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Autoantibodies to centrosomal protein 70 is expected to become a plasma biomarker forearly diagnosis of female breast cancer

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Abstract: Objective Our previous study found that autoantibodies to tumor associated antigens increased specifically in plasma in the early stage of malignant tumors. This study detected and observed the expression of Cep70 autoantibody in the plasma of patients with breast cancer.Methods The improved and optimized ELISA method was used to detect the expression of Cep70 autoantibody in the plasma of 137 patients with breast cancer and 136 healthy people.Results The SBI value of Cep70 antibody in plasma of tumor group was 1.24±0.48, which was significantly higher than that of control group (1.02±0.15) (P<0.01, t=5.11). Further subgroup analysis showed that there was no significant positive correlation between its expression level and clinical stage (P>0.05). The SBI values of Cep70 antibody in plasma from low-risk group to high-risk group were 1.08±0.59, 1.15±0.26 and 1.33±0.38 respectively. There was significant difference between each group and the control group (P<0.05), and its expression level was also positively correlated with the risk grade. Pearson correlation analysis showed that the expression level of Cep70 antibody in the tumor group was correlated with clinical stage and risk classification (P<0.05), and the risk classification factors were most closely related to the positive rate of Ki67. When the specificity is 90%, the sensitivity is 63%, the yoden index is 0.53, the negative predictive value is 0.83, and the SBI value of Cep70 autoantibody is 1.27 as the cut-off value.Conclusion Cep70 autoantibody is expected to become a plasma biomarker for early diagnosis of female breast cancer.

Keywords: Cep70; Breast cancer;autoantibody; Early diagnosis; Tumor markers

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1.Introduction

Centrosomal Protein 70(Cep70) is a member of the centrosome-associated proteins and contains 597 amino acids with a relative molecular mass of 70×103. It is localized to the centrosome and interacts with microtubule proteins through two coiled helix structural domains[1], regulating mitosis by meidiating spindle formation and playing an important role in cell proliferation and migration. Some studies have confirmed that Cep70 is closely related to the development, progression and distant metastasis of breast cancer[2 ～ 3].

Breast cancer is diagnosed with a certain lag, making many patients fail to get a clear diagnosis at an early stage[4].Autoantibodies based on tumor-associated antigens, as plasma tumor markers for the early diagnosis of malignant tumors, have the advantages of high specificity and sensitivity. Studies have demonstrated that autoantibodies can be used to detect 80% of precancerous patients 3 ～ 5years before the malignant tumors that can be detected by modern imaging and laboratory techniques[5].Autoantibodies as a novel diagnostic marker for tumors have received attention from the national and international academic community[6]. In our previous study,we found that autoantibodies to tumor-associated antigens, a novel and accessible biomarker, were significantly expressed in patients with esophageal and cervical cancers and could be detected at an early stage[7 ～ 8].In this study,we propose to use a modified and optimized ELISA technique to detect the expression of Cep70 autoantibodies in plasma of breast cancer patients with different clinical characteristics, and to provide a laboratory basis for the early diagnosis of breast cancer.

2 Materials and Methods

2.1 Patient selection

The inclusion criteria were as follows:(1)Observation group： 1)female patients with pathologically confirmed breast malignant tumor; 2) age range 26 ～ 70 years; 137 cases were enrolled, and plasma samples were taken before receiving any treatment (including surgery,chemotherapy, radiotherapy and endocrine therapy) (2) Control group: 1) healthy volunteers from the community, 2) age range 22 ～ 66 years, 136 cases were recruited, and medical history and family history were asked to exclude the history of breast cancer and other malignant tumors. Patinets with autoimmune diseases

such as autoimmune thyroid disease, anemia, type 1 diabetes, cerebral palsy, multiple sclerosis, ankylosing spondylitis, systemic lupus erythematosus, rheumatoid arthritis, and inflammatory bowel diseases were excluded. All patients were gave informed consent, and were approved by the Ethics Committee of Qingdao University Hospital, in compliance with the Declaration of Helsinki.

