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Identification of novel tumor-associated antigens (TAAs) in ovarian cancer.

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Title: **Identification of novel tumor-associated antigens (TAAs) in ovarian cancer.**

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ABSTRACT

Background-aims: Circulating antibodies directed against self-antigens are a hallmark of several chronic diseases including autoimmune diseases and cancer. There is a growing need for the discovery of new autoantigens useful as biomarkers for the diagnosis, the prognosis and to guide therapeutic strategies. Here we present a high-throughput and unbiased approach to profile the immune responses in a model disease by identifying those antigens recognized by autoantibodies.

Experimental design: Our technological platform combines selection of ORF (Open Reading Frame) filtered cDNA phage display libraries and peptide microarray analyses of readouts. As an initial step, we characterised ascitic fluids from patients for their antibody response targeted against soluble, insoluble intracellular proteins as well as cell surface expressed antigen present in OVCAR cells using various immunological assay. cDNA phage display library selection was performed to isolate ORFs recognised by antibody purified from ascitic fluid of primary ovarian cancer patients which were identified as most reactive. Hundreds of selected peptides were produced and used to construct proteins microarray. These were tested with a "test panel" of ascitic antibodies from cancer patients. Candidate antigens were validated by ELISA screening on ascitic fluids from 35 primary ovarian cancer patients, 13 secondary ovarian cancer, 18 other cancer and 28 non-cancerous controls.

Result and discussion: We have identified and validated a set of different tumour-associated autoantigens that, together, define a "molecular signature" of the disease. These prospective biomarkers present in biological fluids are capable to distinguish ovarian cancer from non cancerous samples. **Therefore, our platform has a great potential for the development of innovative tools for the diagnosis, prognosis and therapeutic guidance of autoantibodies-related pathologies.**

INTRODUCTION

Ovarian cancer is the fifth most common cancer among women and is major causes of death from gynaecologic cancer. This is due to the lack of reliable and effective early detection methods, since majority of early-stage of these cancers are asymptomatic and are detected at later stages, after they have invaded the surrounding tissues. Hence, efficient biomarker for the early detection is an urgent need.

Cancer antigen (CA) -125 is the most extensively investigated and currently been used for the serum based screening of epithelial ovarian cancer (EOC). Although CA-125 is well characterized tumor biomarker specific to EOC, they have a limited ability to detect early stage of this disease and hence used to monitor the effectiveness of the treatment [1, 2]

In past years, studies have revealed the presence of circulating autoantibodies specific to a number of tumor-associated antigens (TAAs) in human [3, 4]. These tumor-associated antigens absent in a normal cell may elicit a very early host immune response in course of tumor development, this feature could be made use to develop biomarkers which can detect autoantibodies which are stable and abundant even at the early stage of tumor [5]. Thus, identification of these TAAs could leads to the discovery of important molecular pathways involved in the development of ovarian cancer, and may give insights regarding the onset of tumorigenesis and therapeutic strategies. Moreover, TAAs has been already demonstrated to be an effective and sensitive means of cancer screening and diagnosis [6]. Using TAAs as biomarker for cancer could be superior of choice, since they are capable of measuring varying level of immune response in reasonably short time. Despite from all these advantages, these TAAs can be used for targeted immunotherapeutic treatments and

might required for vaccine development in the far end of this study [7]. Very few tumor-associated antigens have been identified to-date, offering a promising area yet to be excavated.

Our strategy initially characterised ascitic fluids from various disease condition (Primary/ Secondary ovarian cancer/ other cancer and non-cancerous sample) for their antibody response targeted against soluble, insoluble intracellular proteins as well as cell surface expressed antigen present in OVCAR cells using various immunological assay which includes western blot, whole cell ELISA, cell lysate ELISA, cell surface ELISA and immunofluorescence assay. Thus to identify the most reactive sample which could be used for phage display selection to screen out those peptide displayed phages which are recognised by immobilized antibody present in these ascitic fluids. Western blot analysis of OVCAR cell extract has proven to show the occurrence of cancer specific antibodies. The prevalence of antibody present in cancerous and non cancerous sample targeted against soluble/ insoluble intracellular proteins in their non denatured form present in OVCAR cells were examined by whole OVCAR cell ELISA and was confirmed OVCAR cell lysate ELISA. Cell surface ELISA and immunofluorescence assay were performed for the identification and localization of OVCAR cell surface antigen expression.

Expression library screening especially phage display of cDNA libraries from appropriate tissue sources has proved to be a useful strategy for identification and isolation of novel tumor associated antigen. They have been relied for studies of many different cancer types including ovarian cancer. Unlike the other conventional immunoscreening approaches, phage display technique is much faster as well as more cost and labour effective, requiring no special equipment. Phage display is a system for the high throughput analysis of protein interactions by selecting peptides, proteins, or antibodies with specific binding properties from a pool of variants and uses bacteriophage for the coupling of phenotype to genotype. Comparatively filamentous phage display are much competent than any others and can produce 10^{10}-10^{11} individual clones, each representing a single native protein or protein fragment. Here, cDNAs are expressed as fusion proteins with one of the phage coat proteins and exposed on the surface of the phage thus allowing the selection with antibody from acitic fluid collected from ovarian cancer patient which were identified as most reactive [4-13]. Protein microarrays analysis is well suited for the immunoscreening of these selected antigens for their response to autoantibody. [5, 10-11, 14]. Further these microarray identified antigens were validated by ELISA, since these immuno techniques are able to detect the presence of specific antibody even at very low concentration [15].

In this study we have developed a high-throughput protein expression and screening platform that combines cDNA phage display library selection and proteins microarray approach was used to identify novel TAAs. Phage libraries of open reading frame (ORF) fragments created from mRNA derived from various tissues were used. Ovarian cancer are characterised with accumulation of ascitic fluid in peritoneal cavity. Ascites may have antibodies against the proteins or peptides secreted or leaked from tumorous tissue since they are closer contact with the disease site [15, 16]. A previous work carried out in Trieste laboratory has already been demonstrated that the ascites derived from patients with ovarian cancer are able to activate a mechanism of antibody-dependent cellular cytotoxicity (ADCC) and especially, to activate complement as a mechanism of cell lysis (complement-dependent cytotoxicity). Cancerous ascites and non-cancerous ascite collected from liver cirrhosis patient with no history of cancer were compared to normal serum complement activity. A clear difference with the greater activity of the samples obtained from patients with ovarian cancer was evident from the data obtained. Two rounds of selection were performed to screen out those peptide displayed phages which are recognised by immobilized antibody present in the ascitic fluid from Primary ovarian cancer patients but absent in healthy controls. These selected clones were expressed as GST-fusion proteins and screened for their immunoreactivity by protein microarray analysis. The most reactive peptides were identified by sequencing, 6 predicted autoantigens were expressed and further evaluated. Presence of cancer specific antibody in ascitic fluids (35 primary ovarian cancer patients, 13

secondary ovarian cancer, 18 other cancer and 28 non-cancerous controls) against these identified antigens were tested by indirect antibody sandwich ELISA.

Our approach enables to identify prospective candidates for cancer specific protein biomarkers, so far we have identified and validated six different tumour associated autoantigens, i) CREB3 (Cyclic AMP-responsive element-binding protein 3) ii) MRPL46 (Mitochondrial ribosomal protein L46) iii) BCOR (BCL-6 corepressor) iv) HMGN2 (High mobility group nucleosomal binding domain 2) v) HIP1R (Huntingtin interacting protein 1 related) vi) EXOSC10 (Exosome component 10) and are capable to distinguish ovarian cancer from non cancerous samples. Therefore, our platform has a great potential for the development of innovative tools for the diagnosis, prognosis and therapeutic guidance of autoantibodies-related pathologies.

MATERIALS AND METHODS

Patient samples and cell lines.

Ascitic fluid samples were collected from 94 patients diagnosed with primary (35), secondary (13) ovarian cancer, other cancers (18) (Department of biology, University of Trieste and Istituto Tumori, Milan) and 28 control ascitic fluid from female patients with no known history of cancer were obtained from the Department of Clinical and Experimental Medicine, University of Eastern Piedmont. All ascite samples were centrifuged at 11000 RPM at 4^{o}C for 5minutes and supernatants were stored at -80^{o}C until processing.
Immunoglobulins from all acitic fluid were affinity purified using Protein A Agarose (Roche). 50µl of crude ascitic fluid was mixed with starting buffer containing 100mM TRIS-HCl pH 8, 1% IGEPAL CA630,1mM EDTA,1mM PMSF, 1:100 dilution of protease inhibitor, 40µl Protein A agarose in a final volume of 1ml. Incubated overnight at 4^{o}C on a rotating platform. Washed and eluted to an equal volume of sample used with 100mM glycine pH 2.5, later added 20% 1M TRIS –HCl pH 8 for stability.

Cell lines

The human ovarian adenocarcinoma cell line (OVCAR) was obtained from (Molecular Pathology Lab, University of Eastern Piedmont). Cell lines were grown in RPMI 1640 media containing 10% fetal calf serum, 2mM glutamine, and 1% penicillin/streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing CO2.

Western Blot of OVCAR Cell Extract

Western blot was performed to confirm the presence of tumor specific antigens present in OVCAR cell extract. OVCAR cell extract was prepared under denaturing condition using 8M Urea. After 1hr blocking step with 4%MPBST, 1:50 dilution of antibodies isolated from ascites fluid from 20 patients were used as the source of primary antibodies. These samples were diluted in 2% MPBST and incubated at RT for 45min. Membranes were incubated 1hr 30min at RT and followed by 1 hr incubation with secondary antibody, alkaline phosphatase conjugated anti-human IgG. The membrane was developed using chromogenic substrate (NBT/BCIP, Roche).

Whole Cell-ELISA

OVCAR cells were seeded in 96-well culture plates (Cellstar 3596; greiner bio-one) at a density of 1×10^4 cells/100 µl/well and incubated 24 hrs at 37 °C in presence of CO_2. The plates were subsequently washed 3 times with wash /blocking buffer (1M $MgCl_2$, 1M $CaCl_2$, BSA (FC 2%) in PBS 1X), fixed and permeabilize with chilled 99.8% methanol (pre -incubated at -20^{0}C). After incubation on ice for 5min, the fixative methanol was discarded and washed twice with wash /blocking buffer. The plates were incubated for

1 hr 30 min on ice at 4^0C with 100 µl pre-blocked primary antibody (Purified and normalised Ascitic IgG)/well which were diluted 1:100 in wash /blocking buffer. The plate was subsequently washed 3 times with wash /blocking buffer and incubated for 1 hr on ice at 4^0C with 1:2000 dilution of peroxidase-conjugated secondary antibody in wash /blocking buffer (Polyclonal Rabbit Anti-Human Immunogulobulins/HRP; Dako). Performed 3 washes and dried. 70µl of TMB (3,3′,5,5′-Tetramethylbenzidine liquid substrate, Sigma) was added to each well and dark incubate on ice until proper intensity was observed. This TMB was transferred into a new ELISA plate already containing 35µl 1N H_2SO_4 to stop the enzymatic reaction. The absorbance at 450 nm was measured with a microplate reader. The cutoff value was calculated as cumulative mean + 2 (standard deviation) of the absorbance from non cancerous control wells.

