The correlation and clinical significance of PIK3CA, PIK3CB and HPV infection in esophageal squamous cell carcinoma

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Abstract
Background: Esophageal squamous cell carcinoma (ESCC) is a highly lethal cancer. Currently, human papillomavirus (HPV) is considered as a potential risk factor for ESCC, but this assumption is still contradictory. PI3K/Akt/mTOR network plays a key role in the virus/host cell crosstalk in HPV positive cancer cells. The purpose of this study was to investigate the relationship between the expression of PIK3CA, PIK3CB and HPV infection.

Methods: The expression of PIK3CA, PIK3CB, p16 and p53 in 156 cases of ESCC was detected by immunohistochemistry. Patients are followed up by telephone or clinic.

Results: In this study, the positive rates of PIK3CA, PIK3CB, p53 and p16 in ESCC were significantly higher than in normal esophageal mucosa. The expression of PIK3CA and PIK3CB was related to TNM stage and lymph node metastasis. The expression of p53 is related to the depth of tumor invasion. High p16 expression was found to be significantly correlated with tumor location, TNM stage, differentiation grade and lymph node metastasis. P16 expression is positively correlated with PIK3CA expression but not with PIK3CB and p53. Survival analysis further indicated that that PIK3CA and p53 were markers of poor diagnosis. P16 is a mark of favorable prognosis in ESCC.

Conclusions: The expression of PIK3CA and PIK3CB is related to the development of ESCC. The imbalance of PI3K/Akt signaling pathway is closely related to HPV infection and prognosis. The combined detection of PIK3CA and p16 is of great significance to evaluate the prognosis and optimize the treatment of ESCC.

Background
Esophageal cancer (EC) represents the eighth highest incidence of cancer worldwide (456,000 cases/year) and is usually diagnosed at an advanced stage, showing rapid progression and extremely poor prognosis [1, 2]. The two main histological types of EC are squamous cell carcinoma and adenocarcinoma, and 90% of cases are esophageal squamous cell carcinoma (ESCC). Despite advances in medical technology, such as early screening and surgery combined with chemotherapy or radiotherapy, the prognosis of EC is still very poor [3]. Therefore, elucidating the underlying pathogenesis of EC and identifying effective biological agents are urgently needed.
Viral infections, such as EB virus, nasopharyngeal carcinoma, and Burkitt’s lymphoma, are closely related to tumorigenesis. Studies confirmed that human papilloma virus (HPV) is a risk factor for the occurrence and development of multiple malignant tumors. In particular, HPV16 and HPV18 infections are closely related to the occurrence of multiple tumors [4,5]. Cervical cancer is closely related to HPV infection. The detection of HPV infection is currently used as an important indicator for the diagnosis of cervical intraepithelial neoplasia and the judgement of cancerization [6]. HPV infection is closely related to EC, but its tumorigenic mechanism is not clear [7]. Persistent HPV infection upregulates the expression of oncoproteins, especially E6 and E7. Both proteins lead to genetic instability through the inactivation of p53 and pRB, which is a negative regulator of cyclin-dependent kinase inhibitor p16INK4a (p16). The inactivation of pRB results in the upregulation of p16 [8], which is typically used as an alternative biomarker for HPV oncoprotein activity. In more than 90% of HPV-positive oropharyngeal cancer, p16 was overexpressed. The immunohistochemical evidence of p16 overexpression has been widely used to establish the correlation between HPV carcinogenic activity in cervical cancer and dysplasia [9–11]. The immunohistochemical detection of p16 overexpression may serve as a surrogate marker of functionally relevant HPV infection [10, 12]. This detection is simple, fast, and inexpensive. In our study, p16 expression was detected by immunohistochemistry instead of by HPV detection. Abnormal signal transduction pathway is one of the important mechanisms underlying tumorigenesis and tumor development and has become a popular research topic in the fields of tumorigenesis, target therapy, and drug resistance. The roles of viral infection in cell signaling pathway and oncopgene activation are also popular topics in viral tumorigenic mechanism research. HPV infection can activate the PI3K/AKT signal transduction pathway, upregulate PIK3CA and PIK3CB expression levels, and worsen tumors [13]. The relationship between the PI3K/AKT pathway and tumorigenesis has attracted much attention in recent years [14]. The oncogene PIK3CA is a key molecule with high mutation rate in the PI3K/AKT pathway. PIK3CA mutation exists in 30% of solid tumors, is related to tumorigenesis, and affects targeted therapy [15–17]. Few studies have investigated PIK3CB. In-depth studies on the correlation between HPV infection and PIK3CA and PIK3CB mutations in EC and their
effects on the occurrence, development, and prognosis of EC are also still lacking. Therefore, studying the correlation between HPV infection and PIK3CA and PIK3CB mutations in EC is important for the detection of high-risk factors, judgment of prognosis, and prevention and treatment of EC.

