Original Article

CA125-Tn ELISA assay improves specificity of pre-operative diagnosis of ovarian cancer among patients with elevated serum CA125 levels

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Background: CA125 is the most widely used serum marker for preoperative diagnosis of ovarian cancer. However, CA125 elevation is not specific to ovarian cancer. More than 60% of patients who have elevated CA125 levels do not have ovarian cancer. To overcome the low specificity of CA125, we identified a CA125 glycoform that was specifically elevated in ovarian cancer and that may help in the further triage of patients with elevated serum CA125 levels.

Methods: We used antibody-lectin ELISA to detect various CA125 glycoforms. Among 21 lectins tested, VVA, a plant lectin that preferentially binds Tn antigen, showed significantly stronger binding with ovarian cancer-derived CA125 than benign condition-derived CA125. CA125-Tn levels were tested among patients with elevated CA125 levels (n=328, including 68 ovarian cancer, 15 ovarian borderline tumors, and 245 benign conditions). The efficacy of CA125-Tn in diagnosing ovarian cancer was evaluated using ROC analysis.

Results: Medians and 25th to 75th quartiles of CA125-Tn levels were 0.31 (0.18–0.65) in ovarian cancer, 0.07 (0.02–0.12) in ovarian borderline tumor, and 0.07 (0.01–0.12) in benign conditions. AUC of the ROC curve was 0.890 (95% CI: 0.845, 0.935) for CA125-Tn to discriminate ovarian cancer cases from nonmalignant cases (borderline tumors and benign conditions). Its performance was even better among patients older than 45 y (AUC: 0.905, 95% CI: 0.841, 0.969). Specificity was improved from 35.1% for CA125 to 75.7% for CA125-Tn among patients older than 45 y when sensitivity was fixed at 90%.

Conclusions: CA125-Tn ELISA assay can improve specificity of the preoperative diagnosis of ovarian cancer and serve as a further triage strategy for patients with elevated CA125 levels.

Keywords: Ovarian cancer; CA125; glycoforms; Tn antigen; diagnosis

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Introduction

Ovarian cancer remains the leading cause of death from gynecological malignancies. Symptoms of ovarian cancer are nonspecific. Most patients are diagnosed only when the disease has progressed to an advanced stage (1). Optimal cytoreduction, which requires a well-trained gynecological oncologist, is a key factor for improved survival (2). The quality of first surgery is a strong prognostic indicator. However, due to the large prevalence of ovarian cysts or masses, it is not feasible for all patients with ovarian cysts, whether benign or malignant, to be managed by gynecological oncologists. Patients need to be triaged preoperatively (3,4).

Preoperative diagnosis of ovarian cancer relies mainly on serum CA125 levels and imaging tests such as computed tomography scan and ultrasonography. However, neither sensitivity, nor specificity is satisfactory (5). Elevations in the serum CA125 concentration have been observed in most non-mucinous epithelial ovarian cancer patients (6). The sensitivity for CA125 to detect ovarian cancer is approximately 80% (7). However, patients with benign gynecological diseases such as endometriosis (8) and pelvic inflammatory disease (9), or even apparently healthy women (10) may also have elevated serum CA125 levels (11). It has been reported that endometriosis, which affects more than 10% of women of reproductive age and commonly presents with an adnexal mass, causes increased CA125 levels in up to 67% of patients (12). Previous single center hospital study showed that of 191 patients with abnormal CA125 levels, only 39 (20%) patients were demonstrated to have ovarian cancer by pathological examination (13). Forty-eight (26%) had nonovarian/gynecological malignancies. As many as 54% of the patients had benign diseases or normal results after thorough examination. According to results from the Prostate, Lung, Colorectal and Ovarian (PLCO) screening trial (the largest clinical trial to date assessing the efficacy of ovarian cancer screening in general population), positive predictive value for CA125 alone is only 3.7% (14). Above all, a more accurate preoperative triage strategy for ovarian cancer is urgently needed.

