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Elevation of TP53 Autoantibody Prior to CA125 in Preclinical Invasive Epithelial Ovarian Cancer

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Abstract

Purpose—The TP53 tumor suppressor gene is mutated in >95% of high grade serous ovarian cancers. Detecting an autologous antibody response to TP53 might improve early detection.

Experimental design—An immunoassay was developed to measure TP53 autoantibody in sera from 378 cases of invasive epithelial ovarian cancer and in 944 age-matched healthy controls from the United States, Australia and the United Kingdom. Serial preclinical samples from cases and

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controls were also assayed from the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS).

Results—Using a cut-off of 78 U/mL to achieve a specificity of 97.4%, TP53 autoantibody were elevated in 30% of 50 cases from MD Anderson, 21.3% of 108 cases from the Australian Ovarian Cancer Study and 21% of 220 cases from the UKCTOCS. Among 164 cases with rising CA125 detected with the UKCTOCS risk of ovarian cancer algorithm (ROCA), 20.7% had elevated TP53 autoantibody. In cases missed by the ROCA, 16% of cases had elevated TP53 autoantibody. Of the 34 ovarian cancer cases detected with the ROCA, TP53 autoantibody titers were elevated 11.0 months prior to CA125. In the 9 cases missed by the ROCA, TP53 autoantibody was elevated 22.9 months before cancer diagnosis. Similar sensitivity was obtained using assays with specific mutant and wild-type TP53.

Conclusion—TP53 autoantibody levels provide a biomarker with clinically significant lead time over elevation of CA125 or an elevated ROCA value. Quantitative assessment of autoantibodies in combination with CA125 hold promise for earlier detection of invasive epithelial ovarian cancer.

Keywords

Introduction

Ovarian cancer ranks fifth in cancer deaths among women in 2016 and is the leading cause of death from gynecologic malignancies (1). If detected while still confined to the ovaries (Stage I) or to the pelvis (Stage II), 5-year survival rates are 90% and 70% respectively. Once the disease has spread to the peritoneal cavity (Stage III) or beyond (Stage IV), the 5-year survival decreases sharply to less than 20% (2-4). Computer simulation suggests that earlier detection could reduce mortality by as much as 43% (5). Recent results from the United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) document that screening for ovarian cancer using a multimodal (two-stage) strategy can lead to a stage shift and an estimated 20% reduction in mortality (6).

In the multimodal (two-stage) screening strategy utilized both in UKCTOCS (6) and in the Normal Risk Ovarian Screening Study (NROSS) (7), the level of the serum biomarker CA125 (MUC16) was determined annually in postmenopausal women at average (general population) risk of developing ovarian cancer. A Bayesian statistical method, the Risk of Ovarian Cancer Algorithm (ROCA), was used to calculate each woman's risk of having ovarian cancer based on age specific incidence, dates of blood draws and longitudinal CA125 levels after every new CA125 test. ROCA assesses whether CA125 has significantly increased above an individual's CA125 baseline levels (8,9). If risk has not changed, women return in one year for the next CA125 determination. If the ROCA is strongly elevated, transvaginal ultrasound (TVS) is obtained and, if this suggests malignancy, an operation is performed. If a modest increase in risk is observed, CA125 is repeated in 3 months. This two-stage strategy (ROCA + TVS), where two independent tests are both positive improves the limited specificity of CA125 using a single threshold (e.g. 30 or 35 U/mL) as well as the limited specificity of TVS and results in only 3-4 operations (40% positive predictive value)

for each case of ovarian cancer detected (6,7). It also increases the sensitivity for earlier detection of ovarian cancer to around 85% (10,11). A recent report suggesting that this approach may reduce disease specific mortality (6), adds impetus to the search for new markers that can complement CA125, especially in women who are not detected using CA125 alone.

About 80% of ovarian cancers express significant amounts of CA125 at a tissue level (12). However, only 50-60% of early stage cancers have elevated CA125 serum levels at presentation (13). This suggests that a panel of biomarkers will almost certainly be required to detect all early stage ovarian cancers. During the last two decades, more than 110 different markers have been studied to increase sensitivity of CA125 for detecting ovarian cancer. HE4 and CA72.4 can detect a fraction of cases missed by CA125 (14,15), but in studies with preclinical samples to date, no biomarker has been consistently elevated prior to CA125 before diagnosis of the disease.

The sensitivity of tumor-derived biomarkers for early detection is limited by the expression of a biomarker within a cancer, the rate of biomarker-shedding and the volume of early stage cancers. Small cancers may not shed sufficient amounts of biomarker(s) to raise serum levels, but could evoke an immune response (16). Small volumes of cancer with mutated, overexpressed or aberrantly compartmentalized tumor associated proteins could evoke a humoral immune response with readily detectable titers of specific autoantibody (17). Therefore, detecting an autologous immune response to tumor associated antigens might improve lead time and detect cancers that are too small to be detected by antigen assays.

The *TP53* tumor suppressor gene is frequently mutated in a variety of human cancers. *TP53* is mutated in virtually all high grade serous ovarian cancers (18). Approximately two thirds of *TP53* mutations are missense that stabilize TP53 protein and increase TP53 accumulation (19). Autoantibody reactive with wild-type TP53 have been reported in sera from approximately 15% of women with ovarian cancers, but most reports have studied only a limited number of cases at the time of symptomatic clinical diagnosis (20-36).

The goals of this study are (1) to develop and validate a new and more sensitive immunoassay for autoantibodies against wild-type TP53 protein, (2) to determine the fraction of ovarian cancer patients with early stage disease who have elevated levels of TP53 autoantibody, (3) to discover whether TP53 autoantibody can provide lead time over CA125 and detect cases that do not have elevated CA125, and (4) to test whether autoantibodies against patient specific mutations of TP53 provide more sensitive assays. Availability of serial pre-clinical specimens from UKCTOCS has permitted measurement of TP53 autoantibody in large numbers of women who went on to develop ovarian cancer. We have tested for the first time whether TP53 autoantibody are elevated prior to elevated risk (based on the ROCA). In earlier studies, autoantibody has been detected to wild-type TP53 protein. With access to serum specimens obtained at clinical diagnosis from the Australian Ovarian Cancer Study (AOCS) for which *TP53* sequences are known, we have tested for the first time whether greater sensitivity could be attained by detecting autoantibody to the patient's specific mutant TP53 protein rather than the wild-type TP53 protein.

