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Original Article

Circulating antibody to ANXA1 may be a potential biomarker for early diagnosis of esophageal cancer

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Abstract: Circulating antibodies to linear peptides derived annexin A1 (ANXA1) and mucin type-1 (MUC1) have been found to be significantly increased in some types of cancer. The present study was then designed to test whether circulating antibodies to the above two tumor-associated antigens were altered in esophageal cancer. An enzyme-linked immunosorbent assay was developed in-house to determine circulating IgG against peptide antigens derived from ANXA1 and MUC1, respectively, in 97 patients with esophageal squamous cell carcinoma (ESCC) and 227 healthy subjects. Student's *t*-test revealed that patients with ESCC had significantly higher levels of anti-ANXA1 IgG (t=4.02, $P \leq 0.0001$) and male patients appeared to mainly contribute to the increased levels of anti-ANXA1 IgG in the circulation (t=4.21, $P \leq 0.0001$). However, circulating levels of anti-MUC1 IgG were not significantly altered in ESCC. The anti-ANXA1 IgG levels were decreased with stages of ESCC, of which patients with stage I ESCC had the highest IgG level among all 4 stages (t=4.84, $P \leq 0.0001$, compared to control subjects). Pearson analysis showed a significant correlation between anti-ANXA1 IgG levels and stages of ESCC (r=0.21, df=90, P=0.044) but no correlation between anti-MUC1 IgG levels and stages of ESCC (r=0.01, df=90, P=0.899). In conclusion, circulating IgG to ANXA1 may be a potential biomarker for early diagnosis of esophageal cancer.

Keywords: Autoantibody, ANXA1, MUC1, esophageal cancer, tumor immunity

Introduction

A number of studies demonstrated that circulating antibodies to particular tumor-associated antigens (TAAs) were positive in patients with malignant tumors [1]. The TAAs involved in the specific immune response largely vary between tumor types and between individuals with a tumor, but spontaneous tumor-related antibodies are still useful biomarkers for early diagnosis of malignancy [1-3]. For example, EarlyCDT-Lung was the first autoantibody-based diagnostic tool in lung cancer [2, 4]; up to 50% of patients with lung cancer were positive for circulating antibodies against a panel of 7 TAAs [2]. An effort has been made to identify antibodies to some TAAs as biomarkers for esophageal cancer. The potential biomarkers reported to date include antibodies to p53 [5, 6], peroxiredoxin VI [7], heat shock protein 70 [8], CDC25B phosphatase [9], matrix metalloproteinase-7 [10], ATP-binding cassette transporter C3 [11], CD25 [12] and FOXP3 [13]. A couple of recent studies demonstrated that circulating levels of IgG antibodies to annexin A1 (ANXA1) were significantly increased in lung cancer [14] and breast cancer [15].

Mucins mucs are heavily glycosylated proteins that have been found on the surfaces of both normal epithelial cells and malignantly transformed epithelial cells [16]. MUC1 is a transmembrane mucin and expressed on the apical borders of secretory epithelial cells [17]. The prevalence of anti-MUC1 antibodies has also been found to be increased in some types of cancer [18-21]. The present work was therefore undertaken to investigate whether circulating IgG antibodies for ANXA1 and MUC1 proteins could be altered in esophageal cancer.

Materials and methods

Subjects

A total of 97 patients who were newly diagnosed as having esophageal squamous cell carcino-

Table 1. Information of peptide antigens used for the development of ELISA antibody test

Antigen	Sequence (N -> C)	NCBI accession	Working solution (μg/ml)
ANXA1	H-fntilttrsypqlrrvfqkytlirimvsrseid-OH	NP_000691	10
MUC1	H-pahgvtsapdtrpppgstapaahgvts-OH	NP_877418	15
Control	H-vfqklkdlkdyggvslpewvkiafhtsg-OH	1FKV_A	20

ma (ESCC) were recruited for this study by the Department of Pulmonary Oncology, Third Affiliated Hospital of Harbin Medical University, Harbin, China. Of these 97 patients aged 58.8±7.6 years, 81 were male and 16 were female. Their diagnosis was made based on radiographic examination, esophagoscopy and histological confirmation with staging information. Plasma samples were taken prior to any anticancer treatment. Two hundred and twenty-seven healthy subjects aged 57.1±10.3 years, of whom 135 were male and 92 were female, were also recruited as controls from a local community. Clinical interview and radiographic examination were applied to rule out those control subjects who had history of esophageal cancer or any other malignant tumor. In both the patient and control groups, the individuals who had history of a severe form of autoimmune conditions, such as autoimmune thyroid disease, pernicious anemia, type-1 diabetes, celiac disease, multiple sclerosis, ankylosing spondylitis, systemic lupus erythematosus, rheumatoid arthritis and inflammatory bowel diseases, were excluded from this study. All the subjects were of Chinese Han origin and all gave written informed consent to attend this study as approved by the Ethics Committee of Harbin Medical University and conformed to the requirements of the Declaration of Helsinki.

