Circulating antibodies to p16 protein-derived peptides in breast cancer

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Abstract. Overexpression of the p16 protein has been reported in breast cancer and may trigger the secretion of antibodies against itself. Circulating anti-p16 antibodies that were detected with a recombinant protein have been reported in breast cancer. The present study was designed to determine whether the levels of circulating IgG antibody to p16 protein-derived linear antigens are altered in breast cancer. An enzyme-linked immunosorbent assay (ELISA) was developed in-house to determine circulating IgG against peptide antigens derived from the p16 protein in 152 female breast cancer patients and 160 healthy female subjects. The Student's t-test revealed that breast cancer patients exhibited significantly higher levels of anti-p16 IgG antibody compared to control subjects (t=2.02, P=0.045). In addition, ductal cancer appeared to be the main type contributing to the increased levels of circulating anti-p16 antibodies (t=2.08, P=0.038). Of all four stages of breast cancer, stage I was associated with the highest levels of IgG antibody (t=2.02, P=0.045) and receiver operating characteristic (ROC) analysis demonstrated that the area under the ROC curve was 0.74 (95% confidence interval: 0.65-083) and that the sensitivity against a specificity of 90% was 30.3%. Therefore, the levels of circulating IgG antibody to the p16 protein may be a potential biomarker for early diagnosis of breast cancer.

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Introduction

There is convincing evidence suggesting that circulating autoantibodies for cancer have diagnostic potential (1-6). A successful test has been developed for early diagnosis of lung cancer (7-9). Breast cancer is a common malignant condition, mainly occurring in women, and the leading cause of cancer-related mortality among women, accounting for 23% of all female cancer cases worldwide (10). Although breast cancer is easy to diagnose by microscopic analysis of a sample, i.e., biopsy, early diagnosis of this malignancy is crucial. Circulating autoantibodies have been suggested to serve as biomarkers for the early diagnosis of breast cancer (11,12), but their sensitivity and specificity have not been satisfactory. Thus, it is crucial to identify a panel of useful tumor-associated antigens (TAAs) in order to develop antibody-based tests for the early diagnosis of breast cancer.

The p16 protein is a cyclin-dependent kinase (CDK) inhibitor that has been found to be involved in the downregulation of the cell cycle through the inactivation of CDK (13-15). As this CDK inhibitor mainly plays a role in suppressing CDK4 and CDK6 activity and arresting cells during the G1 phase of the cell cycle (14,16), it is associated with the unrestrained growth that is the hallmark of cancer (17). A number of studies have suggested that the levels of circulating antibodies to the p16 protein are increased in patients with breast cancer (11,18,19). For example, Looi *et al* developed an in-house enzyme-linked immunoassay (ELISA) using recombinant p16 protein as antigens to detect anti-p16 IgG levels in the plasma of cancer patients (18) and they observed a significant increase in the prevalence of IgG antibodies to the p16 protein in breast cancer. Based on our recent studies, the application of linear peptides as antigens may be more sensitive for ELISA in studying circulating antibodies against certain TAAs (20-22). Linear peptide antigens may be completely exposed and specifically bound to the corresponding antibodies. Accordingly, the present study was undertaken to develop an in-house ELISA with human leukocyte antigen class II (HLA-II)-restricted peptide antigens for the detection of circulating antibodies to the p16 protein.

Materials and methods

Subjects. A total of 152 patients, aged 50.1±9.1 years, who were newly diagnosed with breast cancer, were recruited for this study at the Third Affiliated Hospital of Harbin Medical University, Harbin, China. Of these 152 patients, 126 suffered from ductal carcinoma (DC) and 26 from lobular carcinoma (LC). The diagnoses were based on radiographic examination and histological confirmation with staging information. Blood samples were collected prior to any anticancer treatment. A total of 160 healthy subjects, aged 50.9±5.5 years, were also recruited as controls. Clinical interview and radiographic examination were applied to exclude control subjects with a history of breast cancer or any other malignant tumors. All the subjects were of Chinese Han origin and they all provided written informed consent to participate in this study. This study was approved by the Ethics Committee of Harbin Medical University and conformed to the requirements of the Declaration of Helsinki.

Autoantibody testing. ELISA was developed in-house using a linear peptide antigen, as described in our recent publications, in order to detect circulating IgG to linear peptide antigens derived from the p16 protein (21,22). Briefly, the linear peptide antigen was synthesized by solid-phase chemistry, with a purity of >95%; a synthetic peptide (H-VFQKLKDLKDYGGVSLPEWVCIAFHTSG-OH) derived from a goat α-lactalbumin protein (accession 1FKV_A) was used as the control antigen. The two synthetic peptides were dissolved in 67% acetic acid to obtain a concentration of 5 mg/ml as stock solution. The working solution was then prepared by diluting the stock solution with phosphate-buffered saline (PBS) coating buffer (P4417; Sigma-Aldrich, Beijing, China) into 10 μg/ml for the p16 antigen and 20 μg/ml for the control antigen. Coaster 96-Well Microtiter EIA Plates (ImmunoChemistry Technologies, Bloomington, MN, USA) were half-coated in 0.1 ml/well of the p16 antigen (1 μg/well) and half-coated in 0.1 ml/well of the control antigen (2 μg/well). The antigen-coated 96-well microplate was covered and incubated overnight at 4 ˚C. After the coated microplate was washed 3 times with PBS containing 0.05% Tween-20 (PBS-T), 100 μl plasma sample diluted 1:200 in Assay Buffer (DS98200; Life Technologies, Carlsbad, CA, USA) was added to the sample wells and 100 μl Assay Buffer was added to the negative control (NC) wells. Following a 3-h incubation at room temperature, the plate was washed 3 times with PBS-T and 100 μl peroxidase-conjugated goat antibody to human IgG (A8667; Sigma-Aldrich) diluted 1:30,000 in Assay Buffer were added to each well. Following incubation at room temperature for 2 h, color development was initiated by adding 100 μl stabilized chromogen (00-2023; Life Technologies) and terminated 25 min later by adding 50 μl stop solution (SS04; Life Technologies). The measurement of optical density (OD) was completed on a microplate reader (BioTek, Winooski, VT, USA) within 10 min at 450 nm with a reference wavelength of 620 nm.

