

Original Article

Functional mechanism of zinc finger protein 703 in in vitro esophageal carcinoma cell line

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Abstract: Zinc finger protein (ZNF) participates in human cell proliferation and transformation. As one important transcriptional factor, it is closely correlated with occurrence of multiple human tumors. This study investigated the role and mechanism of ZNF703 in esophageal carcinoma cells. Experimental group was prepared using ZNF703 lentivirus transfection in esophageal cancer cell line Eca109, with control group in parallel. Real-time PCR was used to measure ZNF703 mRNA expression, followed by MTT assay to test cell viability, Transwell method to examine cell migration, and Matrigel assay to evaluate cell invasion. TUNEL assay was performed for observing cell apoptosis, followed by Western blot to quantify apoptosis related protein Bcl-2, Survivin and Bax expression. Eca109 cells had elevated ZNF703 expression ($P < 0.05$). Compared to control group, experimental group had enhanced optical density (OD) values of MTT, potentiated invasion or migration ability, lower apoptosis in time dependence ($P < 0.05$). Experimental group had elevated Bcl-2 and Survivin protein expression, and lower Bax expression ($P < 0.05$). ZNF703 is over-expressed in esophageal carcinoma Eca109 cells, and is correlated with enhanced cell viability. It can facilitate cell invasion and migration, and inhibit onset or progression of cell apoptosis, probably via facilitating expression of anti-apoptotic factor Bcl-2 or Survivin, and inhibiting expression of pro-apoptotic factor Bax.

Keywords: Zinc finger protein 703, esophageal carcinoma, cell proliferation, apoptosis, Bcl-2, survivin, Bax

Introduction

Esophageal cancer (EC) mainly derives from esophageal epithelium. China is one epidemic region of EC worldwide. Due to its high incidence and difficult in curable treatment, EC has higher mortality than other malignant tumors [1]. However, large variations still exist across different stages of EC, as early to middle stage cancer is still curable, although it is difficult to treat terminal stage EC patients. Common treatment approaches for EC include surgery, chemotherapy or radiotherapy, which may cause damage on normal organs with major adverse effects, thus compromising treatment efficacy [2]. Recent study of molecular and genetics of malignant tumor has made major progress [3]. Zinc finger protein (ZNF) is one transcriptional factor initially separated from transcriptional factor IIIA of African xenopus laevis by Miller in 1983. It is formed by the binding between amino acid residue and zinc ions, with independent finger like structural domain,

and participates in pathological processes of body cells including differentiation, proliferation and apoptosis [4]. Previous study showed the close correlation between ZNF and occurrence/progression of various human malignant tumors including colorectal carcinoma, gastric cancer, and its value in prognosis prediction. ZNF703 is one newly discovered ZNF family member, and is correlated with cancer cell infiltration or migration [5, 6]. Previous study suggested high expression level of ZNF703 in gastric cancer tissues compared to adjacent or normal tissues, and the close correlation between ZNF703 expression level and infiltration grade, lymph node metastasis and distal metastasis of cancer tissues [7]. The expressional profile of ZNF703 in EC cells, along with its effects of EC cell growth, however, has not been investigated. In this study, we transfected EC cell line Eca109 with lentiviral vector carrying ZNF703 gene, to observe its effects on survival, apoptosis and expression of related pro-

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Table 1. Primer sequences

Gene		Sequence (5'-3')
ZNF703	Forward	5'-AGCCGTC AAGAGCAATAACGAA-3'
	Reverse	5'-GTGCAGGGTCCGAGGT-3'
U6	Primer	5'-CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'

Table 2. ZNF703 expressional profile in Eca109 cells (Relative expression)

Time	Experimental group	Control group
12 h	1.103±0.021*	0.787±0.013
24 h	1.438±0.036*.#	0.768±0.015
48 h	1.894±0.075*.#,&	0.762±0.012

Note: *, $P < 0.05$ compared to control group; #, $P < 0.05$ compared to 12 h; &, $P < 0.05$ compared to 24 h.

teins, to analyze the in vitro role of ZNF703 in EC cells.