Materials and Methods

MagPlex bead-based indirect serological assay development

The MagPlex/xMAP technology (Luminex Corp., Austin, TX) was used to develop an indirect serological assay for detecting human TP53 autoantibody. Coupling recombinant wild-type TP53 antigen to microspheres, confirmation of effective coupling and calibration of the assay were accomplished using protocols modified from the Luminex xMAP Cookbook. In brief, 1×10^6 microspheres were coupled with recombinant human wild-type TP53 protein using an xMAP antibody coupling kit. Coupling was completed by a carbodiimide reaction linking the primary amino groups on TP53 protein and the carboxyl groups on the microsphere surface. The antigen conjugation procedure was performed according to the manufacturer's instructions. Biotin-conjugated anti-human TP53 antibody was used to validate coupling efficiency. A TP53 autoantibody calibrator (10 U/mL) was used to establish a standard curve for quantitating the titer of TP53 autoantibody in human serum samples. The standard operating protocol for performing the assay was established during assay development.

TP53 autoantibody immunoassay performance

A new xMAP bead-based immunoassay for detecting TP53 autoantibody in human serum samples was performed by an established standard operating protocol. In brief, a suspension of TP53 antigen-microspheres was prepared by diluting the coupled microsphere stocks (1×10^6 beads/mL) to a final concentration of 50 beads/μL in PBS buffer. Aliquots of microsphere suspension were placed in each well of a 96-well polystyrene microplate. Standard curves for quantitating TP53 autoantibody were plotted from triplicate assays of half-log dilution series of the TP53 autoantibody calibrator with concentrations ranging from 0.031 U/mL to 0.5 U/mL. Positive and negative controls for TP53 autoantibody were prepared in triplicate according to the manufacturer's instructions. Serum samples for assay (2 μL) were diluted with serum matrix to mimic the native analyte environment. Serum samples were added to 96-well polystyrene microplates and incubated with TP53-coupled microspheres for 1 hour at room temperature with gentle shaking. To wash the beads, each plate was clipped onto a magnetic plate separator for 1 min and the liquid was discarded by inverting the plate. Following washing with PBS buffer, microspheres were incubated with detection antibody biotinylated goat anti-human IgG. The plates were covered to protect them from light for 30 min at room temperature with gentle shaking. Plates were washed two times with PBS buffer and microspheres were incubated with fluorescence reporter Streptavidin-R-phycoerythrin (SAPE). The plates were covered to protect them from light and incubated for 30 min at room temperature with gentle shaking. After a final wash, the beads were re-suspended in PBS buffer and fluorescence measured on the MAGPIX system (Luminex Corp., Austin, TX) with at least 50 beads per well. The data were acquired and analyzed by xPONENT software version 4.2 (Luminex Corp., Austin, TX).

Patient serum sample sets (details available in the Supplementary Materials and Methods)

The MDACC-NROSS samples included 50 preoperative sera from patients with stage III-IV invasive epithelial ovarian/tubal/peritoneal cancer in the MD Anderson Cancer Center Gynecologic Cancer Tissue Bank and 216 sera from healthy controls who did not develop

ovarian cancer on the NROSS coordinated by the MDACC Ovarian SPORE (7). The <u>AOCS</u> sample set included preoperative sera from 108 clinically diagnosed patients with invasive epithelial ovarian/tubal/peritoneal cancer, preoperative sera from 109 patients with benign ovarian tumors and age-matched sera from 464 healthy Australian controls. The <u>UKCTOCS</u> sample set was obtained from participants of this population-based, multi-center randomized controlled trial of ovarian cancer screening in the United Kingdom (10,37). The sample set included 164 screen-detected and 56 screen-negative women with 1,053 preclinical serial samples predating diagnosis of invasive epithelial ovarian/tubal/peritoneal cancer up to 5 years and 619 age-matched controls (3,069 serial samples) who did not develop any type of cancer during follow up. The combined sample sets are generally representative of the performance of multimodal screening in UKCTOCS, but have been enriched for screen negative cases. Ethical approval was obtained from these studies from the appropriate IRB/ ethical committees. All participants had provided consent for use of samples in ethically approved secondary studies.

Statistical analysis

Setting a threshold for detecting TP53 autoantibody positivity in ovarian cancer samples—We chose our cutoff to achieve high specificity in multiple datasets as described below. We applied bootstrapping to construct 95% confidence intervals for the TP53 autoantibody cut-off values associated with 98% specificity in the MDACC, AOCS and UKCTOCS cohorts. A cut-off value of 78 U/mL, which falls within all three of the above 95% confidence intervals and provides an overall specificity of 97.4%, was chosen as the common threshold for detecting ovarian cancer.

Comparing early detection times for CA125 and TP53 autoantibody—Line plots and dot plots were drawn to compare differences in early detection times between TP53 autoantibody, CA125, and ROCA in the longitudinal samples of UKCTOCS cohort. Wilcoxon signed rank tests were performed to assess whether the additional early detection time provided by TP53 autoantibody improved upon CA125 or ROCA when both tests indicated a case. In addition, Fisher exact tests were used to assess the independence of TP53 autoantibody and ROCA in the three sample cohorts. Bootstrapping was applied to determine whether TP53 autoantibody could detect ROCA negative cases in the UCKTOCS cohort.

Comparing single and combination marker panels—Receiver Operating Characteristic (ROC) analysis adapted for early-detection-times was performed to compare performance between single and combination biomarker panels in the UKCTOCS cohort. The R package partial ROC (pROC) was used for ROC computations. Longitudinal sets of values were reduced to single values as follows. First, 35 U/mL and 78 U/mL were specified as cut-off values for CA125 and TP53 autoantibody, respectively. If a participant, either a case or a control, was never detected by one of the biomarkers, the maximum value of that marker was assigned to that participant. If a marker ever exceeded its cut-off, the value seen at the first time that the marker exceeded its cut-off was recorded as the summary value. If a case was detected by both CA125 and TP53 autoantibody assays, and the difference in detection times was larger than a certain threshold, such as 3, 6, or 12 months, a zero for the

biomarker that detected the cancer later was recorded, as an indication that the latter marker had "failed" by not catching the tumor as early as it could have been caught. After this adjustment for early-detection-time, ROC analyses were performed both for single biomarkers and biomarker combinations. For the biomarker combination, a logistic regression model was built with the summary values as inputs and case/control status as targets, and the predicted logistic regression values were applied as the biomarker combination "score". ROC curves were plotted both for the single biomarker and for the biomarker combination. Area under the curve (AUC), partial AUC (pAUC), sensitivity and accuracy were computed at 98% specificity. Next, bootstrapping was used to compute 95% confidence intervals for the AUC, as well as p values for the differences in AUC and pAUC values between single biomarkers and the biomarker combination.

