ATPase H+ Transporting V0 Subunit C regulates Twinfilin Actin Binding Protein 1 and promotes metastatic ability of esophageal cancer

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Research Article

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Abstract

We aimed at investigating the implication of ATP6V0C in esophageal cancer (ECa) and exploring how ATP6V0C participates in this process. ATP6V0C expression in 46 pairs of ECa tissues was determined by RT-PCR analysis, and the relationship between ATP6V0C and clinicopathological indicators as well as prognosis of ECa patients was analyzed. Results demonstrated that ATP6V0C was significantly increased in ECa. Participants with high-ATP6V0C exhibited markedly higher incidence of metastasis and shorter survival rates. Inhibition of ATP6V0C attenuated cell invasive and metastasis abilities. Meanwhile, Luciferase assay confirmed the binding between ATP6V0C and TWF1. TWF1 expression showed an increase in ECa cell lines and tissues, which was positively correlated with ATP6V0C level. In addition, we found that overexpression of TWF1 counteracted the effects of knockdown of ATP6V0C on the proliferation and migration of ECa, and thus affect the malignant progression of ECa. ATP6V0C and TWF1 are both highly expressed and positively correlated in tumor tissues of ECa patients. In addition, ATP6V0C accelerated the malignant progression of ECa cells through interacting with TWF1.

1. Introduction

ECa is the most common malignant tumor of the digestive tract in the world, with the incidence increased year by year [1, 2]. In China, the incidence of ECa rose from 19.24/100,000 in 2003–2007 to 23.62/100,000 in 2018, ranking 6th in malignant tumors [3, 4]. Most of the ECa patients detected and treated clinically are in the middle and late stages, with a five-year survival rate of only 15%~40% [5, 6]. Given this, it’s important to find effective gene or protein targets for early diagnosis and treatment of ECa [7, 8].

ATPase H+ Transporting V0 Subunit C (ATP6V0C) is a multi-subunit protein complex associated with cell membrane. It is a proton pump driven by ATP, and energy obtained by hydrolysis of ATP is used to transport protons across the membrane [9, 10]. Current studies have confirmed that the main functions of V-ATPase are to maintain the acidic environment of vesicles, the fusion of vesicles and microvesicles, energy transport, and changes in cell pH [11, 12]. Among them, there are relatively few reports on the development of ATP6V0C in tumor [9, 13]. Bioinformatics database revealed that ATP6V0C had a high expression in ECa tissues and was thus selected as our study object. Meanwhile, further bioinformatics analysis suggests that Twinfilin Actin Binding Protein 1 (TWF1) is one of the potential target genes of ATP6V0C.

The aim of the current research was to investigate the expression of ATP6V0C and TWF1 in ECa tissues and explored their effects on the biological functions of ECa cells. The purpose of this study was to investigate whether ATP6V0C is implicated in malignant progression of ECa cells through modulating TWF1.

2. Methods
2.1. Patients and ECa samples. 46 pairs of tumor tissue and adjacent non-tumor tissue obtained from patients with ECa undergoing surgical resection were diagnosed as ECa through postoperative pathological analysis. Patients diagnosed with esophageal cancer via cytology and tissue pathology, with no mental illness nor other malignant tumor were included in this study. Patients with severe organic failure or autoimmune system defects, undergoing other anti-tumor treatments within 1 month prior to the treatment were excluded. Each patient signed an informed consent form before the start of the study. This study got the approval from the ethics committee of our hospital.

2.2. Transfection. For transient transfection, cells were treated with Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA) mixed with shRNAs (Keygen, Nanjing, China ) at the cell density of 30-50%.

2.3. Cell Counting Kit-8 (CCK-8) assay. Cells incubated in the plates (96-well) were subjected for CCK-8 assay based on the company’s instructions.

2.4. Transwell cell migration assay. 24 hours after transfection, cells were prepared into cell suspensions, and cell invasion assays were performed using Corning's Transwell chamber (10000 cells/well) according to the instructions. Cells stained with crystal violet were observed in five randomly selected fields under the microscope, and migrating cells were observed by counting.