CA125 is a highly glycosylated mucin encoded by the MUC16 gene (15). In healthy women, it is synthesized mainly by mesothelial cells and inner layer of female genital tract (16,17). Irritations to mesothelium, such as ascites and pelvic inflammatory disease, are common causes of elevated serum CA125 levels (18,19). In contrast, ovarian cancer cells synthesize and secrete CA125 in large amounts. The serum level of CA125 in ovarian cancer patients is positively correlated with cancer tissue staining (20,21), suggesting that cancerous tissue is the main source of serum CA125 in those patients. It is well known that obvious changes occur in glycosylation machinery in malignant tissues, which result in aberrant glycan patterns on glycoproteins synthesized by these cancerous tissues (22-24). Thus, it is reasonable to expect a distinct glycosylation pattern on ovarian cancer-derived CA125 molecules as compared with benign CA125 molecules. Previous studies reported increased glycans, such as truncated O-glycans and sialyl Tn antigen (STn), in ovarian cancer-associated CA125 (25-27). These may serve as biomarkers to help in the differential diagnosis of patients with elevated CA125 levels.

In searching for this, we established an antibody-lectin ELISA assay for detection of glycans attached to CA125 molecules. Of 21 lectins tested, VVA [Vicia Villosa Lectin, preferentially binds Tn antigen (alpha-GalNAc-O-Ser/Thr)] showed the best differentiation efficacy when used as the detecting lectin. Performance of this antibody-lectin ELISA assay (termed the CA125-Tn ELSA assay) was demonstrated to be satisfactory for preoperative triage of patients suspected of having ovarian cancer (with a positive CA125 test result). We present the following article in accordance with the STARD reporting checklist (available at http://dx.doi.org/10.21037/atm-20-8053).

Methods

Patients

Patient recruitment was conducted at multiple sites, including Obstetrics & Gynecology Hospital of Fudan University, Peking University People's Hospital, Cancer Center of Shanxi Province, Dalian Obstetrics & Gynecology Hospital, Huai'an First People's Hospital, and Central Hospital of Fengxian District, from September 2011 to April 2018. Patients who met the following criteria were included in this study: (I) elevated serum CA125 (≥35 U/mL) before treatment; (II) confirmed diagnosis by histopathological examination for whom surgery was performed, or by clinical examination and investigations for those surgery was not required for diagnosis; and (III) more than 300 μL of serum sample remaining after CA125 testing. Pregnant patients were excluded from this study. Leftover serum samples after CA125 testing were collected and stored at −80 ℃ before use. Data concerning each patient's age, menopausal status, imaging findings, diagnosis, histological type, stage, and grade were collected (*Table 1*). This study was conducted in accordance with the

Table 1 Patient characteristics

	N	Age (y), mean ± SD	Post-menopause, N (%)	CA125 (U/mL), mean ± SD	CA125-Tn, mean ± SD	P
Ovarian cancer	68	53±11	35 (50.7)	679.06±993.33	0.44±0.33	Reference
Serous	56	55±10	32 (57.1)	757.60±1,066.23	0.49±0.33	
Mucinous	6	44±15	2 (33.3)	114.90±55.46	0.07±0.03	
Endometrioid	3	44±8	0 (0.0)	215.73±72.02	0.16±0.07	
Clear cell	3	52±8	1 (33.3)	804.53±552.13	0.50±0.49	
Ovarian borderline tumor	15	32±8	1 (6.7)	288.26±185.56	0.10±0.13	<0.001
Serous	13	33±9	1 (7.7)	276.08±173.07	0.08±0.10	
Mucinous	2	28±3	0 (0.0)	367.40±328.94	0.23±0.26	
Benign	245	35±9	4 (1.6)	221.50±247.28	0.10±0.10	<0.001
Endometriosis	143	33±8	1 (0.7)	212.90±276.50	0.07±0.09	
Adenomyoma	44	43±4	0 (0.0)	183.15±98.96	0.07±0.09	
PID	31	36±10	1 (3.2)	315.16±271.71	0.15±0.16	
Teratoma	4	38±13	0 (0.0)	150.77±37.52	0.03±0.06	
Tuberculosis	3	25±5	0 (0.0)	420.45±284.61	0.16±0.12	
Ovarian adenoma	2	52±40	1 (50.0)	235.55±86.06	0.06±0.06	
Others	18	38±14	1 (5.5)	214.20±220.17	0.10±0.08	

PID, pelvic inflammatory disease; P: comparisons of CA125-Tn level means to patients with ovarian cancer.

Declaration of Helsinki (as revised in 2013). This study was approved by the Institutional Review Board of Obstetrics & Gynecology Hospital of Fudan University (2017-52). In this study, we used only serum samples left over after clinical testing. No additional serum sample was collected outside the standard clinical requirement. Thus, written informed consent from participants was not required and was waived by the Institutional Review Board.

