·BASIC RESEARCH·

Dysregulation of Annexin Ⅱ Expression in Esophageal Squamous Cell Cancer and Adjacent Tissues from a High-Incidence Area for Esophageal Cancer in Henan Province

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[ABSTRACT] **BACKGROUND & OBJECTIVE:** Our recent study on proteomics for esophageal cancer has indicated the importance of Annexin Ⅱ as a promising protein to distinguish esophageal cancer patients from healthy subjects. This study was to detect the expression of Annexin Ⅱ in esophageal squamous cell carcinoma (ESCC) and adjacent tissues, and to explore the role of Annexin Ⅱ in ESCC pathogenesis and mechanisms. **METHODS:** The expression of Annexin Ⅱ in 33 specimens of ESCC and adjacent tissues from Linzhou, a high-incidence area for esophageal cancer in Henan province, was detected by ABC immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR). **RESULTS:** Annexin Ⅱ protein was expressed in 90.6% normal esophageal epithelium and decreased with ESCC progression. In carcinoma *in situ* (CIS), 50.0% foci lost Annexin Ⅱ protein expression. The expression of Annexin Ⅱ protein was increased in well differentiated SCC and decreased with loss of differentiation of SCC. In poorly differentiated SCC, 45.4% foci lost Annexin Ⅱ protein expression. However, RT-PCR didn't detect differential expression of Annexin Ⅱ mRNA between normal esophageal epithelium and CIS. **CONCLUSIONS:** Elevated or reduced expression of Annexin Ⅱ may be correlated to reverse or progression of carcinogenesis respectively, and Annexin Ⅱ may be another candidate biomarker for screening of high-risk subjects and early diagnosis of SCC.

KEYWORDS: Esophageal neoplasm, squamous cell carcinoma; Precancerous lesion; Annexin Ⅱ; Immunohistochemistry; Tumor-related gene

1. Introduction

Linzhou (originally Linxian) and its surrounding areas, such as Huixian, Anyang, of Henan province in Northern China is one of the areas with high incidence and mortality rates of esophageal cancer. And esophageal cancer is still the main cause of cancer-related death in this area[1]. The high mortality rate and poor prognosis of esophageal cancer ascribe to the unclear mechanisms of its etiology, formation and development, as well as the lack of sensitive and specific biomarkers for early diagnosis [2]. Our previous study has revealed that Annexin Ⅱ is among the candidate proteins associated with development and progression of esophageal cancer [3]. More researches have suggested that Annexin Ⅱ, a member of the calcium-dependent lipid binding protein/Annexin family, is correlated with

formation, development and metastasis of malignant diseases [4,5]. However, the correlation of Annexin Ⅱ to esophageal cancer, in particular to its precancerous lesions has seldom been reported. In this study, immunohistochemistry and RT-PCR were used to characterize the protein and mRNA expression in esophageal cancer and its surrounding epithelial cells to investigate the role of Annexin Ⅱ in esophageal cancer.

2. Materials and Methods

2.1 Materials and reagents

Thirty-three EC cancer samples were collected from Yaocun Esophageal Cancer Hospital, Linzhou, Henan from 2004 to 2005. The samples consisted of 18 male and 15 female cases, aged from 41 to 75 years, with a median age of 58 years. None of the cases in this study were treated with chemotherapy and/or radiotherapy before operation.

Avidin-biotin-HRP-conjugated-complex IHC (ABC) was used for immunostaining analysis. Rabbit ploy-clonal anti-Annexin Ⅱ antibody and goat anti-rabbit antibody were purchased from Santa Cruz. ABC Kit was bought from Vector Laboratories and DAB Kit from Zhongshan Biotechnology, Beijing.

2.2 Tissue procession

After surgical resection of the esophageal tumor, samples were cut into halves. One half was frozen in liquid-nitrogen or a -80℃ freezer and the other half was fixed in 95% ethanol. Representative tumor samples were extracted from the non-necrosis and non-hemorrhagic area of the ethanol-fixed tissues. Epithelial tissues, 1 cm away from the tumor margin, were isolated without or with a few sub-mucosal tissues from the tumor. Then tissues were dehydrated, embedded, serially cut into 5 μm thick slides for HE and immunohistochemical staining. Tumor and paired normal epithelial tissues were extracted from eight frozen histopathologically confirmed esophageal cancer specimens before RNA extraction.

