

Identifying novel autoantibody signatures in ovarian cancer using high-density protein microarrays

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Abstract

Objectives: To identify autoantibody signatures in ovarian cancer using protein microarray technology.

Design and methods: Protein microarrays were screened using non-malignant peritoneal fluid ($n=30$) and ascites fluid pooled from ovarian cancer patients ($n=30$).

Results: Fifteen potential tumour-associated antigens were discovered. AASDHPPT showed the strongest signal-to-noise ratio.

Conclusions: Protein microarrays are suitable for autoantibody discovery in ovarian cancer but the signatures are of low frequency.

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Introduction

Epithelial ovarian cancer in particular, comprises more than 80% of the ovarian cancer cases [1]. It is often diagnosed in the late stages when the cancer has metastasized to other organs in the peritoneum. The 5-year survival rate for patients with advanced disease (stages III and IV) is 10–20%. In contrast, the 5-year survival rate for patients diagnosed with early-stage disease can be high as 90% [2]. These numbers clearly support the need for early diagnosis.

CA-125 is the clinically accepted biomarker for ovarian cancer and is used routinely to monitor patients' response to therapy and recurrence of disease. However, the test is poor for early detection and risk prediction due to frequent false-positive and false-negative results [3]. Thus, we need to discover new biomarkers that perform better as screening tools. Autoantibody responses to tumour-associated antigens (TAAs) can have both

diagnostic and prognostic value. Some documented autoantibody responses to tumours include ones against p53 [4], NY-ESO-1 [5], MUC-1 [6], and Tyrosinase [7]. In this study we used protein microarray technology to identify autoantibody signatures in ovarian cancer.

Materials and methods

Sample collection

Ascites fluids from ovarian cancer patients with primary and recurrent disease were either collected during surgery or withdrawn at paracentesis. Non-malignant peritoneal fluid was collected from female patients with benign pathologies. All samples were kept frozen at -20°C until analysis. Our protocols have been approved by the Institutional Review Board of Mount Sinai Hospital, Toronto, Canada.

Protein microarray screen

ProtoArrays[®] were purchased from Invitrogen Canada Inc. (Burlington, Ontario, Canada). Microarrays were screened

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according to the instructions provided by the manufacturer. Briefly, the microarrays were blocked for 1 h with blocking buffer. Ascites fluid, pooled from 30 patients with ovarian carcinoma of the serous type, was used as the source of primary antibodies at a dilution of 1:200 in probing buffer, and 120 μ l of sample was overlaid on the microarrays. For the control, 30 specimens of non-malignant peritoneal fluid were pooled together, and the dilution was identical to that of the cancer pool. Arrays were incubated for 2 h at 4 °C, followed by washes with a proprietary wash buffer. Bound antibodies were detected using a mouse anti-human IgG conjugated to Alexa Fluor 647 (Molecular Probes, Carlsbad, CA, USA). Signals were detected using a PE ScanArray Express microarray scanner.

Statistical analysis

Signal analysis was performed using Invitrogen's ProtoArray® Prospector v4. Briefly, the protein-protein interaction mode was selected in the software. ProtoArray® Prospector calculates the mean value and standard deviation of the signals of each protein on the microarray, followed by the calculation of the *z*-score for each protein.

Recombinant AASDHPPT production

The protocol and materials for the production of recombinant protein has been described previously [8]. Primer sequences used for PCR amplification and cloning of AASDHPPT was as follows: Forward primer, 5' CAC CGT TTT CCC TGC CAA ACG GTT CTG 3'; Reverse primer, 5' AGG GAA TCA TCA TGA CTT TGT ACC A 3' (ACGT Corporation, Toronto, ON, Canada). Tandem mass spectroscopy (Thermo Finnigan LTQ) was used to confirm the identity of recombinant AASDHPPT.

Enzyme-linked immunosorbent assay

Briefly, 96-well plates were coated with sheep anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). The ELISA plates were then incubated with mouse antibodies specific to N-terminal His tags, followed by incubations with either recombinant AASDHPPT or BCZ4. The latter protein has no homology to AASDHPPT but was produced in the same system and has a poly-His tag. It was used as a negative control. The wells were then incubated with ascites fluid from patients with serous ovarian carcinoma; 4 wells per ascites sample (2 wells for AASDHPPT and 2 wells for BCZ4). Binding events were detected using mouse anti-human IgG antibodies conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories Inc.).

Antibody isolation and purification

Antibodies were purified from the pooled ascites fluid used to screen the protein microarrays. Protein A affinity purification was performed using the kit system, MAPS (Bio-Rad

Laboratories, Hercules, CA, USA), according to the manufacturers instructions.