Materials and methods

Cells and reagents

Human EC cell line Eca109 (Institute of Medical Science, Chinese Academy of Medicine); MTT (Sigma, US); Fetal bovine serum (FBS, Sijiqing Biotech, China); RPMI-1640 culture medium (Gibco, US); Rabbit anti-human Bcl-2, Bax and Survivin polyclonal antibody, and horseradish peroxidase labelled goat anti-rabbit IgG (Zhongshan Jinqiao Biotech, China); Rabbit anti-human β -actin monoclonal antibody (Sigma, US).

Equipment

Incubator (Thermo, US); CO₂ incubator (SANYO, Japan); Inverted microscope (Nikon, Japan); Biometra T1 type PCR cyler (Biometra, US); Nucleic acid DNA quantification equipment (Eppendorf, Germany); Cold high speed centrifuge (Beckman, US); -80°C fridge (SANYO, Japan).

Normal cell culture

Cells were kept in DMEM medium containing 10% FBS in a 37°C chamber with 5% CO₂. Cells were digested in 0.25% trypsin, and were inoculated into 24-well plate at 10⁵/L density. When cells reached 80% confluence, DMEM containing 1% FBS was applied for 24 h continuous incubation.

Preparation of lentiviral plasmid

Lentivirus was expressed and packaged. Plasmid containing ZNF703 and liposome were added into serum-free, antibiotics-free DMEM for mixture and room temperature for 25 min. Cell transfection was performed in culture dish for 72 h incubation.

The supernatant was collected and filtered for obtaining viral suspensions, which were centrifuged to discard the supernatant. PBS buffer was added to re-suspend viral particles, which were kept at -80°C for further use.

Lentiviral plasmid transfection in EC cells

Cells were cultured and mixed with lentiviral plasmid when reaching 80% attachment growth. Cells were continuously cultured at 37°C with 5% CO₂.

Experimental grouping

Experimental group: EC cells Eca109 at log-phase were inoculated at 1×10⁴/ml into 6-well plate. Lentiviral plasmid was added to transfect cells as described above, followed by 48 h incubation.

Control group: Eca109 cells were transfected by empty plasmid and cultured in normal conditions with RPMI-1640 containing 10% FBS for 48 h.

Real-time PCR for ZNF703 expression in EC cells

Attached cells at 1×10⁶/ml were mixed with TRIzol, followed by homogenization. With addition of chloroform, the mixture was incubated at room temperature for 3 min. The supernatant was collected by centrifugation, and was mixed with 0.5 ml isopropanol for 10 min. After centrifugation, the supernatant was discarded and RNA was solved in 40 μ l DEPC treated water for storage at -80°C for further use. Total RNA was used to synthesize polyA tail of miRNA for generating cDNA. Using cDNA as the template, PCR amplification was performed using primers designed by Sangon (China) as shown in **Table 1**. Reaction conditions were: 95°C 5 min, followed by 30 cycles each containing 95°C 30 s, 58°C 30 s, and 72°C 30 s, ended by 72°C 10 min.

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Table 3. Proliferation activity of Eca109 cells (Relative expression)

Time	Experimental group	Control group
12 h	1.002±0.005*.#	1.001±0.011
24 h	1.034±0.012*.#	1.003±0.008
48 h	1.092±0.019*.#,&	1.002±0.010

Note: *, $P < 0.05$ compared to control group; #, $P < 0.05$ compared to 12 h; &, $P < 0.05$ compared to 24 h.

MTT assay for Eca109 cell viability

Eca109 cells at log-growth phase were selected and seeded into culture plate at 8×10^4 per well density. Cells were cultured in a 37°C with 5% CO_2 . After 12 h, 24 h and 48 h, cell activity was measured by adding 20 μL MTT solution (5 mg/mL) for 4 h incubation. The reaction was quenched by adding 150 μL DMSO. Absorbance (A) values from each well were measured at 570 nm wave length.

Transwell method to record invasion and migration of Eca109 cells

Invasion assay: One day before experiment, matrix gel was dilute using serum-free RPMI-1640 medium and was paved on the chamber. Cells were added at 1×10^6 per ml density, followed by the addition of 200 μL cell suspension in serum-free medium. On the lower membrane, 1300 μL complete culture medium was added for 37°C culture with 5% CO_2 . Cells were fixed in methanol, stained in crystal violet and were counted.