Cell Lysate ELISA

Cell extraction procedure: Fully confluent OVCAR cell line in a 100mm tissue culture plate was washed gently with ice cold PBS. Cells were scraped out into pre-chilled tube by adding 0.5 ml of complete non denaturing extraction buffer containing 100 mM Tris, pH 7.4 ; 150 mM NaCl; 1 mM EGTA; 1 mM EDTA; 1% IGEPAL; Protease inhibitor cocktail p8340 and 1 mM PMSF. Sonicated the cells briefly after incubation on ice for 15-30 min. The soluble cell extract were transferred into new tube following centrifugation at 13,000 x rpm for 10 min at 4°C and the concentration was determined by BCA assay.

Cell Lysate ELISA: 96 well microtiter plate O/N coated with 3.2µg/well of OVCAR cell extract was blocked with 2% BSA in PBST (0.05%) for 1hr at 30°C. The plate was incubated for 1 hr 30 min at *30^oC* with 100 µl primary antibody (1: 50 diluted purified ascitic IgG preblocked for atleast 30 min*)*. The unbound antibodies were removed by washing with PBST (0.05%)and PBS 1X. Incubated for 1hr at 30°C with 1:5000 diluted anti-human IgG/horseradish peroxidase conjugate in each well and subsequently washed. 70 µL of 3,3′,5,5′-Tetramethylbenzidine substrate solution was added and incubated in dark until a proper intensity of colour was observed. The reaction was stopped by adding 35 µL/well of 1 N H_2SO_4. Plates were read spectrophotometrically at 450 nm.

Cell Surface ELISA

Added 100µl of 1x 10^4 OVCAR cells into each well of the 96-well microplates and incubate in 5% CO2 at 37° C in a cell culture incubator for 36hrs. The culture medium was removed from every well and washed once with 100µl RPMI medium containing 10 % FBS (pre warmed at 37^o C). Primary antibody (Purified and normalised Ascitic IgG) were diluted 1:100 in RPMI medium with 10% FBS and ensured proper mixing atleast 30 min at RT. 100 µl of this pre-blocked primary antibody along with 1:200 diluted anti folate receptor antibody (positive control for the assay) were transferred into their appropriate wells in triplicate and incubated 1h 30min at 37^o C in presence of CO_2. The plates were washed thrice with pre warmed RPMI medium and incubated for 1hr at 37^o C in presence of CO_2 with 1:2000 dilution of peroxidase-conjugated secondary antibody in RPMI medium (Polyclonal Rabbit Anti-Human Immunogulobulins/HRP; Dako). A triplicate negative control wells incubated with only secondary antibody was included thus to reduce the non specific signal from the final reading. 3 washes were performed with RPMI medium and once with PBS 1X. Pipette 70µl TMB/well (3,3′,5,5′-Tetramethylbenzidine liquid substrate, Sigma) and dark incubate at RT until proper intensity was observed. This TMB was transferred into a new ELISA plate already containing 35µl 1N H_2SO_4 to stop the enzymatic reaction. The absorbance at 450 nm was measured with a microplate reader. The cutoff value was calculated as cumulative mean + 2 (standard deviation) of the absorbance from non cancerous control wells.

Immunofluorescence Assay

5 x 10^4 OVCAR cells were plated onto poly-L-lysine-coated glass coverslips in a 10cm cuture plate without any overlap. The culture was incubated at 37°C in presence of CO_2 until they attains 80% confluence. This culture plate was washed once and the cover slips were transferred individually to new wells of 24 well culture plate. Two different sets of experiments were performed with these cells.

1) **OVCAR Cells Surface Staining:** The confluent cells on the cover slips were treated with Brefeldin A , an inhibitor of endocytosis, protein synthesis and transport of secretory protein. 10µg/ml concentration of Brefeldin A was prepared in RPMI medium containing 10% serum and incubated with the cells for 5hrs at 37°C. Cells were washed once with plain RPMI and 1: 10 diluted primary antibody in blocking buffer (5% BSA in serum free RPMI medium) was added. Incubated on ice at 4°C for 2 hrs. After primary incubation, the unbound antibodies were removed by washing 3 times with blocking buffer. The cells transferred to new well and were subsequently incubated for another 1 hr on ice at 4°C with Secondary antibody (anti hIgG- cy5) diluted 1:200 in blocking buffer. Performed 2 washes, transferred them to new wells in which the cells were fixed with 200µl of 4% PFA + 4% Sucrose on ice for 15 min, and permeabilised the cells for 2-3min 0.2% triton in PBS 1X. The fixed and permeabilized cells were incubated with 1:2000 dilution of propedium iodide (PI: nuclear stain) in PBS 1X. Cells were washed thrice with PBS 1X and once with dH_2O, mounted to a glass slide with a small drop of mountant and the cells on cover slip facing the mountant and sealed with nail polish. The cells were directly analyzed with a Leica TCS-SP confocal laser scanning microscope using sequential scan tool. Laser power and photomultiplier settings were kept identical for all the samples to be able to compare the results.

2) **OVCAR Cells Starved And Antibody Internalisation:** The confluent cells on the cover slips were let to starve an overnight, thus to compare the specific antibody internalisation by OVCAR cells. The cells were washed twice with prewarmed phosphate-buffered saline (PBS) 1X and cultured in serum-free RPMI medium overnight at 37°C with CO_2. Cells were washed once with plain RPMI and 1: 10 diluted primary antibody in blocking buffer (5% BSA in serum free RPMI medium) was added. After 2hr incubation at 37°C, the unbound antibodies were removed by washing 3 times with blocking buffer. The cells transferred to new well and were subsequently incubated for another 1 hr 37°C with Secondary antibody (anti hIgG- cy5) diluted 1:200 in blocking buffer. Performed 2 washes, transferred them to new wells in which the cells were fixed with 200µl of 4% PFA + 4% Sucrose on ice for 15 min, and permeabilised the cells for 2-3min 0.2% triton in PBS 1X. The fixed and permeabilized cells were incubated with 1:2000 dilution of propedium iodide (PI: nuclear stain) in PBS 1X. Cells were washed thrice with PBS 1X and once with dH_2O, mounted to a glass slide with a small drop of mountant and the cells on cover slip facing the mountant and sealed with nail polish. The cells were directly analyzed with a Leica TCS-SP confocal laser scanning microscope using sequential scan tool. Laser power and photomultiplier settings were kept identical for all the samples to be able to compare the results.

Selection of cDNA Phage Display library

Phage selections were performed as described in Di Niro et al, 2009 [17]. Bacterial stock of library (Human ORF libraries from pancreatic islets (HPI), colon cancer (HCC) and lung fibroblasts (HF)) was grown and infected with wild type helper phage (M13k.07) for replication and recombinant phages were collected. Phage selection and enrichment using G-protein functionalised magnetic beads (dynabeads) coated with ascite IgG. First a pre-clearing step for the removal of the non-reacting phages by incubated with negative bead preparation, ie coated with IgG healthy serum to avoid any sort of background. This was followed by 2

rounds of selection to enrich bound peptide sequence [**Fig 8A**] by separate incubation with seven different IgG purified primary ovarian cancerous ascites (3,14, 18, 12, 39,41,51 in 1:100dil).

After immunoscreening, the selected cDNA fragments were subcloned into an expression vector, a modified pGEX Vector and amplified in the host bacteria_BL21 (DE3) RIPL cells for enhanced protein production. 95 random clones from each of the 7 selections were randomly picked and inoculated into 96 deep well plates inorder to produce GST fusion protein. Protein production was carried out by auto inducing medium ZYM_{5052} in 96 wells format. Purification of recombinant protein was done using GSH magnetic bead. The production and purification of this fusion protein were analysed by coomassie staining and western blot with few random samples. Full length fusion protein were identified by incubating nitrocellulose membrane with 1:5000 dilution of αFLAG primary antibody and screening with alkaline phosphatase-conjugated anti-human IgG secondary antibody.

Microarray Analysis

The immunoreactivity of the phage display antigen library was determined by microarray analysis. With the aid of BioOdissey Calligrapher (Biorad) all the purified GST-fusion proteins were spotted onto a nitrocellulose slide. The proteins along with the controls were printed in duplicate in two identical fields of the same slide maintaining all parameters (such as contact time, distance between spots, wash cycle) and conditions including 15^{o}C temperature and 50% humidity. The slides were incubated for 1hr at room temperature (RT) with blocking buffer (3% non fat dry milk in PBS 0.1% Tween20 (3%MPBST)). Once the sides were saturated, each of the 2 identical fields was incubated with 1:50 dilution for primary antibody (Acitic fluid form cancer patients, non cancerous patients, also anti-GST and Anti-FLAG monoclonal antibody against the fusion Tag) in 2%MPBST for 1hr 30min. The slide assayed with Anti-GST and Anti-FLAG ensures that the proteins a properly spotted on to the slide. Performed 2 washes with PBST for 15 min and once with PBS. Added 1:200 diluted Secondary antibody, Cy5 conjugated anti-Human IgG - Cy5 conjugated (anti-mouse IgG for the slide incubated with anti-GST and Anti-FLAG) in 2% MPBST and incubated at RT for 1 hr. Cy5 is highly sensitive even with very low amount of autoantibody and stabile fluorophore which gets exited at 650 nm wavelength. Washed twice with PBST and once with PBS, 5min for each washing step and dried. All fluorescent signals were detected with the ScanArray Gx (PerkinElmer) scanning device. The sample giving highest florescent signals were selected for next level.

Antigen characterization

The most immunoreactive clones identified by microarray analysis were further processed to decode their sequence. As the first step, these screened clones were PCR amplified using pGEX sense (GGGCTGGCAAGCCACGTTTGGTG) and anti sense primers (GGTGAAAACCTCTG ACACATGCAGCTCCCGG). The PCR amplified products were purified using nucleo spin extract kit II (Macherey_Nagel) and the DNA fragment of varying size (150-800bp) were sequenced. Under optimised condition, a fluorescence based cycle sequencing reaction was performed with the purified PCR amplified fragments/template along with its sequence specific primer (pGEX sense or anti sense) and ABI BigDye Terminator v1.1. The extension products were purified using Centri-Sep Columns (Princeton separations) based on gel filteration. The reaction mixture was added directly on top of the hydrated illustra sephadex fine DNA grade (GE healthcare) gel bed and spun down at 3600RPM. This was done to remove all the free and labelled dNTP's and unwanted buffer salts. The sequencing was carried out using ABI 3100 sequencer.

Western blotting

Western blots were performed using purified GST fusion protein. Membrane was blocked for 1 h at room temperature using 4% MPBST. Then incubated 90 min with 1:5000 dilution anti-FLAG/ anti-GST primary antibody in 2%MPBST. Subsequently were washed using PBST (0.1% v/v) and PBS 1X. Anti mouse IgG coupled to alkaline phosphatase (1:5000 dilution in 2%MPBST) was used as the secondary antibody. Membrane was developed by adding NBT/BCIP substrate.