Results

A sensitive and robust xMAP bead-based immunoassay has been developed to quantify TP53 autoantibody

We have developed a sensitive, rapid and high-throughput MagPlex microsphere-based immunoassay for quantitating anti-TP53-specific autoantibody in small volumes (2 μL) of serum. The procedure for immunoassay development, validation and assessment of performance in nested case-control studies has been outlined in Supplementary Figure 1. After establishing and optimizing of the assay method, a validation study was performed to demonstrate the accuracy, reproducibility and reliability of the TP53 autoantibody immunoassay. Serum samples with three different TP53 autoantibody titers (low, medium and high range of each sample) were measured in triplicate. The intra-assay coefficient of variation was 3.5-4.6% and the inter-assay coefficient of variation was 6.6% on three consecutive days. The linearity (R-square) was 0.9936 and the linear range was between 5.83 and 250 U/mL. The Limit of Detection (LoD) value was 5.83 U/mL and the maximum detectable TP53 autoantibody titer was 250 U/mL for this immunoassay.

A common cut-off value for the TP53 autoantibody immunoassay was chosen at 97.4% specificity across three clinical datasets

To determine a common cut-off value for the TP53 autoantibody immunoassay, we first used a bootstrap technique to construct 95% confidence intervals (CIs) at the 98th percentile of TP53 autoantibody values for healthy controls across three datasets (Supplementary Figure 2). For the MDACC-NROSS dataset, the 98th percentile for controls was 100.9 (95% CIs, 32.4-148.3) U/mL. For the AOCS dataset, the 98th percentile for healthy controls was 48.5 (95% CIs, 35.0-84.4) U/mL. For the UKCTOCS dataset, where serial samples were available from each healthy control, we chose the single highest value from each participant to calculate the 98th percentile of 94.6 (95% Cis, 65.6-128.0). A common cut-off value of 78 U/mL was chosen for TP53 autoantibody levels. This value was included within the 95% CIs for the 98th percentile across all datasets (Supplementary Figure 2). Using all controls, this provided a specificity of 97.4%. TP53 autoantibody levels above 78 U/mL were considered positive.

Elevated TP53 autoantibody levels were detected in 30% of pre-treatment sera from predominantly late stage (III/IV) ovarian cancer patients in the MDACC/NROSS dataset

We first analyzed the MDACC-NROSS set of specimens to validate the newly developed immunoassay for detecting TP53 autoantibody in human serum samples. Patient characteristics and TP53 autoantibody positivity by stage or histology are described in Supplementary Table 1. Results are summarized in Figure 1. With a 78 U/mL cut-off value for TP53 autoantibody, 15 of 50 sera (30%) from cancer cases were positive, whereas 5 of 216 sera (2.3%) of healthy controls exhibited TP53 autoantibody (Figure 1, A).

We also compared CA125 values (the cut-off = 35 U/mL) with TP53 autoantibody levels in each cancer case (Figure 1, A). CA125 was positive in 45 of 50 cases (90%) and 4 of 216 controls (1.9%). The box plot showed that both TP53 autoantibody and CA125 values were significantly elevated in cases when compared to controls. Remarkably, 12 of 15 (80%) ovarian cancer patients had extremely high titers of TP53 autoantibody (>250 U/mL) (Figure 1, B). Elevation of both CA125 and TP53 autoantibody was observed in 13 of 50 (26%). We further evaluated whether TP53 autoantibody correlated with ovarian cancer histology. All TP53 autoantibody positive cases were the serous or serous mixed with endometrioid histotype (Supplementary Table 1).

Elevated TP53 autoantibody levels were detected in 21% of pre-treatment sera from invasive epithelial ovarian/tubal/peritoneal cancer patients in the AOCS dataset

Next we analyzed sera from the AOCS study. Patient characteristics and TP53 autoantibody positivity by stage or histology are described in Supplementary Table 2. TP53 autoantibody values were significantly elevated in ovarian cancer cases when compared to patients with benign ovarian neoplasms or healthy controls. With a 78 U/mL threshold, sera from 23 of 108 invasive epithelial cancer cases (21.3%) had elevated TP53 autoantibody levels (Figure 2). By contrast, 3 of 109 sera (2.8%) from patients with benign ovarian neoplasms and 6 of 464 sera (1.3%) from healthy controls had elevated TP53 autoantibody levels (Figure 2). We further evaluated whether TP53 autoantibody correlated with ovarian cancer stage or histology. Elevated TP53 autoantibody levels were found in 2 of the 12 early stage (I/II) (16.7%) and 19 of 90 late stage (III/IV) (21.1%) patients. All TP53 autoantibody positive cases were the serous histotype (Supplementary Table 2).

Elevated TP53 autoantibody levels were detected in 21% of sera from invasive epithelial ovarian cancer patients in the longitudinal UKCTOCS preclinical dataset

Availability of sera from the UKCTOCS trial permitted detection of TP53 autoantibody in preclinical sera from a large number of women who subsequently developed invasive epithelial ovarian cancer. Patient characteristics and TP53 autoantibody positivity by stage or histology are shown in Table 1. Elevated TP53 autoantibody levels were found in sera from 43 of 220 patients (19.5%) with invasive epithelial ovarian cancer and in sera from 17 of 619 subjects (2.7%) without cancer detected during the study (Figure 3, A). The TP53 autoantibody levels were also compared to ROCA/CA125 values in each of the ovarian cancer cases. In the UKCTOCS dataset, ROCA could detect 164 of 220 (74.5%) cases and 34 cases were detected by both ROCA and anti-TP53 autoantibody assays. In box plots, both values were significantly elevated in cases compared to controls (Figure 3, A). In addition,

scatter plot analysis suggested that CA125 is, as anticipated, a good biomarker to separate cases from controls (Figure 3, B). We further evaluated whether TP53 autoantibody correlated with ovarian cancer stage or histology. Elevated TP53 autoantibody levels were found in 11 of the 81 early stage (I/II) (13.6%) and 32 of 139 late stage (III/IV) (23.0%) patients (Supplementary Table 3). Some 76.7% of cancer cases with elevated TP53 autoantibody levels were of serous histotype, but 1-2 cases with endometrioid, clear cell and mucinous histotype were also associated with elevated TP53 autoantibody (Table 1).