Base on the cellular morphology, tissue structures and the grade of differentiation, esophageal epithelial cells were classified into normal epithelium (NOR), basal cell hyperplasia (BCH), displasia (DYS), carcinoma *in situ* (CIS), well-differentiated SCC (W-SCC), moderately-differentiated SCC (M-SCC) and poorly-differentiated SCC (P-SCC)[1-6].

2.3 Immunohistochemistry (IHC)

Paraffin-embedded slides were deparaffinized in xylene and dehydrated in graded ethanol followed by 0.3% H_2O_2 to quench endogenous peroxidase. Slides were incubated with horse serum at 1:50 to block non-specific binding for 20 min at room temperature (RT). Then tissue slides were incubated with the primary Annexin Ⅱ antibody at 1:200 overnight followed by incubation with goat anti-rabbit antibody at 1:200 for 45 min and subsequent incubation with ABC (1:1:50) for 1 h. Immunostaining signal was detected by DAB followed by hematoxylin counterstaining. Negative control was performed by replacing Annexin Ⅱ with PBS. Proliferation cell nuclear antigen (PCNA) positive slide was used as positive control.

In accordance with the reported immunostaining classification[6], the intensities of the staining were scored as 0, no staining; 1, light yellow granules on the cell membrane or in the cytoplasm; 2, light yellow-brown granules on the cell membrane or in the cytoplasm; 3, a large amount of dark yellow-brown granules. Five fields of each slide were randomly chosen and 100 cells of each field were counted. The percentages of positive cells were scored as 0, <5%; 1, 5-25%; 2, 25-75%; 3, >75%. The sum of the staining intensity and the percentage of the positive cells was the final value of immunostaining of each slide (0-6).

2.5 RNA isolation and RT-PCR

TRIzol (GibocoBRL, Life technologies, USA) was used for RNA isolation. Reverse transcription was performed using ImProm-IITM Reverse Transcription System (Promega) according to the protocol provided by the manufacturer. Annexin Ⅱ primer pairs for PCR amplification were 5-CCAGCGTCATAGAGATCCCG-3 (forward primer); 5-CAGCCTTATCTGGCCACCTG-3 (reverse primer). The primer pairs of Beta-actin used for Annexin Ⅱ normalization were 5-GTGGGGCGCCCCAGGCACCA-3 (forward primer) and 5-CTCCTTAATGTCACGCACGATTTC-3 (reverse primer). Thermal cycle conditions were as follows: pre-denaturation at 94℃ for 10 min, 30 cycles of 94℃ for 45s, 55℃ for 45 s and 72℃ for 60 s, and final extension at 72℃ for 10 min.

2.6 Statistical analysis

SPSS statistical package, Students *t* test with two tails and ordinal regression were used for

statistical evaluation. A P value of less than 0.05 was considered significant.

3. Results

3.1 Results of histopathological diagnosis

All 33 esophageal cancer samples for IHC analysis were diagnosed as squamous cell carcinoma after examination by two independent pathologists. The cancer samples were graded as well-differentiated, moderately-differentiated and poorly-differentiated as listed in Table 1. Surrounding tissues adjacent to the tumor lump consisted of 91 histological types, including NOR, BCH, DYS and CIS (Table1). Another 8 esophageal cancer samples used for RT-PCR were also histopathologically confirmed as squamous cell carcinoma and its surrounding tissues were normal epithelia.

Table 1 Immunoreactivity scores of Annexin Ⅱ in various types of esophageal epithelium [cases (%)]

Histology	Cases	Immunoreactivity score of Annexin Ⅱ						
		0	1	2	3	4	5	6
Adiacent tissue								
NOR	32	3 (9.4)	0	0	0	18(56.2)	8(25.0)	3 (9.4)
BCH	24	4(16.7)	0	0	0	17(70.8)	0	3(12.5)
DYS	21	8(38.1)	0	2 (9.5)	2 (9.5)	8(38.1)	1 (4.8)	0
CIS	14	7(50.0)	0	2(14.3)	3(21.5)	1 (7.1)	1 (7.1)	0
Esophageal carcinoma								
W-SCC	7	0	0	1(14.3)	3(42.9)	2(28.6)	1(14.3)	0
M-SCC	15	4(26.7)	0	4(26.7)	1 (6.7)	3(20.0)	1 (6.7)	2(13.3)
P-SCC	11	5(45.4)	0	3(27.3)	1 (9.1)	2(18.2)	0	0

NOR, normal epithelium; BCH, basal cell hyperplasia; DYS, dysplasia; CIS, carcinoma *in situ*; W-SCC, well differentiated SCC; M-SCC, moderately differentiated SCC; P-SCC, poorly differentiated SCC. $P < 0.05$, NOR vs. DYS, CIS, M-SCC, and P-SCC, BCH vs. DYS, CIS, and P-SCC, CIS vs. W-SCC, and W-SCC vs. P-SCC.

