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Original Article

ALEX1 may be a novel biomarker for human cervical squamous cell carcinoma

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Abstract: The armadillo repeat proteins were first found in armadillo gene of Drosophila. Since then a number of proteins containing armadillo repeats have been noticed and studied. These proteins that consist of 6 to 13 armadillo repeat domains are classified as family of armadillo repeat proteins. Recently, several studies indicated that armadillo repeat family of proteins play an important role in the tumorigenesis and maintenance of tissue integrity. ALEX1 (Arm protein lost in epithelial cancers, on chromosome X), contains two armadillo repeats domains, is expressed different in normal and carcinomas tissues. Several studies have found that ALEX1 protein lost in tumors that originated in epithelial tissues. We evaluated the ALEX1 protein expression in 53 cervical cancers and in 53 non-cancerous cervical tissues from patients and adjacent non-cancerous tissues using immunohistochemistry Results: ALEX1 protein expression is significantly increased in 53 cervical cancers tissues compared with non-cancerous tissues. We found, for the first time, that ALEX1 protein expression in cervical cancers tissues is higher than non-cancerous tissues. It is suggested that the ALEX1 protein is associated with tumorigenesis in cervical cancer and we speculate that the ALEX1 may plays a role as an oncogene in cervical cancer. Moreover, ALEX1 may serve as a novel potential diagnostic biomarker in identifying cervical cancer.

Keyword: Cervical cancer, ALEX1, armadillo repeat protein, immunohistochemistry

Introduction

The World Health Organization (WHO) reported that, cervical cancer comprises 12% of all cancers globally and it is second most common gynecological malignancy after breast cancer in the world [1-3]. It is estimated that there will be 16 million new cases by the year of 2020 [4] and between 15 and 44 years of age. Cervical cancer is characterized by uncontrolled cell division and tissue invasiveness due to genetic and epigenetic changes. Eighty to ninety percent of cervical cancers are caused by infection with human papilloma virus (HPV) [1, 3, 5, 6]. PHV can activate the proto-oncogenes to oncogenes or deactivated of tumor suppressor genes; thus enhances the rate of cell proliferation and lead to cervical neoplasms by the integration of viral DNA into the chromosomal DNA [1, 3].

The armadillo gene was first found in the segment polarity genes of Drosophila [7]. Since then a growing number of related proteins have been identified based on sequence homologies and is classified as armadillo repeat family of proteins. These proteins' common feature is an amino acid motif of about 42 residues, which has been identified in 6-13 repeated units in all members of this family [8, 9] and each repeat domain consists of three a helices, designated H1, H2, and H3 [10-12]. The armadillo domain proteins have roles as cell-contact and cytoskeleton-associated proteins and signaling functions by generating and transducing signals affecting gene expression [10, 12]. Studies have shown that armadillo repeat proteins participate in protein-protein interactions with the armadillo domain play an important role in the recognition process [13].

ALEX is a novel subgroup within the armadillo family, including ALEX1, ALEX2 and ALEX3, which has one or two Arm repeats domains and is located at chromosome X [14-17]. The pres-

Table 1. Clinicopathologic characteristics of 53 patients and expression of ALEX1

Factors	N (%)
Age (y)	
<60	44 (83.9)
≥60	9 (16.1)
Clinical stage	
I	33 (62.3)
II	9 (17.0)
III	11 (20.7)
Histological type	
Squamous cell carcinoma	
Pathological grading	
1	7 (13.2)
2	38 (71.7)
3	8 (15.1)
T classification	
T1	43 (81.1)
T2	7 (13.2)
T3	3 (5.7)
N classification	
N0	40 (75.6)
N1	13 (24.5)
Expression of ALEX1	
Low	9 (17.0)
High	44 (83.0)

ent study suggests that ALEX1 mRNA is widely higher expressed in human normal tissues compared with in several human carcinoma cell lines and tissues which is significantly reduced or even not expressed [14, 17].

However, it is first time that our study suggests that ALEX1 protein is significantly higher in cervical cancer tissues compared with non-cancerous tissues by immunohistochemistry. It is suggested that the ALEX1 protein is associated with tumorigenesis in cervical cancer and we speculate that the ALEX1 may play a role as an oncogene in cervical cancer.

Materials and methods

Patients and collection of general information

The samples of this study included 53 cervical squamous cell carcinoma and 53 adjacent non-cancerous cervical squamous epithelium tissues. Cervical cancer tissues were collected from 53 patients who were pathologically diagnosed with cervical cancer in the First Affiliated Hospital of Chongqing Medical University from November 2013 to October 2014. In addition, adjacent non-cancerous tissues were collect from these cervical cancers of 53 patients, with a median age of 48 years (ranging from 29 to 73 years). The clinical characteristic of the cervical cancer patients are described in detail in **Table 1**.