2.5. Cell wound Healing. After 48 hr of transfection, the density of the plated cells was determined according to the size of the cells, and the confluency of the cells reached 90% or more the next day. After stroke, cells were washed 2-3 times with PBS and observed.

2.6. RT-PCR. Total RNA was extracted from cells using TRIzol reagent, followed by being reverse-transcribed to cDNA by PrimeScript RT reagent kit. Reverse transcription was performed by PrimeScript ™ RT kit. qPCR was carried out with the SYBR Green kit. The primers were listed in Table 1. The relative expression was normalized to that of GAPDH according to 2−ΔΔCt methods.

2.7. Dual-luciferase reporter assay. The plasmid was constructed into pmir with the mutation binding site. ECa cell lines OE19 and OE33 were co-transfected with luciferase reporter plasmids. The plasmid were then introduced into the cells using Lipofectamine 3000. The luciferase activity of each group was measured after 48 h of transfection.

2.8. Statistical analysis. Statistical analysis was performed using SPSS 22.0 software. χ² test and the exact probability Fisher test were used for univariate analysis, while COX regression was for multivariate analysis. Data are presented as X ± SD, and P less than 0.05 was statistically significant.
Table 1
The following primers were used in the qPCR reaction

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP6V0C</td>
<td>Forward 5'-CAACGCTGCGGAGATCCA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CCCAGGGCGCTGAAGAC-3'</td>
</tr>
<tr>
<td>TWF1</td>
<td>Forward 5'-CAATGAGAGCCCAGAGGATCA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-ACTGCACATAGTTGAGCTGTCT-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward 5'-CCTGGCACCCAGCACAAT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GCTGATCCACATCTGCTGGAA-3'</td>
</tr>
</tbody>
</table>

3. Results

3.1. ATP6V0C was closely related to the incidence of metastasis and prognosis of ECa patients. In comparison to the normal control group, ATP6V0C showed abnormally high expression in ECa tumor tissue samples (Fig. 1A). According to qPCR results of ATP6V0C expression, we split the 46 pairs of tissue samples into high expression group and low expression group. Table 2 indicated that lymphatic and distant metastases were positively correlated with high ATP6V0C expression, but not with age or pathological stage (Fig. 1B). Therefore, the above findings indicated that ATP6V0C acted as a novel biological index for the prediction of the malignant progression of ECa. Furthermore, compared to HEEC cells, ATP6V0C was significantly overexpressed in ECa cells, specially in OE19 and OE33 (Fig. 1C). Subsequently, Kaplan–Meier survival curve revealed that the poor prognosis of ECa patients was closely related to the high expression of ATP6V0C.
3.2. Silencing ATP6V0C inhibits the proliferation and invasiveness of ECa cells. To clarify the role of ATP6V0C in ECa cell function, knockdown of ATP6V0C was established in OE19 and OE33 cell lines (Fig. 1D). Afterwards, it was found that knockdown of ATP6V0C remarkably attenuated the proliferative capacity as well as the migratory or invasion abilities of ECa cells, measured by CCK8 (Fig. 2A), cell wound healing test and Transwell experiment (Fig. 2B-C).

3.3. Interaction between ATP6V0C and TWF1. We performed a bioinformatics analysis (https://gtrd.biouml.org/) and found that TWF1 may be a target gene of ATP6V0C, which in turn affects ECa malignant progression. Figure 3A indicates that knocking down ATP6V0C resulted in a reduction in TWF1 expression in ECa cell lines both in mRNA and protein levels. Meanwhile, qPCR results showed an increased TWF1 expression in ECa tumor tissues (Fig. 3B), and a positive correlation between ATP6V0C and TWF1 was found. In addition, the luciferase reporter assay confirmed that ATP6V0C can target the TWF1 gene-specific sequence (Fig. 3C).