Enzyme linked Immunosorbent assay (ELISA)

Presence of a specific antibody in ascitic fluids were tested by indirect antibody sandwich ELISA. 96 well ELISA plate was pre coated (O/N at 4°C) with 1µg of purified full length GST fusion protein in 100 µL of PBS was saturated with blocking buffer (2% BSA in PBST(0.05%)) for 1 hr at 30°C.1: 50 diluted primary antibodies in blocking buffer were incubated at RT for 45 min enabled with proper mixing and were subsequently transferred into their appropriate well and incubated at 30°C for 90 min. Primary antibody used were affinity purified IgG from 94 ascitic fluid which includes 35 primary ovarian cancer/ 13 secondary ovarian cancer/ 18 other cancer/ 28 non cancerous control. After incubation at 30^{o}C, the unbound primary antibodies were removed by washing 3 times with PBST 0.05% and PBS 1x. Incubation at 30°C for 1hr with 1:5000 dilution of peroxidase-conjugated secondary antibody in 2% blocking buffer. 70 µL of 3,3′,5,5′-Tetramethylbenzidine was added to all well after final wash (3 times) with PBST(0.05%) and PBS 1x. The plate was incubated in dark until they have attained proper intensity of colour. The reaction was stopped by adding 35 µL/well of 1 N H_2SO_4. Plates were read spectrophotometrically at 450 nm. For each antigen validation, the cutoff value was independently calculated as 'sum of (cumulative mean + twice the standard deviation) of the absorbance from non cancerous control wells.

Cross inhibition assay

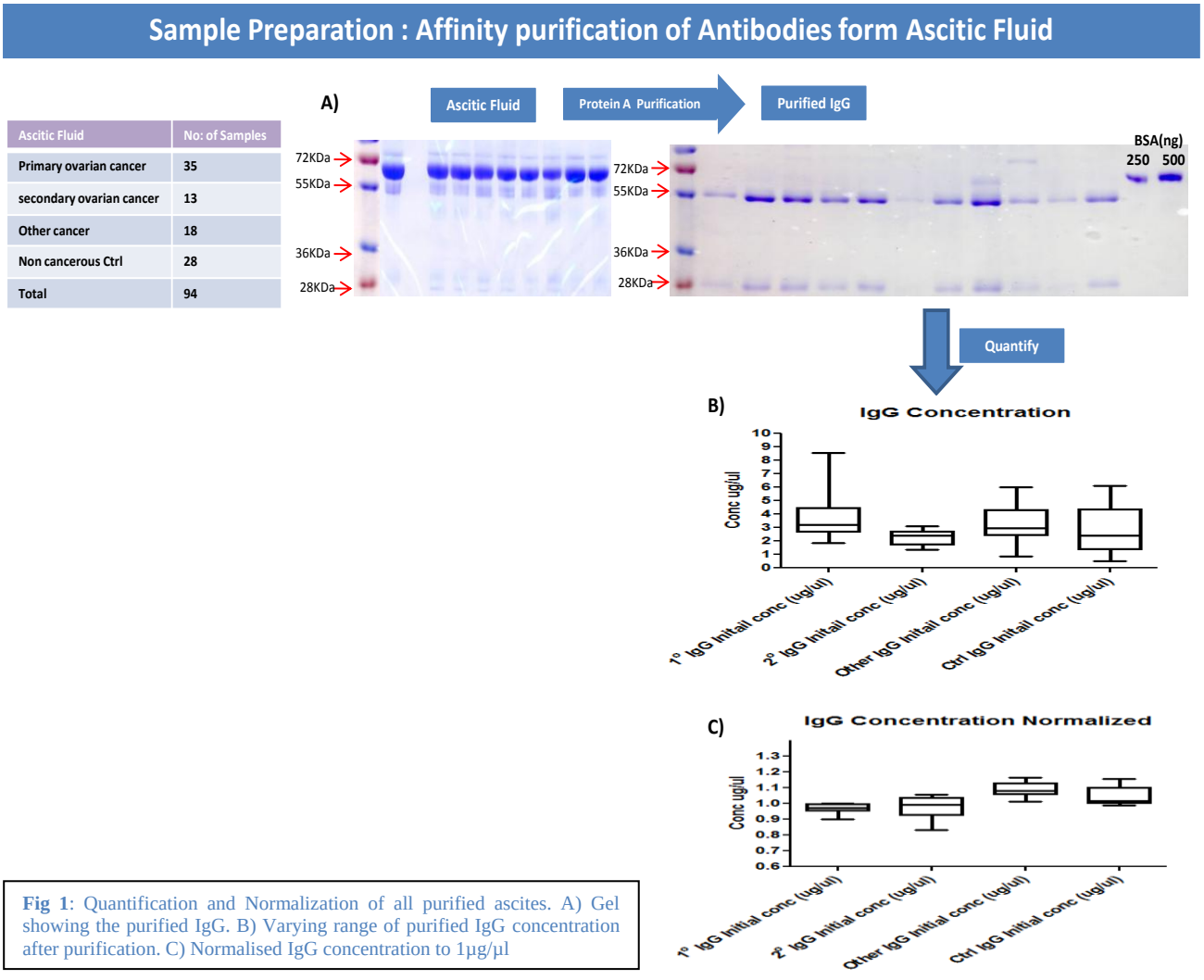
80% confluent OVCAR cell were fixed with a 1:1 mixture of 4%paraformaldehyde (PFA) and 4% sucrose in PBS at RT for 20min. These fixed cells were permeabilized by 3min incubation with 0.02%TX-100 and washed with PBS 1X. Blocked for 1hr at RT with 1% BSA. 1 control and 2 different IgG purified ascites which has been identified most reactive to the antigenic proteins were chosen as primary antibody. 2 different set up were prepared for each test sample. i) Normal IgG sample, ii) IgG preincubated with combination of antigenic protein (atleast 1hr at RT). These primary antibodies were incubated separately with preblocked OVCAR cells for 1hr at RT. Removed the unbound antibody by washing with PBS 1X and incubated 1 hr with 1:200 Cy5 conjugated anti-human IgG. Washed and added 1:2000 dilution of propedium iodide (Nuclear stain) and incubated for 2min at RT. Dried after the final wash and mounted on a slide. Images were captured using Confocal microscope.

RESULT AND DISCUSSION:

We applied cDNA phage display library system and protein microarray to identify targets involved in humoral and cell mediated immunity in ovarian cancer. After the phage display selection rounds, the immunoreactivity of the screened antigenic proteins from the display library were determined by protein microarray analysis. Further, the identified TAAs were validated by ELISA.

Sample processing

Ascites from 94 patients including 28 controls were collected from cancer and non cancerous patients. The IgG fractions of the ascitic fluids samples were purified with Protein A, according to the manufacturer's instructions. All IgG purified ascites were quantified using both nanadrop and ImageJ (measures the intensity of coomassie staining band in pixels). Quantified IgG were normalised to 1µg/µl as shown in **figure: 1** and were used for the proceeding experiments.



Ascitic Fluid	No: of Samples
Primary ovarian cancer	35
secondary ovarian cancer	13
Other cancer	18
Non cancerous Ctrl	28
Total	94

Fig 1: Quantification and Normalization of all purified ascites. A) Gel showing the purified IgG. B) Varying range of purified IgG concentration after purification. C) Normalised IgG concentration to 1µg/µl

Western Blot of OVCAR cell extract

The occurrence of antibodies to any specific antigenic protein in OVCAR cell extract was investigated by western blot. An equal amount of OVCAR cell lysate was loaded into each well. Recognition of the antigen was compared between 2 IgG purified cancerous acites and 2 controls. The result (**Fig 2**) shows the variation in amount of auto antigen present against specific antigenic protein in OVCAR cells.

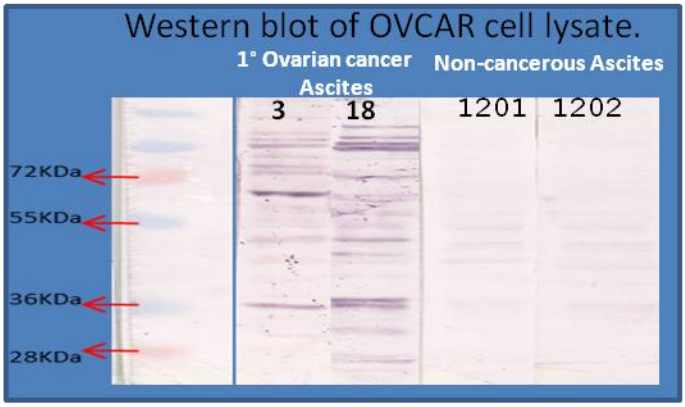


Fig 2: Western blot of OVCAR cell lysate. Each strip contains 15µg of OVCAR lysate and were incubated separately with IgG purified ascites. Samples 3 and18 form primary ovarian cancer patient. 1201-1202 are non cancerous controls.

OVCAR whole Cell ELISA

Whole cell enzyme-linked immunosorbent assay is convenient method that can accurately quantify both soluble as well as insoluble intracellular proteins/ tumor associated antigens in cultured adherent cells as well as a possible alternative to immunohistochemical characterization of tumors [18]. The OVCAR cells were grown in and fixed to the bottom of a 96-well plate. Targets of interest are detected by primary antibodies purified form ascitic fluid, which are in turn are quantified with α-human immunoglubulin labelled secondary antibody.

The cell-ELISA experiments were performed in triplicate for each sample and the results were reproducible. As far as immuno reactivity was concerned, we detected 20/35 IgG from Primary and 11/12 from secondary ovarian cancer samples to be positive and recognizes their target present in OVCAR cells. While IgG from other cancer types were less reactive and detected to be 6/18. Except 1/28 non_cancerous samples, others were shown to be below the cutoff value and have no tumor specific antibody.

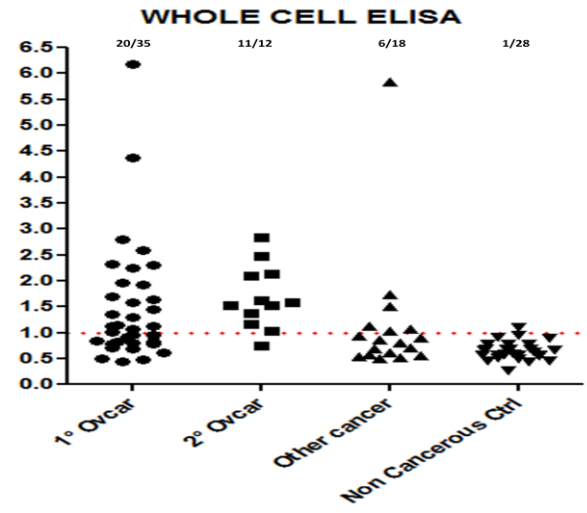


Fig 3 Whole cell ELISA: Fixed and permeabilized OVCAR cells were incubated with 1:100 dil of purified IgG from 1°, 2° Ovarian cancer ascites, other cancers and non cancerous controls. 1:2000 dil of detection antibody αhIgG-HRP was added. Developed using TMB substrate and stopped with 1N H_2SO_4. OD was measured at 450nm.