Elevated TP53 autoantibody levels were found in sera from 16% of ovarian cancer patients whose disease was not detected with increasing CA125 and the ROCA

In the UKCTOCS dataset, among the 220 women who went on to develop ovarian cancer, 164 cases (74.5%) were detected with rising CA125 using the ROCA and TVS (screen positives) and 56 cases (25.5%) were not detected by ROCA (screen negative). Thirty-four of 164 (20.7%) screen positive cases and 9 of 56 (16.1%) screen negative cases had elevated levels of TP53 autoantibody (Figure 3, A). Thus, in retrospect, TP53 autoantibody could identify 16% of ovarian cancer cases missed by the ROCA.

TP53 autoantibody levels can increase prior to elevation of CA125

To determine whether TP53 autoantibody titers might increase prior to elevation of CA125 above the 35 U/mL threshold or to an indication of elevated risk determined by the ROCA, we measured TP53 autoantibody in serial serum specimens from ovarian cancer cases in the UKCTOCS dataset. Serial values for CA125 and TP53 autoantibody from two representative early-stage cancer cases are plotted in Supplementary Figure 3, A and B. TP53 autoantibody titers were markedly elevated (>250 U/mL) 8 and 7 months before cancer diagnosis, while CA125 levels remained within the normal range (<35 U/mL) during the entire period. In addition, the TP53 autoantibody titers were elevated in cancer cases at late stage (Supplementary Figure 3, C and D). While CA125 remained within normal range, autoantibody titers increased dramatically (190~250 U/mL) at 4 years 4 months and 1 year 6 months prior to cancer diagnosis.

Among 43 cases with autoantibody elevation, TP53 autoantibody was detected 11.8 months (mean) prior to an elevation of the ROCA and 11.0 months prior to CA125 elevation (35 U/mL) (Figure 4, A). In 34 cases with both autoantibody elevation and ROCA positivity, TP53 autoantibody were detected 9.2 months prior to ROCA positivity, and 8.1 months prior to CA125 elevation (Figure 4, B). In 9 cases with autoantibody positivity that were not detected by the ROCA, TP53 autoantibody titers rose 22.9 months (mean) prior to cancer diagnosis (Figure 4, C). Thus TP53 autoantibody levels provide a promising biomarker with clinically significant lead time over either elevation of CA125 levels alone or elevation of risk using the ROCA algorithm to analyze CA125 levels.

A combination of TP53 autoantibody and CA125 enhanced detection of ovarian cancer in the UKCTOCS trial

To determine whether TP53 autoantibody levels enhanced the ability of CA125 to detect ovarian cancer, we generated ROC curves to discriminate cancer cases from controls for the UKCTOCS. The values first reaching the TP53 autoantibody cut-off (78 U/mL) and CA125

cut-off (35 U/mL) were chosen for ROC analysis. The AUC for TP53 autoantibody, CA125 and the combination of these two markers have been calculated. In the UKCTOCS dataset (Figure 5, A), the AUC of TP53 autoantibody was 0.699. Adding TP53 autoantibody values to the panel can significantly increase the AUC (CA125, 0.838 vs. TP53 AAb + CA125, 0.867, p= 0.007).

Since the regular ROC analysis may not reflect the real situation that TP53 autoantibody has lead time prior to CA125, we performed new ROC analyses incorporating lead time. ROC analysis adapted for early-detection-times was performed to compare performance between single and combination marker panels in the UKCTOCS cohort. Partial ROC curves for TP53 autoantibody and CA125 in the UKCTOCS study were calculated when 3, 6 and 12 months were chosen as the cut-off to define the "significant early detection". In ROC result with 3 months cut-off (Figure 5, B), the AUC of TP53 autoantibody was 0.636. Adding TP53 autoantibody to the panel can significantly increase the AUC (CA125, 0.751 vs TP53 AAb + CA125, 0.861, P= 0.000). Using 6 and 12 months cut-off did not obviously change AUCs of single and combination biomarker panels (Supplementary Figure 4, A and B). Thus, a combination of CA125 and TP53 autoantibody significantly enhance the ROC curve relative to either biomarker alone.

Similar sensitivity for detecting TP53 autoantibody was obtained with patient specific mutant TP53 and wild-type TP53

At a set cut-off value (78 U/mL) for TP53 autoantibody, our immunoassay using wild-type TP53 detected autoantibody in 21~30% of invasive epithelial ovarian cancer patients in three data sets. As virtually all high grade serous cancers, which comprise approximately 60% of all ovarian cancers, have TP53 mutations at a variety of sites, we may have missed autoantibody against epitopes expressed by these mutant TP53 proteins, but not by wild-type TP53. Indeed, our previous screening result in the AOCS trial showed that cancer cases who harbored *TP53* mutant gene have higher TP53 autoantibody immune responses compared with *TP53* wild-type cancer cases (Supplementary Figure 5). This result is consistent with previous reports (34). Therefore, we asked whether assays incorporating the specific mutant TP53 proteins might detect antibody in a larger fraction of patients or at higher titer.

At present, 95 different TP53 mutations have been identified in high grade serous ovarian cancers (18). Since *TP5*3 gene status has been sequenced for each case in the AOCS trial, we expressed the specific mutant proteins from the ovarian cancers of these patients using a mammalian protein expression system. We then established immunoassays and compared autoantibody titers to those obtained with wild-type TP53 with every mutant TP53 assay's reference level set at 98% specificity in AOCS controls. We selected 15 *TP53* point mutation candidates for multiplex immunoassay development based on previous screening result in the AOCS trial (Supplementary Figure 6). Candidates were chosen where mutations at the same locus were associated with anti-TP53 antibody or no antibodies in different patients or mutations that were associated with intermediate TP53 wild-type autoantibody titers.

When sera from 29 patients in the AOCS study were assayed against 15 corresponding mutant TP53 proteins and the results compared to those from assays against wild-type TP53 protein, increased sensitivity was not observed (Supplementary Table 4). Patients with

undetectable TP53 wild-type autoantibody had undetectable TP53 mutant autoantibody titers. Differences in titers, when present, did not exceed thresholds for either TP53 wild-type or TP53 mutant for positivity at 98% specificity. Subsequently, we analyzed sera from the AOCS trial against TP53 wild-type and 9 mutant protein candidates. When compared to titers against TP53 wild-type protein, most of the TP53 mutant proteins detected the same number or fewer cases (19.4-22.2%) with the thresholds set at 98% specificity (Supplementary Table 5). In general, they detected exactly the same cases as TP53 wild-type protein. Only TP53 R248W, R273H and R273L mutant proteins detected an additional 2-3 more cases (24.0-25.0%) compared with TP53 wild-type protein (Supplementary Table 5). Consequently, it appears that the predominant epitope(s) recognized by the autologous humoral response is likely to be expressed on the wild-type TP53 protein.