Migration assay: Cells were added at 1×10^6 per ml density, followed by the addition of 200 μL cell suspension in serum-free medium. On the lower membrane, 1300 μL complete culture medium was added for 37°C culture with 5% CO_2 . Cells were fixed in methanol, stained in crystal violet and were counted.

TUNEL assay for apoptosis of Eca109 cells

Tissue samples were dehydrated and immersed in paraffin for preparing tissue sections, which were immersed in xylene and gradient ethanol. Proteinase K working solution was used to process tissues, followed by the addition of TUNEL reaction mixture. Number of apoptotic cells

was counted. Converter-POD as added, followed by DAB culture, hematoxylin counterstaining and cell count.

Western blot assay for Bcl-2, Bax and Survivin protein expression in Eca109 cells

Cells were lysed in lysis buffer to extract proteins. In brief, cells were ruptured by ultrasound and centrifuged at 12,000 rpm to collect the supernatant, which was stored at -80°C . Protein samples were mixed with loading buffer for boiling, and were separated by gel electrophoresis. Proteins were transferred to PVDF membrane, which was rinsed in TBST and blocked overnight. Primary antibody (1:100 dilution factors) was added for 2 h room temperature incubation, followed by 1 h room temperature in horseradish peroxidase labelled secondary antibody. The membrane was then developed, exposed and imaged.

Data analysis

SPSS 17.0 statistical software was used for data processing. Enumeration data were analyzed by chi-square test, whilst measurement data were processed in analysis of variance (ANOVA) and presented as mean \pm standard deviation (SD). A statistical significance was defined when $P < 0.05$.

Results

ZNF703 expression increased in Eca109 cells after transfection

To test ZNF703 expression in Eca109 cells, we used real-time PCR for assays. Results showed that, compared to control group, experimental group had significantly elevated ZNF703 expression ($P < 0.05$). With elongated treatment time, the increase of ZNF703 expression in experimental group was more significant ($P < 0.05$, **Table 2**).

Proliferation activity of Eca109 cells enhanced after transfection

To compare proliferation activity of Eca109 cells, MTT assay was performed. Results showed that experimental group had elevated OD values ($P < 0.05$ compared to control group). With elongated treatment time, experimental

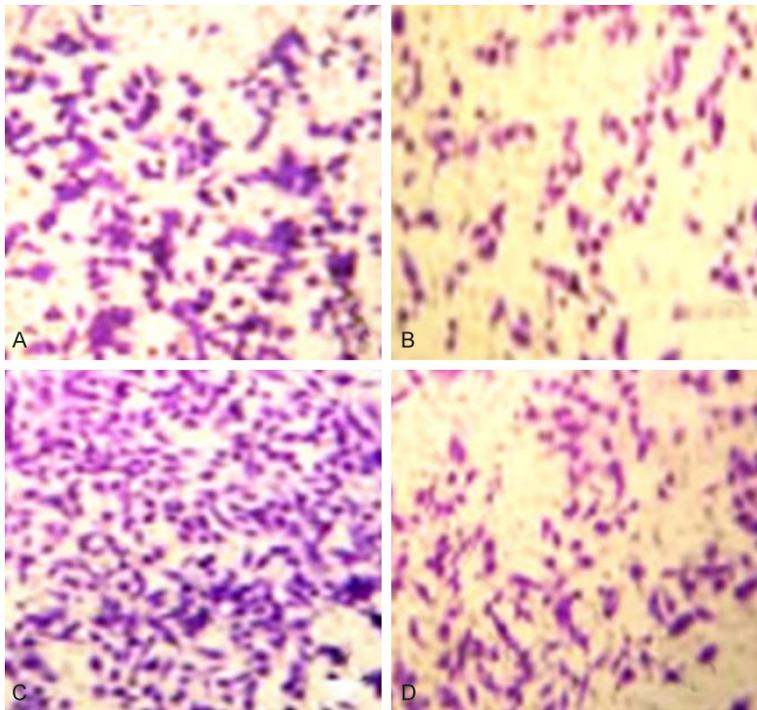


Figure 1. Cell invasion and migration potency (40×). A. Invasion in experimental group; B. Invasion in control group; C. Migration in experimental group; D. Migration in control group.

