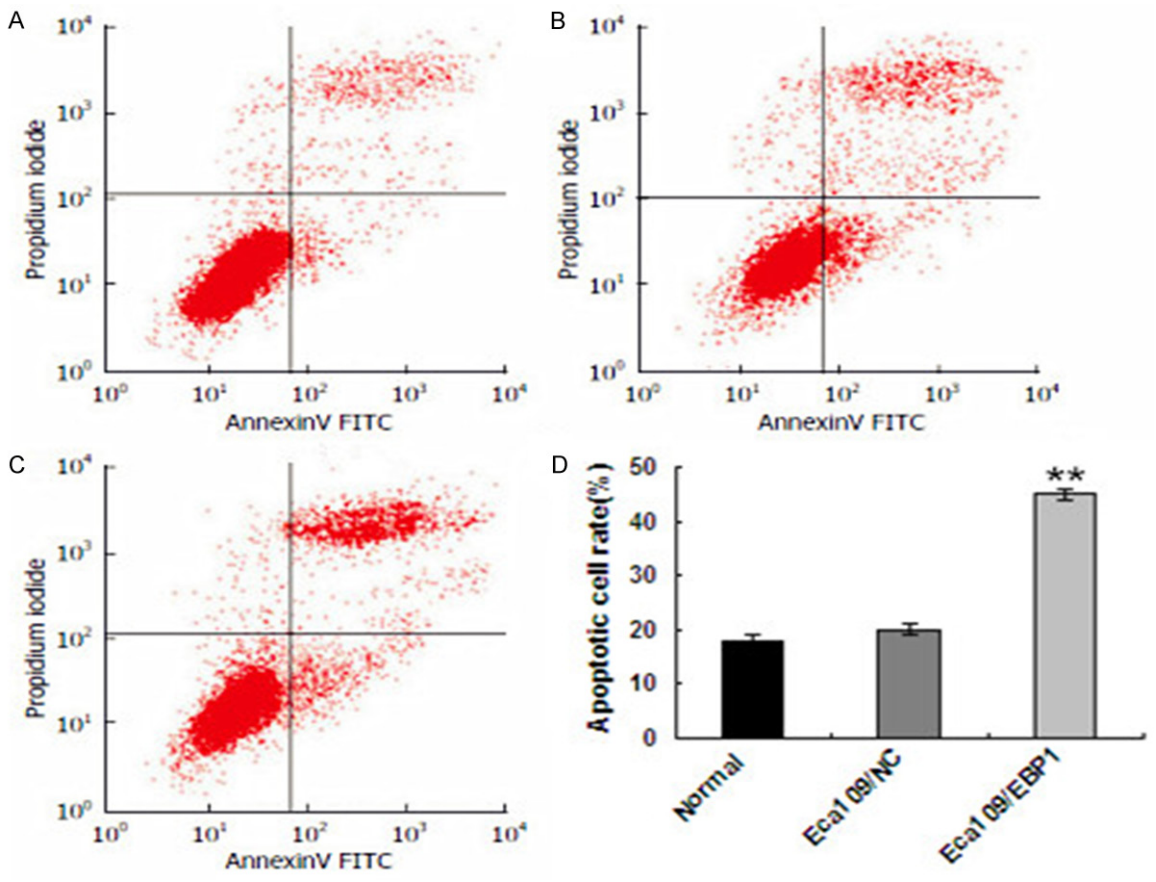

Figure 5. The effects of EBP1 on cell apoptosis were detected by Flow Cytometry. **, p<0.01 versus the Eca109/NC group and Normal group.

