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Please cite this article as: Murphy Mairead A., OConnell David J., OKane Sara L., OBrien John K., OToole Sharon, Martin Cara, S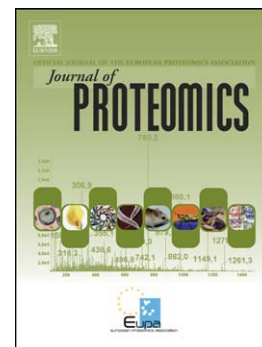heils Orla, OLeary John J., Cahill Dolores J., Epitope presentation is an important determinant of the utility of antigens identified from protein arrays in the development of autoantibody diagnostic assays, *Journal of Proteomics* (2012), doi: 10.1016/j.jprot.2012.02.031

Epitope presentation is an important determinant of the utility of antigens identified from protein arrays in the development of autoantibody diagnostic assays

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Autoantibodies represent an attractive biomarker for diagnostic assays principally due to the stability of immunoglobulin in patient serum facilitating measurement with conventional assays. Immune responses to tumorigenesis may facilitate detection of ovarian cancer in the early stages of the disease with identification of a panel of tumour specific autoantibodies. Despite the reporting of many tumour associated autoantibodies using arrays of tumour antigens, this has not led to the advance in diagnostic capability as rapidly as was initially expected. Here we examine the potential diagnostic utility of candidate autoantibody biomarkers identified via screening of serum samples on a high content human protein array from a unique cohort of early stage and late stage ovarian cancer patients. We analyse the performance of autoantibodies to the tumour suppressor protein p53 and the novel autoantigens alpha adducin and endosulfine alpha identified in our array screen. Each antigen has different performance characteristics using conventional ELISA format and Western blot immunoassay. The high attrition rate of promising autoantigens identified by array screening can in part be explained by the presentation of the epitope of the antigen in the subsequent method of validation and this study provides directions on maximising the potential of candidate biomarkers.

Introduction

Profiling of the circulating antibody repertoire in human serum against protein expression libraries has assisted in the identification of autoantibodies associated with neoplastic events in a wide variety of human cancers [1-4]. It is generally accepted that there is a humoral immune response to intracellular antigens released at the site of tumorigenesis and this may arise in advance of clinically detectable disease using conventional diagnostic techniques. Ovarian cancer is a pronounced example, where there is an urgent unmet need to identify early markers of ovarian tumorigenesis. This disease has proven especially refractory to diagnosis with late diagnosis particularly prevalent and this is associated with a poor prognosis for the patient. Detection of immune surveillance of neoplastic events with a blood based assay of tumour associated autoantibodies is a highly attractive diagnostic entity, which has yet to materialise in the hands of the clinician [5].

Ovarian cancer is a leading cause of death from gynaecological malignancy worldwide. It is the fifth leading cause of cancer related deaths in women worldwide. During 2000-2004 there were, on average 374 cases of ovarian cancer diagnosed in the Rep. of Ireland each year (National Cancer Registry Ireland) and approximately 140,000 women worldwide died from the disease in 2008 (Cancer Research UK). The survival rate has remained relatively constant over the past 30 years for ovarian cancer patients (cancerresearchuk.org). This static level is disappointing when compared with cancers such as breast cancer, where earlier diagnosis and tailored therapies have lead to steep falls in mortality rates [6].

One of the most important determinants of ovarian cancer survival is early stage diagnosis however indications are very subtle and can be easily overlooked due to the unspecific nature of the symptoms of the condition [7]. Ovarian cancer when diagnosed in early stages results in over 90% 5-year survival rate, however if diagnosed in late stage this drops to only 30% 5-year survival rate [8].

The most commonly used biomarker for ovarian cancer is Cancer Antigen 125 (CA125) or Mucin16 which is thought to provide a protective, lubricating barrier against particles and infectious agents at mucosal surfaces [9]. Elevated levels of this antigen are detected in over 90% of sera of disseminated ovarian cancer cases but only in 50% of patients in the early stages of the disease [10]. CA 125 screening is used with the addition of ultrasound screening. Although these combined approaches

complement each other and increase the specificity [11] of earlier detection a simple, blood based detection method using robust biomarkers available in the clinic is needed to assist with ovarian cancer diagnosis