Microplate fabrication and preparation

Antibodies are glycoproteins. Glycans attached to the Fc fragment will bind lectins and thus interfere with the detection of glycans on antigens in the antibody-lectin assay. To eliminate interference from antibody glycans, we performed derivatization of the anti-CA125 antibody after coating. The procedure was performed as described by Chen *et al.* in 2007 with modifications (*Figure 1*) (28). First, a 96-well microplate was coated with 1 μg/mL anti-MUC16 antibody (Abcam, USA, clone X325) in Na_2CO_3-$NaHCO_3$ buffer (0.05 M, pH 9.6) overnight at 4 ℃. After washing with PBST0.1 three times to remove unbound antibody, the plate was incubated in a blocking solution of PBST0.1 containing 1% BSA at 37 ℃ for 1 hour. Then, 20 mM NaIO4 in coupling buffer (0.1 M sodium acetate, pH 5.5) was added to oxidize the sugar groups on the antibody, and the plate was incubated in the dark at 4 ℃ for 2.5 hours. After a brief rinse, the plate was incubated with 1 mM 4-(4-N-maleimidophenyl)butyric acid hydrazide hydrochloride (MPBH; Pierce Biotechnology) for 2 hours at room temperature to derivatize the carbonyl groups. Finally, 1 mM Cys-Gly dipeptide (Sigma Aldrich, USA) in PBST0.1 was reacted with maleimide on MPBH, and sugar groups were blocked overnight at 4 ℃.

Antibody-lectin assay

Microplates coated with derivatized anti-CA125 (X325) were used for the antibody-lectin assay. Briefly, 100 μL of serum (100 μL of PBS as blank) was added to the plate and incubated at 37 ℃ for 90 min. The plate was washed 3 times with PBST0.1 to remove unbound proteins and then incubated with 10 μg/mL of biotin-lectins (Vector, USA) at room temperature for 1 hour. After 6 washes with PBST0.1,

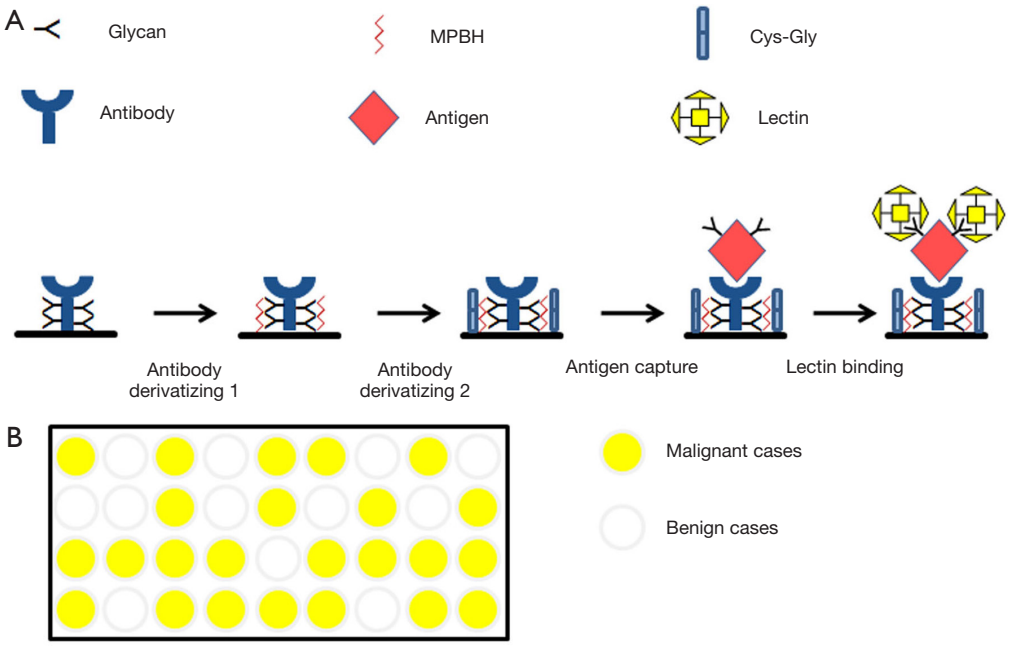

Figure 1 Derivatizing of glycans on IgG Fc fragments. (A) Experimental scheme; (B) Example of results of the antibody-lectin ELISA assay.

streptavidin-HRP (Vector, USA) was added to bind with biotin-lectins. After another 6 washes, color development was performed using TMB solution (Excel, China) in the dark for 10 min and stopped by 1 N HCL. The optical density (OD) of each well was estimated at A_{405} using a microplate reader (Perkin Elmer, USA). Each sample was tested in duplicate, and the results were averaged.