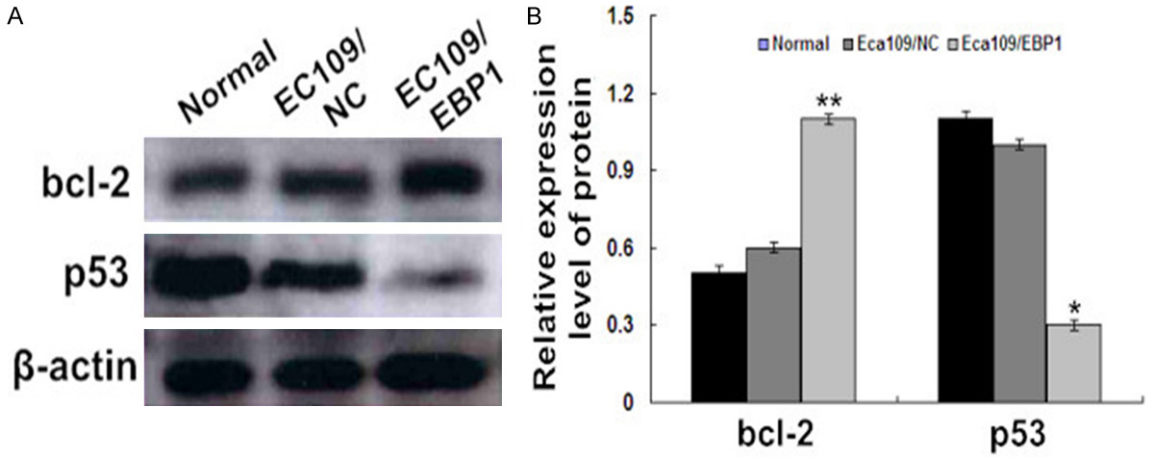

Figure 6. The expression of p53 and bcl-2 were detected by Western-blot. A. Protein blotting stripe. B. The relative protein content. **, p<0.01, *, p<0.05 versus the Eca109/NC group and Normal group.

Discussion

Esophageal squamous cell carcinoma (ESCC) is one of the common malignant tumors with high fatality rate and morbidity. And also, China is one of the countries with high incidence. ErbB3-binding protein 1 (EBP1) is the intracellular binding protein of ErbB3, which participates in

a series of important physiological processes and signal transduction, and is closely correlated with cell differentiation and proliferation. The abnormal expression (low or high expression) of EBP1 can be seen in multiple tumors. However, the role of EBP1 in esophageal cancer remains unclear. Therefore, we speculate that EBP1 may also exert the function of tumor suppressor in esophageal cancer, and it could be served as the potential biomarker or molecular target.

Results in this research indicate that expression of EBP1 is reduced in human esophageal cancer tissues, while over-expression of EBP1 can inhibit the proliferation significantly, viability and colony formation ability of esophageal cancer Eca109 cells in vivo, and can effectively inhibit tumor formation ability of esophageal cancer cells in vivo. Our results have not been verified in normal esophageal cells, but it is verified in this research that EBP1 expression is remarkably higher in normal tissues than in esophageal cancer tissues. The above research results suggest that EBP1 can inhibit the genesis and development of esophageal cancer. As has been verified in research, EBP1 expression is down-regulated in multiple cancer tissues, such as bladder cancer, prostate cancer, breast cancer and oral cancer, while over-expression of EBP1 can markedly inhibit proliferation of multiple cancer cells described above [7, 10, 15, 16]. In the meantime, over-expression of EBP1 can inhibit tumor cell proliferation in gastrointestinal cancer adjacent to esophagus, such as tongue squamous cell carcinoma [17]. These studies have strongly verified our results and confirmed that, EBP1 can effectively inhibit growth of esophageal cancer cells in vivo and in vitro, and exerts the function of tumor suppressor. So how can EBP1 inhibit the growth and tumor formation of esophageal cancer cells? It is demonstrated that EBP1 can regulate cell cycle and apoptosis, EBP1 expression is closely related to cell apoptosis, and decreasing EBP1 expression renders enhanced cell viability while weakened apoptosis [18, 19]. Therefore, cell cycle and apoptosis are detected in this research, the results indicate that EBP1 expression can markedly induce apoptosis of esophageal cancer cells. In terms of cell cycle, it is observed in Eca109/EBP1H cells that EBP1 can remarkably inhibit G2 stage of cell cycle, but it has no influence on other cells, suggesting that the effects of EBP1 on cell cycle may be dose- and cell origin-dependent. Therefore, the above results indicate that EBP1 controlling esophageal cancer cell proliferation through inducing apoptosis is associated with universal significance. EBP1 can form transcriptional regulation complex with bcl-2, and p53 [20-22]. Apoptosis-associated factor detection results suggest that EBP1 up-regulates expression of bcl-2 and P53 genes, and suppresses esophageal cancer cell growth. Bcl-2 and P53 can induce apoptosis and inhibits proliferation and tumor formation of cancer cells; in addition, down-regulated expression of Rb and up-regulated expression of P53 genes may be involved in the effects of EBP1 on esophageal cancer cells.