The best characterised autoantigen/autoantibody relationship is that of the tumour suppressor protein p53 [12-16]. A number of studies have demonstrated that the half-life of mutated p53 (several hours) is markedly increased compared to wild-type (several minutes) which may result in its accumulation in the cell nucleus and the accumulation of protein rather than the mutations has been suggested as the immunogenic trigger that results in autoantibody generation [17]. Clinicopathological and molecular studies suggest that ovarian carcinoma can be divided in two broad categories, "Type I" and "Type II" tumors [8]. The terms, "Type I" and "Type II" refer to different tumourigenic pathways rather than specific histopathologies . "Type I" tumours are typified by somatic mutations in genes encoding protein kinases such as kRAS and BRAF. These growths are often low grade, slow growing and they develop from a well characterized precursor lesion in a step by step fashion. "Type II" tumours are characterized by a high frequency of p53 mutation. "Type II" tumors are high grade with a rapid growth rate and have almost always spread beyond the ovaries at presentation. "Type II" tumours include high-grade serous and endometrioid carcinomas.

Our laboratory has developed protein array based technologies and methodologies since first developing the hEx1 protein expression library [18] with which we have performed studies on the binding of autoantibodies to arrayed proteins in *Alopecia areata* and *Dilated Cardiomyopathy* [19, 20] , binding of antibodies to proteins identified from tumour neovasculature in humans [21],context independent motif identification in the human proteome [22] and of identification of novel protein-protein interaction networks [23, 24]. We have employed this library screening method as part of an ovarian cancer research consortium, *Discovary*, performing a pilot study on autoantibody identification screening the hEx1 protein library with ovarian cancer serum samples from a well characterised patient cohort with stage I ovarian cancer of mixed histology, stage III serous papillary adenocarcinoma, primary peritoneal carcinoma and normal/healthy individuals. We have selected promising autoantigen candidates bound by antibodies from the serum of patients not identified in control samples. We then expressed and purified the antigen from the parent clone

and assessed their diagnostic utility with both direct ELISA and Western blot immunoassay. Additional validation was performed on an increased number of early stage I ovarian cancer samples and a unique cohort of late stage serous papillary ovarian cancer patient sera. We show that each antigen has unique performance characteristics and that Western blot was more sensitive than ELISA immunoassay, which suggests that antigens identified by autoantibody binding in an array screen are detected with greater sensitivity upon presentation of linear epitopes in the subsequent validation assay.

Materials and Methods

Serum Sample Details

Serum samples and clinical information was obtained with informed consent and study approval was obtained from St. James's Hospital and Adelaide and Meath incorporating the National Childerns Hospital research ethics committee. Pre-operative bloods were obtained from patients undergoing cytoreductive surgery for possible ovarian neoplasm. Blood was collected into a non-heparinised tube and allowed to clot, then centrifuged at 400 x g for 15 min. The serum supernatant was removed and dispensed into labelled cryovial tubes. Serum was stored at -80°C until further use.

hEx1 Serum Screening of Ovarian Cancer Samples

The hEx1 array PVDF membranes (each hEx1 array is made up of 2 PVDF membranes, pt 8 and pt 9) were activated in ethanol for 1 min, rinsed in deionised water and washed in tris-buffered saline-T-T (500mM NaCl, 10mM Tris-HCl pH 7.5, 0.05% v/v Tween, 0.5% v/v Triton X). Dessicated bacterial colonies were removed with tissue. The arrays washed in TBS-T-T for 10 min, TBS-T (500mM NaCl, 10mM Tris-HCl pH 7.5, 0.05% v/v Tween) for 10 min and TBS (150mM NaCl, 10mM Tris-HCl pH 7.5) for 10 min. Arrays were then blocked for 2 hours at room temperature in 2% milk-TBS. The arrays were incubated in serum diluted in 20ml 2% BSA TBS-T and a serum dilution of 1:100 was used. The arrays were incubated overnight at room temperature.

Arrays were washed 3 times for 30 min in TBS-Tween. Secondary mouse anti-human Fc antibody (Sigma) diluted 1:5000 in 2% BSA TBS-T. The arrays were then incubated for 1 hour at room temperature. Arrays were washed 3 times for 30 min in

TBS-Tween. Tertiary goat anti-mouse Fc AP conjugate antibody (Sigma) was diluted 1:5000 in 2% BSA TBS-Tween. Arrays were incubated in antibody for 1 hour at room temperature.