Western blotting

Western blots for AASDHPPT were performed using recombinant AASDHPPT. Membranes were blocked for 1 h at room temperature using 5% non-fat milk in PBS-Tween (0.1% v/v). Antibodies isolated from ascites fluid pooled from 30 patients were used as the source of primary antibodies (1:500 dilution in PBS containing 0.1% Tween) and membranes were incubated overnight at 4 °C. Individual ascites samples were also diluted 1:500 in PBS containing 0.1% Tween. Membranes were washed using PBS-Tween (0.1% v/v). Goat anti-human IgG coupled to alkaline phosphatase (1:10000 dilution in PBS containing 0.1% Tween and 5% non-fat milk) was used as the secondary antibody. Signals were developed and captured on film by using a chemiluminescent substrate (Diagnostics Product Corporation, Los Angeles, CA, USA).

Table 1
List of proteins found on microarray screen

Proteins	Accession number	Cellular function
Identified on both microarrays		
E3 ubiquitin-protein ligase HECW2	Q9P2P5	Mediates ubiquitination of TP73
Homo sapiens cDNA clone IMAGE:4829245	BC021189.2 [‡]	Unknown
Regulator of G-protein signalling 13 (RGS13)	O14921	Increases GTPase activity of G-protein alpha
Homo sapiens lymphocyte cytosolic protein 2 (LCP2)	Q13094	T-cell antigen receptor signalling
Serine/threonine kinase 25	O00506	Oxidative stress activated kinase
Vacuolar sorting protein SNF8	Q96H20	regulation of transcription from RNA polymerase II promoter/multivesicular body formation
L-aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase	Q9NRN7	post-translational modification of target proteins by phosphopantetheine
Coiled-coil domain-containing protein 101	Q96ES7	Unknown
High mobility group nucleosomal binding domain 3 (HMGN3)	Q15651	Thyroid receptor binding
Chromosome 18 open reading frame 25	NM_145055 [‡]	Unknown
Identified on one microarray		
Large proline-rich protein BAT3	P46379	Unknown
IMPACT protein	Q49AM0	Unknown
F-box/WD repeat protein 1B	Q9UKB1	Protein ubiquitination
Homo sapiens cDNA clone MGC:18299	BC008624.1 [‡]	Unknown
Twinfilin-2	Q6IBS0	Inhibits actin polymerization

Accession numbers shown are Swiss-Prot identifiers, except ones denoted with a [‡], which are NCBI identifiers.

Results

Screening microarrays with ascites fluid

Two identical human protein microarrays were screened with ascites fluid pooled from 30 patients with ovarian cancer. The first experiment identified 10 proteins that were potentially antigenic. Replicate protein features whose mean signals were 3 standard deviations or greater than the mean signals of all protein features were considered as true binding events. Table 1 lists these proteins, their corresponding database identifiers, and the normalized signals (in arbitrary units). In the second identical experiment, the same 10 proteins plus 5 other proteins were identified. As a control, we screened a microarray with non-malignant peritoneal fluid. All proteins identified in the control experiment were eliminated from the compiled list of potential antigenic proteins. Of the 15 candidates, L-aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase (AASDHPPT) had the highest signal-to-noise ratio.

Verification of AASDHPPT as an immunogenic protein

To confirm that AASDHPPT was indeed immunogenic, we conducted Western blots with recombinant AASDHPPT, and the pooled ascites fluid as the primary antibody source (Fig. 1). BCZ4 (His-tagged protein produced in *E. coli*) was used as a negative control to verify that non-specific binding to the His-tag was not occurring. The estimated size of the recombinant AASDHPPT was 39 kDa, and it was detected on the Western blot. There was no binding to BCZ4 (~37 kDa protein).

Ascites samples used to create the pooled sample were used in Western blots to examine which samples contained anti-

AASDHPPT antibodies. One sample contained a high-titre of anti-AADHPPT antibodies relative to the other samples, as indicated in Fig. 1B. A dose-response experiment was conducted using this particular ascites, to verify that the binding was specific (Fig. 1C). For comparison, the same experiment was repeated using an anti-AASDHPPT negative ascites. Binding to recombinant AASDHPPT increased with increasing amount of protein. This was not seen in the Western blot with the anti-AASDHPPT negative ascites.

Validation of AASDHPPT by ELISA

We screened 100 ascites samples from patients with serous ovarian carcinoma by ELISA, for anti-AASDHPPT activity. Raw counts for AASDHPPT were compared with the corresponding counts for BCZ4. Raw counts for AASDHPPT that were at least twice that of the corresponding counts for BCZ4 indicated presence of anti-AASDHPPT antibodies. From the 100 ascites samples screened, only one was positive. This was the same sample that showed positive reactivity in the Western blots (Fig. 1B). The average raw count for the anti-AASDHPPT positive ascites was 8 times greater than that measured for corresponding BCZ4 control (49637 versus 6199 arbitrary units respectively).