To sum up, it is verified in this research that EBP1 is down-expressed in esophageal cancer, which can regulate expression of bcl-2 and P53, induces esophageal cancer cell apoptosis, thus inhibiting the growth and tumor formation ability of esophageal cancer cells in vivo and in vitro. It is also revealed that EBP1 may play a certain role in the genesis and development of esophageal cancer. Therefore, EBP1 expression may be served as a potential biomarker or therapeutic target of esophageal cancer, which provides new thought and novel strategy for the clinical research and treatment of esophageal cancer.

Acknowledgements

This work was supported by a grant from Science and Technology program of *Shandong province* (No. SD2015ly112).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Peiliang Zhang, Department of Radiotherapy, Linyi Central Hospital, 17 Health Road, Yishui County, Shandong Province, China. Tel: +86 543 3258720; Fax: +86 543 3257792; E-mail: zhangplsd123@163.com

References

[1] De Santis CE, Lin CC, Mariotto AB. Cancer treatment and survivorship statistics. CA Cancer J Clin 2014; 64: 252-271.

[2] Han HJ, Russo J, Kohwi Y, Kohwi-Shigematsu T. SATB1 reprogrammes gene expression to pro-

mote breast tumour growth and metastasis. Nature 2008; 452: 187-193.
[3] Salehi M, Moradi-Lakeh M, Salehi MH, Nojomi M, Kolahdooz F. Meat, fish, and esophageal cancer risk: a systematic review and dose-response meta-analysis. Nutr Rev 2013; 71: 257-267.
[4] Lamartine J, Seri M, Cinti R, Heitzmann F, Creaven M, Radomski N, Jost E, Lenoir GM, Romeo G, Sylla BS. Molecular cloning and mapping of a human cDNA(PA2G4) that encodes a protein highly homologous to the mouse cell cycle protein p38-2G4. Cytogenet Cell Genet 1997; 78: 31-35.
[5] Zhang Y, Woodford N, Xia X, Hamburger AW. Repression of E2F1-mediated transcription by the ErbB3 binding protein Ebp1 involves histone deacetylases. Nucleic Acids Res 2003; 31: 2168-2177.
[6] Lessor TJ, Hamburger AW. Regulation of the ErbB3 binding protein Ebp1 by protein kinase C. Mol Cell Endocrinol 2001; 175: 185-191.
[7] He HC, Ling XH, Zhu JG, Fu X, Han ZD, Liang YX, Deng YH, Lin ZY, Chen G, Chen YF, Mo RJ, Zhong WD. Down-regulation of the ErbB3 binding protein 1 in human bladder cancer promotes tumor progression and cell proliferation. Mol Biol Rep 2013; 40: 3799-3805.
[8] Hu B, Xiong Y, Ni R, Wei L, Jiang D, Wang G, Wu D, Xu T, Zhao F, Zhu M, Wan C. The downregulation of ErbB3 binding protein 1 (EBP1) is associated with poor prognosis and enhanced cell proliferation in hepatocellular carcinoma. Mol Cell Biochem 2014; 396: 175-185.