Discussion

In this study, we have developed a new, high-throughput xMAP bead-based immunoassay for quantitating TP53-specific autoantibody in ultra-low volumes of human serum. This assay was used to analyze sera from patients with invasive ovarian, tubal and primary peritoneal cancer from three large data sets to test whether TP53 autoantibody might serve as a biomarker for early detection of ovarian cancer. We set a cut-off value of TP53 autoantibody titers (78 U/mL) for each study, reflecting a specificity of 97.4%. Our assay detected elevated levels of TP53 autoantibody in 21% to 30% of cases at the time of clinical diagnosis, whereas previous studies had found TP53 autoantibody in around 15% of cases (20-36). In the UKCTOCS trial, TP53 autoantibody could detect 16% of ovarian cancer patients who were not detected using the CA125 based multimodal strategy. Remarkably, in cases in the UKCTOCS set in screen detected cases that were TP53 autoantibody positive, levels rose a mean of 11.0 months earlier than did CA125 (>35 U/mL) and 11.8 months earlier than the ROCA. In cases that were not screen detected using the CA125-based ROCA screening strategy, TP53 autoantibody titers rose an average of 22.9 months before cancer diagnosis. Among more than 110 ovarian cancer biomarkers identified to date, this is the first promising candidate that is able to detect invasive epithelial ovarian cancer at an earlier time than CA125 (38). Recently, Russell *et. al.* identified new biomarkers Protein Z, Fibronectin and C-reactive protein by the proteomic platform and those biomarkers showed a lead time on CA125 in small UKCTOCS sample sets (39,40).

The *TP53* gene is frequently mutated in a variety of human cancers and plays a crucial role in their development, continued growth and genetic instability. Our immunoassay for detecting TP53 autoantibody may have potential applications for early detection of other types of cancer. Indeed, detection of TP53 autoantibody has also been reported in other cancers such as esophageal cancer (41,42), head and neck cancer (43,44), colorectal cancer (45-47), hepatocellular carcinoma (48,49), lung cancer (50-52) and breast cancer (53,54), etc. If TP53 autoantibody is used for screening in a general population, more prevalent cancers may also be detected. This could be an asset rather than a limitation, if a cost-effective diagnostic algorithm were developed to differentiate other types of cancer in addition to ovarian cancer in women with an elevated anti-TP53 autoantibody titer.

We also evaluated the potential benefit in combining CA125 with TP53 autoantibody for detecting ovarian cancer. ROC curve analysis for the UKCTOCS dataset showed that combination of CA125 with TP53 autoantibody can significantly increase the AUC (Figure 5, A). To further confirm those results, we performed new ROC analyses incorporating 3, 6 and 12 months lead time of CA125 and TP53 autoantibody (Figure 5, B and Supplementary Figure 4, A and B). Combination of CA125 with TP53 autoantibody can significantly increase the AUC (Figure 5, B; CA125, 0.751 v.s. TP53 AAb + CA125, 0.861, P= 0.000). Our data suggest that the addition of TP53 autoantibody may improve upon CA125 alone or the ROCA for identifying patients with early stage ovarian cancer. Further large-scale clinical studies will be required to evaluate the clinical efficacy of a combination of CA125 with TP53 autoantibody assays for detecting ovarian cancer.

We tested whether using assays that incorporated the mutant TP53 protein corresponding to that in the ovarian cancer from each patient might provide a more sensitive assay than wild-type TP53. Similar sensitivity was obtained with specific mutant and wild-type TP53. Given current technology, mutant proteins and peptides could be incorporated in assays for early detection, if there were an advantage to this approach. However, we did not find such an advantage in this study. Consequently, this was an important issue to resolve. This also suggests that the autoantibody might be directed against wild-typeTP53 and the amount of non-degraded TP53 in ovarian cancer cells might be an important factor in developing autoantibody.

To our knowledge, this study is the first to assay TP53 autoantibody in large numbers of preclinical sera. As only 20-25% of patients with invasive epithelial ovarian cancer have elevated levels of autoantibody against TP53, this biomarker may have only a limited, but potentially significant, impact on the ability to detect early stage disease. Our study does demonstrate that TP53 autoantibody levels: (1) can complement CA125 at the time of diagnosis; (2) provide substantial lead time over CA125 in a fraction of cases; and (3) could contribute one important member of a panel of biomarkers that would improve substantially upon the performance of CA125. Given the proof of concept that TP53 autoantibody can be elevated in advance of CA125, we are identifying other autoantibodies that would complement TP53 autoantibody levels and create a panel of tests that would detect CA125 negative cases and provide lead time in a greater fraction of cases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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TRANSLATIONAL RELEVANCE