Statistical analysis

The CA125-Tn value for serum samples was calculated as the logarithm of A_{405} in sample cells minus the value from blank cells. CA125-Tn levels in ovarian cancer, ovarian borderline tumor, and benign conditions were compared using Student's *t* test. Comparisons of CA125-Tn levels between different histological types, stages, and grades were performed using one-way analysis of variance (ANOVA). Diagnostic performance of the CA125-Tn ELISA assay was evaluated with receiver operating characteristic (ROC) curves analysis. All statistical analyses were performed using PASW Statistics software, version 17.0.

Results

Selection of lectins for antibody-lectin assay

Twenty-one lectins (see Table S1) were screened for the detection of CA125 glycoforms using an antibody-lectin assay. To reduce background signals caused by lectin binding with antibody glycans, we first performed chemical derivatization of the coating antibody before antigen capture. As shown in *Figure 2A*, strong binding of AAL, ConA, LCA, ACL, MAL-II, PSA, and SNA with the coating antibody was observed. After derivatization, background signals in LCA, PSA, and SNA were significantly reduced. However, moderate reductions in ConA and MAL-II and no reductions in AAL and ACL were observed. Thus, LCA, PSA, and SNA, together with DBA, ECL, E-PHA, L-PHA, MAL-I, PNA, RCA, UEA, VVA, BPL, DSL, EEL, GNL, LTL, and SBA, which showed weak binding with the coating antibody, had background signals low enough for the antibody-lectin assay. After incubation of serum with the derivatized antibody, lectin binding signals were enhanced, indicating that the antibody retained its capacity for CA125 binding after derivatization.

To confirm that lectins bind with CA125 through specific glycan recognition, we preincubated lectins with their corresponding inhibiting monosaccharides before they were used in the antibody-lectin assay. Binding of DBA, ECL, MAL-I, RCA, VVA, BPL, EEL, LTL, PSA, SBA, or UEA with CA125 were significantly inhibited by monosaccharide in a concentration-dependent manner (*Figure 2B* and Table S1).

Then, we sought to find whether lectins bind specifically with glycans carried by CA125 molecules but not by