Materials And Methods
Clinical specimens and patient data
A total of 156 ESCC tissue samples at different stages were collected from the Department of Pathology of Binzhou Medical University Hospital between January 2011 and May 2013. Tissue samples were routinely fixed in 10% buffered neutral formalin. Cancer tissues were cut into wedge shapes, whereas normal tissues were cut at least 5 cm away from the tumor margin. All ESCC patients were clinically and pathologically proven to have not received preoperative chemotherapy or radiotherapy. All specimens were collected with the informed consent of the patients, and the Ethical Committee of Binzhou Medical University Hospital approved the protocols used in this study. Clinicopathologic classification was performed according to the National Comprehensive Cancer Network classification parameters. Demographic and clinicopathological parameters were prospectively recorded using a chart review. Patients were followed-up annually by telephone or at outpatient clinics until December 2019 or death.

Immunohistochemistry and scoring
Before staining, paraffin-embedded tissue blocks were cut into 4 μm-thick sections. The sections were deparaffinized in an oven at 60 °C for 2 h and then rehydrated with two and three changes of xylene and ethanol, respectively. Antigen retrieval was performed using the microwave retrieval method. Endogenous peroxidase activity was quenched through incubation with 3% hydrogen peroxide for 10 min at room temperature. Nonspecific binding was blocked by incubating the sections with 10% normal goat serum in PBS for 30 min at room temperature. Without washing, the sections were incubated with rabbit monoclonal antibody against human PIK3CA (1:150; Abcam, Cambridge, MA, USA), PIK3CB (1:250; Abcam, Cambridge, MA, USA), p16 (Fuzhou Maixin Biotech Co.), and p53 (Fuzhou Maixin Biotech Co.) at 4 °C overnight. The sections were then incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (Abcam, San Francisco, USA) for 1 h at
room temperature. They were washed with PBS and treated with the metal-enhanced DAB substrate kit (Thermo Scientific, USA) to visualize the antigen-antibody complex. Two researchers who were unaware of the clinicopathological status of the specimens, scored each section separately. The percentage of stained cells on each section was scored as follows: 0 (less than 5%), 1 (5%-25%), 2 (26%-50%), and 3 (> 50%). Staining intensity was scored as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The final score of each specimen was calculated by the multiplying stained cell score with the staining intensity score. Thus, the final score ranged from 0 to 9. Low expression was defined as a final score of <4, whereas a score ≥4 was determined to be high expression.

Statistical analysis
All statistical analyses were conducted with the SPSS 22.0 software (SPSS Inc., Chicago, USA). The correlation of PIK3CA, PIK3CB, p16, and p53 expression with clinical parameters was analyzed using X and Fisher’s exact tests. Overall survival (OS) was plotted using the Kaplan-Meier method. The analyses of prognostic factors for OS were determined using multivariate Cox proportional hazards regression method. P value ≤0.05 was considered statistically significant.

Results
Expression levels of PIK3CA, PIK3CB, p53, and p16 in ESCC samples
Immunohistochemistry was used in human ESCC and matched adjacent non-tumor tissues to explore the expression levels of PIK3CA, PIK3CB, p53, and p16. The immunohistochemical results are shown in Figure 1. The expression levels of PIK3CA and PIK3CB, p53, and p16 were positive in the cytoplasm, nuclei, and cytoplasm and nuclei of the ESCC samples, respectively. The positive ratios of PIK3CA, PIK3CB, p53, and p16 were 20.50%, 15.4%, 30.8%, and 65.4%, respectively. As shown in Table 1, PIK3CA, PIK3CB, p53, and p16 expression levels were significantly higher in the ESCC samples than in the non-tumor tissues (P < 0.05).