Arrays were washed twice in TBS-T for 30 min and in TBS for 30 min. Arrays were equilibrated in attophos buffer (100mM Tris-HCL pH 9.5, 1mM MgCl) for 10 min and then incubated in the dark for 5 min in 1:40 dilution of attophos substrate in attophos buffer. The arrays were imaged using a LAS3000 Fuji imager, using tray position 3, filter 3 and 460nm blue light to detect positive binding events that indicated autoantibody binding.

Expression of hEx1 Recombinant Proteins

Escherichia coli bacterial colonies from the hEx cDNA library were picked under sterile conditions and used to inoculate centrifuge tubes containing 1ml sterile 2 x YT-AKG media. Media were agitated overnight on a shaker (220rpm) at 37°C to promote bacterial growth. For well expressing clones 100μl of these cultures were then used to inoculate 10ml of sterile autoinduction media (Overnight Express, Novagen). For poorly expressing clones 500μl of cultures were used to inoculate 50ml of sterile autoinduction media. Inoculated media was agitated overnight on a shaker (220rpm) at 37°C to promote bacterial growth. Bacterial cultures were centrifuged at 1780 x *g* for 35 min at 4°C and the supernatant removed. The bacterial pellet was stored at -80°C for at least 1 hour.

Purification of His-tagged proteins

Bacterial pellets were removed from -80°C. 2ml of 6M guanidine hydrochloride lysis buffer solution was added to each pellet. Pellets were resuspended by vortex and placed on a rocker for an hour. Resuspended pellet solutions were then centrifuged at 16,100 x *g* for 25 min at 4°C. Meanwhile, 400μl of Ni^{+}-NTA resin agarose beads (Merck) were aliquoted into microcentrifuge tubes centrifuged at 400 x *g* for 2 min. Supernatant was discarded and the nickel pellets were resuspended in 1ml of lysis buffer and centrifuged at 400 x *g* for 2 min, the supernatant was discarded. This pellet washing step with lysis buffer was repeated 3 times. The supernatant from the bacterial solutions was used to resuspend the nickel pellets, this was placed on a rocker for 30 min. The resuspended pellet solutions were added to polypropylene columns and extra liquid allowed to run off. The nickel columns were then washed

twice with 4ml of 8M urea wash buffer (pH 6.3). Columns were stoppered and 200μl of 8M elution buffer (pH 4.5) was added to the column and incubated for 10 min. Columns were unstoppered and the run-off discarded. 500μl of elution buffer was passed through columns and purified protein eluate was collected. Purified proteins were stored at 4°C until further use.

Analysis by SDS polyacrylamide gel electrophoresis (SDS-PAGE)
Protein quantification was carried out using a BCA protein assay kit, (Pierce BSA Kit II, catalog number 500-0002). Absorbance readings at 540nm were recorded. A standard curve of the BSA standards was generated and used to determine the concentration of the unknown proteins.

Proteins were normalized relative to each other and ran on a 12% SDS polyacrylamide gel. The gels were stained in coomassie stain solution for 2 hours on slow rock. Gels were destained overnight

Gels were scanned and images were stored for subsequent analysis.

Commercial recombinant p53 Western Blot Analysis
Recombinant p53 protein was purchased from Aviva systems Biology (Cat no: A902005P - refolded and purified from E. coli inclusion bodies). SDS-gels were loaded with 200ng/well or 50ng/well of protein and electrophoresed. The proteins on the gel were transferred to a PVDF membrane at 100 volts for 1 hour. The blot was rinsed in TBS-T (150mM NaCl, 10mM Tris-HCl pH 7.5, 0.05% v/v Tween), then blocked in 5% milk-TBS-T for 1 hour at room temperature. The blots were then incubated with either, commercial mouse anti-p53 antibody (Calbiochem, # OP43) or human serum.

Commercial anti-p53 antibody incubation of 200ng per lane Western blots
Anti-p53 antibody was diluted (1:1000 dilution) in 10ml 2% milk-TBS-T and blot was incubated in antibody for 1 hour on slow rock at RT. (Figure S1)

Human serum incubation
Serum was diluted (1:870 dilution for 200ng/lane blots or 1:100 dilution for 50ng/lane blots) in 10ml 2% milk-TBS-T blots were incubated overnight on slow rock at RT. Blot washed 3 times for 10min in TBS-T and then incubated in mouse anti-human antibody at a 1:5000 dilution in 10ml 2% milk TBS-T for 1 hour.