Each sample was tested in duplicate. To reduce the interference from a non-specific signal produced by passive absorption of various antibodies in the plasma to the surface of the 96-well microplate, a specific binding index (SBI) was used to express the levels of circulating antibodies to the p16 protein. SBI was calculated as follows:

$$SBI = (OD_{p16\ antigen}-OD_{NC})/(OD_{control\ antigen}-OD_{NC})$$

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

Data analysis. Data are presented as mean ± standard deviation in SBI. IBM SPSS Statistics 21.0 software was used to perform the Student's t-test for the difference in SBI between the patient group and the control group and the receiver operating characteristic (ROC) analysis was used to estimate the area under the ROC curve (AUC) with 95% confidence interval (CI) and the sensitivity of the ELISA antibody test against a specificity of >90%.

Results

Levels of circulating IgG to p16-derived peptide antigens. Patients with breast cancer had a significant higher level of circulating IgG to the p16 protein compared to control subjects (t=2.02, P=0.045). As shown in Table I, ductal carcinoma appeared to be the main type contributing to the increased levels of anti-p16 IgG (t=2.08, P=0.038).

Association between breast cancer stage and levels of circulating IgG to p16-derived peptide antigens. Of all four stages of breast cancer, patients with stage I disease had the highest levels of circulating anti-p16 IgG (t=2.02, P=0.045, compared to control subjects), whereas in the other 3 stages here was not significant increase in anti-p16 IgG levels (Table II).

ROC analysis of anti-p16 IgG antibodies. In patients with stage I breast cancer, the ROC analysis revealed an AUC of 0.74 (95% CI: 0.65-083) for anti-p16 IgG; the sensitivity against a specificity of 90% was 30.3% (Table III).

Discussion

The p16 protein plays a crucial role in downregulating cell proliferation (13). Overexpression of p16 protein has been reported in several types of cancer, although normal tissues exhibit low or undetectable levels (18,23). This is possibly a feedback reaction to the development of malignancy in the body. In this study, we identified a significant increase in anti-p16 IgG levels in patients with breast cancer compared to control subjects (Table I), particularly in those with stage I disease (Table II). This finding suggests that the anti-p16 IgG antibody may be a potential marker for early diagnosis of breast cancer. There remains the question of the mechanism underlying the increased anti-p16 IgG levels in breast cancer. It has been widely recognized that the development of cancer is a multistep process that may involve abnormal regulation of cell growth, differentiation and apoptosis. The immune system is also involved in identifying potential cancer cells through immune surveillance. It is possible that the overexpression

Table I. Levels of circulating IgG to p16-derived peptide antigens in patients with breast cancer.

Type of cancer	Patient (n)	Control (n)	t[a]	P-value
Ductal	1.18±0.23 (126)[a]	1.10±0.41 (160)	2.08	0.038
Lobular	1.14±0.18 (26)	1.10±0.41 (160)	0.58	0.565
Combined	1.17±0.26 (152)	1.10±0.41 (160)	2.02	0.044

The antibody levels are expressed as mean ± standard deviation in specific binding index. [a]Student's t-test (two-tailed).

Table II. Association between the stages of breast cancer and the levels of circulating IgG to p16-derived peptide antigens.

Stage	Patient (n)	Control (n)	t[a]	P-value
I	1.28±0.26 (33)	1.10±0.41 (160)	2.02	0.045
II	1.15±0.17 (49)	1.10±0.41 (160)	0.86	0.388
III	1.12±0.22 (62)	1.10±0.41 (160)	0.52	0.607
IV	1.27±0.23 (7)	1.10±0.41 (160)	1.10	0.271

The antibody levels are expressed as mean ± standard deviation in specific binding index. [a]Student's t-test (two-tailed).

Table III. ROC analysis of anti-p16 IgG antibodies in patients with breast cancer.

Stage	AUC	95% CI	SE	Sensitivity (%)[a]
I	0.74	0.65-0.83	0.04	30.3
II	0.63	0.54-0.71	0.04	6.1
III+IV	0.59	0.52-0.67	0.04	15.9

[a]Against a specificity of 90%. AUC, area under the curve; CI, confidence interval; SE, standard error.

of such a TAA may stimulate the immune system to secrete antibodies against itself (24). In addition, somatic p16 mutation in primary tumors may be another possible reason for an autoimmune response to the p16 protein due to increased immunogenicity (25).

Although the circulating levels of anti-p16 IgG were significantly increased in breast cancer, its low positivity rate in the patient group suggests that such an antibody alone is unlikely to serve as a biomarker for the diagnosis and prognosis of malignancy. Therefore, it is crucial to identify a panel of TAA-derived linear peptide antigens for the development of diagnostic testing with a high sensitivity. Similar to the EarlyCDT-Lung test, the panel antibody positivity is a more powerful diagnostic tool compared to individual antibody positivity (6-8). Moreover, synthetic peptides are significantly more cost-effective compared to recombinant TAAs. The peptide-based ELISA antibody test may be a more suitable option for population screening.

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