Immunohistochemical staining

Cervical sections obtained from all cancer and non-cancerous tissues that all samples tissues were paraffin embedded, were cut into 4 μm thick and 2 mm diameter sections to construct tissue microarrays. Tissue samples of the selection criteria were histologically proven diagnosis of cervical cancer and the proportion of tumor tissues exceeded 50% on microscopic slides.

The mouse monoclonal antibody to ALEX1 was used to immunohistochemistry and antibody dilution of 1:200 according to the manufacturer instructions. The slides were processed as follow steps: 1) dewaxed in xylene, rehydrated in graded alcohol, 2) incubated in citrate buffer at 95°C for 20 min as antigen retrieval, 3) blocked for the endogenous peroxidase in 3% H_2O_2 in citrate buffer for 20 min, 4) washed with washing buffer TBST in three 5 min cycles, 5) slides were then preincubated with 3% normal Goat serum albumin at 37°C for 30 min, 6) incubated with 1:200 dilution of anti-ALEX1 antibody in PBS at 4°C for 16 h, 7) rewarmed for 30 min at 37°C, 8) washed with washing buffer TBST in three 5 min cycles, 9) incubated with anti-mouse secondary antibody for 30 min at 37°C, 10) washed with washing buffer TBST in three 5 min cycles, 11) marked with streptavidin horseradish peroxidase at 37°C for 30 min, 12) washed with washing buffer TBST in three 5 min cycles, 13) for color reaction.

Staining analysis

We used the staining intensity and the percentage of positive stained to evaluate ALEX1 expression. Staining intensity was scored as 0 (no staining), 1 (pale yellow), 2 (yellow), 3 (brown). The percentage of positive stains was scored as 0 (no staining), 1 (<25% of staining), 2 (26%-50% of staining), 3 (51%-75% staining), or 4 (75%-100% staining) [18]. The sum of the intensity and percentage of positive scores was used as the final staining score (0 to 7) of ALEX.

Table 2. Correlation between the clinicopathologic features and expression of ALEX1 protein

Characteristics	ALEX1 (%)		*P*-Value
	Low expression	High expression	
Age (y)			0.65
<60	7 (15.9)	37 (84.1)	
≥60	2 (22.2)	7 (77.78)	
Clinical stage			0.32
I	4 (12.1)	29 (87.9)	
II	3 (33.3)	6 (66.7)	
III	2 (18.2)	9 (81.8)	
Histological type			<0.0001
Malignant	9 (17.0)	44 (83.0)	
Normal	40 (75.5)	13 (24.5)	
Pathological grading			0.80
1	1 (14.3)	6 (85.7)	
2	6 (15.8)	32 (84.2)	
3	2 (25.0)	6 (75.0)	
T classification			0.52
T1	7 (16.3)	36 (83.7)	
T2	2 (28.6)	5 (71.4)	
T3	0 (0.0)	3 (100.0)	
N classification			0.86
N0	7 (17.5)	33 (82.5)	
N1-3	2 (15.4)	11 (84.4)	

Section obtaining a final staining score (<3) were grouped into low ALEX1 expression and those with scores (≥3) were grouped into high ALEX1 expression.

Statistical analysis

SPSS.18 software was used for statistical analysis. Chi-square was used to analyze the relationship between ALEX1 expression and clinicopathologic characteristics. $P<0.05$ was considered statistically significant.

Results

Increased expression of ALEX1 in cervical cancer tissues

ALEX1 protein expression was examined in 53 cervical squamous cell carcinoma and adjacent non-cancerous cervical squamous epithelium tissues by immunohistochemical analysis. ALEX1 protein was detected in all cervical s carcinoma tissues and non-cancerous tissues samples. The results show that ALEX1 protein expression is high in 44 of 53 (83.0%) cervical carcinoma tissues and in 13 of 53 (24.5%) non-cancerous tissues (**Table 2**). As shown in **Figure 1**, ALEX1 showed high expression in cervical cancer tissue samples (**Figure 1A**). ALEX1 showed low expression in non-cancerous tissues samples (**Figure 1B**).