OVCAR Cell Lysate ELISA

A total Cell lysate ELISA of OVCAR cell line was performed as a confirmatory assay for the whole cell ELISA. With an intention to examine the prevalence of antibody present in test and control sample targeted against soluble intracellular proteins/ TAA's in OVCAR cells. The cell extract was prepared under non denaturing conditions and the soluble fraction was quantified by BCA. The microtiter plate coated with the soluble fraction of OVCAR cell extract (3.2µg/well) were challenged with 1: 50 diluted human IgG (1 µg/µl) from 35 primary ovarian cancer patient , 13 secondary ovarian cancer patient, 18 patients having other cancers and 28 non cancerous control. Resulting in the specific antibody sandwiched between the immobilized antigen and enzyme conjugate anti-human antibody. Substrate was added to measure the amount of antibody targeting /bound specifically against/to antigens present in the soluble fraction of OVCAR cell extract and read the optical density at 450nm. It was clear from the result (**Fig 4**) that presence of antibodies particularly in primary ovarian cancer sample (29/35) which recognizes TAA's as compared to secondary ovarian cancer, other cancer or Non cancerous control (0/13, 0/18 and 3/28 respectively)

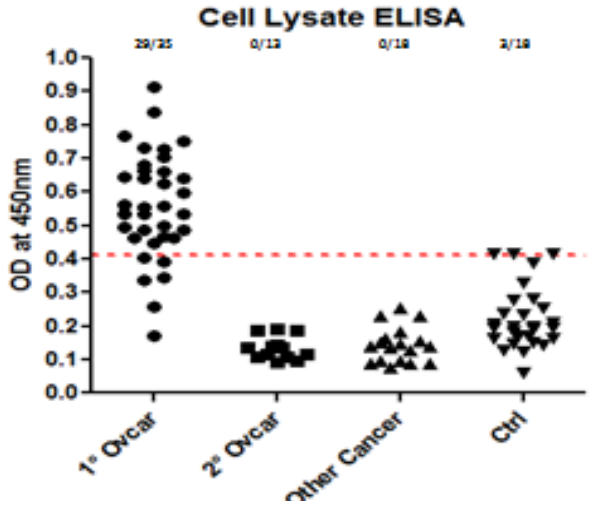


Fig 4 Cell Lysate ELISA: .O/N coated the microtiter palate at 4° C with 3.2µg/well of OVCAR cell Lysate. Blocked with 2% BSA in PBST (0.05%). Incubated with 1: 50 diluted purified primary IgG for 1 hr 30min at 30° C. 1hr at 30° C with 1:5000 diluted secondary αhIgG-HRP was added to each well. Added TMB substrate and stopped wit 1N H2SO4. Read at 450nm.

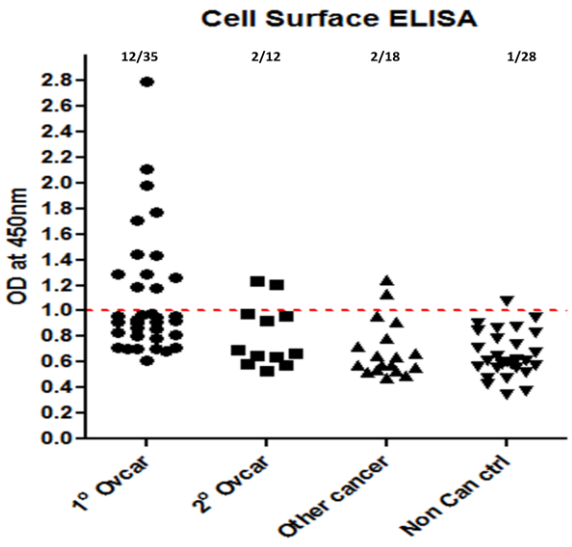


Fig 5 Cell Surface ELISA: OVCAR cells were incubated at 37° C in presence of CO_2 with 1:100 dil of purified IgG from 1° , 2° Ovarian cancer ascites, other cancers and non cancerous controls. 1:2000 dil of detection antibody αhIgG-HRP was added. Developed using TMB substrate and stopped with 1N H_2SO_4. OD was measured at 450nm.

Cell Surface ELISA

OVCAR cells were diluted to 1×10^4 cells/100 µl in RPMI medium and were cultured in triplicate to wells of a 96-well culture plates (Cellstar 3596; greiner bio-one) and incubate in presence of 5% CO2 at 37° C. 80% confluent cells were incubated with 1:50 dilution of primary antibody (affinity-purified antibodies from primary ovarian, secondary ovarian cancer, other cancer and non-cancerous control). Later detection antibody was added to determine the presence of cell surface antigen. The results indicated that all 12/35 affinity-purified antibodies from primary ovarian cancer patients had reactivity towards OVCAR cells surface antigen. Antibodies from secondary ovarian cancer (2/12) and other cancer patients (2/18) had comparatively less reactivity. The only positive (1/28) non cancerous control produced a weak signal as compared to all the other groups, leaving rest (27/28) below the cut off.

Cell surface ELISA is an immuno-enzymatic technique for the identification and localization of target antigens as well as the quantitative analysis of cell surface antigen expression. This technique allows for the quantification of immunoreactive antibodies present in samples targeted specifically against tumor associated antigens expressed by immobilized/ adherent OVCAR cell on their cell surface [18].

Cell surface Staining: Brefeldin A, an endocytosis inhibitor and effect of low temperature.

Living OVCAR-3 cells were incubated for 5 hrs at 37°C with the endocytosis inhibitor brefeldin A [19-21] in the incubation medium thus to inhibits the uptake of antibody. To exclude the possibility that cellular uptake of antibodies in the presence of endocytosis inhibitors, incubations were performed at 4°C which, only resulted in binding to the cell membrane since cellular uptake does not occur at this temperature. Fluorescence image shows the surface localization in OVCAR cells which were incubated with ascitic antibody at 4°C, followed by secondary antibody conjugated to Cy5 as described in materials and methods. Membrane-bound fluorescence was detected and directly thereafter visualised using confocal laser scanning microscopy. (A, B) Image A and B represents cells incubated with cancerous ascites (46 and 44 respectively). The cell surface localization of tumor specific antibody was detected with α-human immunoglubulin conjugated to Cy5 (in blue). Nucleus were stained with Propedium iodide (in red), the phase contrast image shows the cell morphology and finally the merged image. (C) Cells incubated with non Cancerous ascite. (D) Cells incubated with healthy human IgG. (E) Positive control humanised α-Folate antibody. (F) Negative control with no primary to find non specific binding of secondary antibody.

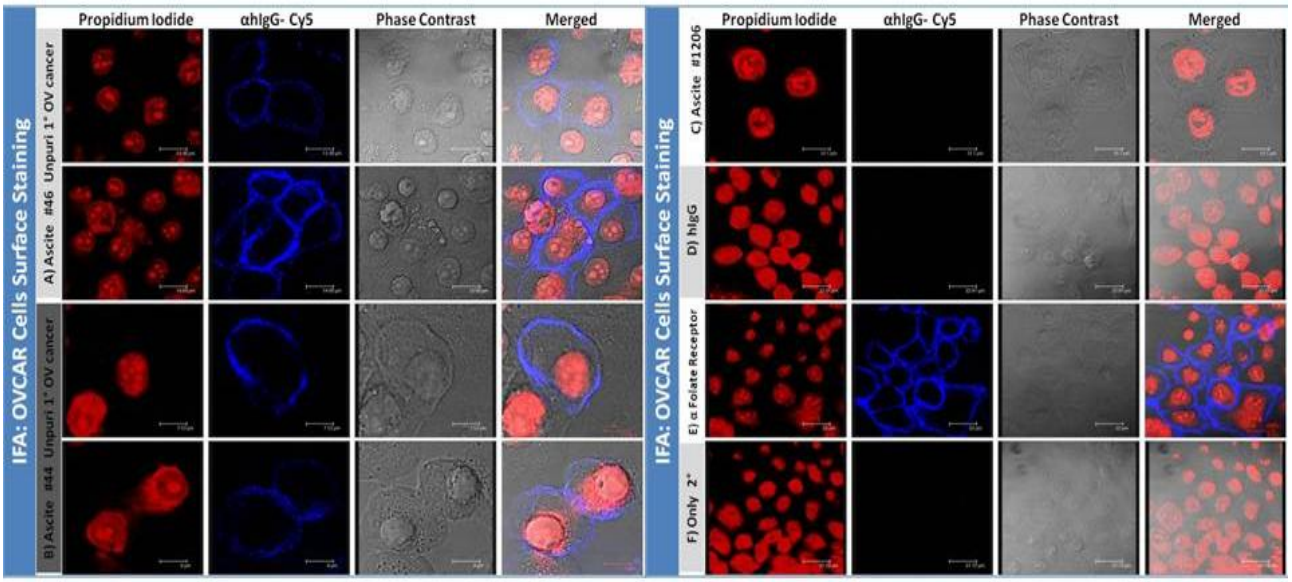


Fig 6 Cell Surface Staining: (A, B) Image A and B represents cells incubated with cancerous ascites (46 and 44 respectively). The cell surface localization of tumor specific antibody was detected with α-human immunoglubulin conjugated to Cy5 (in blue). Nucleus were stained with Propedium iodide (in red), the phase contrast image shows the cell morphology and finally the merged image. (C) Cells incubated with non Cancerous ascite. (D) Cells incubated with healthy human IgG. (E) Positive control humanised α-Folate antibody. (F) Negative control with no primary to find non specific binding of secondary antibody.

Internalisation of Antibody

Antibody uptake in OVCAR cells were revealed by indirect immunofluorescence with double staining (**Fig 7**). Primary antibodies were added to non-permeabilized live OVCAR after overnight serum starvation. (A, B) Image A and B represents cells incubated with cancerous ascites (46 and 44 respectively). The up take of tumor specific antibody by the cell was detected with α-human immunoglubulin conjugated to Cy5 (in blue). Nucleus were stained with Propedium iodide (in red), the phase contrast image shows the cell morphology and finally the merged image. (C) Cells incubated with non Cancerous ascite. (D) Cells incubated with healthy human IgG. (E) Positive control humanised α-Folate antibody. (F) Negative control with no primary to find non specific binding of secondary antibody.

To determine whether OVCAR cells can take up specific antibody, we examined acitic IgG staining of live, non-permeabilized OVCAR cells using an indirect immunofluorescence assay. Live cells that were serum-starved have been already known to show higher efficacy in taking up antibody into the cells [22, 23]. When OVCAR cells were serum-starved overnight, the living, non-permeabilized OVCAR cells were able to internalise antibodies with higher efficiency. The indirect immunofluorescence double staining confirms that the antibody can access into cells internally. The data indicate that cancerous ascitic IgG are still able to either partially or completely penetrate into non-permeabilized cancer cells. Where as, cells incubated with either acitic fluid form non-cancerous patient or healthy human IgG failed to produce any signal of internalisation. The results suggest that the presence of tumor specific antibody targeting intracellular proteins. The precise mechanism by which these antibodies getting internalised has to be further investigated.