2.2 Testing Method

Cep70 linear polypeptide fragment was prepared by chemical synthesis and dissolved in 67% acetic acid to obtain a storage solution at a concentration of 5 mg/ml. The antigen was diluted in phosphate buffer containing 0.1% sodium azide at pH 7.4 Our preliminary study concluded that the optimal working solution was 10ug/ml of Cep70-derived antigen and 20ug/ml of goat control antigen.96-well microtiter plates were coated half with 0.1ml/well of Cep70 antigen (1ug/well) and the other half with 0.1ml/well of goat control antigen (2ug/well), then it was incubated overnight at 4°C. The plates were washed 3 times with Phosphate Buffered Saline (PBS) containing 0.05% TWEEN 20. 100ul of plasma sample diluted 1:200 in Analytical Buffer was added to the sample wells,while 100ul of analytical buffer was added to the negative control wells(NC). Incubate for 3 hours at room temperature and then wash 3times with PBS solution. Add 100ul of peroxidase-conjugated goat antibody in 1:40,000 human IgG diluted in assay buffer or 1:30,000 human IgA diluted in assay buffer to each well. After incubation for 2 hours at room temperature, 100ul of color developer was added to each well for color development and the reaction was terminated by adding 50ul of termination solution after 25minutes. The optical density (OD) values were measured by an enzyme marker for 10minutes at a detection wavelength of 450nm and a reference wavelength of 620nm. To reduce the interference of non-specific signals generated by various antibodies in plasma adsorbed on the surface of 96-well microtiter plates, the expression of circulating autoantibody levels of Cep70 protein was determined using the Specific Binding Index (SBI).SBI=[Cep70antigen(OD)-NC(OD)]/[control antigen(OD)-NC(OD)]

2.3 Statistical analysis

Statistical processing of the data was performed using GraphPad Prism v7.0software,the measurement data were derived as the mean±standard deviation of SBI, and the t-test was used to detect the difference in SBI values between the tumor and control groups, and to analyze the interrelationship between the autoantibody levels of Cep70 in plasma of breast cancer with different clinical characteristics (clinical stage, risk factor classification). The fraction of antibody levels above 99% in the control group was used as the positive thresholdfor anti-Cep70 antibodies, and the $\chi 2$ test was used tocom-

Table 1. Clinical characteristics and statistical results of the tumor andcontrol groups

Clinicalcharacteristics	Tumor group	Control group
Age		
＜40years	21	24
40～60 years	72	72
＞60years	44	40
Stage		
Phase I	21	21
Phase IIA	7	7
Phase IIB	13	13
Phase IIIA	27	27
Phase IIIB	31	30
Phase IIIC	11	11
Phase IV	27	27
Risk factors classification		
Low risk	24	24
Medium risk	72	71
High risk	41	41

pare the difference in antibody positivity between the tumor and control groups, and theirodds ratio (OR) and 95% confidence interval (95% CI). To reduce intra-batch variation, the ratio of ODs between double replicate wells was used as a method to assess the precision of each sample. If the intra-lot variation was >10%, the processing of this sample was considered invalid and could not be used for data analysis. Between-lot variation was assessed using combined plasma samples, i.e. quality control samples, collected in a randomized manner from >100 unrelated healthy volunteers and tested in each 96-well plate (detection level of 0.05)

Table 2 Expression levels of Cep70 antibody in peripheral blood plasma of eachclinical stage.

Clinicalcharacteristics	Tumor group	Control group	t	P
All patients	1.24±0.48 (n=137)	1.02±0.15 (n=136)	5.11	＜0.01
Phase I	1.21±0.19 (n=21)	1.02±0.15 (n=136)	5.21	＜0.01
Phase II	1.19±0.31 (n=20)	1.02±0.15 (n=136)	3.99	＜0.01
Phase III	1.25±0.18 (n=69)	1.02±0.15 (n=136)	9.69	＜0.01
Phase IV	1.28±0.24 (n=27)	1.02±0.15 (n=136)	7.35	＜0.01

3 Results

3.1 Clinical characteristics

From the results in Table 1, 137 cases were enrolled in the tumor group and 136 cases were enrolled in the control group, with a peak of 40 to 60 years old. The patients in the tumor group were enrolled in 21, 20, 69, and 27 cases from stage I to IV, respectively, and the proportion of stage III patients was the highest, accounting for about 50.4% of all tumor groups. According to the risk factor classification group, 24, 72, and 41 cases were enrolled in the low-risk, medium-risk, and high-risk groups, respectively, and the proportion of medium and high risk patients accounted for 82.5%.