Correlation of PIK3CA, PIK3CB, p53, and p16 with different clinicopathologic parameters
The correlation between the PIK3CA, PIK3CB, p53, and p16 expression levels of the ESCC samples and a set of clinicopathologic parameters, including age, gender, tumor location, TNM stage, differentiation grade, and lymph node metastasis was analyzed (Table 2). The expression of p16 was
significantly correlated with esophageal location, differentiation grade, TNM stage, and lymph node metastasis. The expression rate of p16 in the cervical/upper part (64.71%) of the esophagus was significantly higher than that in the middle (30.36%) and low (24.10%) parts. In addition, p16 expression was higher in well-differentiated ESCC (43.14%) than in moderately and poorly differentiated ESCCs (27.40% and 18.75%, respectively). The p16 expression levels in stages I and II (37.5%) were higher than those in stages III and IV (20%). The expression of p16 was lower in the presence of lymph node metastasis (20.97%) than in its absence (37.23%). PIK3CA and PIK3CB expression levels were significantly correlated with TNM stage and lymph node metastasis and higher in the presence of lymph node metastasis (32.26% and 24.19%, respectively) than in its absence (12.77% and 9.57%, respectively). The expression of p53 was significantly correlated with the depth of invasion and higher in the myometrium and adventitia (68.29% and 70.79%, respectively) than in the mucosa and submucosa (42.31%). Other characteristics were not associated with PIK3CA, PIK3CB, p53, and p16 expression levels.

Correlation of PIK3CA, PIK3CB, and p53 with p16
As shown in Table 3, PIK3CA expression was positively correlated with p16 (rs = 0.175 P = 0.027). However, PIK3CB and p53 expression levels did not show any correlation with p16.

Correlation between PIK3CA, PIK3CB, p53, and p16 expression levels and patient OS
The median survival time of 156 patients was 44 months, and their 5-year survival rate was 28.2%. Kaplan–Meier analysis (Figure 3) revealed that PIK3CA, p16, p53, depth of invasion, TNM stage, and lymph node metastasis could be relevant predictive factors of OS. The 5-year survival rates of patients with positive and negative PIK3CA expression levels were 18.7% and 30.6%, respectively (P < 0.01, Figure 3A). Thus, PIK3CA and p53 were markers of poor prognosis in ESCC. The 5-year survival rates of patients with positive and negative p16 expression levels were 39.6% and 23.1%, respectively (P < 0.05, Figure 3B). Therefore, p16-positive ESCC was a distinct entity with a favorable prognosis compared with p16-negative ESCC. Cox regression analysis showed that PIK3CA and p16 expression levels, differentiation grade, and depth of invasion were independent prognostic factors of ESCC (Table 3).
Discussion
EC is a tumor with high invasive ability. It is usually diagnosed in the late stage due to the lack of specific initial symptoms. For late-stage EC, the main treatment is surgery combined with chemotherapy [18, 19]. However, the cure and survival rates of patients are still very low, and other methods are needed to help predict the survival rate and identify potential responders of a given treatment.
Clinicopathological factors, molecular biomarkers, and HPV infection may be predictive variables of ESCC [20, 21]. In head and neck cancer, patients with HPV-positive cancer have better therapeutic outcome and higher survival rate than patients with HPV-negative cancer [22–24]. A similar correlation possibly exists, because the esophagus may also be infected with HPV [25]. HPV-infected tumors are characterized by a high expression of p16, which is widely considered as an alternative marker for HPV infection in squamous cell carcinoma [10, 12]. In the present study, the expression rate of p16 in ESCC was 30.8%, which was significantly higher than in normal esophageal mucosa. In the correlation analysis with clinical pathological factors, p16 expression was significantly correlated with esophageal location, and the expression rate of p16 in the cervical/upper part was significantly higher than in the middle and lower parts. In addition, p16 expression was negatively correlated with the degree of differentiation, invasion depth, and lymph node metastasis of ESCC. Thus, p16 was highly expressed in patients with a high degree of differentiation, shallow invasion depth, and no lymph node metastasis. Prognosis analysis showed that the 5-year survival rate of p16-positive group was 39.6%, whereas that of p16-negative group was 23.1%. Cox regression analysis of OS and PFS showed that p16 status was an independent prognostic factor for OS and PFS in patients with ESCC. The present study clearly showed that p16-positive tumor was related to high differentiation, TNM stage, and lymph node metastasis. These three differences may have enhanced the prognosis in patients with p16-positive tumor. Another hypothesis was that HPV infection leads to the high expression of tumor protein E6/E7, which weakens the functions of p53 and RB proteins and promotes genome rearrangement [26], and DNA rearrangement is theoretically more sensitive to radiotherapy and chemotherapy [27]. This hypothesis explains the indications of increased survival rate in patients
with HPV-positive tumors.