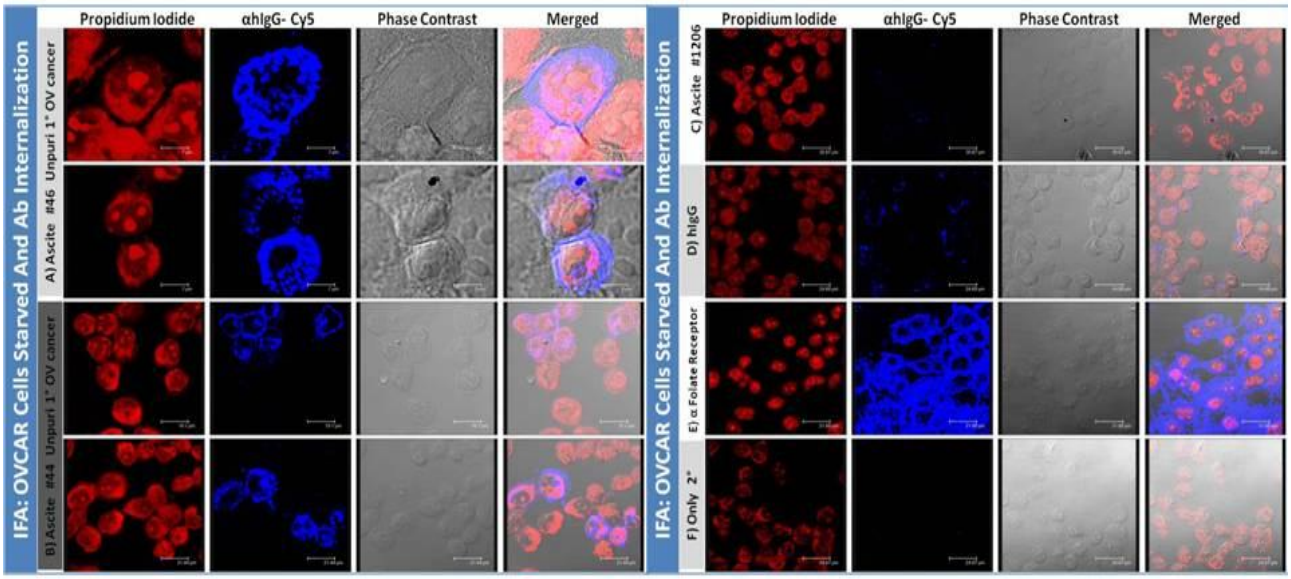


Fig 7 Internalisation of Antibody: (A, B) Image A and B represents cells incubated with cancerous ascites (46 and 44 respectively). The up take of tumor specific antibody by the cell was detected with α-human immunoglubulin conjugated to Cy5 (in blue). Nucleus were stained with Propedium iodide (in red), the phase contrast image shows the cell morphology and finally the merged image. (C) Cells incubated with non Cancerous ascite. (D) Cells incubated with healthy human IgG. (E) Positive control humanised α-Folate antibody. (F) Negative control with no primary to find non specific binding of secondary antibody.

Immunoscreening of phage display cDNA Expression

The Phage Display technology approach was used to identify novel TAAs. A phage library of open reading frame (ORF) fragments created from mRNA derived from various tissues was used. ORF displaying phages were immunoprecipitated with ascitic fluid collected from patients diagnosed with ovarian cancer (**Fig 8A**) which has already been characterised as most reactive based on the result obtained from the initial immunological assays . The presence of the inset was confirmed by PCR analysis (**Fig 8B**) before they were cloned into an pGEX expression vector. Immunoprecipitated ORF clones were expressed as GST-fusion proteins. Western blot of few random clones was carried out to check whether the expressed proteins were full length fusion protein (**Fig 8C**).

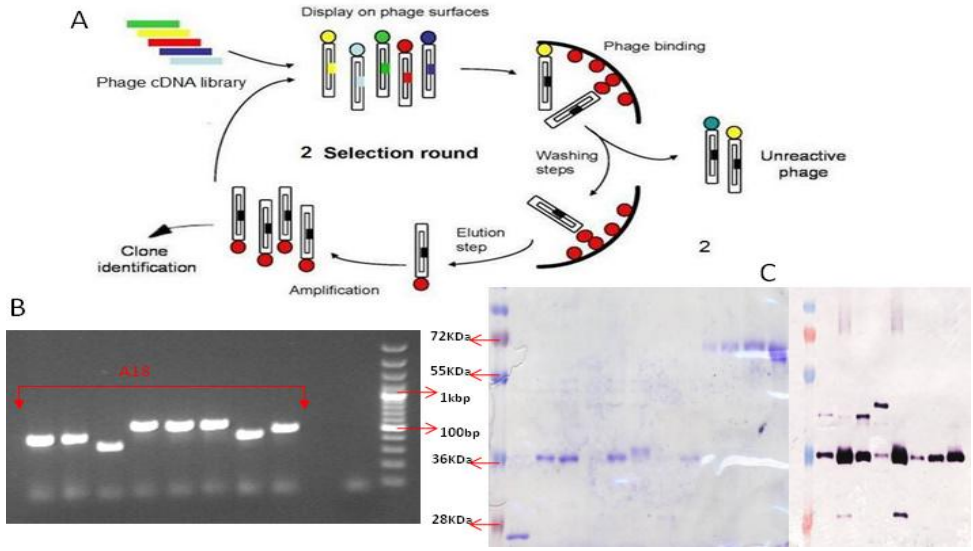


Fig 8: A) Selection of cDNA Phage Display library. B) PCR with VLPT2 and VHPT2 primers to check the presence of the insert. C) Coomassie staining and Western blot of GST fusion protein.

Microoarray Analysis

7 different purified antigen libraries (96X7 different proteins) were spotted on to nitrocellulose slides and screened for their immunoreactivity. The spotted slides were challenged against 1:50 dilution of 17 different Ascitic fluid collected from patients representing different disease conditions.13 ascitic fluid out of 17 were collected form patients suffering primary ovarian cancer, 2 from cancer patients other than ovarian or secondary ovarian cancer patients and 2 ascitic fluid collected from non cancerous conditions [**Fig 9**]. Thus, leading to the identification of 54 most immunoreactive clones.

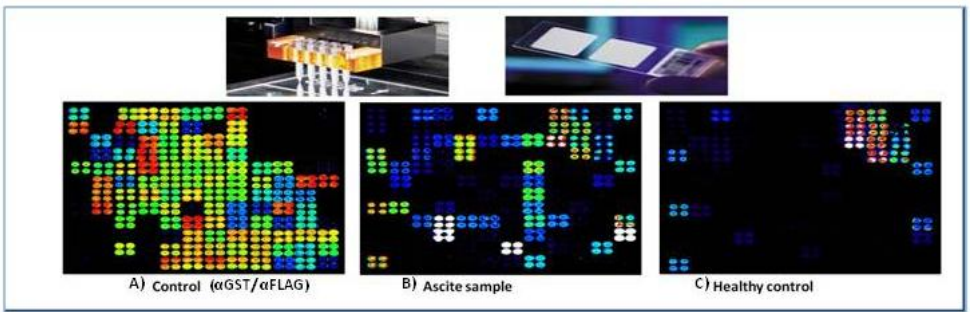


Fig 9: A) Fluorescent signal from A18 library incubated with 1:2000 dilution of anti-GST. B) Fluorescent signal from A18 library challenged with 1:50 diluted antibody isolated from primary ovarian cancer patient. C) The same library when incubated with 1:50 diluted non-cancerous ascite antibody produced a weak signal.

Antigen sequence analysis

All the 54 immunoreactive clones were PCR amplified with pGEX primers and were run on a 1% agarose gel [**Fig 10**].

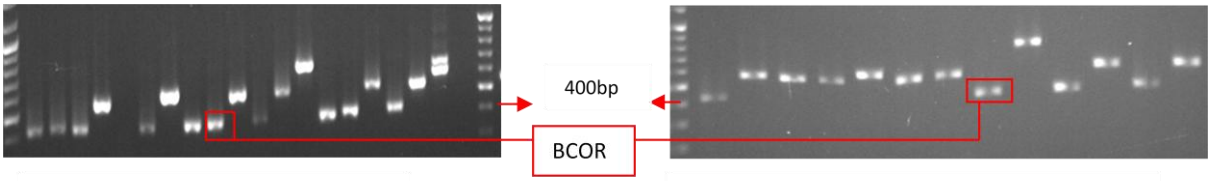


Fig 10:PCR Amplified fragments Purified fragments for sequencing

It was observed from the gel that the size of the amplified fragments vary between 150-800bp, which complies with the initial dimension of the cDNA fragments (this size range will be correctly displayed by the phage on the caspid) used for the construction of phage display library. Fragments representing each size were further purified using nucleo spin extract kit II (Macherey_Nagel) and subjected to a sequencing reaction and later sequenced using ABI 3100 sequencer. 54 clones were sequenced.

NAME	REF ID	NO: OF CLONES	BSSHII/NheI	LENGTH	STRAND ORIENTATION	GENIC
Homo sapiens BCL6 corepressor (BCOR)	ref|NM_001123385.1|	4	yes/yes	228	plus/plus	yes
Homo sapiens cAMP responsive element binding protein 3 (CREB3)	ref|NM_006368.4|	1	yes/yes	303	plus/plus	yes
Homo sapiens exosome component 10 (EXOSC10)	ref|NM_002685.2|	1	yes/yes	231	plus/plus	yes
Homo sapiens huntingtin interacting protein 1 related (HIP1R)	ref|NM_003959.1|	1	yes/yes	396	plus/plus	yes
Homo sapiens mitochondrial ribosomal protein L46 (MRPL46)	ref|NM_022163.3|	1	yes/yes	204	plus/plus	yes
Homo sapiens high mobility group nucleosomal binding domain 2 (HMGN2)	ref|NM_005517.3|	1	yes/yes	262	plus/plus	yes
Homo sapiens heterogeneous nuclear ribonucleoprotein C (C1/C2) (HNRNPC)	ref|NM_001077443.1|	1	yes/yes	288	plus/plus	yes
Homo sapiens chromogranin A (parathyroid secretory protein 1) (CHGA)	ref|NM_001275.3|	1	yes/yes	459	plus/plus	yes
Homo sapiens olfactomedin 4 (OLFM4)	ref|NM_006418.4|	1	yes/yes	129	plus/plus	yes
Homo sapiens flightless I homolog (Drosophila) (FLII)	ref|NM_002018.2|	1	yes/yes	423	plus/plus	yes
Homo sapiens KIAA1755 (KIAA1755)	ref|NM_001029864.1|	1	yes/yes	156	plus/plus	yes
Homo sapiens inositol-3-phosphate synthase 1 (ISYNA1)	ref|NM_001170939.1|	1	yes/yes	63	plus/plus	yes
Homo sapiens plexin B2 (PLXNB2)	ref|NM_012401.2|	1	yes/yes	420	plus/plus	yes
Homo sapiens inhibitor of DNA binding 1, dominant negative helix-loop-helix protein (ID1)	ref|NM_181353.1|	1	yes/yes	159	plus/plus	yes
Homo sapiens KH-type splicing regulatory protein (KHSRP)	ref|NM_003685.2|	1	yes/yes	576	plus/plus	Yes

Table 1: Final antigenic clones selected. Highlighted in red are already been validated by ELISA

Gene fragment display suffers from the problem that only one clone in 18 is functional, due to the orientation of the fragment and the reading frame in which they are translated. It has been noticed from the result that majority of the fragments are composed of 3N bases suggesting the cDNA library was well ORF filtered. From the sequencing result we matched our sequences to the genetic sequences available in blast database (www.ncbi.nlm.nih.gov/BLAST/) and later translated to identify the protein expressed by this cDNA. Hence, decided to evaluate those cloned fragment with 3N bases,+1 reading frame of translation carrying both the BsshII and Nhe I restriction sites. We identified a set of antigenic clones [**Table 1**]

Production of GST fusion proteins and Western blot analysis

Out of 54 most reactive peptides which were sequenced, set of predicted autoantigens were expressed and further evaluated. 100ml culture of these clones was induced for 2hrs at 25^{o}C with 0.1mM IPTG. Purification was done using GSH agarose and eluted with 750µl of GSH buffer at pH 8. All the expressed and purified proteins were quantified and were analysed by coomassie staining and western blot [**Fig 12**] with both αGST and αFLAG primary antibody. 1:5000 dilution of alkaline phosphatase conjugated α mouse IgG was used as the seconday antibody. The reaction was stopped once the proper bands appeared when incubated with NBT/BCIP substrate.