3.2 Testing results

Spearman rank correlation coefficient analysis showed that the expression level of Cep70 antibody in tumor group was correlated with clinical stage and risk grade (Spearman r=0.819/0.836, P<0.01). As shown in Table 2 and Figure 1, the SBI value of Cep70 antibody in plasma of breast cancer patients was significantly higher than that of the control group(1.24±0.48 vs 1.02±0.15), difference between these two group is statistically significant. (P<0.01, t=5.11). Further by subgroup analysis based on clinical staging, it was found that the tumor groupI ～ IV The SBI values of Cep70 antibodies in plasma of stage patients were 1.21±0.19 (P<0.01, t=5.21), 1.19±0.31 (P<0.01, t=3.99), 1.25±0.18 (P<0.01, t=9.69), and 1.28±0.24 (P<0.01, t=7.35), respectively, which were significantly higher than those of the control group, The differences were statistically significant. Among the stages, the highest value was found in stage IV and the lowest value in stage II. The ANOVA was performed for each phase, and the P value was greater than 0.05, which did not show a dose-dependent relationship and statistical significance.

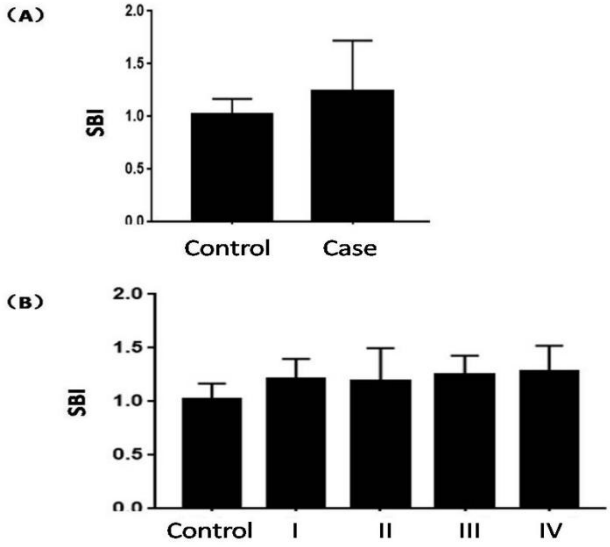

Figure 1. Changes of SBI value of Cep70 antibody in peripheral plasma.(A)control group and tumor group; (B) control group and different stages of tumor group.

To further understand the correlation between risk

factors and early screening for breast cancer, the risk level of all patients in the tumor group was graded with reference to NCCN and CSCO guidelines. As seen in Table 3 and Figure 2, the SBI values of Cep70 antibodies in plasma of low-, medium-, and high-risk patients in the tumor group were 1.08±0.59 (P>0.05, t = 1.03), 1.15±0.26 (P<0.01, t=4.57), and 1.33±0.38 (P<0.01, t=7.75), respectively, and their values were dose-dependently increased. Comparison of the subgroups with the control group revealed that medium and high risk groups have statistical difference with the control group (P<0.01), while no statistical difference was seen between the low risk group and the control group (P>0.05). The subgroups were subjected to variance analysis with a P>0.05, which did not show a dose-dependently relationship and statistical significance.

Table 3 Expression levels of Cep70 antibody in peripheral blood plasma of each risk level classification

Clinicalcharacteristics	Tumor group	Control group	t	P
All patients	1.24±0.48（n=137）	1.02±0.15（n=136）	5.11	＜0.01
Low risk group	1.08±0.59（n=24）	1.02±0.15（n=136）	1.02	0.31
Medium risk group	1.15±0.26（n=72）	1.02±0.15（n=136）	4.57	＜0.01
High risk group	1.33±0.38（n=41）	1.02±0.15（n=136）	7.75	＜0.01