Western completion and imaging

Blot washed 3 times for 10 min in TBS-T and incubated in goat anti-mouse AP conjugate antibody at a 1:5000 dilution in 10ml 2% milk TBS-T for an hour. The blot was washed twice for 10 min in TBS-T and once for 10 min in TBS.

The blot then equilibrated in attophos buffer for 10 min and incubated in attophos substrate solution (1:40 dilution in attophos buffer) for 5 min in the dark. The blot was then imaged using a LAS3000 Fuji imager, using tray position 1, filter 3 and 460nm blue light.

To compare effect of different imaging approaches a standard horseradish peroxidise detection with chemiluminescence and X-ray film was also used for some Western blots to compare to previously obtained results with alkaline phosphatase/attophos detection. For this a Mouse anti-human Fc spec IgG HRP-conjugate antibody (Sigma) was used as a detection antibody after the serum incubation step (Figure S2).

hEx1 Protein (p53, alpha adducin, endosulfine alpha) Western Blot Analysis

These blots were as per previous blot with the following exceptions: SDS-gels were loaded with 500ng/well of protein and electrophoresed. Serum was diluted 1:100 dilution for p53, alpha adducin and endosulfine alpha blots. Blots were incubated for 2 hours in serum. The blot was washed 3 times for 10 min in TBS-T and then incubated in goat anti-human Fc AP conjugate antibody (Sigma) at a 1:5000 dilution in 10ml 2% milk TBS-T for 1 hour. Blot was washed 2 times for 10 min in TBS-T and once for 10 min in TBS. The blot then treated with attophos buffer and substrate as previous and imaged.

Direct E.L.I.S.A. Analysis

Antigens were diluted in deionised water to a 1ng/μl concentration. 50μl of diluted antigens were aliquoted into wells of a 96 well maxisorb plate (Nunc) and were left overnight at 4°C to coat. Antigens were ejected and the plate was washed in an ELISA plate washer twice with 400μl per well of TBS-Tween (150mM NaCl, 10mM Tris-HCl pH 7.50, 0.1% Tween). For blocking 400μl of 5% milk TBS-T was aliquoted into each well to block for 2 hours. The plate was washed twice with TBS-T. Serum was diluted (1:100) in 5% milk TBS-T and 50μl of diluted serum was added to wells, the plate was then incubated on the plate shaker for 2 hours at RT. Plate was washed 5

times with TBS-T. Mouse anti-human Fc spec IgG HRP-conjugate antibody was diluted to 1:5000 dilution in 5% milk TBS-T. 50μl of diluted antibody was aliquoted into wells and left to incubate for 1 hour at RT. Plate was washed 5 times with TBS-T. 50μl of TMB substrate was added to wells and incubated for 30 min in the dark. 25μl of 1N sulfuric acid was added to wells and the plate shaken briefly to mix contents. Absorbance was read at 450nm using a plate reader.

Results

Identification of autoantigens from screening of sera on human protein arrays

A high density human protein array containing 37,200 redundant, recombinant proteins expressed in E. coli was screened for autoantibody binding with sera obtained from the patient and control cohort (Figure 1). Serum autoantibody screening was performed on 20 late stage ovarian adenocarcinoma, 13 early stage ovarian cancer and 5 primary peritoneal carcinoma sera with 15 sex matched normal controls enrolled for the study (Table 1). 41 protein expressing clones on the array were bound by antibodies from late stage ovarian patient sera that were not bound by antibodies in any of the control sera samples. 17 protein expressing clones were bound by early ovarian cancer serum samples not bound by antibodies in serum from the control group. 22 protein expressing clones were bound by antibodies from primary peritoneal patient sera not bound by antibodies in serum from the control group. Among this collection of redundant protein expressing clones we identified autoantibodies to the tumour suppressor protein p53 in 4/20 late stage serous papillary ovarian cancer sera. No autoantibodies to p53 were seen in the early stage (0/13) ovarian cancer serum samples or in any of the control serum samples (0/15). We identified autoantibodies to novel autoantigens, including a protein phosphatase inhibitor endosulfine alpha (ENSA) in 4/20 late stage serous papillary not in control sera and to a cytoskeleton assembly protein alpha adducin (ADD1) in 3/20 late stage ovarian cancer sera and 3/13 early stage ovarian cancer (Stage 1-II) (Figure 1 and Table 2).

