A Novel Algorithm to Improve Specificity in Ovarian Cancer Detection
Audrey Arjomandi, Michelle L. Delanoy, Roger P. Walker, Steven R. Binder

Abstract
We report the use of a novel algorithm that can increase specificity, and potentially sensitivity, of a screening test. We used this new algorithm to detect more autoantibodies to p53 in sera of patients with ovarian cancer than when we use a traditional multiplex approach, by combining p53 protein and selected confirmatory epitopes.

Background: Measurement of autoantibodies (AAbs) to tumor-associated antigens has been proposed to aid in the early detection of ovarian cancer with high specificity. Here, we describe a multiplex approach to evaluate selected peptide epitopes of p53 protein and propose a novel approach to increase specificity and potentially sensitivity for discrimination between healthy women and women with cancerous masses. Materials and Methods: Twenty-mer overlapping peptide epitopes of p53, generated by mapping the complete p53 sequence, were evaluated in a multiplex immunoassay for their detection of serum AAbs in patients with ovarian cancer, using Luminex technology. AAbs to the selected peptides and to p53 full-length protein were then detected in a multiplex immunoassay evaluating 359 sera from healthy women and 285 sera from patients with early- and late-stage ovarian cancer. CA-125 levels were measured in all p53 AAb-positive sera. Results: We considered the AAb results together to identify sera where both the full-length protein and at least one selected peptide epitope were positive and chose cutoffs that reduced false positives from these AAbs to 1 of 359 samples, improving specificity. Using this combined approach, we could identify 7 AAb-positive patients who were negative for CA-125 (concentrations below 35 IU/mL); this represents 26% of the p53-positive patients in the total population. Conclusion: By detecting p53 AAbs in CA-125–negative sera, we demonstrated that combining measurement of AAbs to the full-length p53 protein and one or more selected epitopes can potentially improve sensitivity and specificity for ovarian cancer detection.

Introduction
Ovarian cancer is the leading cause of gynecologic cancer death worldwide. Serum CA-125 levels, combined with imaging techniques, are currently used for monitoring disease recurrence and therapeutic response. CA-125 as a diagnosis marker has limited specificity and sensitivity for ovarian cancer. Although many additional biomarkers have been proposed for ovarian cancer, with the emergence of new proteomic technologies, no marker has been proven sensitive and specific enough for screening. Previous studies have demonstrated that combining biomarkers has the potential to improve specificity and sensitivity and increase predictive value for detecting ovarian cancer. The use of autoantibodies (AAbs) to tumor-associated antigens is a relatively new strategy that has been proposed to support early detection with high specificity. AAbs to p53 protein have been detected in sera of patients with various carcinomas, including ovarian cancer, making p53 an attractive AAb biomarker for diagnosis of ovarian cancer. It was shown that p53 AAbs can be detected up to 3.8 years before diagnosis in a cohort of normal-risk women with colorectal cancer, and p53 AAbs were studied for their high predictive value in high-risk populations. p53 AAbs were also evaluated for monitoring residual ovarian cancer and were found to be more sensitive than CA-125. Previous investigators studied immuno-dominant epitopes recognized by p53 antibodies; earlier attempts to develop an immunoassay with synthetic peptides corresponding to these epitopes were unsuccessful because they led to a high level of false-negative results.

In this report, we have developed a multiplex bead-based assay, using Luminex technology, for the detection of AAbs...
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simultaneously against wild-type p53 full-length protein and selected peptide epitopes. We describe the use of an algorithm combining AAbs against both the protein and 1 or 2 peptides to increase the specificity and sensitivity for discriminating between healthy women and women with cancerous masses. To determine whether the use of this algorithm could complement the CA-125 serum test by multiplexing the p53 markers from our study with serum CA-125 measurements, we further evaluated p53 AAb-positive samples in CA-125—negative population.

Materials and Methods

Patient Sera

Sera from healthy female blood donors (n = 359, median age of 65 years) were obtained from Gulf Coast Regional Blood Center. Sera from women with ovarian cancer (n = 285, median age of 61 years) were obtained from various vendors including Bio-reclamation (Westbury, NY), ProteoGenex (Culver City, CA), Asterand (Detroit, MI), or Cureline (South San Francisco, CA). Sera were collected after diagnosis but before any treatment. Cancer sera were divided between early (stage I/II) and late (stage III/IV) disease (FIGO stage 1, n = 88; stage 2, n = 106; stage 3, n = 74; stage 4, n = 17). After collection, all sera were stored at −80°C. Demographics are summarized in Table 1.