The differential expression of tumor suppressor protein p53 is one of the most common abnormal expressions in cancer. Mutation is mainly related to cell invasion, metastasis, and late-stage tumor. The expression of HPV E6/E7 oncoprotein can induce the transformation of HPV transformed cells, which leads to the degradation and instability of p53 and pRB. In this study, the expression of p53 protein was related to invasion depth and the poor prognosis of ESCC. As a tumor suppressor gene, wild-type p53 can regulate cell cycle and avoid cell carcinogenesis. After p53 gene mutates, it loses its regulatory effect on cell growth, apoptosis, and DNA repair and increases the tumor’s adaptability to hypoxic microenvironment due to the change in its spatial conformation [28, 29]. In the present study, no correlation was found between p53 and p16 expression levels, possibly due to the insufficient number of samples.

As one of the important signal transduction pathways in cells, the PI3K/Akt signaling pathway is closely related to the occurrence and development of various human tumors. Its abnormal activation promotes the normal development of cells to cancer cells. An abnormal PI3K/Akt signal transduction is present in many human malignant tumors [30–33]. As a catalytic subunit of PI3K, PI3Kp110 has four members, namely, P110α, P110β, P110δ, and P110γ. P110α and P110β, which are encoded by PIK3CA and PIK3CB, respectively, are widely found in cells. They can regulate DNA synthesis and promote normal cell development with normal expression; moreover, they are closely related to the occurrence and development of tumors with abnormal expression [34]. The mutation of PIK3CA gene can increase the expression of p110α, which receives the EGF signal. In addition, p110α is activated by Ras protein, phosphorylates downstream Akt protein, and continuously activates the PI3K/Akt signal pathway, leading to cell overproliferation. The role of p110β in tumorigenesis and its mechanism are unclear. In the present study, the positive rates of PIK3CA and PIK3CB in ESCC were significantly higher than those in the normal mucosa, suggesting the abnormal PIK3CA and PIK3CB expression levels in ESCC. The PIK3CA and PIK3CB expression levels in ESCC were also correlated with TNM stage and lymph node metastasis but not with age, gender, tumor location, tumor differentiation, and depth of invasion. Thus, the positive rates of PIK3CA and PIK3CB in esophageal
carcinoma with low differentiation, deep invasion, high TNM stage, and lymph node metastasis were higher than those with high differentiation, shallow invasion, and low TNM stage. High PIK3CA and PIK3CB expression levels were related to the malignant potential of EC. Thus, PIK3CA overexpression resulted in the abnormal activation of the PI3K/Akt pathway. This abnormal activation could promote the growth and proliferation of tumor cells, inhibit apoptosis, promote invasion and metastasis, regulate endothelial growth and angiogenesis, and affect the efficacy of chemotherapy by catalyzing a series of protein phosphorylation.