In addition, we found that ALEX1 was mainly expressed in the cytoplasm of cancer tissues, but non-cancerous tissues showed mainly expression in the nuclear. As shown in **Figure 2**, ALEX1 showing mainly cytoplasm expression in cervical cancer tissue sample (**Figure 2A**). ALEX1 showing mainly nuclear expression in non-cancerous tissues sample (**Figure 2B**). The results suggest that ALEX1 differentially expressed sites in cervical cancer (mainly cytoplasm expression) and non-cancerous tissues (mainly nuclear expression), these indicated that change in ALEX1 protein expression may was associated with cervical tumorigenesis.

ALEX1 protein expression and clinical feature

Immunohistochemistry analysis of ALEX1 expression in cervical cancer tissues was association with the clinicopathologic features (**Table 2**). As shown in **Table 2**, ALEX1 expression was significantly correlated with pathological type ($P<0.05$). However, there was no significant correlation with age, clinical stage, pathological grading, T classification, or N classification. The result firstly indicated that the ALEX1 protein is association with tumorigenesis of cervical cancer and ALEX1 plays a different role in various tumors.

Discussion

ALEX1 as a member of the armadillo family, located at Xq21.33-q22.2, is composed of four exons which encode a protein with a molecular weight of 49 KD that has two Arms repeats domains and 453 amino acids [14]. ALEX1 includes eight potential protein kinase C sites and five potential casein kinase II sites [14, 19]. Armadillo repeat proteins are abundant eukaryotic proteins and diverse cellular locations have diverse functions, including cell junction

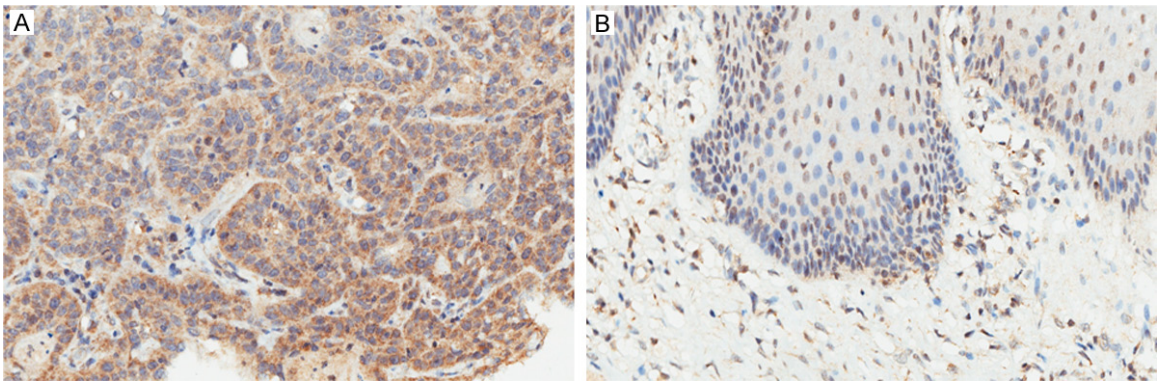

Figure 1. Immunohistochemistry analysis of ALEX1 expression was staining with anti-ALEX1 antibody in cervical cancer tissue sections and non-cancerous tissue sections. A. ALEX1 showing high expression in cervical cancer tissue sample. B. ALEX1 showing low expression in non-cancerous tissues tissue sample.

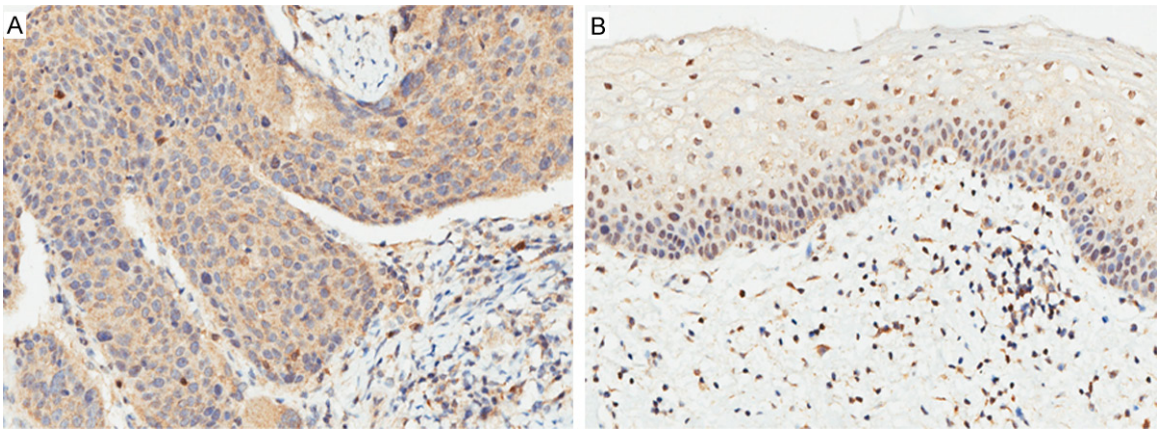

Figure 2. Nuclear expression of ALEX1 Protein in cervical cancer and non-cancerous tissues (A) ALEX1 showing cytoplasm expression in cervical cancer tissue sample. (B) ALEX1 showing nuclear expression in non-cancerous tissues sample.