3.2 Results of immunohistochemistry

The Annexin Ⅱ immunocomplex was located mainly on the cellular membrane, occasionally in cytoplasm but rarely in nucleus. The complex exhibited light to dark yellow granules in accordance with the amount of immunocomplex or the intensity of immunoreactivity (Figure 1). In morphologically normal epithelial cells surrounding the tumor, the intensity of immunostaining of Annexin Ⅱ was from moderate to intense; while with the progression of the disease, the expression of Annexin Ⅱ in esophageal cancer reduced accordingly. Especially in DYS and CIS, the intensity of immunostaining was mild. The final score of morphologically normal esophageal epithelia was above 4. In BCH, about 70.8% lesions scored 4.

When the esophageal lesions progressed to DYS and CIS, about 38.1% and 50% disease foci lost the expression of Annexin Ⅱ protein respectively. Interestingly, Annexin Ⅱ protein was decreased in malignant esophageal cells and the immunostaining scores were less than 4. Statistical analysis revealed that alteration of Annexin Ⅱ protein expression in the following paired groups was significant: NOR vs. DYS, NOR vs. CIS, NOR vs. M-SCC, NOR vs. P-SCC, BCH vs. DYS, CIS vs. P-SCC (P<0.01). Meanwhile, the expression of Annexin Ⅱ protein decreased progressively from well-differentiated SCC to poorly-differentiated SCC (Table 1). Annexin Ⅱ protein was lost in 26.7% M-SCC and 45.4% P-SCC. The expressions of Annexin Ⅱ protein were statistically different between NOR and ESCC, BCH and ESCC in 33 samples (P<0.05).

3.3 Results of RT-PCR

Figure 2 shows the mRNA expression of Annexin Ⅱ and beta-actin in eight pairs of esophageal cancer and matched nearby normal epithelia. No differential expression of Annexin Ⅱ mRNA was detected between them.

4. Discussion

Dynamically differential expression of Annexin Ⅱ protein was observed in morphologically normal epithelia and precancerous lesions nearby esophageal cancer. The highest amount of Annexin Ⅱ was observed in normal esophageal epithelia,