Protein and Peptides

Full-length p53 recombinant protein was obtained from GenScript (Piscataway, NJ). The p53 (361-393) 33-mer C-terminal peptide (sequence GSRAHSSHLKSKQSTSRHKKLMFKTE-GPDSD) was obtained from Biomer Technology (Pleasanton, CA). Epitope mapping was done to select peptide epitopes and utilized overlapping 20-mer peptides each offset by 7 residues encompassing the entire sequence of the p53 protein, was screened for immunoreactivity using an epitope mapping approach against 116 sera from healthy women and sera from 267 women with ovarian cancer. Each 20-mer peptide was interrogated independently in the multiplex assay. Two 20-mer peptides (amino acids 40-59 and 209-228) showed detection of high numbers of AAbs. The p53 peptide (40-59), localized toward the N-terminus of the protein, detected 33 of 267 AAbs, using a 99th percentile cutoff, consistent with the full-length protein (data not shown); this peptide was selected as a confirmatory epitope for the whole protein for our study. A 33-mer C-terminal peptide (361-393), which showed

<table>
<thead>
<tr>
<th>Disease Group</th>
<th>Histology</th>
<th>Number of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early-stage (I/II) ovarian cancer patient cases</td>
<td>Serous</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>Mucinous</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Endometrioid</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Clear cell</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Other epithelial (subtype unknown)</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>195</td>
</tr>
<tr>
<td>Late-stage (III/IV) ovarian cancer patient cases</td>
<td>Serous</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>Mucinous</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Endometrioid</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Clear-cell</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>2</td>
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<tr>
<td></td>
<td>Total</td>
<td>90</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>NA</td>
<td>359</td>
</tr>
</tbody>
</table>

Abbreviation: NA = not applicable.

Table 1 Distribution of Stage and Histology for Evaluated Sample Set

CA-125 Measurement and Multiplex p53 AAb Assay

All CA-125 values for patient samples were obtained using the Food and Drug Administration—approved LOCI CA-125 II method on the Dimension Vista 500 system (Siemens Healthcare Diagnostics, Erlangen, Germany). AAb measurement was made using a multiplexed bead-based immunoassay. p53 protein and peptides were covalently coupled individually to dyed carboxylated magnetic beads using carbodiimide chemistry, according to a protocol described elsewhere. Patient samples were diluted 1:60 in a triethanolamine buffer with 3% BSA and 50 µL was mixed with antigen-coated magnetic beads, followed by incubation for 60 minutes with continuous mixing. After washing off excess sera 3 times with phosphate buffered saline/0.1% Tween-20, the beads were mixed with an antihuman IgG antibody, labeled with phycoerythrin, and incubated for 30 minutes with continuous mixing. After 3 more wash steps, the bead fluorescence was read using a Bio-Plex 200 reader and Bio-Plex Manager 6.0 software (Bio-Rad, Hercules, CA). Fluorescence intensity data were acquired with the following advanced settings: 200 beads/region, 50 µL sample size, and doublet discriminator gate values of 5000 to 25,000, and RFI values were typically between 10 and 27,000. Cutoffs were determined using the 99.9th percentile RFI of the healthy population for each analyte. Cutoffs for the analytes in the algorithm described in this report were assigned manually to obtain only one false-positive sample in the healthy population (of 359 patient samples); this healthy sample was positive for p53 protein and for one of the p53 peptides at the assigned cutoffs.

Statistical Analysis

All statistical analyses and receiver operating characteristic (ROC) curves for evaluation of analyte performance were performed using JMP 10 software (SAS, Cary, NC).

Results

Peptide Selection for Multiplex Assay

A complete set of overlapping 20-mer peptides, spanning the entire sequence of the p53 protein, was screened for immunoreactivity using an epitope mapping approach against 116 sera from healthy women and sera from 267 women with ovarian cancer. Each 20-mer peptide was interrogated independently in the multiplex assay. Two 20-mer peptides (amino acids 40-59 and 209-228) showed detection of high numbers of AAbs. The p53 peptide (40-59), localized toward the N-terminus of the protein, detected 33 of 267 AAbs, using a 99th percentile cutoff, consistent with the full-length protein (data not shown); this peptide was selected as a confirmatory epitope for the whole protein for our study. A 33-mer C-terminal peptide (361-393), which showed
good performance in preliminary studies (data not shown), was selected as a second epitope for the study.