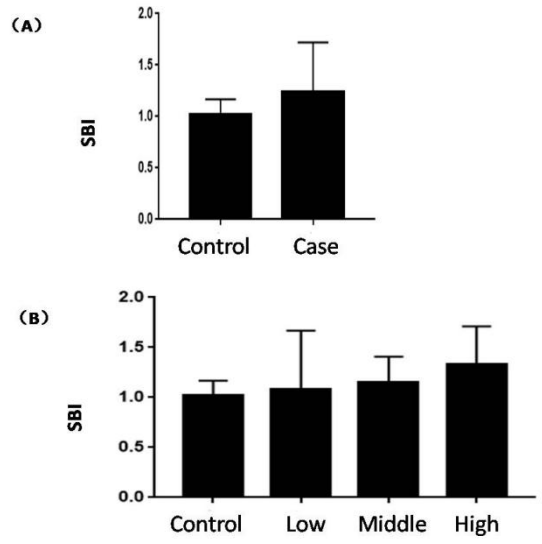

Figure 2. Changes of SBI value of Cep70 antibody in peripheral plasma.(A) control group and tumor group; (B) control group and different risk classification of tumor group.

ROC curve analysis was performed between the tumor group and the control group, as shown in Figure 3 The area under the ROC curve (AUC) was 0.84, with a 95% confidence limit range (CI) of 1.24 to 1.28. When the specificity was 90%, the sensitivity was 63%, the Yordon index was 0.53, and the negative predictive value was 0.83, with the SBI value of Cep70 autoantibody 1.27 was the cut-off value.

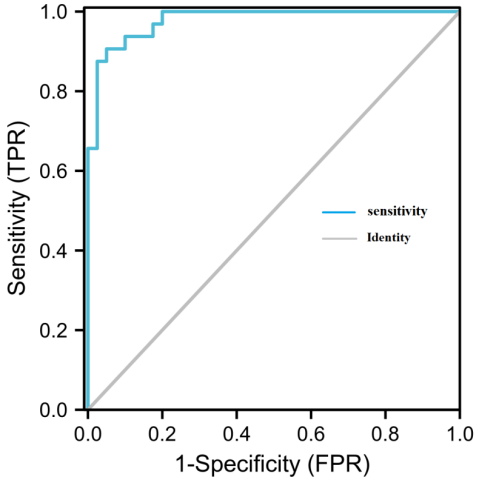

Figure 3. the ROC curve

ROC curve analysis was performed between the tumor group and the control group, as shown in Figure 3 The area under the ROC curve (AUC) was 0.84, with a 95% confidence limit range (CI) of 1.24 to 1.28. When the specificity was 90%, the sensitivity was 63%, the Yordon index was 0.53, and the negative predictive value was 0.83, with the SBI value of Cep70 autoantibody 1.27 was the cut-off value.

4 Discussion

According to the data released by NCCN, the 5-year survival rate of breast cancer in stage I ～ II is 85% ～ 90%, the 5-year survival rate in stageIII is about 50%, and the 5-year survival rate in stage iv is only 10% ～ 18%[9]. Therefore, “early detection, early diagnosis and early treatment” is the basic strategy for the diagnosis and treatment of female breast cancer, which

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has positive significance for the prognosis of female breast cancer patients. In recent years, the research on the early diagnosis of female breast cancer has become a hot spot of clinical oncology research. Traditional imaging methods have a certain lag in the diagnosis of female breast cancer, and the current clinical tumor markers lack specificity and sensitivity. Development of basic disciplines such as tumor immunology, medical genetics, molecular biology, and computer engineering, the diagnostic methods of malignant tumors have also been improved, and the diagnostic methods are closer to the level of cells, molecules and genes. The research on Circulating Tumor Cells (CTCs) and MicroRNA(miRNA) is still immature and has not yet reached clinical standards. In fact, there is currently no specific tumor marker for female breast cancer in the true sense, and the research on early diagnosis of breast cancer has encountered a "bottleneck". Exploring reliable early diagnosis indicators with high sensitivity and specificity is essential for improving the early diagnosis rate of female breast cancer. A study published in the New England reported that the sensitivity of 22 prostate cancer antigens obtained by detecting antibodies for the diagnosis of prostate cancer was 81.6% and the specificity was 88.2%, which was significantly higher than that of Prostate Specific Antigen(PSA), which was also rated as the most important progress in molecular diagnostic markers in recent years[10].EarlyCDT-Lung is a kit for early diagnosis of lung cancer based on the detection of autoantibodies, which has been generally recognized by the academic community.