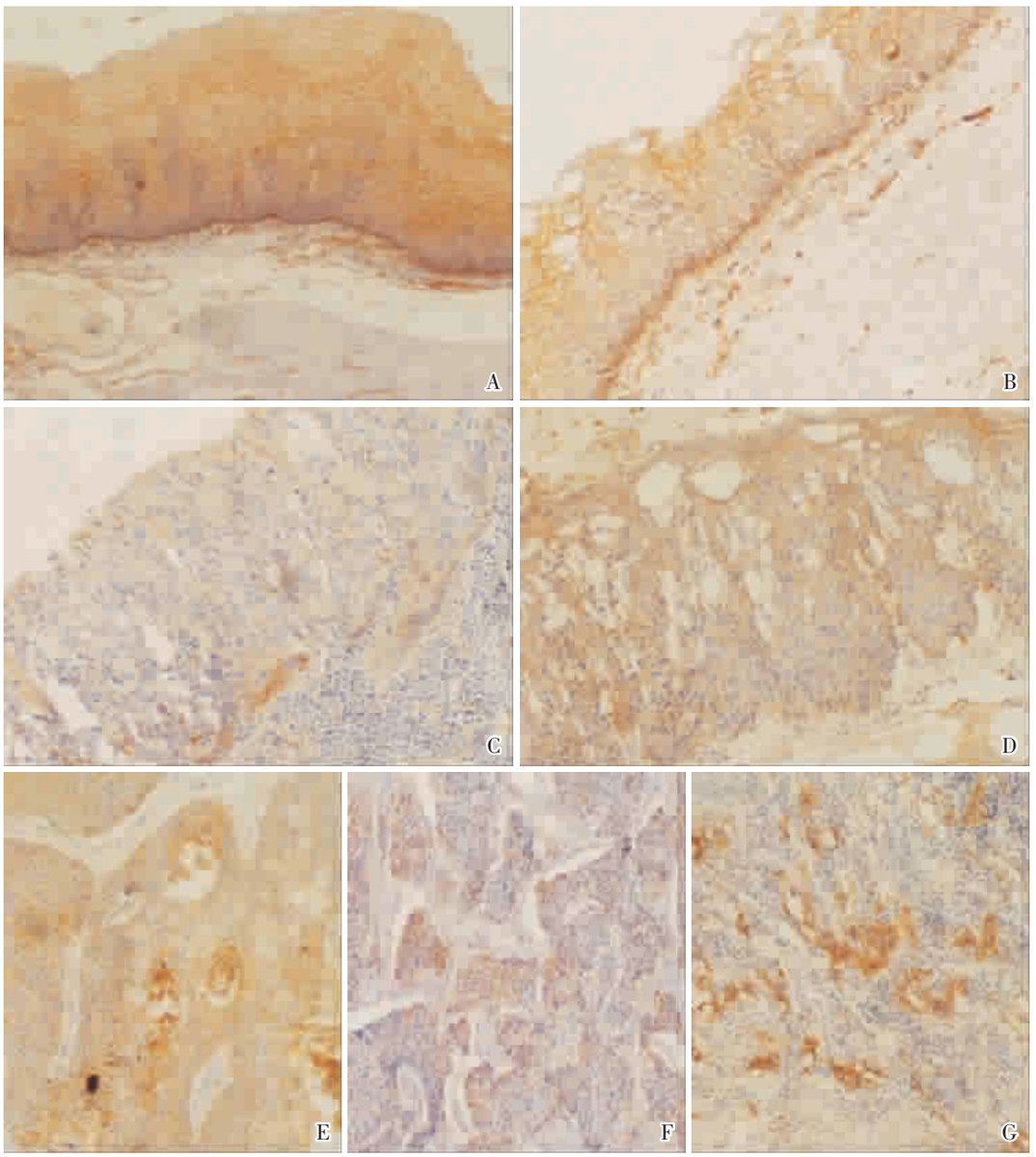

Figure 1　Immunoreactivity of Annexin Ⅱ in various types of esophageal epithelium (DAB ×200)

A: In normal esophageal epithelium close to esophageal carcinoma, Annexin Ⅱ protein is expressed mainly in cytoplasm and on membrane of basal cells, on membrane of suprabasal and superficial cells (score 6).

B: In esophageal basal cell hyperplasia, Annexin Ⅱ protein is moderately expressed in hyperproliferating cells (score 4).

C: In esophageal dysplasia, dysplastic cells lost Annexin Ⅱ expression (score 0).

D: In carcinoma *in situ*, Annexin Ⅱ protein is weakly expressed (score 2).

E: In well differentiated esophageal squamous cell carcinoma, Annexin Ⅱ protein is expressed maily in the middle of cancer nest, particularly in horny pearl (score 3).

F: In moderately differentiated esophageal squamous cell carcinoma, Annexin Ⅱ protein expression is decreased obviously (score 2).

G: In poorly differentiated esophageal squamous cell carcinoma, Annexin Ⅱ protein is expressed mainly in diffused cancer cells.

Figure 2　Expression of Annexin Ⅱ mRNA in 8 specimens of esophageal squamous cell carcinoma (T) and matched normal esophageal epithelium tissues (N)

whereas a lower level of Annexin Ⅱ was observed in the diseased epithelia. Expression of Annexin Ⅱ was decreased progressively with the progress of precancerous lesions.