The biomarker CA125 can be detected in 80% of patients with advanced stage invasive epithelial ovarian cancer and in 50-60% of patients with early stage disease. Whereas small volumes of ovarian cancer may not shed sufficient CA125 to be detected, small amounts of tumor associated antigen(s) could stimulate production of detectable autoantibody. We have detected TP53 autoantibody in approximately 20% of ovarian cancers and in 16% that could not be detected with CA125 alone. We have observed elevated titers of TP53 autoantibody 11 months prior to elevation of CA125 and 23 months prior to diagnosis in patients not detected with CA125. Our observations provide proof of concept that autoantibodies can detect ovarian cancer in advance of shed tumor associated antigens such as CA125. Measurement of CA125 in combination with TP53 autoantibody and other autoantibodies could provide an effective strategy for earlier detection of patients with invasive epithelial ovarian cancer.
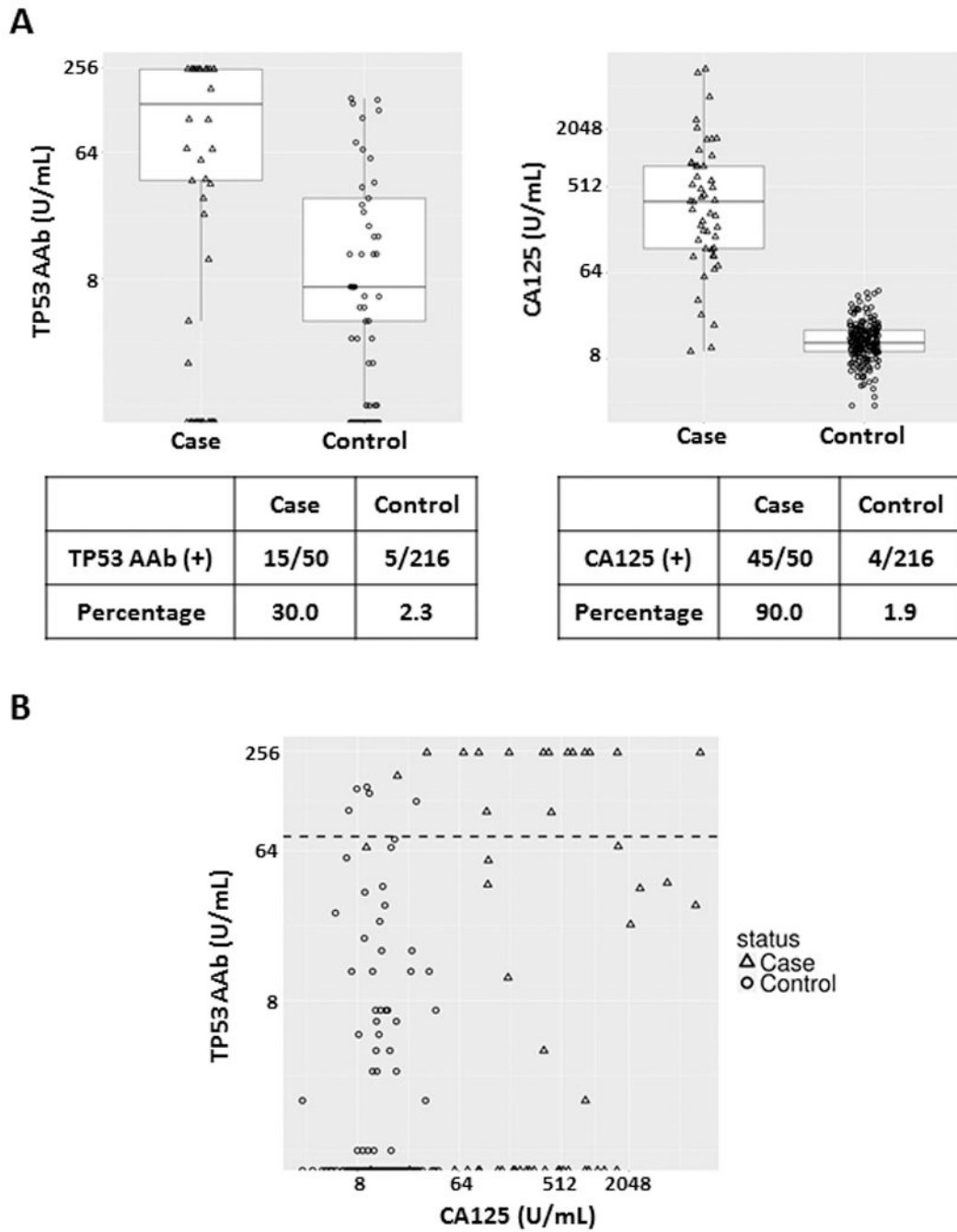
Figure 1.
TP53 autoantibody titers and CA125 values in serum samples from the MDACC-NROSS study. **A**, Box plots for TP53 autoantibody and CA125. Each box exhibits maximum, upper quartile, median, lower quartile and minimum values. Triangles represent cases and circles represent controls. The lower tables display TP53 autoantibody and CA125 positive numbers and percentages in the case and control groups. **B**, Scatter plot of TP53 autoantibody titers and CA125 values. Triangles represent ovarian cancer cases and circles

represent controls. The black dashed line represents the common cut-off value (78 U/mL) of TP53 autoantibody.

	Case	Benign	Control
TP53 AAb (+)	23/108	3/109	6/464
Percentage	21.3	2.8	1.3
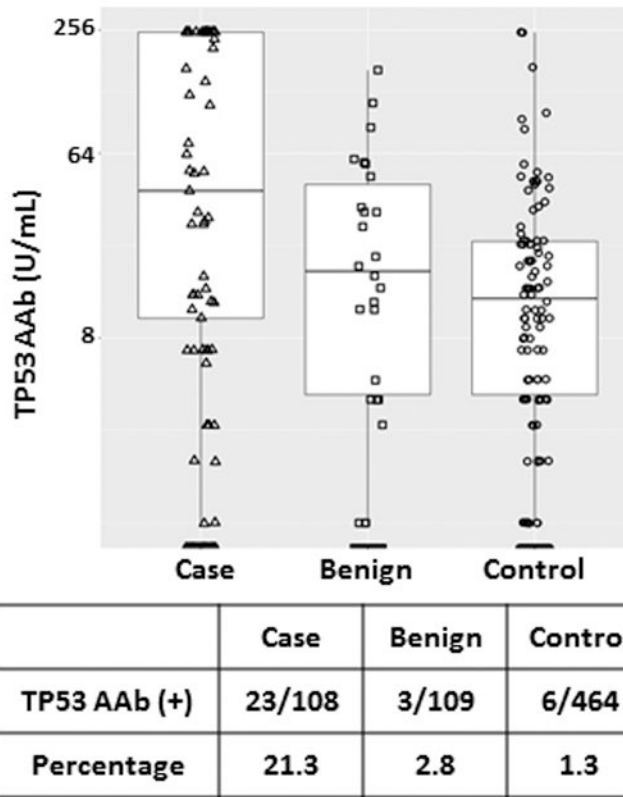
Figure 2.
TP53 autoantibody titers in serum samples from the AOCS biobank data set. Box plots for TP53 autoantibody titers. Each box exhibits maximum, upper quartile, median, lower quartile and minimum values. Triangle symbols represent cases, square symbols represent benign ovarian neoplasms, and circle symbols represent controls. The lower table displays TP53 autoantibody positive (above common cut-off 78 U/mL) numbers and percentages in case, benign, and control groups.