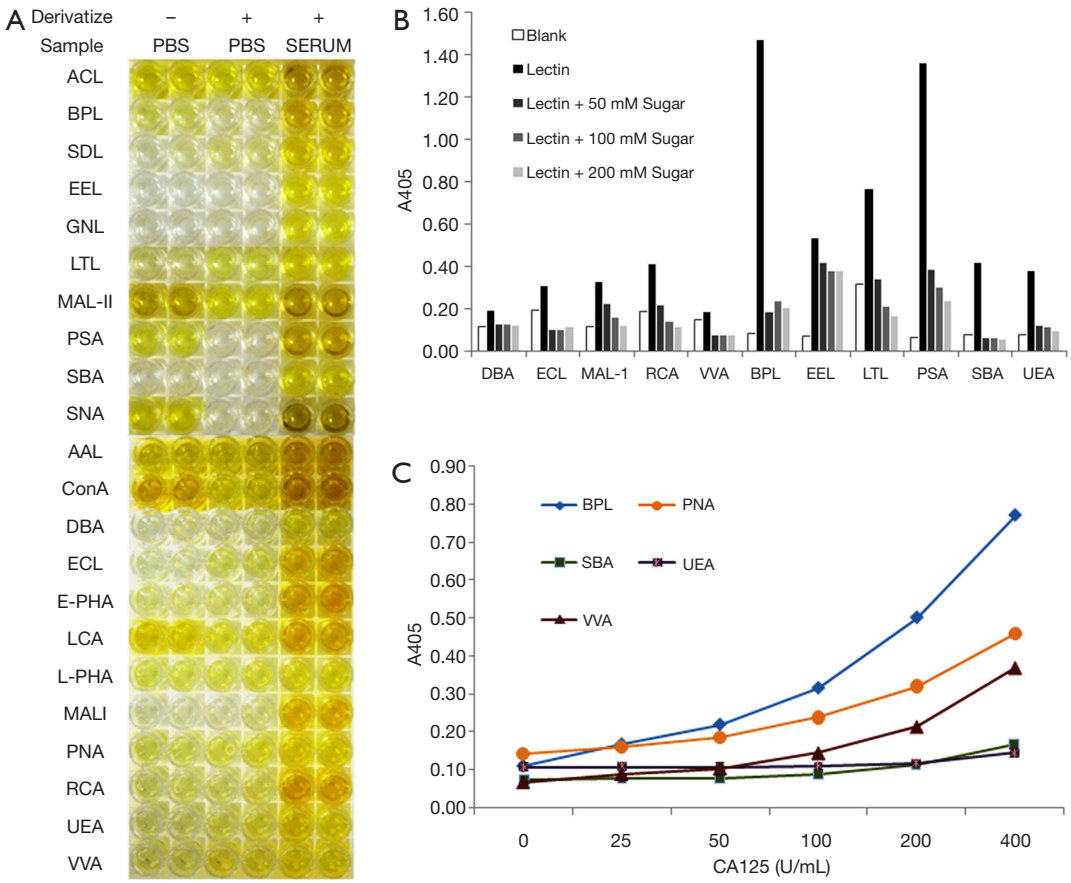

Figure 2 Microplate fabrication. (A) Derivatizing glycans attached to the Fc fragment of the antibody significantly reduced the background signal in the antibody-lectin ELISA assay; (B) Lectin binding with antigens was inhibited by its corresponding inhibiting sugars; (C) Lectin binding had a linear relationship with the concentration of CA125.

antibodies or other nonspecific serum proteins. To investigate this, we assessed the linear relationship between CA125 concentration and signal intensity of lectins. CA125 was first immunodepleted from serum, and then purified CA125 was incorporated at varying concentrations. Signal intensities of BPL, PNA, SBA, UEA, and VVA showed a positive correlation with the concentration of CA125 (*Figure 2C*).

VVA binds preferentially with ovarian cancer-derived CA125

Generally, CA125 levels in patients with ovarian cancer are higher than those in patients with benign diseases. Thus, glycans that are overexpressed in ovarian cancer-derived CA125 may enlarge the gap and therefore help in the differential diagnosis of patients with elevated CA125 levels. We chose 7 benign gynecological disease patients with CA125 higher than 100 U/mL and 7 ovarian cancer patients with CA125 lower than 200 U/mL to examine the differential efficacies of these five lectins in the antibody-lectin assay. Among them, VVA displayed the most significant difference in signal intensity between the two groups (Figure S1). VVA is a lectin that specifically recognizes Tn antigen (29). Therefore, an antibody-lectin assay using VVA as the detecting lectin (termed the as CA125-Tn ELISA) was further tested for its differential efficacy in a larger population. The intra- and interassay variation for CA125-Tn ELISA were estimated to range from 8% to 10%.

CA125-Tn level was higher in ovarian cancer patients

We enrolled 328 patients with serum CA125 levels equal to or greater than 35 U/ml, including 68 ovarian cancers, 15 ovarian borderline tumors, and 245 benign conditions (*Table 1*). Endometriosis, adenomyosis, and pelvic