Invasion and migration ability of Eca109 cells enhanced after transfection

We examined cell invasion and migration ability of Eca109 cells. Results showed enhanced invasion and migration potency of Eca109 cells in experimental group ($P < 0.05$ compared to control group, **Figure 1**).

Eca109 cell apoptosis reduced after transfection

To compare any difference of cell apoptosis between two groups, TUNEL assay was employed to investigate cell apoptosis condition at 48 h after intervention. Results showed that, compared to control group, Eca109 cells in experimental group had decreased cell apoptosis ($P < 0.05$, **Figure 2**; **Table 4**).

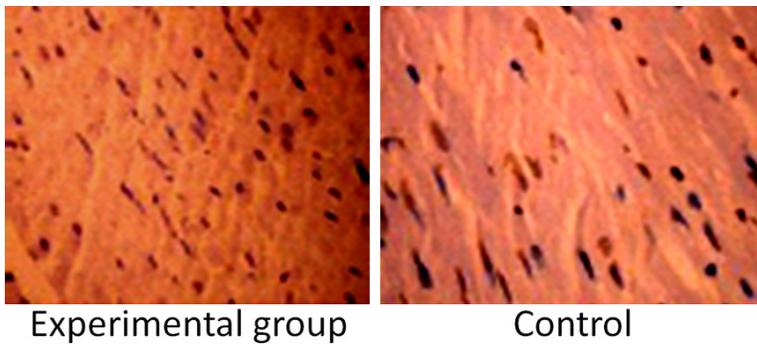


Figure 2. Eca109 cell apoptosis detected by TUNEL assay (40×).

Bcl-2, Bax and Survivin protein expression changed in Eca109 cells after transfection

To compare Bcl-2, Bax and Survivin protein expression in Eca109 cells, Western blot results showed elevated Bcl-2 and Survivin protein expression in experimental group, which also had decreased Bax protein expression ($P < 0.05$ compared to control group). With elongated treatment time, the extent of elevated Bcl-2 or Survivin and decreased Bax protein expression became more potent with significant difference among different time points ($P < 0.05$, **Table 5**; **Figure 3**).

Table 4. Apoptosis of Eca109 cells between two groups (%)

Time	Experimental group	Control group
12 h	5.01±1.56*	10.27±1.44
24 h	3.81±1.23*.#	11.48±1.37
48 h	2.32±0.49*.#,&	11.85±1.12

Note: *, $P < 0.05$ compared to control group; #, $P < 0.05$ compared to 12 h; &, $P < 0.05$ compared to 24 h.

group had even higher OD values ($P < 0.05$, **Table 3**).

ment time, the extent of elevated Bcl-2 or Survivin and decreased Bax protein expression became more potent with significant difference among different time points ($P < 0.05$, **Table 5**; **Figure 3**).

Discussion

Occurrence and progression of malignant tumor is one pathological process involving multiple factors and steps. Moreover, various body genes are involved in this process to exert

Table 5. Bcl-2, Bax and Survivin protein expression in Eca109 cells (Relative expression)

Item	Experimental group	Control group
Bcl-2		
12 h	0.08±0.01*	0.64±0.37
24 h	0.10±0.03*,#	0.68±0.38
48 h	0.18±0.11*,#,&	0.69±0.41
Bax		
12 h	0.39±0.17*	0.10±0.09
24 h	0.22±0.13*,#	0.10±0.08
48 h	0.13±0.10*,#,&	0.11±0.07
Survivin		
12 h	0.17±0.02*	0.12±0.06
24 h	0.26±0.08*,#	0.10±0.09
48 h	0.38±0.09*,#,&	0.12±0.07

Note: *, $P < 0.05$ compared to control group; #, $P < 0.05$ compared to 12 h; &, $P < 0.05$ compared to 24 h.