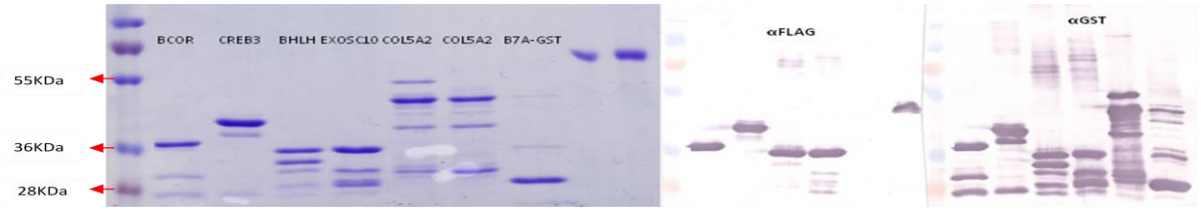


Fig 12: Coomassie staining and western blot of predicted antigens. 0.5µg of each protein/well was loaded

Enzyme linked immunosorbent assay

ELISA was performed to determine whether the antibodies present in the ascitic fluid from the ovarian cancer patients were specifically recognized by the selected peptides derived from the screenings. These techniques offer a higher level of sensitivity measured by ELISA even at a very low concentration of antibody. Each well of elisa plate was coated with 1µg in 100µl PBS 1X. A total of 94 protein A purified acites (35 Primary ovarian cancer/ 13 secondary ovarian/ 18 other cancer /and 28 non cancerous controls) were evaluated for the presence of cancer specific antibody against those identified antigens. The HRP conjugated α-human IgG secondary antibody was used for the detection. The plate was read spectrophotometrically at 450nm.

Out of all antigens tested, 6 were identified as novel putative TAAs that includes **A**) CREB3 (Cyclic AMP-responsive element-binding protein 3) **B**) MRPL46 (Mitochondrial ribosomal protein L46) **C**) BCOR (BCL-6 corepressor) **D**) HMGN2 (High mobility group nucleosomal binding domain 2) **E**) HIP1R (Huntingtin interacting protein 1 related) **F**) EXOSC10 (Exosome component 10). Each antigen were validated separately by ELISA against a set of affinity purified antibody from various disease condition which includes 35 primary ovarian cancer, 13 secondary ovarian cancer, 18 other cancer and 28 non cancerous cancer (**Fig 13**). Out of 35 primary ovarian cancer, CREB3 detected 20 as positive. Were as, MRPL46 and BCOR detected 14 out of 35 as positive. 8/35 and 10/35 were predicted to be above the cutoff by HMGN2 and HIP1R respectively. EXOSC10 was the least sensitive among the panel and predicted 4/35 to be positive. 12/13 secondary ovarian cancer remained above the cutoff where as 50% of the other cancerous sample produced a signal above the cutoff. It was noticed that only 5 out of 28 of the control sample were predicted to be positive by our panel. 2 among the 5 reactive non-cancerous sample appear to have pathology related to appendicitis.

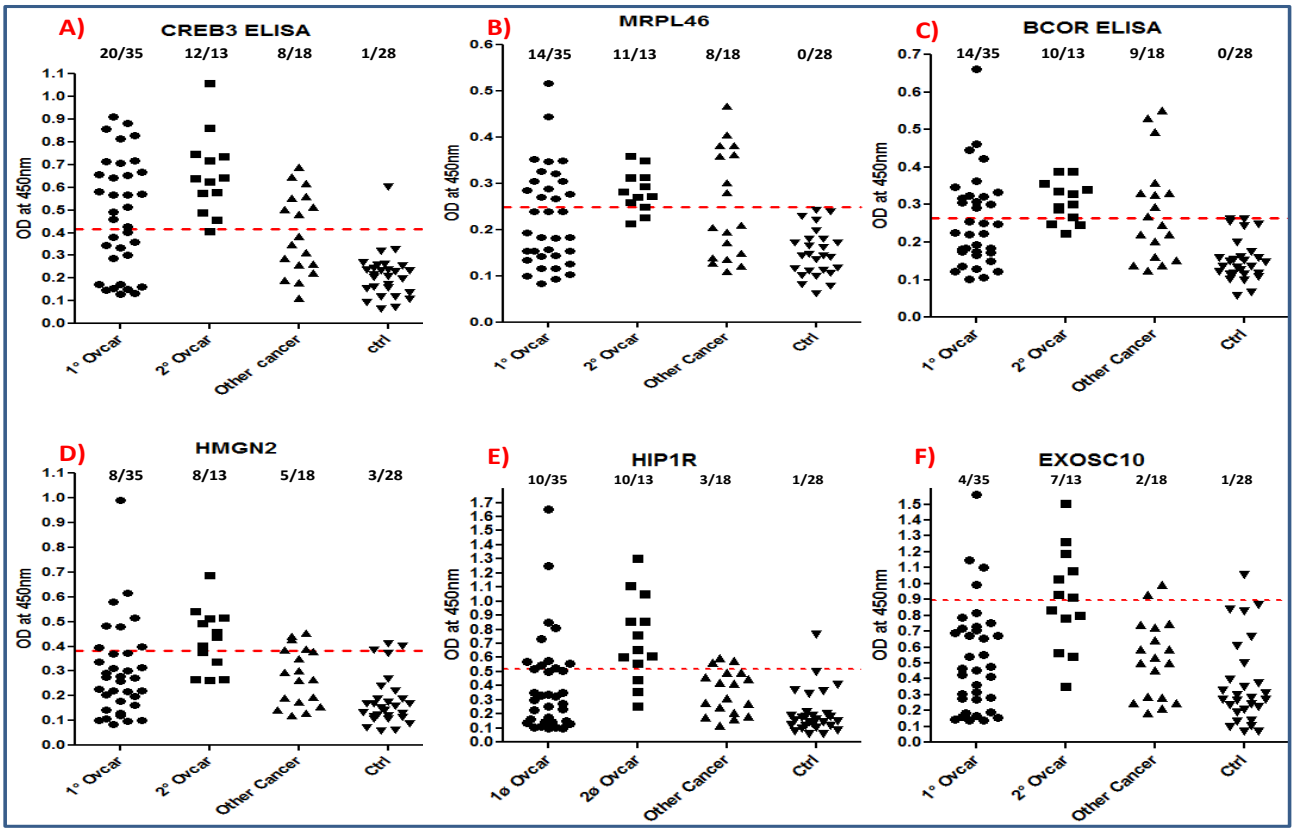


Fig 13: validation of antigenic protein by ELISA. Each antigen was challenged with 1: 50 dilution of a total 94 IgG purified ascites (1µg/µl) which further is divided into 4 different groups i) Primary ovarian cancer (35) ii) Secondary ovarian cancer (13) iii) Other cancer (18) iv) Non-cancerous control (28). Cut-off value was calculated separately for each antigen as mean + 2(standard deviation) of the OD from all non cancerous control wells.

Western Blot of Fractionated OVCAR Cell

Further we prepared 5 different fraction of OVCAR cell. Since the protein concentration were very low, these samples were approximately normalized base on their proportion resulted from bradfords assay . The fractions are listed below in the order as they were loaded into the gel.1- cytosolic, 2- nuclear extract , 3- nuclear membrane, 4- soluble protein, 5- total memrane protein. Two separate incubation was performed with 1:75 dil 1^{o} Ab as: Strip 1: protein A purified 13 (CREB +) and Strip 2 : protein a purified 1208 (Ctrl). Membrane incubated with sample 13, a band of 55KDa was observed in the cytosolic fraction and couple of bands in the range 30-35KDa were seen for the nuclear extract [**Fig 14**]. The size of full length CREB3 protein is estimated to be 41.39KDa.

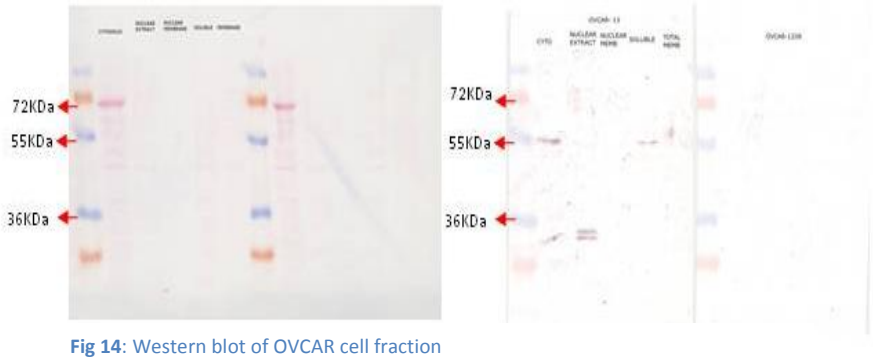


Fig 14: Western blot of OVCAR cell fraction

Cross inhibition assay

We assessed the expression of antigenic protein, as well as their subcellular localization in OVCAR cell line. To demonstrate the presence of tumor specific anibody in ascites collected from overian cancer, we inhibited the antibody by pre incubating them with a combination of GST fusion protein. Affinity purified sample 20 was preincubated with HMGN2/ HIP1R /EXOSC10, where as sample 44 was incubated with CREB3/BCOR/HIP1R. Non cancerous control 1209 was preincubated with a set of 5 proteins which includes HMGN2/HIP1R/EXOSC10/CREB3/BCOR. The results are in concordance with the ELISA result suggesting that presence of these intracellular proteins in OVCAR cells. The inhibition experiment performed localised staining to peri-nuclear region when preincubated antigen set. Non cancerous control 1209 produced a weak signal regardless whether or not preincubated with antitigenic set. Thus, suggesting the presence of autoantibody capable of binding to the certain intracellular protein produced by tumerous cells.