Detection of p53 AAb by ELISA

Comparison of serum autoantibody binding/recognition to purified hEx1 p53 was compared to a commercially available recombinant p53 protein (A902005P, Aviva Systems Biology) with an optimised direct ELISA developed in the laboratory (Figure S3). ELISA analysis of the hEx1 p53 his-tagged protein fragment and the full length commercial protein revealed almost identical binding patterns relating to intensity and frequency in serum (Figure S3).

Characterisation of AAb by ELISA and Western blot

p53 – TP53

We evaluated the performance of each antigen using the direct ELISA that was optimised for detection of the p53AAb. In the 20 late stage serous papillary adenocarcinoma group we detected p53 autoantibodies in 4/20 sera (20%) (Figure 2A and Table 3). The ELISA positive sera matched 4 of 5 sera identified as positive by array screening. Western immunoblots of the expressed hEx1 protein with this patient group identified p53 AAb immunoreactivity in 5/20 patient sera (25%) (Figure 2B and Table 3). The sera positive for p53 immunoreactivity by Western blot matched exactly the sera positive for p53 immunoreactivity by protein array screening.

In an additional unique cohort of 14 late stage serous papillary ovarian cancer patients ELISA and Western immunoblotting was also performed. ELISA analysis identified p53 AAb immunoreactivity in 3/14 (21%) patient sera. Western blotting also identified the same 3/14 (21%) patient sera as reactive to the p53 protein (Table 4).

Alpha Adducin - ADD1

In the 20 late stage serous papillary adenocarcinoma group we detected alpha adducin autoantibodies in 1/20 sera (5%) by ELISA (Table 3). The ELISA positive serum matched 1 of 3 sera identified as positive by array screening. Western immunoblots of the expressed hEx1 protein with this patient group identified alpha adducin AAb immunoreactivity in 6/20 patient sera (30%) (Figure S4b and Table 3). The sera positive for alpha adducin immunoreactivity by Western blot contained those sera that were positive for alpha adducin immunoreactivity by protein array screening.

In the expanded early stage ovarian cancer group we failed to detect alpha adducin autoantibodies in any of the 20 patient sera by ELISA (Table 3). By contrast we detected alpha adducin autoantibodies in 4/20 (20%) patient samples by Western immunoblot (Figure S4b and Table 3). The four sera that were immunoreactive to alpha adducin with the Western immunoblotting were from the original 13 patient

samples screened on the hEx1 array i.e., the additional alpha adducin immunoreactive serum detected by Western blot was negative by array screening.

In an additional unique cohort of 14 late stage ovarian cancer patients ELISA analysis identified alpha adducin AAb immunoreactivity in 0/14 patient sera. Western blotting identified AAb to alpha adducin in 3/14 (21%) of the patient sera (Table 4).

Endosulfine alpha - ENSA

For the endosulfine alpha autoantigen, we detected autoantibody immunoreactivity in 1/20 late stage serous papillary adenocarcinoma patient sera (5%) by ELISA (Table 3). The ELISA positive serum matched 1 of the 4 sera identified as positive by array screening. Western immunoblot of the expressed hEx1 protein with this patient group identified endosulfine alpha AAb immunoreactivity in 3/20 patient sera (15%) (Figure S4c and Table 3). 2 of the 3 endosulfine alpha AAb positive sera matched those identified in the protein array screen. In addition a further two healthy (nonremarkable) control sera were found to be positive for autoantibodies to endosulfine alpha with Western immunoblotting (Figure S4c and Table 3).

In the expanded early stage ovarian cancer group we failed to detect endosulfine alpha autoantibodies in any of the 20 patient sera by ELISA (Table 3). By contrast we detected endosulfine alpha autoantibodies in 2/20 (10%) patient samples by Western immunoblots (Figure S4c and Table 3). Of the two sera that were immunoreactive to endosulfine alpha with the Western immunoblotting one was from the original 13 patient samples screened on the hEx1 array and one was a serum sample from the additional 8 early ovarian cancer sera. In an additional unique cohort of 14 late stage ovarian cancer patients ELISA analysis identified endosulfine alpha AAb immunoreactivity in 0/14 patient sera. Western blotting identified AAb to endosulfine alpha in 7/14 (50%) of the patient sera (Table 4).