[9] Zhang Y, Linn D, Liu Z, Melamed J, Tavora F, Young CY, Burger AM, Hamburger AW. EBP1, an ErbB3-binding protein, is decreased in prostate cancer and implicated in hormone resistance. Mol Cancer Ther 2008; 7: 3176-3186.
[10] Zhang Y, Ali TZ, Zhou H, D'Souza DR, Lu Y, Jaffe J, Liu Z, Passaniti A, Hamburger AW. ErbB3 binding protein 1 represses metastasis- promoting gene anterior gradient protein 2 in prostate cancer. Cancer Res 2010; 70: 240-248.
[11] Lu Y, Zhou H, Chen W, Zhang Y, Hamburger AW. The ErbB3 binding protein EBP1 regulates ErbB2 protein levels and tamoxifen sensitivity in breast cancer cells. Breast Cancer Res Treat 2011; 126: 27-36.
[12] Sun J, Luo Y, Tian Z, Gu L, Xia SC, Yu Y. Expression of ERBB3 binding protein 1 (EBP1) in salivary adenoid cystic carcinoma and its clinicopathological relevance. BMC cancer 2012; 12: 499.
[13] Zhou X, Chen W, Zhang Y, Sun J, Wang Q, Yu Y. Potential therapeutic strategy for oral squamous cell carcinoma by ErbB3-binding protein 1 gene transfer. J Cancer Res Clin Oncol 2010; 136: 891-896.
[14] Kim CK, Nguyen TL, Joo KM, Nam DH, Park J, Lee KH, Cho SW, Ahn JY. Negative regulation of p53 by the long isoform of ErbB3 binding protein Ebp1 in brain tumors. Cancer Res 2010; 70: 9730-9741.
[15] Lessor TJ, Yoo JY, Xia X, Woodford N, Hmaburger AW. Ectopic expression of the ErbB-3 binding protein ebp1 inhibits growth and induces differentiation of human breast cancer cell lines. J Cell Physiol 2000; 183: 321-329.
[16] Mei Y, Zhang P, Zuo H, Clark D, Xia R, Li J, Liu Z, Mao L. Ebp1 activates podoplanin expression and contributes to oral tumorigenesis. Oncogene 2014; 33: 3839-3850.
[17] Wang Q, Yu YC, Chen WT, Pan HY, Zang ZY, Tao FJ. The inhibitory effects of ebp1 gene on proliferation of tongue squamous cell carcinoma cell lineTca8113 cells. Fudan Univ J Med Sci 2009; 36: 201-205.
[18] Ahn JY, Liu X, Liu Z, Pereira L, Cheng D, Peng J, Wade PA, Hamburger AW, Ye K. Nuclear Akt associates with PKC-phosphorylated Ebp1, preventing DNA fragmentation by inhibition of caspase-activated DNase. EMBO J 2006; 25: 2083-2095.
[19] Liu Z, Liu X, Nakayama KI, Nakayama K, Ye K. Protein kinase C-delta phosphorylates Ebp1 and prevents its proteolytic degradation, enhancing cell survival. J Neurochem 2007; 100: 1278-1288.
[20] Xia X, Cheng A, Lessor T, Zhang Y, Hamburger AW. Ebp1, an ErbB-3 binding protein, interacts with Rb and affects Rb transcriptional regulation. J Cell Physiol 2001; 187: 209-217.
[21] Zhang Y, Akinmade D, Hamburger AW. The ErbB3 binding protein Ebp1 interacts with Sin3A to repress E2F1 and AR-mediated transcription. Nucleic Acids Res 2005; 33: 6024-6033.
[22] Akinmade D, Lee M, Zhang Y, Hamburger AW. Ebp1-mediated I nhibition of cell growth requires serine 363 phosphorylation. Int J Oncol 2007; 31: 851-858.