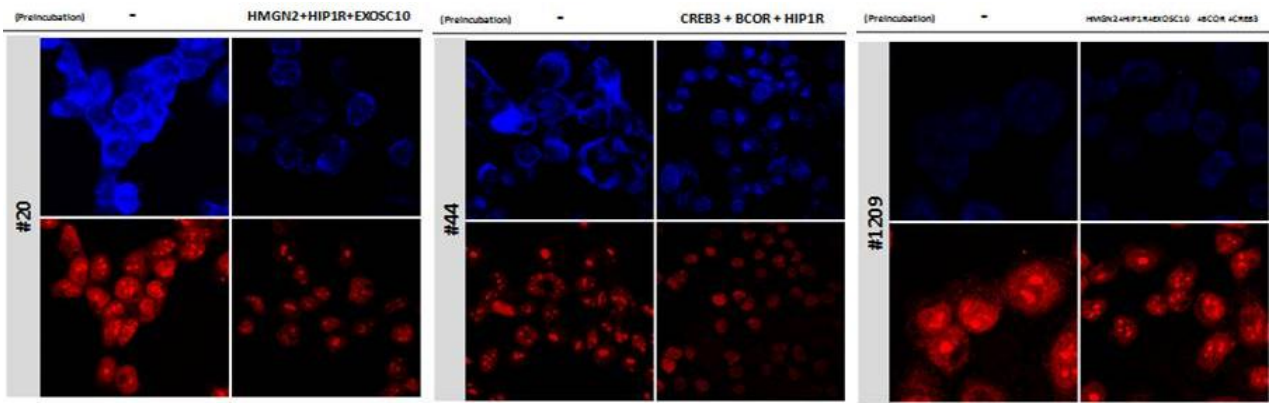


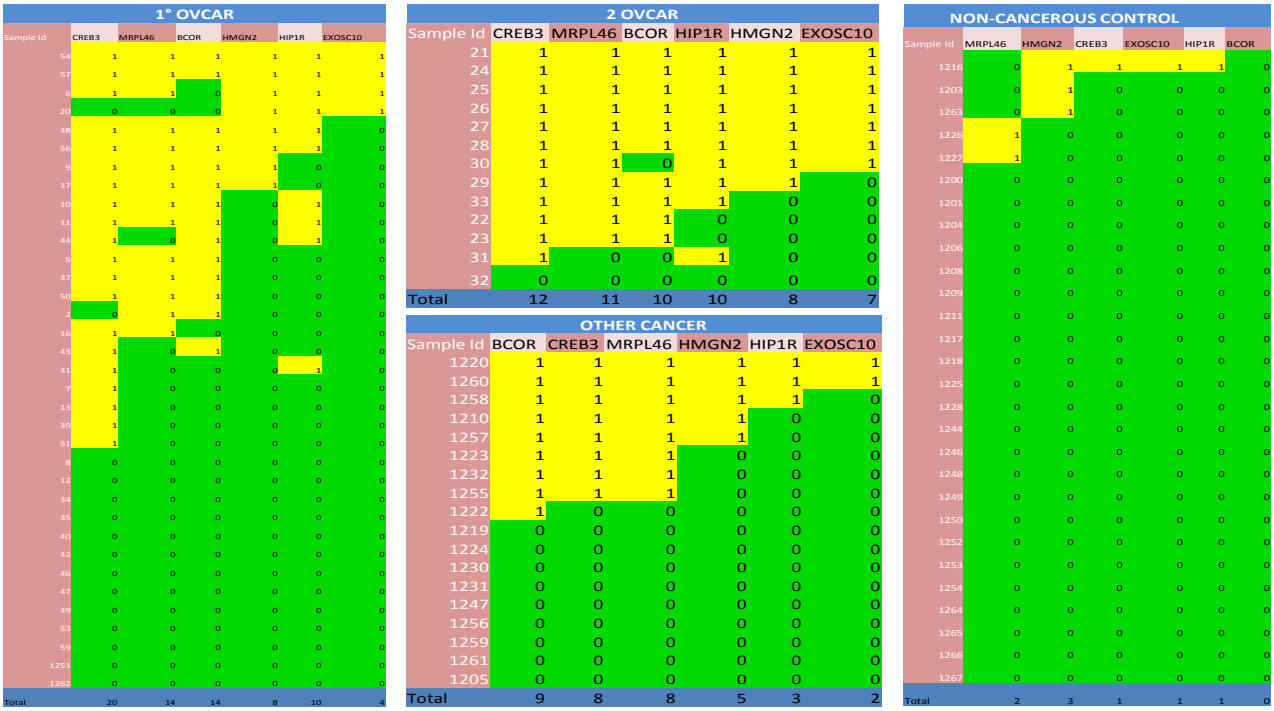
Fig 15: Cross inhibition immunofluorescence assay. A) Image A represents cells incubated with cancerous ascites 20. B) Image B represents cells incubated with cancerous ascites 44. The up take of tumor specific antibody by the cell was detected with α-human immunoglubulin conjugated to Cy5 (in blue) . The intensity of the signal shown to reduce drastically and were localised to perinuclear region when preincubated with a combination of antigens. Nucleus were stained with Propedium iodide (in red), the phase contrast image shows the cell morphology and finally the merged image. (C) Cells incubated with non Cancerous ascite produced a weak and non significant signal even with or without preincubation.

DISCUSSION AND CONCLUSION

Currently there are no efficient methods available for early diagnosis of ovarian cancer; thus still remains as a challenge for the cancer researchers. By early detection, a tremendous decrease in mortality can be achieved. Keeping this as our fundamental objective, we have identified 6 TAAs with very high potential to be a biomarker for ovarian cancer. To this extent, we employed a high-throughput protein expression and screening platform that combines cDNA phage display library selection and proteins microarray. Phage display of cDNA libraries from pancreatic islets (HPI), colon cancer (HCC) and lung fibroblasts (HF) used for identification and isolation of novel tumor associated antigen. This cDNA library was filtered for the presence of open reading frames (ORFs) and when characterized by massive sequencing resulted in 51,071 sequences had at least 95% identity and 90% overlap. 7,576 genes were identified by at least once while 83% of these genes were represented by no more than 10 reads in the library. Thereby, confirming the high diversity of the library and the efficacy of cDNA normalization. The efficacy of the ORF filtration was analysed by matching the obtained sequence with the annotated genome sequence. 91.2 % were found to be genenic sequence with 3N bases, +1 reading frame of translation carrying both the BsshII / Nhe I restriction sites at both ends and 99% of these contained no stop codons, confirming the quality of the filtering procedure [24]. We performed 2 rounds of selection for the enrichment of the reactive peptide and involve a pre-clearing step/ negative selection prior to selection with IgG from cancerous ascites, subsequently eliminating all non specific binding.

After two rounds of selections, the immuno screened phages were sub cloned into an expression vector, modified pGEX Vector. This modified vector was constructed with BssHII and NheI restriction sites and fusion tags, the N-terminal fusion with GST, a highly soluble protein and C-terminal FLAG protein was introduced to improve the quality of protein by permitting better folding of the downstream polypeptide thereby avoiding inclusion bodies formation and to monitor the degradation. In addition GST fusion proteins facilitate the purification by affinity separation. BL21 (DE3) RIPL cells were transfected with this expression vector. Induction of Lac promoter of the bacteria causes the production of T7 polymerase thus leading to the transcription of cDNA fragment located downstream of the T7 promoter in pGEX vector and an enhanced protein production. 700 random clones were produced in GST format and screened by protein microarray. Protein microarrays are very reliable and extensively been used for the identifying novel antigens in cancer [5,11,14]. Besides their ease of use to analyze a large set of biomolecules, they requires less amount of sample and reagent, making them a choice of interest. Here we took the advantage of this technology to pick out 54 most reactive peptides recognized by antibody present in ascites from cancerous patient. These clones were characterized by sequencing and identified the expressed antigenic protein.

Further, we validated the antigenic protein by ELISA. Each predicted antigen were challenged with 1: 50 dilution of a total 94 IgG purified ascites (1µg/µl) which further divided into 4 different groups i) Primary ovarian cancer (35) ii) Secondary ovarian cancer (13) iii) Other cancer (18) iv) Non-cancerous control (28). i) CREB3 (Cyclic AMP-responsive element-binding protein 3) ii) MRPL46 (Mitochondrial ribosomal protein L46) iii) BCOR (BCL-6 corepressor) iv) HMGN2 (High mobility group nucleosomal binding domain 2) v) HIP1R (Huntingtin interacting protein 1 related) vi) EXOSC10 (Exosome component 10) were identified as six novel putative TAAs. The data we obtained implying these identified antigens clearly differentiate cancerous from non cancerous test sample. 13 samples were recognised by none of the 6 tested antigens and remain as always negative. Hence, we require to identify and validate more number of antigenic proteins. Our panel of 6 tumor associated antigen were able to detect majority of samples as positive which originated from primary ovarian cancer patient atleast by once with any of the 6 antigen. It is clear from **chart 1** that a combination of multiple markers can increase the sensitivity of detection. A combine sensitivity of 63% and 82-100% specificity was achieved from this assay.



1° OVCAR

Sample Id	CREB3	MRPL46	BCOR	HMGN2	HIP1R	EXOSC10
54	1	1	1	1	1	1
57	1	1	1	1	1	1
6	1	1	0	1	1	1
20	0	0	0	1	1	1
48	1	1	1	1	1	0
56	1	1	1	1	1	0
9	1	1	1	1	0	0
17	1	1	1	1	0	0
10	1	1	1	0	1	0
11	1	1	1	0	1	0
44	1	0	1	0	1	0
5	1	1	1	0	0	0
37	1	1	1	0	0	0
50	1	1	1	0	0	0
2	0	1	1	0	0	0
16	1	1	0	0	0	0
43	1	0	1	0	0	0
41	1	0	0	0	1	0
7	1	0	0	0	0	0
13	1	0	0	0	0	0
39	1	0	0	0	0	0
51	1	0	0	0	0	0
8	0	0	0	0	0	0
12	0	0	0	0	0	0
34	0	0	0	0	0	0
35	0	0	0	0	0	0
40	0	0	0	0	0	0
42	0	0	0	0	0	0
46	0	0	0	0	0	0
47	0	0	0	0	0	0
49	0	0	0	0	0	0
53	0	0	0	0	0	0
59	0	0	0	0	0	0
1251	0	0	0	0	0	0
1262	0	0	0	0	0	0
Total	20	14	14	8	10	4

2 OVCAR

Sample Id	CREB3	MRPL46	BCOR	HIP1R	HMGN2	EXOSC10
21	1	1	1	1	1	1
24	1	1	1	1	1	1
25	1	1	1	1	1	1
26	1	1	1	1	1	1
27	1	1	1	1	1	1
28	1	1	1	1	1	1
30	1	1	0	1	1	1
29	1	1	1	1	1	0
33	1	1	1	1	0	0
22	1	1	1	0	0	0
23	1	1	1	0	0	0
31	1	0	0	1	0	0
32	0	0	0	0	0	0
Total	12	11	10	10	8	7