Discussion

We amongst others are interested in the application of high content protein arrays as a screening method to identify novel protein-protein interactions. In human disease the potential power of this approach is in the identification of novel protein interactions specific to the disease state, interactions not normally seen in healthy individuals. One such application of this approach is in the screening of patient serum for circulating autoantibodies present as a function of an immune response to the disease-causing

process itself. In human cancers it is generally accepted that there is a humoral immune response to tumorigenesis and that this response may arise in advance of clinically detectable disease using conventional diagnostic techniques [25, 26]. Progress in this area has been underwhelming, with numerous reports of autoantibody biomarker candidate identification but with little evidence of translation to clinical utilities.

We performed a pilot study on autoantibody identification for early and late stage ovarian cancer. Diagnosis of ovarian cancer would particularly benefit from a blood based diagnostic assay that could be employed as a routine test, given poorly defined symptoms and the low power of current diagnostic methods. We designed a screen to identify candidate autoantibody biomarkers and evaluated the effect of epitope presentation on autoantibody detection by ELISA and Western blot.

The array screening identified a panel of autoantigens from which the well known autoantigen p53 (TP53) and novel candidates, alpha adducin (ADD1) and endosulfine alpha (ENSA), were selected to evaluate the effect of epitope presentation on their potential utility in diagnostic assays.

Mutated p53 is found in 50% of human cancers [27] and of cancer patients with somatically mutated p53 up to 30% have circulating autoantibodies to p53 [12]. In ovarian cancer these autoantibodies are associated with high grade ovarian cancer which is characterised by predominantly Type II tumours [28]. Serous papillary adenocarcinoma is a Type II ovarian cancer and in our cohort of late stage serous papillary adenocarcinoma we measured an incidence of 25% of patients with p53 autoantibodies by protein array and Western blot, in line with published data [12, 28]. This serves to confirm the validity of the array screening approach taken. However, using ELISA immunoassay detection was lower at 20%. Importantly, we further identified 2/20 (10%) early stage ovarian cancer patients as p53 autoantibody positive with Western blot immunoassay. These patients were negative for this autoantibody status by ELISA. We validated the sensitivity and specificity of p53 AAb in a second unique cohort of late stage serous papillary ovarian cancer patient sera (n=14) detecting 21% positive immunoreactivity.

For the novel candidate autoantibodies studied there was higher sensitivity of detection of AAb with Western immunoblot assays. For alpha adducin we detect the highest number of autoantibody positive sera (6/20 ~ 30%) in the initial late stage

patient cohort by Western blot as compared to only 1/20 identified by ELISA . Indeed this is higher sensitivity than that for the detection of p53 autoantibodies. We further identified 3/14 ~ 21% patients positive for alpha adducin AAb in the second cohort of late stage patient sera where no positive sera were identified by ELISA. Importantly 4/20 ~ 20% of early stage ovarian cancer patients were identified as alpha adducin AAb positive by Western blot with none positive using ELISA. This highlights the potential of alpha adducin autoantibodies to perform as a powerful biomarker in early stage ovarian cancer diagnosis and also indicates that epitope presentation is a critical factor in determining this performance.

For endosulfine alpha, 2/20 early and 3/20 late stage ovarian cancer sera were positive by Western blot while 0/20 early and 1/20 late were positive by ELISA in the initial cohort tested. There was an increased sensitivity of detection of immunoreactivity by Western blot 7/14 v. 0/14 detected by ELISA for the additional late stage cohort. This high sensitivity of detection (~50%) is due to the method of epitope presentation but the improved performance is offset by the decreased specificity of endosulfine alpha AAb to ovarian cancer patients, with 2/15 control sera also positive for this autoantibody.

Allowing for the small size of the study cohort this data is compelling and clearly indicates that the presentation of the epitope is a significant factor in recognition of autoantigens from a protein array screen where the proteins are significantly denatured. The human immune response can generate antibodies to protein regions presented as discontinuous, conformational or linear epitopes. The superior sensitivity of the Western blot, for alpha adducin in particular, appears to be associated with optimal recognition of a linear epitope. It is likely that proteins expressed in E. coli, denatured and immobilised on membranes *in situ* on protein arrays, as is the case for hEx1 proteins, are highly unfolded polypeptides. Upon expression and purification of soluble protein and with subsequent dilution for coating the wells of ELISA plates there is opportunity for the polypeptide to refold, which may lead to burial of epitopes recognised by the patient autoantibodies resulting in reduced sensitivity of the assay. In contrast, Western blot involves completely denatured protein that is then transferred as a linear polypeptide to the membrane prior to incubation with the patient serum. This may enhance a linear epitope increasing the sensitivity of this assay.