Autoantibody testing

Two human peptide-based antigens (hAgs) were applied to develop an in-house enzyme-linked immunosorbent assay (ELISA) as described in our recent publications [22-24] to detect circulating IgG antibodies to linear peptide antigens derived from ANXA1 and MUC1 (**Table 1**); a peptide fragment derived from a maize protein (NCBI accession 1BFA_A) was used as the control antigen (cAg). Briefly, synthetic peptides were dissolved in 67% acetic acid to obtain a concentration of 5 mg/ml (stock solution kept at -20°C), and were diluted with phosphate-buffered saline (PBS)-based coating buffer (P4417, Sigma-Aldrich) containing 0.1% sodium azide. Coaster 96-Well Microtiter EIA Plates (ImmunoChemistry Technologies, USA) were half-coated in 0.1 ml/well of each hAg and half-coated in 0.1 ml/well of cAg. The antigen-coated 96-well microplate was incubated overnight at 4°C. The plate was washed 3 times with wash buffer made from Tris-buffered saline with Tween® 20 (T9039, Sigma-Aldrich), and 100 μl plasma sample diluted 1:150 in assay buffer (PBS containing 1.5% BSA) was then added and 100 μl assay buffer was also added to the negative control (NC) wells. Following 2 hour incubation at room temperature, the plate was washed 3 times and 100 μl peroxidase-conjugated goat antibody to human IgG (A8667, Sigma-Aldrich) diluted 1:30000 in assay buffer was added to each well. After incubation at room temperature for an hour, color development was initiated by adding 100 μl Stabilized Chromogen (SB02, Life Technologies) and terminated 25 min later by adding 50 μl Stop Solution (SS04, Life Technologies). The measurement of the optical density (OD) was completed within 10 min at 450 nm with a reference wavelength of 620 nm. To reduce the interference from a non-specific signal produced by passive absorption of various IgG antibodies in plasma to the surface of 96-well microplate, a specific binding index (SBI) was used to express the levels of circulating antibodies to ANXA1 and MUC1. Each sample was tested in duplicate and SBI was calculated as follows: $SBI = (OD_{hAg}-OD_{NC})/(OD_{cAg}-OD_{NC})$.

To minimize an intra-assay deviation, the ratio of the difference between duplicated OD values to their sum was used to assess the precision for assay of each sample. If the ratio was >10%, the test of this sample was treated as being invalid and would not be used for data analysis.

Data analysis

The mean ± standard deviation (SD) in SBI was used to present data. Student's *t*-test was performed to examine the difference in SBI

Table 2. The levels of circulating IgG to ANXA1 and MUC1 peptide antigens in patients with ESCC

Antibody[1]	Patient (n)	Control (n)	t^2	*P*
ANXA1				
Male	1.343±0.334 (81)	1.170±0.266 (135)	4.21	<0.0001
Female	1.337±0.307 (16)	1.253±0.252 (92)	1.18	0.240
Combined	1.342±0.328 (97)	1.204±0.262 (227)	4.02	<0.0001
MUC1				
Male	1.276±0.103 (81)	1.290±0.180 (135)	0.64	0.523
Female	1.300±0.099 (16)	1.306±0.102 (92)	0.22	0.830
Combined	1.280±0.104 (97)	1.296±0.153 (227)	0.98	0.329

[1]The antibody levels are expressed as mean ± SD in SBI. [2]Student's *t*-test (two-tailed).