### Biomarker Performance in a Traditional Multiplex Assay and Using a New Algorithm

To evaluate selected biomarker performance, a total of 359 sera from healthy women and 285 sera from patients with ovarian cancer were tested in the multiplex bead-based assay described previously.

The best discrimination of cases versus controls for detected AABs was achieved for p53 (40-59) peptide (mean RFI, 875 vs. 30, previously. AAbs was achieved for p53 (40-59) peptide (mean RFI, 875 vs. 30, previously. The 99.9th percentile was used as cutoff value for each analyte in the multiplex. The 99.9th percentile was used as cutoff value, which gave 1 false positive of 359, ie, 99.7% sensitivity.

To potentially increase the assay specificity and sensitivity, we analyzed the data using a new algorithm. We observed that samples with markedly elevated signals for the peptide AABs consistently showed a strong signal for the protein AAb as well, suggesting that the peptides represented dominant epitopes.

In this study, we sought to identify peptide AABs that would be combined with the CA-125 serum assay to add value to the test by increasing specificity and sensitivity. For this purpose, levels of CA-125 were measured in all p53 AAb-positive sera. An ovarian cancer test requires high specificity; to obtain a 10% positive predictive value, it has been proposed that the specificity of a laboratory test should be 99.7%, with a sensitivity of at least 80%.19

### Discussion

Our goal was to determine whether p53 AAB biomarkers could be combined with the CA-125 serum assay to add value to the test by increasing specificity and sensitivity. For this purpose, levels of CA-125 were measured in all p53 AAB-positive sera. An ovarian cancer test requires high specificity; to obtain a 10% positive predictive value, it has been proposed that the specificity of a laboratory test should be 99.7%, with a sensitivity of at least 80%.19

The study described in this report included sera from women with different stages of ovarian cancer (I-IV). Our results confirm previous findings by other investigators that p53 AABs do not possess sufficient diagnostic sensitivity to be used as the sole screening test,14,20 showing AUC values between 0.50 and 0.70 from ROC curve analysis. Our data also suggest that multiplexing p53 protein and peptide epitopes in a traditional approach does not improve assay performance compared with the use of one individual biomarker.

In this study, we sought to identify peptide AABs that would increase the specificity of p53 AAB determination. We found an epitope from the N-terminus of p53 protein (amino acids 40-59) and 99.9th percentile cutoff for each analyte (Table 3). Using the 99.9th percentile cutoff for each of the 3 AAB analyte in the multiplex assay, 2 sera were positive in the healthy population, which corresponded to an assay specificity of 99.4%.

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To potentially increase the assay specificity and sensitivity, we analyzed the data using a new algorithm. We observed that samples with markedly elevated signals for the peptide AABs consistently showed a strong signal for the protein AAB as well, suggesting that the peptides represented dominant epitopes. By defining a positive result to be when both an AAB to full-length protein and an AAB to one or more peptide epitopes were obtained, we were able to use a lower signal cutoff, as described in the Materials and Methods section. Furthermore, the specificity increased because there was only one healthy control sample where both p53 protein and peptide AABs were simultaneously observed. By reducing the number of allowed AAB false positives from 2 to 1 and therefore decreasing considerably the cutoffs for each analyte, we improved specificity from 99.4% to 99.7% using this new algorithm. In the total cancer population studied, 27 sera were AAB positive using the algorithm, which corresponded to 9% of the population, compared with 21 positive sera using the traditional approach (Table 3). Seven of the 27 p53 AAB-positive patients were negative for CA-125 (at a cutoff of 35 IU/mL); only 2 of these samples were AAB positive using the traditional approach based on higher cutoffs (Table 3).

### Table 2

<table>
<thead>
<tr>
<th>Autoantibody Analyte</th>
<th>Healthy Controls ($n = 359$); Mean RFI ($95%$ CI)</th>
<th>OVCA Cases ($n = 285$); Mean RFI ($95%$ CI)</th>
<th>P Value for Comparison of the Population Means</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 protein</td>
<td>519 (331-708)</td>
<td>2272 (1567-2978)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>p53 (361-393) peptide</td>
<td>207 (31-383)</td>
<td>918 (466-1369)</td>
<td>.002</td>
</tr>
<tr>
<td>p53 (40-59) peptide</td>
<td>30 (17-43)</td>
<td>875 (437-1314)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>All 3 markers</td>
<td>—</td>
<td>—</td>
<td>0.663</td>
</tr>
</tbody>
</table>

Population means are given in RFI and were compared using a Student t test. AUCs were calculated from the receiver operating characteristic curves of individual and multiplexed AAB biomarkers. Abbreviations: AAB = autoantibody; AUC = area under the curve; CI = confidence interval; OVCA = ovarian cancer; RFI = relative fluorescence intensity.