Discussion

Our screens yielded 15 proteins that were candidates for further study as TAAs, 10 of which were reproducible in the cancer set. To date, none of these proteins have been studied in ovarian cancer. AASDHPPT was a good candidate for further study, given the high signal-to-noise ratio. However, testing individual ascites samples revealed that only one sample out of the 100 ascites tested positive for anti-AASDHPPT antibodies.

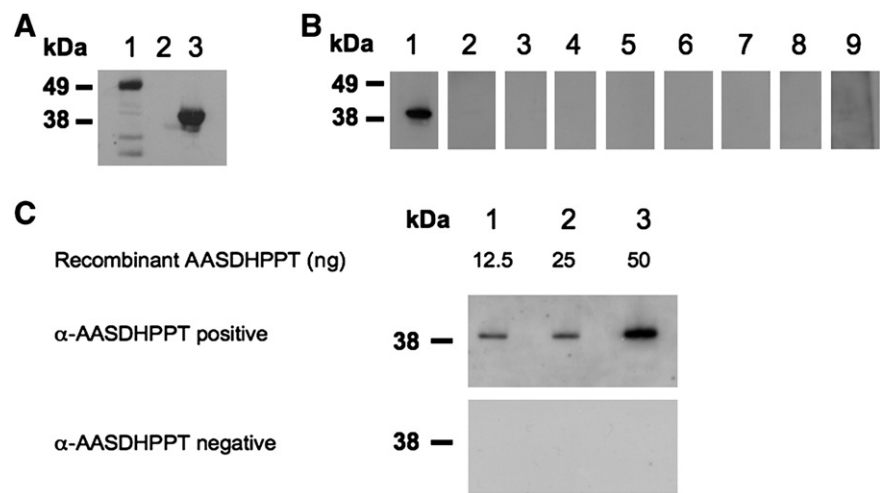


Fig. 1. (A) Western blot analysis for anti-AASDHPPT antibodies in the pooled ascites sample from ovarian cancer patients. Lane 1: human IgG (control); Lane 2: recombinant BCZ4 produced in *E. coli*; Lane 3: recombinant AASDHPPT produced in *E. coli*. (B) Western blot analysis for anti-AASDHPPT antibodies in individual ascites samples. Recombinant AASDHPPT was used as the antigen. Panels 1–9 represent individual Western blots. (C) Western blot for AASDHPPT using an anti-AASDHPPT antibody positive and negative ascites. Lanes 1–3 have increasing amounts of AASDHPPT. Top panel is the Western blot using the anti-AASDHPPT positive ascites. The bottom is the Western blot using anti-AASDHPPT negative ascites.

This sample was also part of the pooled ascites sample used in the two discovery screens. Thus, the relatively high signal seen on the microarrays for AASDHPPT is likely due to the contribution from this single ascites sample, rather than a cumulative contribution from several ascites. The Western blots for AASDHPPT using individual ascites fluids confirm this observation. The strong signal present in our two microarrays was also validated by the strong signal detected by ELISA. Although the experiments did not succeed in demonstrating AASDHPPT as a frequent TAA of ovarian cancer, we demonstrated that protein microarrays are suitable for uncovering autoantibody responses, as also reported by others [9].

The genesis of the autoantibody response in malignancy is still poorly understood. This is complicated further by the rarity of autoantibody responses to tumours. For example, anti-p53 antibodies are seen in only 18% of ovarian cancer patients [4] and anti-NY-ESO-1 antibodies are detected in no more than 20% of lung cancer patients [5]. Our results substantiate this observation.

Based on the low frequency of autoantibody responses to tumours and the difficulties in detecting new responses, it is unlikely that an autoantibody response to a single TAA will provide the superior sensitivity and specificity needed for an early detection tool. This was clearly seen with AASDHPPT. The prevailing view is the use of multiparametric analysis in microarray format. Success with a multiparametric detection assay has been demonstrated in prostate cancer where a peptide microarray constructed from 22 putative TAAs, detected prostate cancer with a specificity of 88.2% and a sensitivity of 81.6% [10]. These results were better than using PSA alone.

To conclude, protein microarrays are suited for detecting autoantibody responses. Moreover, their ease of use, the low amounts of reagents and sample required and the number of molecules that can be tested per array make them ideal for multiparametric testing. Although we did not succeed in finding

a TAA that was a good biomarker, this is not due to the limitations of the protein microarray, but rather due to the nature of autoantibody responses in cancer.

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