Table 3. The levels of IgG to ANXA1 and MUC1 peptide antigens in staged ESCC

Antibody[1]	Patient (n)	Control (n)	t^2	*P*
ANXA1				
Stage I	1.478±0.358 (26)	1.204±0.263 (227)	4.84	<0.0001
Stage II	1.340±0.311 (47)	1.204±0.263 (227)	3.12	0.002
Stage III+[3]	1.246±0.276 (19)	1.204±0.263 (227)	0.68	0.500
Unknown	1.027±0.202 (5)	1.204±0.263 (227)	-1.49	0.138
MUC1				
Stage I	1.284±0.079 (26)	1.292±0.153 (227)	0.40	0.688
Stage II	1.271±0.113 (47)	1.292±0.153 (227)	1.06	0.289
Stage III+[4]	1.293±0.105 (19)	1.292±0.153 (227)	0.08	0.935
Unknown	1.285±0.127 (5)	1.292±0.153 (227)	0.17	0.866

[1]The antibody levels are expressed as mean ± SD in SBI. [2]Student's t-test (two-tailed). [3]Combination of stages III (17) and IV (2). [4]Combination of stages III (17) and IV (2).

between the patient group and the control group; Pearson correlation analysis was performed to examine the correlation between circulating IgG level and stages of ESCC. Receiver operating characteristic (ROC) analysis was applied to work out the area under the ROC curve (AUC) with 95% confidence interval (CI) and ELISA sensitivity against a specificity of >90%.

Results

Patients with ESCC had significant higher levels of circulating IgG to ANXA1 than control subjects (t=4.02, P<0.0001); male patients appeared to mainly contribute to the increased levels of anti-ANXA1 IgG antibodies in the circulation (t=4.21, P≤0.0001). However, circulating anti-MUC1 IgG levels were not significantly altered in ESCC (**Table 2**).

Circulating anti-ANXA1 IgG levels were decreased with stages of ESCC (**Table 3** and **Figure 1**), of which patients with stage I ESCC had the highest IgG levels among all 4 stages (t=4.84, P≤0.0001, compared to control subjects). Pearson correlation analysis showed a significant correlation between anti-ANXA1 IgG levels and stages of ESCC (r=0.21, df=90, P=0.044) but no correlation between anti-MUC1 IgG levels and stages of ESCC (r=0.01, df=90, P=0.899).

As shown in **Table 4**, ROC analysis showed an AUC of 0.62 with 95% CI 0.55-0.69 for anti-ANXA1 IgG and 0.46 with 95% CI 0.40-0.53 for anti-MUC1 IgG. The sensitivity of test was 30.9% against specificity of 90.3% in the anti-ANXA1 IgG assay and 11.3% against specificity of 90.2% in the anti-MUC1 IgG assay.

Discussion

The present work confirmed that circulating IgG antibodies for ANXA1 were significantly increased in ESCC. Interestingly, patients with stage I ESCC showed the highest levels of anti-ANXA1 IgG antibodies among all 4 stages of the malignant tumor, suggesting that this autoantibody may be a useful serological biomarker for early diagnosis of esophageal cancer. However, testing of circulating IgG antibodies for each peptide antigen has a low sensitivity and a panel of antigens is needed to develop a highly sensitive test. What mechanism underlies the elevation of IgG antibodies for ANXA1 remains unknown, but overexpression of TAAs could stimulate the immune system to secret antibodies against themselves [25]. The role of ANXA1 in tumorigenesis of esophageal cancer has been suggested [26] and overexpression of ANXA1 has also been reported in esophageal cancer cell lines [27]. Therefore, the increased levels of anti-ANXA1 IgG may result from overexpression of ANXA1 by esophageal cancer cells.