### Table 3

| AAB Positivity in the Total Ovarian Cancer Population and in CA-125-negative Ovarian Cancer Patient Samples (< 35 IU/mL) Using Individual AAB Markers, a Traditional Approach, and the Algorithm |
|-------------------------------------------------|-------------------------------------------------|---------------------------------|---------------------------------------------|
| AAB to p53 protein                              | Number of Positive Results in Total Population ($n = 285$) | Number of CA-125–negative Patients (Cutoff 35), Out of p53–Positive Results |
| 14 (5%)                                        | 2 Of 14 (14%)                                    | 2 Of 14 (14%)                   |
| AAB to p53 (361-393) peptide                   | 5 (2%)                                         | 1 Of 5 (20%)                    |
| AAB to p53 (40-59) peptide                     | 21 (7%)                                        | 2 Of 21 (10%)                   |
| All 3 markers multiplexed (traditional approach; 99.4% specificity) | 21 (7%)                                        | 2 Of 21 (10%)                   |
| Algorithm (99.7% specificity)                   | 27 (9%)                                        | 7 Of 27 (26%)                   |

*The 99.9th percentile was used as cutoff value, which gave 1 false positive of 359, ie, 99.7% specificity.

*The 99.9th percentile was used as cutoff value for each analyte in the multiplex.

*Algorithm allowing 1 false-positive patient sample in the healthy population and identifying positives when full-length protein and at least 1 epitope were positive at the selected cutoff.
which can discriminate between healthy and OVCA patient samples with a similar pattern for AAb detection as the protein. Previous epitope mapping studies also demonstrated the immunogenicity of the N-terminal portion of the protein.10,11,17,21 We chose to use this peptide epitope, together with the 33-mer epitope at the C-terminus of the protein, in a multiplex assay in combination with the full-length protein. We used an algorithm specifying that a positive result was defined by AAb to full-length p53 and at least 1 of the 2 AAbs to a p53 epitope, at the chosen cutoff. This algorithm allowed the use of lower cutoffs at 99.7% specificity, which resulted in a higher p53 positivity rate in the total population and detection of more patients with ovarian cancer who were negative for the CA-125 marker compared with a traditional approach at 99.4% specificity. The rationale of this approach was based on the fact that a positive assay result was established to be concordant with the clinical result when both the full-length protein and at least one of its epitopes could be detected, using the peptide as a confirmatory marker. This method allowed avoiding some of the protein limitations; it mainly reduced the number of false-positive samples in the healthy population.

This study has some limitations. A larger study is needed to verify, and modify if needed, these cutoffs and should include CA-125 protein level measurement for all the samples in the study. In the traditional multiplex format, at least 2000 healthy patients would be needed to obtain 99.9% specificity for each marker. We acknowledge that there is no “test” set in this study, and a separate investigation should include “pedigreed” samples rather than samples of convenience. This study does not have impact on new biomarker discovery but rather shows the importance of multiplexing biomarkers effectively.

In conclusion, the use of protein and peptide epitopes together is a novel way to identify AAbs more specific for ovarian cancer screening. Additional studies are required to confirm these observations and to determine whether this approach could be useful with other AAb targets and serum markers.

**Clinical Practice Points**

- Autoantibody (AAb) measurement to p53 protein for ovarian cancer detection has previously been studied and has not been shown to be successful as a sole screening test for specific AAb detection. p53 AAb detection combined with measurement of the known marker CA-125 is not sufficient to achieve a good screening test performance.

- Here, we report the use of a novel algorithm that can increase specificity, and potentially sensitivity, of a screening test by combining p53 protein and selected confirmatory epitopes. We used this new algorithm to detect more AAbs to p53 in sera of patients with ovarian cancer (vs. control sera) than when we use a traditional multiplex approach.

- We believe that this approach can be applied to other biomarkers as well and make screening tests more efficient.

**Acknowledgments**

The authors thank Lois Weir and Elizabeth Umelo-Njaka (Bio-Rad Laboratories, Irvine, CA) for the determination of CA-125 levels in patient samples.

**Disclosure**

The authors are employees of Bio-Rad Laboratories, a manufacturer of in vitro diagnostic reagents and instrumentation.

**References**