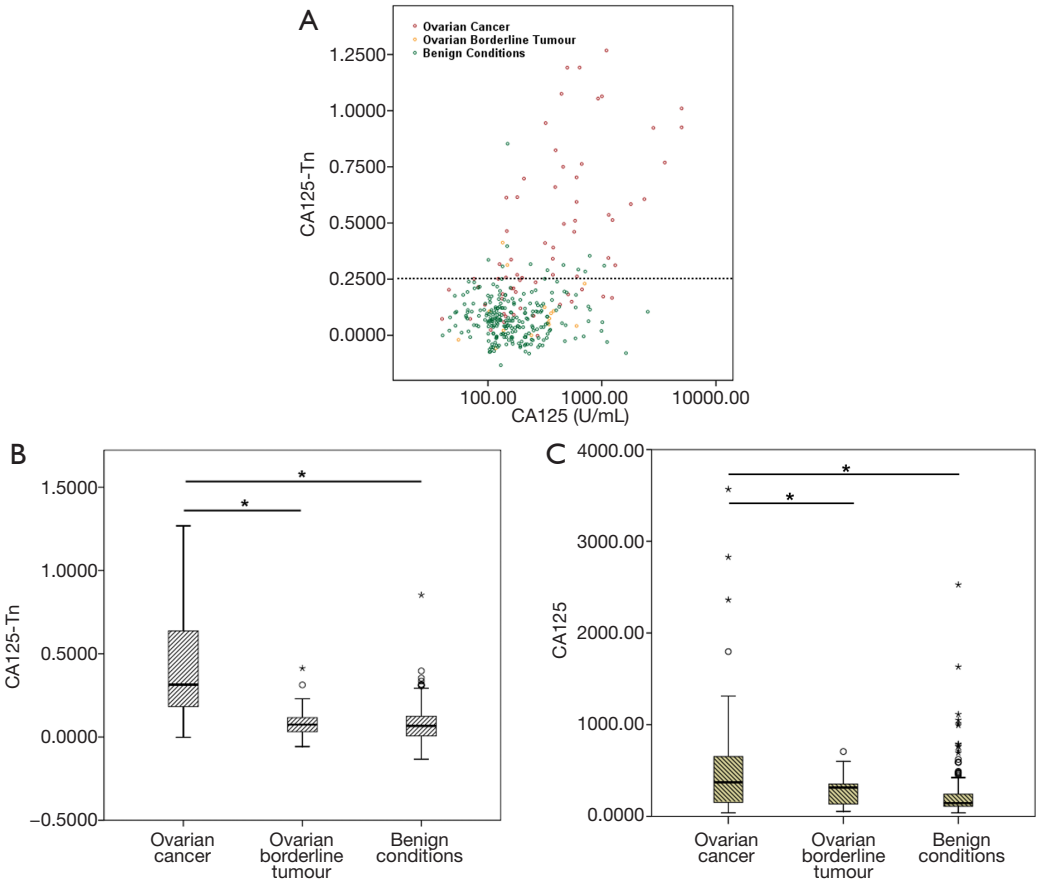

Figure 3 CA125-Tn and CA125 levels in ovarian cancer, ovarian borderline tumor, and benign conditions. (A) Most ovarian cancer patients have CA125-Tn levels above 0.25; ovarian cancer patients have higher CA125-Tn (B) and CA125 (C) levels than patients with borderline ovarian tumor or benign gynecological conditions. *, P<0.05.

inflammatory disease comprised 89% of the benign condition group. CA125-Tn levels were significantly higher in patients with ovarian cancer than in those with ovarian borderline tumor or benign conditions (*Figure 3*). No significant difference between the benign and borderline groups was observed. Medians, 25th and 75th quartiles of CA125-Tn levels were 0.31 (0.18–0.65) for ovarian cancer, 0.07 (0.02–0.12) for ovarian borderline tumor, and 0.07 (0.01–0.12) for benign conditions. Among the different histotypes, serous (n=56) and clear cell ovarian cancer (n=6) showed higher CA125-Tn levels than mucinous (n=3) or endometrioid (n=3) ovarian cancer (*Table 1*).

CA125-Tn ELISA improves ovarian cancer diagnosis among patients with elevated CA125 levels

For patients with positive CA125, receiver operating characteristic (ROC) curves analysis showed that the area under the curve (AUC) for CA125-Tn and CA125 to discriminate ovarian cancer patients from nonmalignant patients (both borderline tumors and benign conditions) was 0.890 (95% CI: 0.845, 0.935) and 0.721 (95% CI: 0.648, 0.795), respectively (*Figure 4A*). Sensitivity for CA125-Tn to detect ovarian cancer was 72.1%, with a fixed specificity of 90%. However, it was only 44.1% for CA125. When sensitivity was fixed at 90%, specificity was improved from 20.1% for CA125 to 58.3% for CA125-Tn.

Patients with ovarian cancer were generally older than patients with borderline tumors or benign conditions in our cohort (*Table 1*). To avoid the effects of age bias on CA125 and CA125-Tn levels, we analyzed performance in two age subsets (>45 y and ≤45 y). For patients older than 45 y, AUCs of the ROC curves for CA125-Tn and CA125 to discriminate ovarian cancer patients from nonmalignant