The expression of HPV E6/E7 oncoprotein induced the carcinogenesis of HPV-transformed cells, inhibiting the expression levels of tumor suppressor genes p53 and Rb and changing various cell and molecular events by activating the PI3K/Akt signaling pathway. Xie J et al. demonstrated that ALA-PDT treatment inhibits the proliferation of HeLa cells in a dose-dependent manner and effectively reduces HPV viral load via autophagy and apoptosis by regulating the Ras/Raf/MEK/ERK and PI3K/AKT/mTOR pathways [35]. Madera D et al. suggested that inhibiting PIK3CA expression and HPV can slow down the further growth of OSCC [36]. p16 expression was positively correlated with PIK3CA expression but was not correlated with pik3cb expression. We believe that the PI3K/Akt/mTOR signaling pathway in HPV-infected cells was activated by mutations in pathway components and the activation of upstream signaling molecules. Activating this pathway contributes to the dysregulation of proliferation and changes in anti-apoptosis and metabolic characteristics and ultimately leads to the malignant transformation of infected cells. This signaling pathway may be a huge therapeutic opportunity and practical challenge for the treatment of HPV-induced cancer. The PI3K/Akt pathway plays a key role in many HPV-related human cancers, and the HPV infection expressed in E6/E7 activates the PI3K/Akt signaling pathway by changing various cell and molecular events that lead to cancer.

In conclusion, our study clearly demonstrated that the increased expression levels of PIK3CA and PIK3CB was related to the development of EC. Imbalance in the PI3K/Akt signaling pathway was closely related to HPV infection and prognosis. In ESCC, PIK3CA, and p53 were markers of poor prognosis, whereas P16 was a marker of good prognosis. The combined detection of PIK3CA and p16 is very important for prognostic evaluation and optimization of ESCC treatment.
Declarations

Ethics approval and consent to participate

The experimental protocol was established, according to the ethical guidelines of the Helsinki Declaration and was approved by the Human Ethics Committee of Binzhou Medical University Hospital. Written informed consent was obtained from individual or guardian participants.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Feifei Wen and Shuhua Wu conceived the idea of the study; Xiaoyang Xu, Yuanhang Liu, Liu Liu, Ningjie Guo, Zhenzhen Jia, and Haiqing Liu performed the research; Feifei Wen, Shuhua Wu, Yangyang Li, and Shuang He analysed the data; Feifei Wen, Shuhua Wu, and Xiangfang Shu interpreted the results; Feifei Wen wrote the paper; all authors discussed the results and revised the manuscript.

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No.

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Table

Table 1. Expression of PIK3CA, PIK3CB, p16, and p53 protein in esophageal squamous cell carcinoma and normal mucosa

| Group     | Normal mucosa | Cancer       | P value |
|-----------|---------------|--------------|---------|
| PIK3CA    | 2.5(4/156)    | 20.5(32/156) | 0.001*  |
| PIK3CB    | 1.3(2/156)    | 15.4(24/156) | 0.001*  |
| p16       | 0(0/156)      | 30.8(48/156) | 0.001*  |
| p53       | 1.9(3/156)    | 65.4 (102/156)| 0.000*  |

* P<0.05

Table 2. Correlation of PIK3CA, PIK3CB, p53, and p16 with different clinicopathologic parameters