3.4. ATP6V0C can mediate TWF1 expression and promote malignant progression of ECa. To further understand how ATP6V0C and TWF1 inhibit the malignant progression of ECa cells, we performed co-transfection of sh-ATP6V0C sequences and TWF1 overexpression plasmid in ECa cell lines OE19 and

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### Table 2
Basic information of esophageal cancer patients with high ATP6V0C and low ATP6V0C expression.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Number of cases</th>
<th>ATP6V0C expression</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low (n = 29)</td>
<td>High (n = 17)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>18</td>
<td>10</td>
<td>0.399</td>
</tr>
<tr>
<td>≥60</td>
<td>28</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>T stage</td>
<td></td>
<td></td>
<td>0.828</td>
</tr>
<tr>
<td>T1-T2</td>
<td>28</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>T3-T4</td>
<td>18</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td>0.009</td>
</tr>
<tr>
<td>No</td>
<td>30</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>16</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Distance metastasis</td>
<td></td>
<td></td>
<td>0.004</td>
</tr>
<tr>
<td>No</td>
<td>33</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>13</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
OE33. It was found that TWF1 expression was increased in co-transfection group as compared to sh-ATP6V0C group, but reduced in comparison to TWF1 overexpression group (Fig. 4A). CCK-8 and Transwell experiments have demonstrated that TWF1-overexpression partially reversed the suppressive effects of ATP6V0C-knockdown on cell invasiveness and metastasis (Fig. 4B & 4C).

4. Discussion

The latest tumor statistics in China in 2015 showed that the incidence of ECa ranked the third among malignant tumors [3, 4]. The occurrence of ECa was the result of the mutual effect between environmental and genetic factors, among which the former mainly include smoking, excessive drinking, excessive intake of nitrite, and lack of micronutrients [5–8]. Environmental factors are initiating factors, however, the incidence of ECa varies among individuals exposed to the same environment, indicating that individual genetic factors play a key role in the progression of ECa [14].

In recent years, despite advances in diagnosis, surgical treatment, patient care and adjuvant therapy, morbidity and mortality have remained high [15]. In addition to the unclear pathogenesis of ECa, which leads to the failure to formulate and carry out effective preventive measures, the lack of clinical diagnosis and treatment of ECa is also one of the main reasons [15, 16]. At present, clinically targeted tumors such as esophageal cancer still lack very effective molecular diagnostic markers. At the same time, in addition to traditional surgery plus radiotherapy and chemotherapy, the application of gene therapy has been very poor [17, 18]. Fundamentally speaking, the main reason for this situation is the lack of clinical research on target genes. Therefore, identifying marker molecules to detect early esophageal cancer and precancerous lesions, looking for target genes that may be used in gene therapy, and explore the feasibility of their use as therapeutic targets are hot spots in the field of tumor research [7, 8].

The tumor database indicates that ATP6V0C in tumor tissues of ECa patients is dysregulated, indicating that ATP6V0C may act as a carcinogen to cause the occurrence of this cancer, or as a tumor suppressor to prevent it. At present, ATP6V0C was reported to be over-expressed in various cancer tissues, such as prostate cancer and colorectal cancer, and its expression level is closely relevant to clinicopathological characteristics and clinical prognosis. Consistently, this study found that ATP6V0C in ECa tissues was significantly up-regulated and was correlated with the incidence of patients' lymph node and distant metastasis, indicating that ATP6V0C may serve as a cancer-suppressing gene in esophageal cancer. Subsequently, we proved that ATP6V0C enhances the proliferation and invasiveness of ECa and thus play an essential role in this disease.

The innovation of this study lies that we performed a variety of molecular biology technologies and bioinformatics analysis and explored the functions of ATP6V0C in ECa and the potential molecular mechanism. Based on the basic data of a large number of clinical cases with esophageal cancer, the present work elucidated whether the age of esophageal cancer patients and the clinical and pathological staging are correlated with ATP6V0C level. All these findings provided theoretical basis and reference for ATP6V0C as a possible molecular logo that predicts the occurrence of esophageal cancer. Through online
data query and prediction, TWF1 was finally predicted to be the target binding gene of ATP6V0C. In this study, luciferase reporter gene experiment confirmed ATP6V0C can bind to the specific sequence of TWF1 gene. Meanwhile, it was confirmed by qPCR that ATP6V0C and TWF1 were positively correlated in ECa tissues, indicating that ATP6V0C may directly target TWF1 and play a positive regulatory role. Consistently, Western Blotting suggested a reduction in TWF1 expression induced by ATP6V0C knockdown. In addition, overexpression of TWF1 offset the inhibitory effect of ATP6V0C knockdown on cell functions, suggesting that ATP6V0C/TWF1 regulatory axis may exert crucial effects on the progression of ECa.