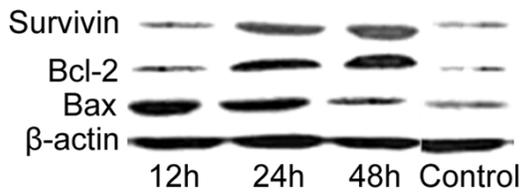


Figure 3. Bcl-2, Bax and Survivin protein expression in Eca109 cells from all groups.

biological functions. Gene treatment for tumors has arisen recently, and has become a focus for clinical and basic study. It mainly aims to repair or correct mutated gene expression, and mediates certain regulatory function on growth, proliferation and metastasis of malignant tumors [8]. Study showed that gene therapy has become a novel approach with high potency for treating malignant tumors [9]. ZNF703 is one newly discovered NET family member, and can regulate DNA recognition, RNA packaging, transcriptional activation, protein folding, lipid binding, and formation, growth of malignant tumor plus body immunity. During these processes, ZNF703 also participate in various signal transduction pathway, exerting synergistic effects for mediating growth and differentiation of malignant tumors [10-13]. In this study, we selected EC cell line Eca109 to analyze the effect of ZNF703 on cancer cell growth.

Firstly, ZNF703 lentiviral plasmid was prepared and used to transfect Eca109 cells as the

experimental group, in parallel with untreated control group for normal culture. Assays showed elevated ZNF703 expression in experimental group in a time dependent manner, indicating successful transfection of ZNF703 viral vector and over-expression in transfected Eca109 cells. Sircoulomb et al studied breast cancer and found certain correlation between ZNF703 expression and clinical or pathological features in breast cancer [14]. ZNF703 was found to be highly expressed in breast cancer cells, but with lower expression level in normal breast gland epithelium cells. Those breast cancer cells with ZNF703 over-expression had significantly potentiated gene instability, with potent tumor invasion or metastasis potency.

Secondly, to compare proliferation activity of Eca109 cells between two groups, and any change regarding invasion or migration ability, we employed MTT and transwell approach and found enhanced OD values in experimental group, with potentiated invasion or migration potency. With elongated intervention time, experimental cells showed more potent increase of OD values or invasion/migration potency. These results indicated that the existence of ZNF703 over-expression in Eca109 cells, and its role in facilitating cell proliferation activity, and cell invasion nor migration. Previous study showed the over-expression of ZNF703 gene could bind with promoter site of transformation growth factor beta (TGF- β) receptor II, and block the inhibitory role of TGF- β on tumor cell proliferation, thus suppressing expression level of cell cycle inhibitory protein, modifying cell cycle to accelerate the entry into S phase from G1 phase, thus potentiating proliferation activity of malignant tumor cells [15, 16]. Moreover, over-expression of ZNF703 participates in the induction of WNT and NOTCH signal pathway transduction, to facilitate expression of related proteins, thus modifying differentiation and self-renewal ability of breast cancer stem cells [17, 18], as similar with our results.

Moreover, to compare apoptosis condition of Eca109 cells between experimental and control group, we performed assays and found decreased apoptosis of tumor cells in experimental group. Further assay on apoptosis related proteins revealed elevated Bcl-2 and Survivin protein expression in experimental

group, plus lower Bax protein expression. With elongated intervention time, the potent of elevated Bcl-2 and Survivin expression or decreased Bax expression became more significant. As one important anti-apoptotic protein, Survivin participates in apoptosis suppression by various factor inductions [19]. Bcl-2 also has certain apoptotic suppression effects and can elongate median survival time of cultured cells [20]. Bax as one important pro-apoptotic factor, can down-regulate Bcl-2 expression level, thus inducing cell apoptosis [21]. Results of this study showed that over-expression of Eca109 cells can facilitate Bcl-2 or Survivin expression, and inhibit Bax expression, thus inhibiting cell apoptosis to certain extents.

Conclusion

EC cell line Eca109 has over-expression of ZNF703, which can enhance cell proliferation activity, facilitate cell invasion or migration, and inhibit cell apoptosis occurrence or progression, probably related with the facilitation of anti-apoptotic factor Bcl-2/Survivin expression, and inhibition of pro-apoptotic factor Bax. Future study can focus on animal model, gene and protein levels to assist the optimization of treatment plan for clinics, thus achieving better efficacy and improving patient prognosis.

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Disclosure of conflict of interest

None.

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