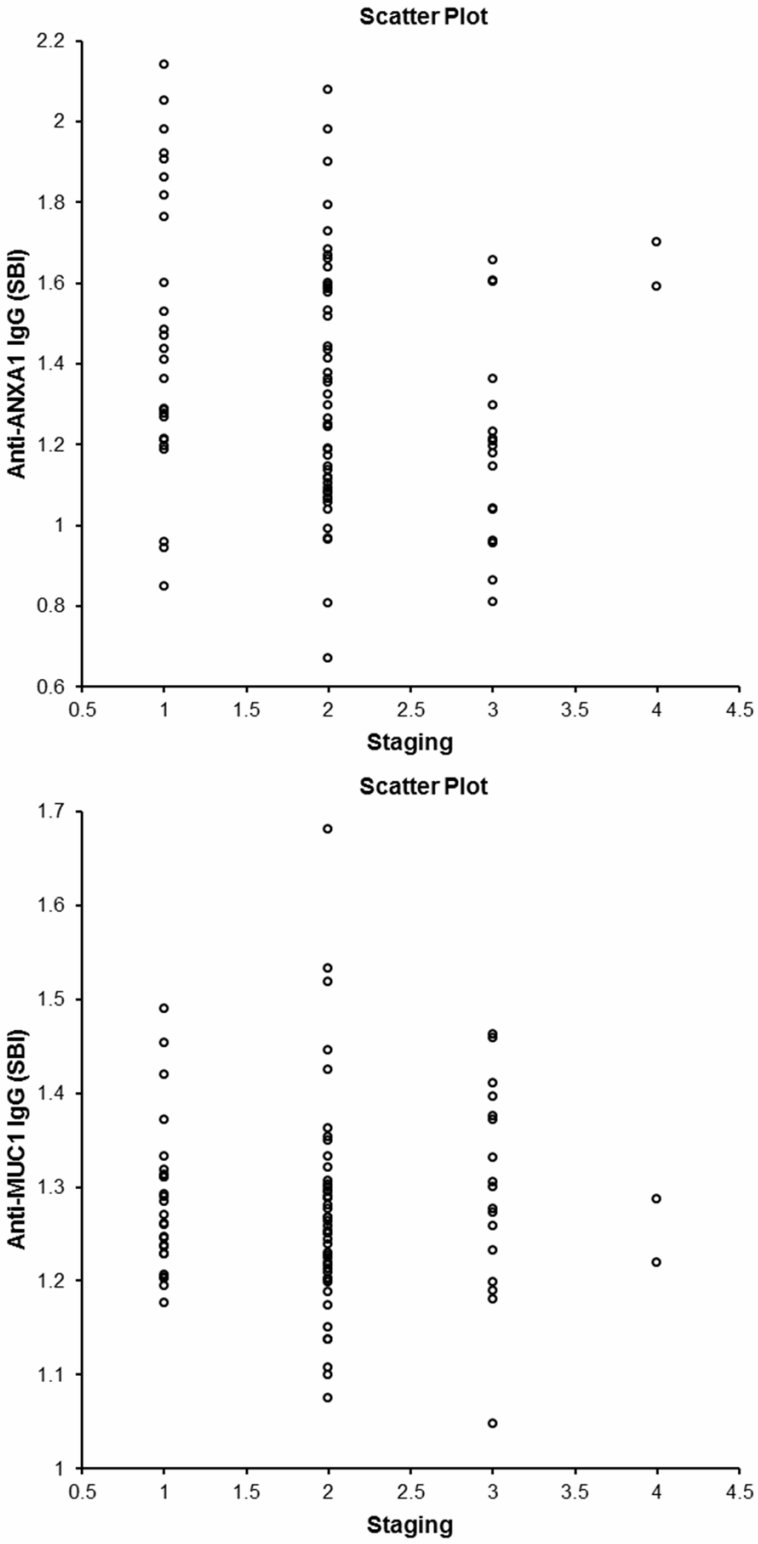

Figure 1. Correlation between circulating IgG levels and stages of ESCC. Anti-ANXA1 IgG: r=0.21, df=90, *P*=0.044. Anti-MUC1 IgG: r=0.01, df=90, *P*=0.899.

While the elevated levels of circulating anti-MUC1 antibodies have been found in breast cancer, ovarian cancer, prostate cancer and colorectal cancer [18-21], a recent study suggests that autoantibodies to MUC1 glycopeptides cannot be used as a screening assay for early detection of breast, ovarian, lung or pancreatic cancer [28]. The present also failed to show an increase in circulating anti-MUC1 IgG levels in patients with ESCC as compared with control subjects (**Table 2**). Whether circulating antibodies to MUC1 can be used as a biomarker for early diagnosis of malignant tumours remains in debate.

These study has a couple of limitations. First, the sample size used for antibody testing in the female group is rather small. Therefore, this initial work needs to be replicated in a large sample size. Second, the control subjects were recruited from the local communities and thus, they might represent a healthy group instead of the population at a high risk of ESCC. Investigation of the healthy relatives of patients with ESCC may be useful to draw a firm conclusion.

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Disclosure of conflict of interest

None.

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Table 4. ROC analysis of IgG antibodies to ANXA1 and MUC1 in ESCC

Autoantibody	AUC	95% CI	SE	Sensitivity (%)	Specificity (%)
ANXA1	0.62	0.55-0.69	0.035	30.8	90.3
MUC1	0.46	0.40-0.53	0.035	11.3	90.3

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