As the most important microtubule tissue center in mammalian cells, Cep is composed of a pair of centrioles and high electron density surrounding centrioles, which play an important role in cell proliferation and cell migration[11]. Cep57 is the most studied Cep family, which is closely related to microtubule stability[12],while there are few reports on functional studies of Cep70.Autoantibodies have a longer half-life and are easier to detect in serum, and changes in expression level can occur at the early stage of tumor occurrence[13]. As a tumor marker, tumor-associated antigen autoantibody has the advantages of strong specificity, high sensitivity and low false positive rate, and can be used for early screening and prognosis evaluation of tumors. Studies have shown that about 80% of precancerous patients can be detected by autoantibody technology 3 ～ 5 years before malignant tumors develop to be detected by modern imaging and laboratory techniques[14]. In this study, Cep70 autoantibodies in peripheral blood plasma of breast cancer patients were detected by an improved and optimized ELISA method(National Patent Number:201110388134.1），Table 2 and Figure 1 showed that the level of Cep70 autoantibodies in peripheral blood plasma of breast cancer patients was significantly higher than that of the control group, and the difference was statistically significant（$P < 0.05$）,it is consistent with our previous research results on early screening of esophageal cancer and cervical cancer[7 ～ 8]. ROC curve analysis showed that the results were in line with the detection rule of plasma autoantibodies, and this study showed good specificity, sensitivity and negative predictive value, which was higher than the average detection effect of domestic and foreign counterparts, and had good diagnostic and evaluation value. Further subgroup analysis based on clinical stage of breast cancer showed that stage IV had the highest value and stage II had the lowest value, showing no dose-dependent relationship.No statistical difference was found between stage I and stage II, III and IV（$P > 0.05$）. These results indicate that Cep70 circulating autoantibody is highly expressed in breast cancer patients, significantly higher than that in healthy controls, and its expression level is not correlated with clinical stage, but can be highly expressed at early stage, suggesting that it is expected to be a serological tumor marker for early diagnosis of breast cancer. In addition, the prognosis and treatment strategy of breast cancer are closely related to risk factors in addition to clinical stage. Early assessment of risk factors in breast cancer patients is of great importance to improve the cure rate and survival benefit. Risk factors include pathological type,degree of differentiation,histological grade,molecular typing,Ki67 positive rate,and external invasion status (vascular,nerve,lymph node),etc.,which can be divided into low risk,medium risk,and high risk according to the score.As shown in Table 3 and Figure 2,the expression level of high-risk group to low-risk group decreased in a dose-dependent manner,and the expression level of medium-risk group and high-risk group was significantly higher than that of control group($P < 0.05$）,while there was no significant difference between low-risk group and control group（$P > 0.05$）. These results suggest that Cep70 circulating autoantibody is positively correlated with risk factors, and its expression level can indicate the risk degree and the risk of recurrence and metastasis of breast cancer.

In conclusion,Cep70 circulating autoantibodies are significantly higher in breast cancer patients than in healthy controls,and their expression level is not significantly correlated with clinical stage,namely tumor load, but is closely correlated with risk factors,showing positive correlation.Tumor microenvironment is a prerequisite for tumor development and is also a critical risk factor,which can reflect the early changes of tumor earlier than tumor load.As a sensitive biochemical indicator,Cep70 peripheral blood autoantibodies can be reflected and detected earlier than imaging examinations,which is also consistent with the detection

results of this study.Therefore,Cep70 circulating autoantibody is suitable for early screening of breast cancer and is expected to be a serological biomarker for early diagnosis of breast cancer.At present,the specific molecular mechanism through which Cep70 promotes the occurrence and development of breast cancer is not clear.Mitosis regulated by Cep70 is related to cell proliferation and invasion[15].Xie et al.reported thatCep70promoted the proliferation of pancreatic cancer cells through genomic instability,while it was significantly inhibited after interfering with Cep70,suggesting that Cep70 could become a biomarker for pancreatic cancer[16]. Shi et al.confirmed through mouse animal model that Cep70 is closely related to the occurrence and development of breast cancer,and its expression is particularly obvious in advanced breast cancer with lung metastasis[17].Pedroet al. reported that Cep70 may undergo epitheliar mesenchymal transformation through the TGF-βsignaling pathway, resulting in changes in the number and size of microtubules[18],ultimately promoting proliferation and invasion of breast cancer cells[19].This study still has certain limitations,and in the future,it is still necessary to further clarify the relationship between cep70 and the signaling pathways for the occurrence and development of breast cance.

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