There are also obvious limitations in this article. For example, we have not verified the role of ATP6V0C through TWF1 to regulate the development of esophageal cancer tumors. In addition, we did not verify the role of ATP6V0C in the process by blocking TWF1. In the future, we will conduct animal experiments and further explore and verify the molecular mechanism. At the same time, we will also collect more clinical samples and patient data, the predictive value of ATP6V0C for the prognosis of patients with esophageal cancer was further studied and analyzed.

5. Conclusion

In summary, ATP6V0C and TWF1 are both highly expressed and positively correlated in tumor tissues of patients with ECa. In addition, ATP6V0C can promote the malignant progression of ECa cells through its interaction with TWF1.

Abbreviations

Esophageal cancer (ECa); ATPase H+ Transporting V0 Subunit C (ATP6V0C); V0 domain of microencapsulated ATPase (V-ATPase); Twinfilin Actin Binding Protein 1 (TWF1); human normal esophageal epithelial cell (HEEC); American Type Culture Collection (ATCC); dulbecco’s modified eagle medium (DMEM); fetal bovine serum (FBS); Cell Counting Kit-8 (CCK-8); phosphate buffered saline (PBS); standard deviation (SD).

Declarations

Ethical Approval

This study was in accordance with the Helsinki Declaration and approved by the Medical Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (Ethical approval NO. 20190531-03).

Competing interests

The authors declare that they have no competing interests.
Authors' contributions

IS conceived and designed the experiments; WJY collected clinical samples and corresponding clinical data; BZQ performed the experiments and wrote the manuscript; MN provided technical assistance; JA and LWZ performed the statistical analysis. All the authors made substantial contributions to the other works in this paper, including collection and analysis of the data and results. All authors agree to the publication of this paper.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

References


Figures
Figure 1

ATP6V0C is highly expressed in esophageal cancer tissues and cell lines. (A) Compared with the tissue of cancer, the level of ATP6V0C in the tumor tissue of the esophageal cancer patients increased significantly; (B) ATP6V0C was highly expressed in esophageal cancer tissues with lymph node metastasis or distant metastasis; (C) QRT-PCR detected the expression level of ATP6V0C in esophageal cancer cell lines; (D) qRT-PCR and Western Blotting verified the transfection efficiency in esophageal cancer cell lines OE19 and OE33. ATP6V0C expression was reduced after ATP6V0C was knocked down. Data are average ± SD, * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 2

Silencing ATP6V0C inhibits esophageal cancer cell proliferation and invasion and migration. (A) Transfection of ATP6V0C knockdown vectors significantly decreased the proliferation ability of esophageal cancer cells; (B) Knockdown of ATP6V0C significantly inhibited the invasive and migrative abilities of esophageal cancer cells; (C) Cell wound healing test detected the crawling ability of esophageal cancer cells after transfection of ATP6V0C knockdown vector in esophageal cancer cell lines OE19 and OE33. Data are average ± SD, * P < 0.05.
ATP6V0C directly regulates TWF1. (A) TWF1 expression level in the esophagus cell lines was significantly reduced after knocking down ATP6V0C; (B) qRT-PCR detected the expression of TWF1 in tumor tissues and adjacent tissues of esophageal cancer patients; (C) The luciferase reporter gene experiment suggested that ATP6V0C can directly bind to TWF1. Data are average ± SD, * P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 4

ATP6V0C can regulate TWF1 to promote the occurrence and development of esophageal cancer. (A) qRT-PCR detection of TWF1 expression levels after co-transfection of ATP6V0C knockdown vector and TWF1 overexpression vector in esophageal cancer cell lines OE19 and OE33; (B) Overexpression of TWF1 counteracted the ability of ATP6V0C knockdown to promote the proliferation and invasion of esophageal cancer cells; (C) Transwell invasion test detected invasion and migration of esophageal cancer cells after transfection of ATP6V0C knockdown vector and TWF1 overexpression vector. Data are average ± SD, * P < 0.05.