	ROCA/CA125 (+)	ROCA/CA125 (-)	Total Cases	Controls
TP53 AAb (+)	34/164	9/56	43/220	17/619
Percentage	20.7	16.1	19.5	2.7
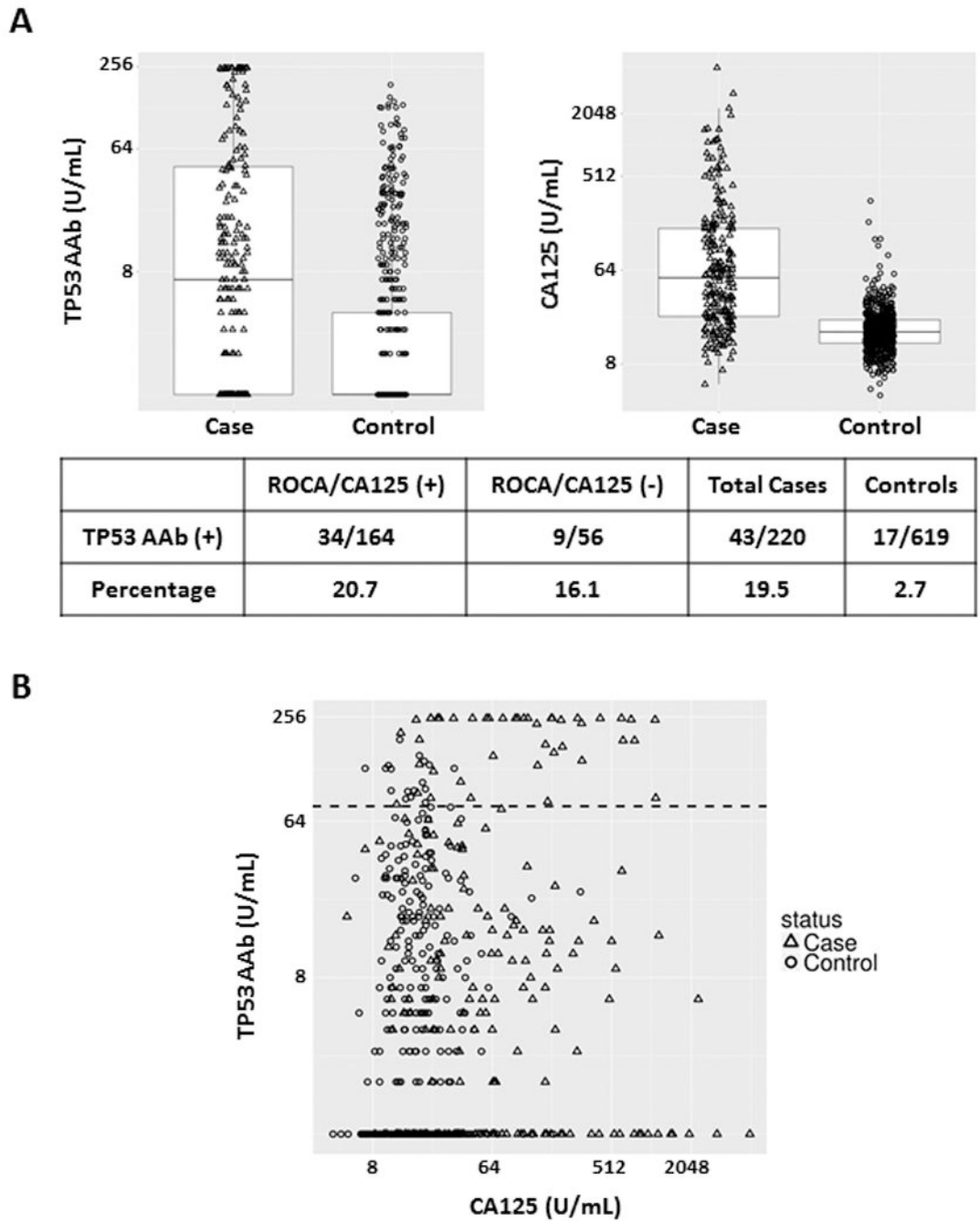
Figure 3.
TP53 autoantibody titers and CA125 values in serum samples from the UKCTOCS
screening trial. **A,** Box plots for TP53 autoantibody and CA125. Each box exhibits
maximum, upper quartile, median, lower quartile and minimum values. Triangles represent
cases and circles represent controls. The lower table displays TP53 autoantibody positive
numbers and percentages in ROCA/CA125 (+) case, ROCA/CA125 (-) case and control
groups. **B,** Scatter plots of TP53 autoantibody levels and CA125 values. Triangles represent

ovarian cancer cases and circles represent controls. The black dashed line represents the common cut-off value (78 U/mL) for TP53 autoantibody.
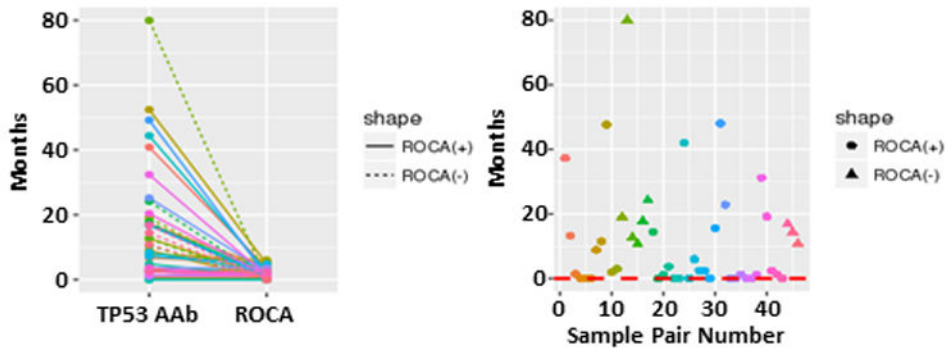
Figure 4.