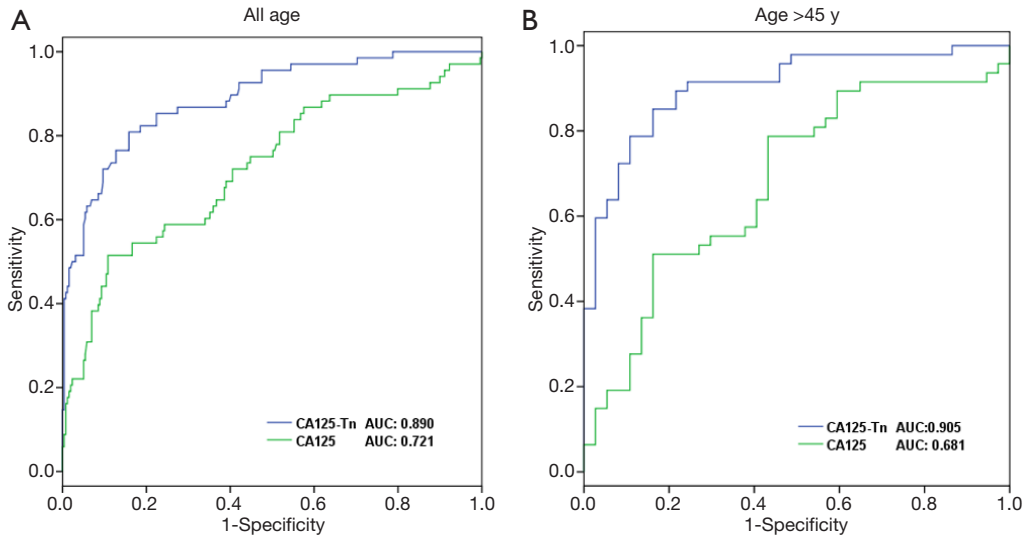

Figure 4 ROC curve of CA125-Tn and CA125 to detect ovarian cancer among patients with elevated CA125 levels. (A) Cases of all age, (B) Cases older than 45 y.

patients (both borderline tumors and benign conditions) were 0.905 (95% CI: 0.841, 0.969) and 0.681 (95% CI: 0.565, 0.798), respectively (*Figure 4B*). At a fixed sensitivity of 90%, specificity was improved from 35.1% for CA125 to 75.7% for CA125-Tn. Due to the low case number of ovarian cancer patients, ROC analysis could not be performed among patients aged 45 y or younger (data not shown).

Discussion

Despite many promising new markers, CA125 remains the single-best biomarker for ovarian cancer (30). A significant drawback of CA125 is its low specificity. To address this problem, various combinations of ovarian cancer biomarkers were tested. However, none of them provided significant improvement in diagnostic accuracy (31,32). In this study, we specifically focused on patients with elevated serum CA125 levels, aiming to find a CA125 glycoform that is elevated in patients with ovarian cancer, which will help in the preoperative triage of patients with abnormal CA125 levels. We employed an antibody-lectin ELISA assay to detect various CA125 glycoforms. Among 21 lectins, VVA, a plant lectin that specifically binds Tn antigen, was demonstrated to be the most powerful in differentiating patients with ovarian cancer from those with benign conditions or ovarian borderline tumors. For patients older than 45 y, CA125-Tn performed even better in improving specificity. This novel assay will be useful for the differential diagnosis of patients with positive CA125 levels. However, it has no effects in discriminating borderline ovarian tumors from benign gynecological patients.

Tn antigen is an O-glycan structure demonstrated to be upregulated in many malignant tissues (33,34). In ovarian cancer, upregulation of Tn antigen was also manifested in cancerous tissues (35). Additionally, CA125, the predominant mucin secreted by ovarian cancer was reported to carry Tn antigen in large amounts (25,36). Unsurprisingly, CA125-Tn, which combined both CA125 level and Tn level, improved the diagnosis of ovarian cancer. This was also demonstrated by Salminen *et al.*, who found that CA125-Tn (detected with macrophage galactose-type lectin) was helpful in differential diagnosis of pelvic masses (37).