assembly and nuclear transport and transcriptional activation, by interactions of its armadillo repeat domain with diverse binding partners [8, 11, 13, 20]. Such as β-catenin and adenomatous polyposis coli (APC) [21], armadillo family members, are essential components of the Wnt signaling pathway [19, 22-25]. And β-catenin plays an important roles in cellular and developmental as an adhesion protein and a signaling protein [26].

The present study showed that members of the armadillo family of proteins exert diverse functions such as signal transduction, cell adhesion, development, and tumorigenesis by interactions of their armadillo repeat domain with several binding partners [27-31]. In 2001, Kurochkin and Natsumi Yonemitsu have confirmed that expression of ALEX1 mRNA is lost or significantly reduced in human lung, prostate, colon, pancreas, and ovarian carcinomas [14]. And they had found ALEX1 gene is normally expressed in cell lines derived from other types of tumors such as sarcomas, neuroblastomas, and gliomas [14]. In 2008, Zender L and Xue W found that knockdown of ALEX1 gene accelerated liver cancer in mice [32]. In 2010, Hiroyoshi Iseki and Akihiko Takeda studied found that ALEX1 is a target gene of CREB, and ALEX1 gene expression is regulated by Wnt/β-catenin signaling pathway. And they also found that cyclic AMP response element is important for basal promoter activity and the transcriptional activation of ALEX1 gene response to β-catenin [19]. In addition, overexpression of ALEX1 suppresses colony formation of human carcinoma cells [17]. These results indicated that ALEX1 plays a role in suppression of carci-

nomas originating from epithelial tissue. But we found, for the first time, that ALEX1 protein expression in cervical cancers tissues is higher than normal cervical tissues. It is speculated that the ALEX1 protein is associated with tumorigenesis in cervical cancer and may play a role as an oncogene in cervical cancer. ALEX1 is associated with tumorigenesis that has been more and more attention.

In this study, we detected expression of ALEX1 protein in 53 cervical squamous cell carcinoma and adjacent non-cancerous cervical squamous epithelium tissues by immunohistochemistry. We found that ALEX1 protein expression is significantly increased in cervical cancerous tissues compared with noncancerous tissues. The results are different from previous studies that expression of ALEX1 was reduced in human lung, prostate, colon, pancreas, and ovarian carcinomas [14]. So the result firstly indicated that the ALEX1 protein plays an important role in tumorigenesis of cervical cancer and may plays a role as an oncogene in cervical tissues. The results indicated that ALEX1 pays a different role in various tumors.

In addition, we found that ALEX1 was mainly expressed in the cytoplasm of cancer tissues, but non-cancerous tissues showed nuclear expression of ALEX1 protein. These results speculated that ALEX1, in normal tissue, mainly expressed in the nuclear and regulation of the downstream genes, to maintain the normal function of cells. However, ALEX1 overexpression in tumor tissues that may ALEX1 protein transferred to the cytoplasm, leading to ALEX1 accumulated in the cytoplasm. We speculated possible reasons for ALEX1 protein accumulated in the cytoplasm that spatial structure of the protein is changed which leading to ALEX1 protein is recognized by nuclear localization signal, or ALEX1 gene mutation associated with nuclear localization signal which leading to ALEX1 protein is recognize the receptor of nuclear localization. These results leaded that ALEX1 protein enter the cytoplasm and accumulated in the cytoplasm. We speculated that ALEX1 accumulated in tumor tissues may plays a role in the occurrence and development of cervical cancer.

This different in cancer tissues and adjacent non-cancerous tissues could make ALEX1 as a potential diagnostic biomarker identification in identifying cervical cancer and provide a possibility gene therapy instead of traditional treatment of cancer by chemotherapy and radiotherapy.

Due to the limited cases of cervical cancer samples collected in the study, the function and mechanism of ALEX1 in cervical cancer remained to be answered. We will collect more clinical specimens and concentrate on exploring the function of ALEX1 in cervical cancer.

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Disclosure of conflict of interest

None.

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