Longitudinal analysis of CA125 values and TP53 autoantibody titers in pre-diagnostic serial serum samples from ovarian cancer patients in the UKCTOCS study. **A**, Lead time prior to diagnosis for elevated TP53 autoantibody, ROCA and CA125 (>35 U/mL) in 43 cases with elevated TP53 autoantibody. In the left panel solid lines were detected with the ROCA and the dashed lines were not. In the middle panel, circles were detected with the ROCA and the triangles were not. **B**, Lead time prior to diagnosis for elevated TP53 autoantibody, ROCA and CA125 (>35 U/mL) in 34 cases with elevated TP53 autoantibody who were diagnosed

with the ROCA. **C,** Lead time prior to diagnosis for elevated TP53 autoantibody, ROCA and CA125 (>35 U/mL) in 9 cases with elevated TP53 autoantibody who were not diagnosed with the ROCA. Sample pair number indicates the index of a given sample pairing within each set, ranging from 1 to 46 in A, 1 to 37 in B, and 1 to 9 in C. Colors match between the two plots in each panel, so the dramatic downward segment in C is seen to correspond to sample pair 2, with a difference in detection times of around 80 months.
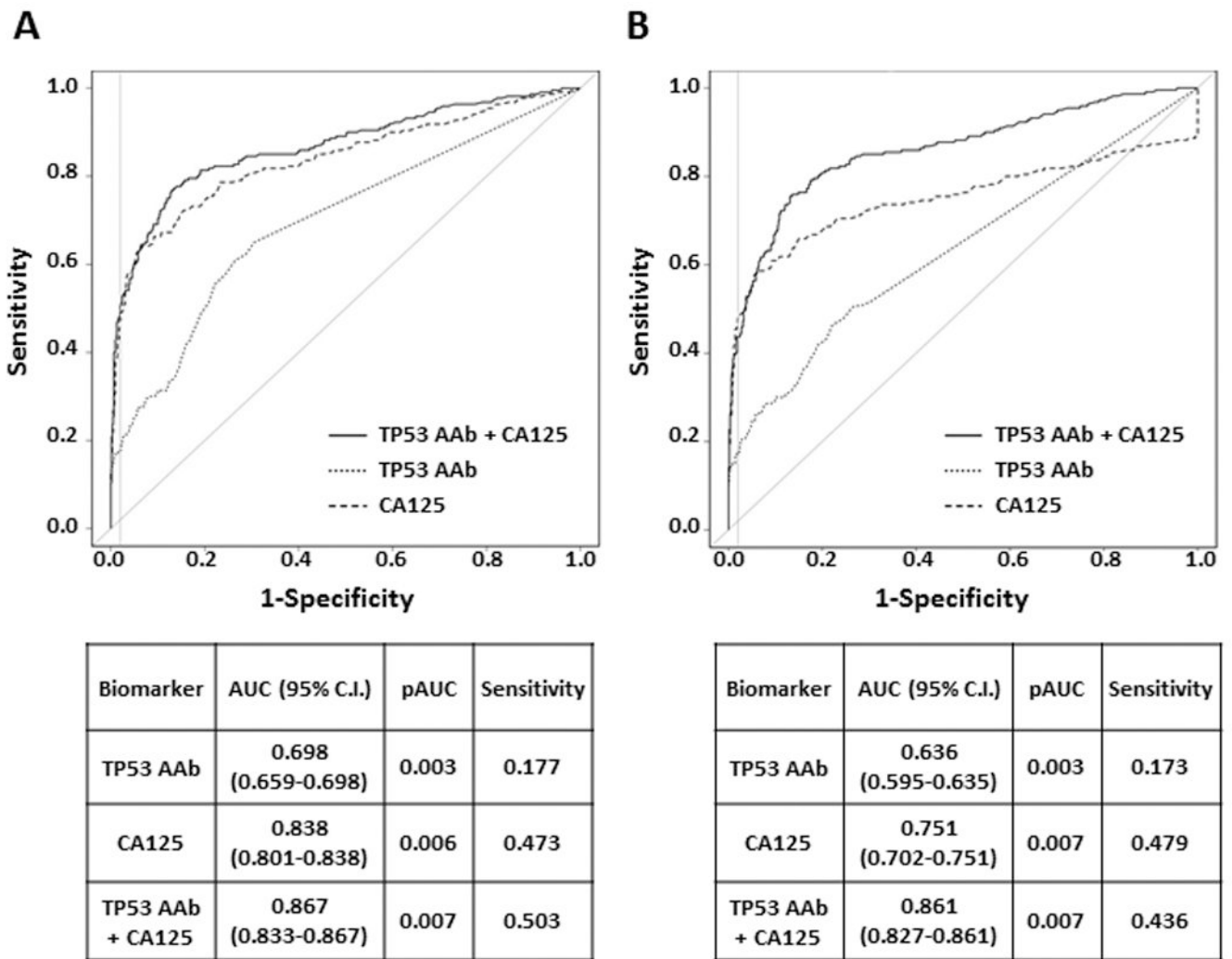
Figure 5.
ROC curve analysis for TP53 autoantibody and CA125 biomarkers in the UKCTOCS study. **A**, The AUC, partial AUC (pAUC) and sensitivity for TP53 autoantibody, CA125 and the combination of these two markers are shown in the table (p value = 0.001 for AUC of CA125 vs CA125+TP53 AAb; p value = 0.097 for pAUC of CA125 vs CA125+TP53 AAb). Dotted line, ROC curve for TP53 autoantibody; dashed line, ROC curve for CA125; solid line, ROC curve for the combination of two biomarkers. The gray vertical lines are corresponding to 0.98 specificity. **B**, ROC curves for TP53 autoantibody and CA125 in the UKCTOCS study were calculated when 3 months was chosen as the cut-off to define the "significant early detection". The AUC, partial AUC (pAUC) and sensitivity for TP53 autoantibody, CA125 and the combination of these two markers are shown in the table (p value = 0.000 for AUC of CA125 vs CA125+TP53 AAb; p value = 0.983 for pAUC of CA125 vs CA125+TP53 AAb).

Table 1

Characteristics of patients with invasive epithelial ovarian/tubal/peritoneal cancer by ROCA/CA125 status, histotype and TP53 autoantibody positivity in the UKCTOCS study. *TP53 AAb (+): TP53 autoantibody screen positive; N/A: Not available

ROCA/CA125 (+) N= 164			ROCA/CA125 (-) N = 56		
Histology			Histology		
Type	No.	TP53 AAb (+)* No. (%)	Type	No.	TP53 AAb (+)* No. (%)
Serous	119	27 (22.7%)	Serous	28	6 (21.4%)
Mucinous	1	0 (0.0%)	Mucinous	1	1 (100.0%)
Endometrioid	19	2 (10.5%)	Endometrioid	6	0 (0.0%)
Clear cell	7	0 (0.0%)	Clear cell	7	1 (14.3%)
Poorly differentiated	17	4 (23.5%)	Poorly differentiated	11	0 (0.0%)
Mixed	1	1 (100.0%)	N/A	3	1 (33.3%)
Total	164	34 (20.7%)	Total	56	9 (16.1%)