CA125 is a highly glycosylated, huge mucin with a molecular weight of 200 to 4,000 KD. Glycans account for up to 70% of its molecular weight (15). CA125 molecules are highly heterogeneous in terms of both molecular weight and glycan modification, which makes structural investigation difficult (38-40). Variations in glycoforms on CA125 molecules from different sources have long been recognized. In 2008, Jankovic *et al.* analyzed the lectin binding profile of CA125 purified from an ovarian cancer cell line and amniotic fluid (36). They demonstrated increased RCA-, WFA-, PHA-E-, PHA-L-, SBA-, and MAA-binding fractions and decreased WGA- and UEA-binding fractions in CA125 purified from ovarian cancer cell line, indicating

increased bisecting N-glycan, multiantennary N-glycan and Tn antigen modification on ovarian cancer-derived CA125 molecules. In contrast to CA125 purified from the placenta, in which only core fucose was detected, Wong *et al.* showed both core and outer fucose on ovarian cancer-derived CA125 (39,41). Most of these investigations employed chromatography or mass spectrometry or both for CA125 glycoform profiling, which requires a large amount of purified CA125. However, these time- and material-consuming methods are not appropriate for routine clinical sample testing. Therefore, we turned to an antibody-lectin ELSA assay, in which only 200 μL of serum sample was required for glycosylation pattern analysis of CA125. The major disadvantage of this method is its high background signal caused by glycans on coating antibodies or nonspecifically binding proteins, which increases the lower limit of the test (42,43). In this study, we used a glycan derivatizing strategy developed by Chen *et al.* (28) to reduce signals from antibody glycans, which made the detection of CA125-Tn in low abundance possible.

Conclusions

In this study, we developed a novel immunoassay, the CA125-Tn assay, for detecting ovarian cancer among patients with positive CA125 results. It improved specificity while maintaining satisfactory sensitivity. This assay warrants further validation in a larger population.

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Footnote

Reporting Checklist: The authors have completed the STARD reporting checklist. Available at http://dx.doi.org/10.21037/atm-20-8053

Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-20-8053

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-20-8053). Dr. YSW, Dr. JQL, Dr. XYZ, Dr. XPL, Dr. RC, and Dr. CJX report that they have a patent CA125-Tn ELISA kit licensed to Obstetrics and Gynecology Hospital of Fudan University. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the Institutional Review Board of Obstetrics & Gynecology Hospital of Fudan University (2017-52). In this study, we used only serum samples left over after clinical testing. No additional serum sample was collected outside the standard clinical requirement. Thus, written informed consent from participants was not required and was waived by the Institutional Review Board.

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Supplementary

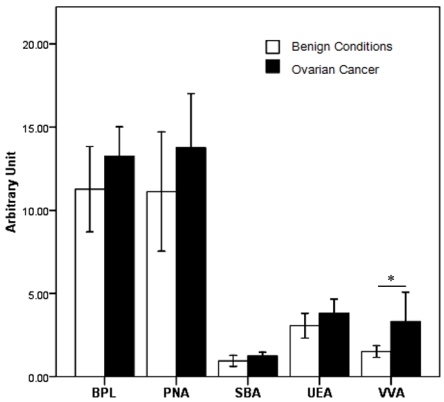

Figure S1 Antibody-lectin ELISA assay showed increased binding of VVA to CA125 in ovarian cancer patients (n=7). *, P<0.05.

Table S1 Lectins and their corresponding inhibiting sugars

Lectins	Abbreviation	Inhibiting Sugars	Recommended Concentration of Sugars (mmol/L)
Aleuria Aurantia Lectin	AAL	Fuc	100
Amaranthus Caudatus Lectin	ACL	/	/
Bauhinia Purpurea Lectin	BPL	Lac	100
Concanavalin A	ConA	Man/Glc	200
Dolichos Biflorus Agglutinin	DBA	GalNAc	200
Datura Stramonium Lectin	DSL	/	/
Erythrina Cristagalli Lectin	ECL	Lac	200
Euonymus Europaeus Lectin	EEL	Lac	500
Phaseolus vulgaris Erythroagglutinin	E-PHA	/	/
Galanthus Nivalis Lectin	GNL	Man/Glc	100-200
Lens Culinaris Agglutinin	LCA	Man/Glc	200
Phaseolus vulgaris Leucoagglutinin	L-PHA	/	/
Lotus Tetragonolobus Lectin	LTL	Fuc	50-100
Maackia Amurensis Lectin I	MAL-I	Lac	200
Maackia Amurensis Lectin II	MAL-II	/	/
Peanut Agglutinin	PNA	Gal	200
Pisum Sativum Agglutinin	PSA	Man/Glc	200
Ricinus Communis Agglutinin	RCA120	Gal or Lac	200
Soybean Agglutinin	SBA	GalNAc	200
Sambucus Nigra Lectin	SNA	/	/
Ulex Europaeus Agglutinin I	UEA	Fuc	50-100
Vicia Villosa Lectin	VVA	GalNAc	200

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