| Parameters | N     | PIK3CA (+) | r value | P value | PIK3CB (+) | r value | P value |
|------------|-------|------------|---------|---------|------------|---------|---------|
| Age (years)|       |            |         |         |            |         |         |
| <60        | 53    | 12         | 0.038   | 0.678   | 10         | 0.069   | 0.46    |
| ≥60        | 103   | 20         |         |         | 14         |         |         |
| Gender     |       |            |         |         |            |         |         |
| Male       | 110   | 20         | 0.089   | 0.265   | 15         | 0.075   | 0.34    |
| Female     | 46    | 12         |         |         | 9          |         |         |
| Tumor location                | 17 | 4  | 0.051 | 0.816 | 6 | 0.191 | 0.051 |
|------------------------------|----|----|-------|-------|---|-------|-------|
| Cervical/Upper               |    |    |       |       |   |       |       |
| Middle                       | 56 | 10 | 8     |       |   |       |       |
| Low                          | 83 | 18 | 10    |       |   |       |       |
| TNM stage                    |    |    |       |       |   |       |       |
| I-II                         | 96 | 14 | -0.183| 0.025*| 10| -0.172| 0.04  |
| III-IV                       | 60 | 18 | 14    |       |   |       |       |
| Differentiation grade        |    |    |       |       |   |       |       |
| Well                         | 51 | 9  | 0.172 | 0.092 | 7 | 0.114 | 0.37% |
| Moderate                     | 73 | 12 | 10    |       |   |       |       |
| Poor                         | 32 | 11 | 7     |       |   |       |       |
| Depth of invasion            |    |    |       |       |   |       |       |
| Mucosa and submucosa         | 26 | 5  | 0.023 | 0.959 | 4 | 0.071 | 0.67% |
| Myometrium                   | 41 | 9  | 8     |       |   |       |       |
| Adventitia                   | 89 | 18 | 12    |       |   |       |       |
| Lymph node metastasis        |    |    |       |       |   |       |       |
| Yes                          | 62 | 20 | 0.230 | 0.003*| 15| 0.194 | 0.02  |
| No                           | 94 | 12 | 9     |       |   |       |       |
* $P<0.05$

Table 3. Correlation of PIK3CA, PIK3CB, p53 with p16

|        | P16 (+) | P16 (-) | $X^2$ value | r value | $P$ value |
|--------|---------|---------|-------------|---------|-----------|
| PIK3CA (+) | 15      | 17      | 4.902       | 0.175   | 0.027*    |
| PIK3CA (-)  | 33      | 91      |             |         |           |
| PIK3CB (+)  | 11      | 13      | 3.022       | 0.138   | 0.138     |
| PIK3CB (-)  | 37      | 95      |             |         |           |
| P53 (+)     | 30      | 72      | 0.255       | 0.040   | 0.614     |
| P53 (-)     | 18      | 36      |             |         |           |

* $P<0.05$

Table 4. Univariate and multivariate analysis of clinicopathologic parameters with OS by Cox proportional hazards regression
| Parameters                                      | β    | SE   | Wald  | Exp(β) | 95%CI   |
|------------------------------------------------|------|------|-------|--------|---------|
|                                                |      |      |       |        | Lower   |
| Age (<60, ≥60)                                 | 0.219| 0.198| 1.221 | 1.245  | 0.844   |
| Gender (male, female)                          | 0.325| 0.208| 2.453 | 1.384  | 0.922   |
| Tumor location                                 |      | 3.026|       |        |         |
| Tumor location (Cervical/Upper, Middle)        | 0.307| 0.345| .790  | 0.736  | 0.374   |
| Tumor location (Middle, Lower)                 | 0.319| 0.198| 2.590 | 0.727  | 0.493   |
| Lymph node metastasis (yes, no)                | 1.194| 1.073| 1.237 | 0.303  | 0.037   |
| Differentiation grade                          |      |      |       |        |         |
| Differentiation grade (Well, Moderate)         | 0.101| 0.289| .123  | 0.903  | 0.513   |
| Differentiation grade (Moderate, Poor)         | 0.539| 0.235| 5.271 | 0.583  | 0.368   |
| Depth of invasion                              |      |      |       |        |         |
| Depth of invasion (Mucosa and submucosa,       | 0.959| 0.289| 10.997| 0.383  | 0.217   |
| Myometrium)                                    |      |      |       |        |         |
| Depth of invasion (Myometrium, Epicardial)     | 0.535| 0.237| 5.091 | 0.586  | 0.368   |
| TNM stage (I-II, III-IV)                       | 0.791| 1.071| .546  | 2.207  | 0.271   |
| PIK3CA                                         | 0.796| 0.239| 11.107| 2.217  | 1.388   |
| PIK3CB                                         | 0.340| 0.255| 1.779 | 1.405  | 0.852   |
| P16                                            | -0.524| 0.251| 4.371 | 0.592  | 0.362   |
| P53                                            | 0.266| 0.207| 1.642 | 1.304  | 0.869   |

*P<0.05

Figures
Figure 1

Immunohistochemical results
Figure 2

Kaplan-Meier analysis