Annexin Ⅱ is located on chromosome 15 and has a 1.4 kb of highly conserved gene-coding sequence, which encodes a phosphorylated protein with a molecular weight of 36 kD [7]. Annexin Ⅱ protein serves as the substrate after activation of tyrosine receptor kinases, such as pp60v^{src}, protein kinase C, epidermal growth factor, platelet-derived growth factor and insulin. It also plays functions in multiple physiological processes, such as modulation of phospholipidase activation, conjugation of cellular membrane skeleton, cell growth and differentiation, cellular secretion, substance transportation on cellular membrane, inter-cellular adhesion, signal transduction induced by adhesive molecules, anti-inflammatory and coagulation roles, and so on[8-12]. Two Annexin Ⅱ proteins serving as heavy chains always form a hetero-tetramer with two p11 light chains, which link cellular skeleton and membrane. Additionally, p11 protein is able to regulate binding affinity of Annexin Ⅱ with calcium and lipid. Recent studies have indicated that high expression of Annexin Ⅱ occurs in hyper-proliferating cells and malignantly transformed cells, and its reduced expression correlates with cell growth retardation and terminal differentiation [7, 13-15]. Annexin Ⅱ protein is not expressed in normal human central nervous system, but is highly expressed in primary neuroectodermal tumor [16]. In addition, up-regulation of Annexin Ⅱ was reported in multiple forms of human tumors, such as colon cancer, breast cancer, liver cancer, pancreas cancer, cerebral astrocytoma, acute promyelocytic leukemia, gastric cancer, head and neck squamous cell carcinoma and small cell lung carcinoma[4-5, 13-14, 17-22]. After transfected with the anti-sense of Annexin Ⅱ, human lung adenocarcinoma cell line SPC-A-1 abated expression of Annexin Ⅱ mRNA, colony formation and ^{3}H-TdR incorporation. In addition, SPC-A-1 grew slowly and cell cycle was arrested at G_0-G_1 phase [23]. However, other studies reported conflicting results. In prostate cancer and precursors, 65% prostatic intraepithelial neoplasia (PIN) and all prostate cancer lost Annexin Ⅱ protein expression (31/31)[24]. In esophageal carcinoma, Annexin Ⅱ protein expression was down-regulated compared to nearby normal epithelia (P <0.05) [25]. Furthermore, Annexin Ⅱ protein was decreased significantly in M-SCC and P-SCC compared with W-SCC (P<0.05). Gillette reported that expression of Annexin Ⅱ mRNA and protein was correlated with metastasis of human osteosarcoma. Enhanced expression of Annexin Ⅱ could induce differentiation of osteosarcoma cells, reduce tumorigenous and metastatic ability [26]. The above conflicting findings may be due to tissue specificity, cellular compartmentalization of Annexin, which is responsible for differential expression and various biological functions.

The dynamic change of Annexin Ⅱ protein in precancerous lesions of esophageal cancer implied that Annexin Ⅱ may be linked to multi-stage of esophageal carcinogenesis and is an inhibitory element in development and progression of cancer. Follow-up investigation has indicated that esophageal carcinogenesis implicates a multi-stage process, including normal esophageal epithelium → mild dysplasia → severe dysplasia → CIS → SCC [27]. Annexin Ⅱ protein was reported to link to differentiation of epithelial cells, such as increased calcium concentration (1.5 mmol/L) in human primary keratinocyte culture induced cell differentiation[28].

The expression of Annexin Ⅱ protein which decreased with progression of precancerous lesions seemed to have a turning point at CIS, as Annexin Ⅱ increased mildly in W-SCC and decreased with dedifferentiation of ESCC. Expression of Annexin Ⅱ from NOR to P-SCC presents an inverse "S" type. We speculate that Annexin Ⅱ is one of biomarkers associated with cell growth and differentiation in ESCC and negatively reflects the proportion of proliferating cells to differentiated cells. In precancerous stages, the proportion of proliferating cells to differentiated cells is increased which would lead to decreased expression of Annexin Ⅱ. From CIS to SCC, the proportion decreases despite the malignantly transformed cells grow rapidly. With progression of W-SCC to P-SCC, the number of proliferating cells exceeds differentiated cells, which would cause decreased expression of Annexin.

There were no differences in mRNA expression between cancer and its nearby normal epithelial cells of ESCC, indicating that post-transcription regulation might be responsible for distinct expression of Annexin Ⅱ. It was reported that Annexin Ⅱ protein forms hetero-tetramer with p11 protein, or

with neurofilament or MAP-2, which would increase the half-life and stability of Annexin Ⅱ protein.

For a long period, the sample source of precancerous lesions was procured from biopsy. But the limited amount does not meet study requirements. Based on the notion of multifocal cancerization, independent lesions may evolve through each of the precancerous stage and develop into carcinoma ultimately. Our studies on double cancer derived from esophagus (SCC) and cardia (adenocarcinoma) support multifocal cancerization. The para-cancerous tissues harbor a variety of independent precancerous lesions which can expand our resources of precancerous samples. Concentrically rolling of epithelial tissues surrounding esophageal cancer after dissection permits pathologists to view more epithelial tissues on a single slide, which would save time and cost.

In conclusion, Annexin Ⅱ may be one of the key molecules during multi-stage esophageal carcinogenesis. Up-regulation or down-regulation of Annexin Ⅱ protein may correlate with regression or progression of precancerous lesions of esophageal cancer. Annexin Ⅱ may be one of the suitable candidate biomarkers for detection and early diagnosis of esophageal cancer. Furthermore, Annexin Ⅱ may also correlate with clinical parameters and prognosis of EC.

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