In conclusion it is of great importance to consider exactly what the autoantibody is binding to when identified using high throughput protein arrays. More complete analysis of the exact epitope is merited to afford the optimal performance in diagnostic assay format. Presentation of short peptide sequences of specific antigens in diagnostic assay format has the potential to perform with a higher level of specificity and sensitivity than we have seen to date. In conclusion, the future of autoantibodies as biomarkers, particularly in human cancers, requires better knowledge of epitope recognition and superior presentation of the epitope to which the autoantibody binds.

Figure S1. Western blot probed with commercial anti-p53 antibody. A Western immunoblot image of commercial p53 protein detected with commercial monoclonal p53 antibody.

Figure S2. Comparison of Western blot detection methods. (A) Horseradish peroxidase and enhanced chemiluminescence detection on X ray film **(B)** Alkaline phosphatase and Attophos substrate detection with CCD camera.

Figure S3. ELISA coating concentration optimisation for commercial and hEx1 p53. ELISA using p53 protein, hEx1 purified p53 protein and additional hEx1 purified protein at different coating concentrations.

Figure S4. Positive Western blot images. (A) Serum positive for AAb to commercial recombinant p53 antigen. **(B)** Serum positive for AAb to hEx1 purified alpha adducin antigen. **(C)** Serum positive for AAb to hEx1 purified endosulfine alpha antigen.

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Figure legends

Figure 1. Field of hEx1 protein array showing binding of OC AAb. (i) Late stage ovarian cancer serum 71 binding to the p53 expressing clone (ii) late stage ovarian cancer serum 37 binding to the alpha adducin expressing clone and (iii) late stage ovarian cancer serum 36 binding to the endosulfine alpha expressing clone. The grid used for array scoring is outlined below with the antigen spotting pattern identified in yellow.

Figure 2. Comparison of ELISA and Western blot antigen concentrations.
(A) ELISA analysis and comparison for hEx1 p53 antigen (i) 100ng/well and (ii) 50ng/well. **(B)** Western immunoblotting analysis and comparison for the commercial p53 antigen at different concentrations (i) 200ng/lane and (ii) 50ng/lane.
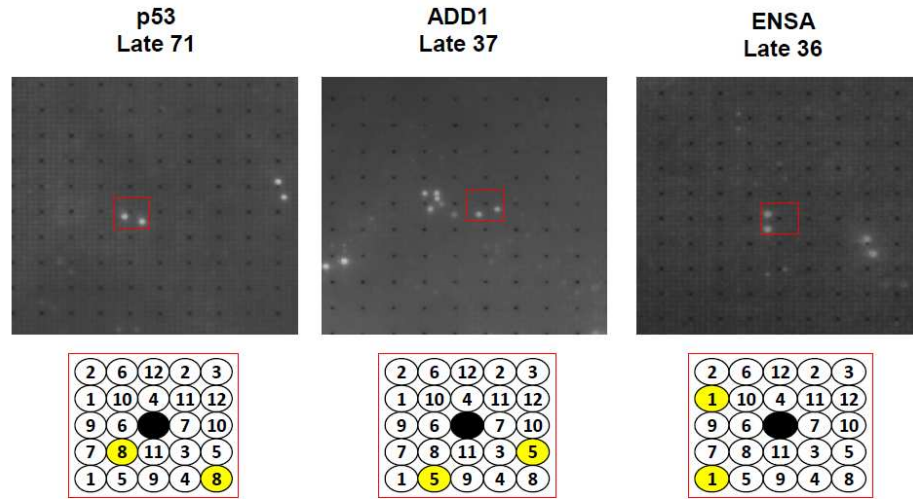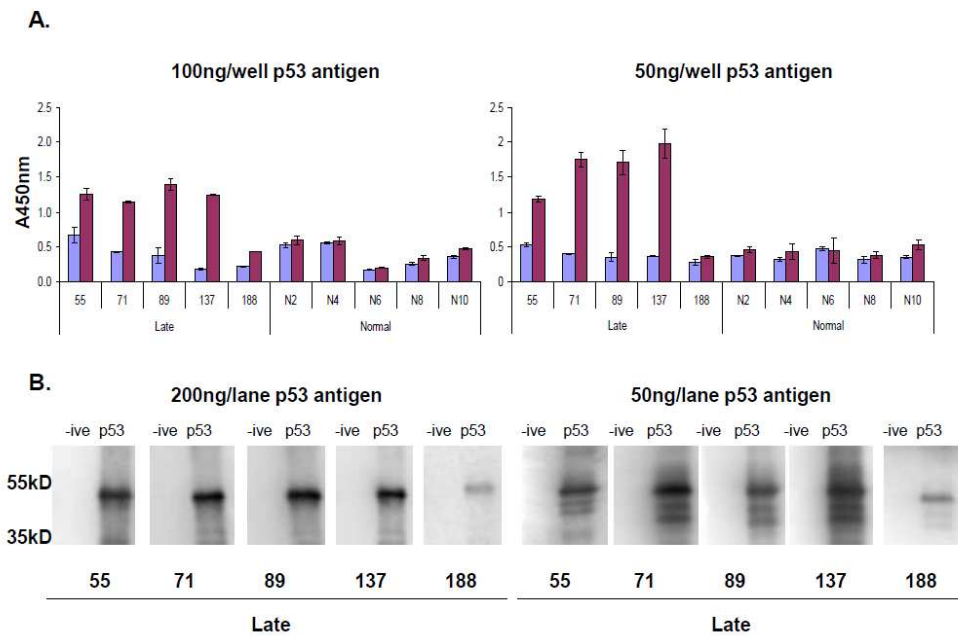
Figure 1

A.

100ng/well p53 antigen

50ng/well p53 antigen

B.

200ng/lane p53 antigen

50ng/lane p53 antigen

Figure 2

Table 1. Details of patient and control serum samples screened on the hEx1 protein array

'N' of samples	Stage	Histology	Mean Age
13	Early OC (I- II)	Mixed histology	54.4
20	Late OC (III-IV)	Serous papillary Adenocarcinoma	62.5
5	Primary Peritoneal Carcinoma	Serous histology	64.6
15	Non-remarkable	-	63.6

Table 2. Frequency of autoantibodies to the three hEx1 purified proteins in serum as determined by hEx1 array screening

	Late OC	Early OC	Peritoneal Carcinoma	Non-remarkable/Healthy
p53	5/20	0/13	0/5	0/15
ADD1	3/20	3/13	0/5	0/15
ENSA	4/20	0/13	0/5	0/15

Table 3. Comparative frequency of autoantibody detection in late stage serous papillary ovarian cancer, early ovarian cancer and non-remarkable control sera with ELISA and Western blot.

	Late OC		Early OC		Non-remarkable	
	ELISA	Western blot	ELISA	Western blot	ELISA	Western blot
p53	4/20	5/20	0/20	2/20	0/15	0/15
ADD1	1/20	6/20	0/20	4/20	0/15	0/15
ENSA	1/20	3/20	0/20	2/20	0/15	2/15

Table 4. Comparative frequency of autoantibody detection in the additional unique late stage serous papillary ovarian cancer cohort with ELISA and Western blot.
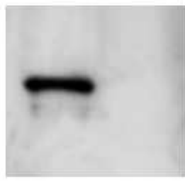
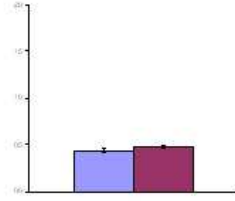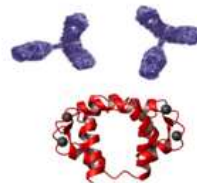|	Additional Cohort - Late OC	
	ELISA	**Western blot**
p53	3/14	3/14
ADD1	0/14	3/14
ENSA	0/14	7/14
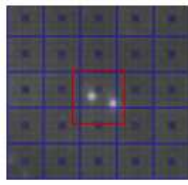
Protein Array

Western blot
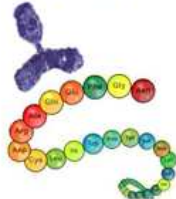
ELISA

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Graphical abstract

Highlights

- Assessment of diagnostic utility of early and late stage ovarian cancer candidate biomarkers

- Epitope presentation in validation phase as partly explaining high attrition rate of autoantigens

- Results of autoantibodies to tumour suppressor p53 & to novel Adducin &

Endosulphine alpha proteins