OTHER CANCER

Sample Id	BCOR	CREB3	MRPL46	HMGN2	HIP1R	EXOSC10
1220	1	1	1	1	1	1
1260	1	1	1	1	1	1
1258	1	1	1	1	1	0
1210	1	1	1	1	0	0
1257	1	1	1	1	0	0
1223	1	1	1	0	0	0
1232	1	1	1	0	0	0
1255	1	1	1	0	0	0
1222	1	0	0	0	0	0
1219	0	0	0	0	0	0
1224	0	0	0	0	0	0
1230	0	0	0	0	0	0
1231	0	0	0	0	0	0
1247	0	0	0	0	0	0
1256	0	0	0	0	0	0
1259	0	0	0	0	0	0
1261	0	0	0	0	0	0
1205	0	0	0	0	0	0
Total	9	8	8	5	3	2

NON-CANCEROUS CONTROL

Sample Id	MRPL46	HMGN2	CREB3	EXOSC10	HIP1R	BCOR
1216	0	1	1	1	1	0
1203	0	1	0	0	0	0
1263	0	1	0	0	0	0
1226	1	0	0	0	0	0
1227	1	0	0	0	0	0
1200	0	0	0	0	0	0
1201	0	0	0	0	0	0
1204	0	0	0	0	0	0
1206	0	0	0	0	0	0
1208	0	0	0	0	0	0
1209	0	0	0	0	0	0
1211	0	0	0	0	0	0
1217	0	0	0	0	0	0
1218	0	0	0	0	0	0
1225	0	0	0	0	0	0
1228	0	0	0	0	0	0
1244	0	0	0	0	0	0
1246	0	0	0	0	0	0
1248	0	0	0	0	0	0
1249	0	0	0	0	0	0
1250	0	0	0	0	0	0
1252	0	0	0	0	0	0
1253	0	0	0	0	0	0
1254	0	0	0	0	0	0
1264	0	0	0	0	0	0
1265	0	0	0	0	0	0
1266	0	0	0	0	0	0
1267	0	0	0	0	0	0
Total	2	3	1	1	1	0

Chart 1: comparison chart of reactivity of antigenic protein panel validated by ELISA challenged with i) Primary ovarian cancer (35) ii) Secondary ovarian cancer (13) iii) Other cancer (18) iv) Non-cancerous control (28). Each sample is represented by yellow (above the cutoff) and green (below the cutoff) boxes depending on the reactivity towards a specific antigen across the column.

We have identified and validated six TAA which have never been reported in context of ovarian cancer. The phage display library screening facilitates enrichment of the clones carrying same cDNA as well as selection of overlapping sequences of varying length sharing same epitopes. As expected, this phenomenon was clearly observed in view of the fact that multiple copies of BCOR clones were immunoscreened. On further analysis of these BCOR clones it was noticed that they occur in varying length with an overlapping epitope in common (**Fig 16**). All forms of BCOR were expressed and ELISA was performed with BCOR proteins of varying length and found D5 Short BCOR (36.69KDa) is recognised by most number of ascitic fluid [data not shown]. Consequently, leading to the identification of restricted epitopes.

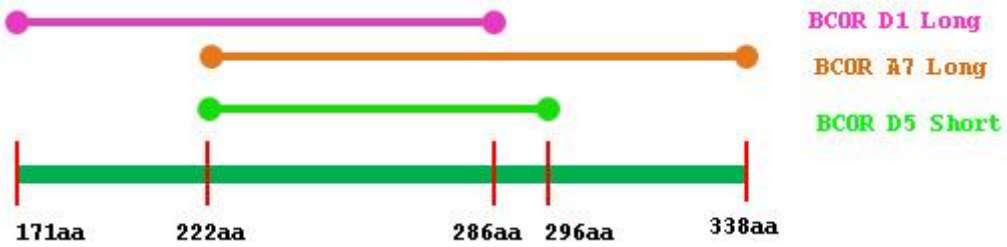


Fig 16: Overlapping epitopes of BCOR.

BCOR was originally identified as a novel corepressor of BCL-6 repressor and selectively potentiate BCL-6 repression. BCoR gene (127514 bps) is located in X chromosome at Xp11.4, encodes for 1755 AA BCL6 corepressor protein (MWt 192.19KDa) and are known to have 4 isoforms produced by alternative splicing. BCL-6 corepressor function as a novel transcriptional corepressor, specifically inhibit gene expression of B-cell lymphoma 6 (BCL6). BCL-6 encodes a POZ/zinc finger transcriptional repressor that is required for germinal center formation and may influence apoptosis. When BCL6 is aberrantly expressed it leads to the development of diffuse large B cell lymphomas (DLBCL). The interaction of BCL6 with their novel corepressor suggests a strong correlation between BCOR and the oncogenic mechanism underlying the BCL-6-associated lymphomas [26, 27]. BCOR mutations are associated with the X-linked inherited diseases Lenz

microphthalmia and Oculofaciocardiodental syndrome [28] as well as in acute myeloid leukemia with normal karyotype [29]. BCoR is ubiquitously expressed in all tissues examined, including the spleen and lymph nodes, where BCL-6 expressing mature B cells are found.The impression of our Immunofluorescence assay performed on OVCAR cells is in accordance with the work done by Khanh D. Huynh et.al , 2000 [30] implies their localization in nucleus.

CREB3 gene spans 4669 bps of chromosome 9 at 9p13.3 encodes for 395AA cAMP responsive element binding protein 3 (MWt 43.9KDa) and exists 3 isoform. cAMP responsive element binding protein 3 (CREB3) is a transcription factor belonging to leucine zipper family of DNA binding proteins. CREB3 subfamily proteins are anchored to the ER membrane in an inactive form. Only upon stimulation, the CREB3 subfamily proteins are translocated by COPII vesicles from the ER to the Golgi apparatus where they will proteolytically cleaved there by site 1 protease (S1P) and S2P sequentially to release the N-terminal fragment, which translocates into the nucleus and might work as homodimers or heterodimers to activate the transcription of genes. [31].This transcription factors activated upon intramembrane proteolysis (RIP), binds the cAMP via a conserved gene promoter element CRE (cAMP response element), which has the sequence 5'-TGACGTCA-3' and regulates cell proliferation. Nuclear form of CREB3/Luman specifically binds the UPRE consensus sequence and activates transcription [32]. A previous study has demonstrated that HDAC3 selectively represses CREB3-mediated transcriptional activation and chemotactic signalling in human metastatic breast cancer cells [33]. CREB3 was known to be a binding partner of hepatitis C virus core protein. This interaction with the viral oncoprotein might interfere with a tumor suppressive function of CREB3 to promote cellular transformation in hepatocellular carcinoma [34]. Dendritic cells (DCs) are antigen-presenting cells (APC) of the immune system. Recently CREB3/Luman was identified to interact with as a DC-STAMP (Dendritic Cell Specific TrAnsMembrane Protein (DC-STAMP). Same group also demonstrated that CREB3/ Luman coimmunoprecipitated OS9, DC-STAMP-interacting protein. Suggesting LUMAN and OS9 are part of the same complex and interact with each other at the cytosolic site of the ER. Thus leading to a new proposed Model of DC-STAMP/LUMAN/OS-9 pathway in which DC-STAMP/LUMAN/OS9 complex resides in the ER in immature DC and upon DC maturation, DC-STAMP/LUMAN complex translocates to the Golgi, where LUMAN is subsequently cleaved and its amino-terminal region is liberated, which then relocates to the nucleus. Where as, OS9 does not alter their localization [35]. The CREB3 epitope (40.15KDa) resulted from library screening fits in activated nuclear region as well as they overlap with a part of minimal region of CREB3/LUMAN needed for DC-STAMP interaction.

37.36KDa fragment which was pulled down by phage display selection fit within the HRDC domain of human exosome component 10 (EXOSC10). EXOSC10 (100.831KDa) also known as Polymyositis/scleroderma autoantigen 2or PM/Scl-100 was identified as 100–110 kDa protein reactive with PM/Scl autoantibodies [36-38] with a high specificity [39]. Approximately 5–8% of the sera from myositis patients, 3% of those from scleroderma patients, and 24% of those from patients with PM/Scl overlap syndrome contain the anti- PM/Scl autoantibody. The autoantibodies that characterize this specificity are predominantly directed against the PM/Scl-100 antigen.Immunolocalization studies showed that the PM/Scl autoantigens are present in the nucleoplasm and, at higher concentrations, in the nucleolus. The nucleolus is the site of ribosome synthesis, which involves the transcription and nucleolytic processing of precursor rRNAs, the nucleotide modification of rRNAs, and the assembly of mature rRNAs with approximately 80 ribosomal proteins into small and large ribosomal subunits .In yeast (*Saccharomyces cerevisiae*), 11 proteins, 10 of which are known or predicted to have 3'-5' exoribonuclease activity. Yeast exosome components, namely Rrp6p are homologous to the PM/Scl-100 autoantigens. Functional studies, suggest their presence in the nuclear and cytoplasmic compartments. Exosome is involved in the processing and degradation of several RNA species. The cytoplasmic exosome subfraction is probably involved in the degradation of mature cytoplasmic mRNAs. The human counterpart of the yeast exosome exosome was shown toexhibit 3'-5' exoribonuclease activity [40].

HIP1R (119.388KDa) is a multi-domain protein consisting of an N-terminal phospholipid binding domain (ANTH) which mediating phosphoinositides-interaction, a central coiled-coil, and a C-terminal actin-binding domain (THATCH). The coiled-coil domains of comprise the binding site for clathrin light chains that regulates actin assembly and the clathrin-mediated endocytic machinery in cells. Isolated fragment of of HIP1R (43.06KDa) constitute a region of central coiled-coil domain. Inside the cell, they are mainly localized at the endocytic compartments and in the perinuclear region were well demonstrated in our cross inhibition assay (**Fig 15**). HIP1R has been previously described as a specific binding partner of BCL2L10 and induces BAK-dependent Cell Death [41, 42]. In 2009, a study conducted at Medical University of Vienna, Austria [43] to define a gene signature in chronic lymphocytic leukaemia (CLL) patients with trisomy 12 lead to the identification of HIP1R. HIP1R is considered as the best potential surrogate marker for +12 in CLL. Expression of this gene has a high sensitivity and specificity for detection.HIP1R identified as antigens associated with a colon cancer-related serological response [44], reacted exclusively with serum IgG derived from colon cancer patients but not with serum IgG from normal controls.

In 2002, Kimmo Porkka et.al identified a 31 AA peptide identical to nucleosomal binding domain present **in** the N-terminal part of HMGN2 protein. When internalised, this peptide binds to the nuclei of tumor cells and tumor endothelial cells in vivo. This nuclear-targeting capability of the peptide could be utilized to deliver anti cancer drugs that can act in nucleus [45]. Further XIONG Wen-bi and colleagues in 2008 coupled pseudomonas exotoxin domain III with the same HMGN2 fragment. The *in vivo* anti-tumor activity of this recombinant chimeric protein was determined in female Balb/c nude mice bearing HeLa tumor cells. Chimeric protein significantly inhibited the growth of xenograft HeLa tumor in nude mice. Remarkable necrosis and apoptosis of tumors were seen in treated mice. This peptide has the potential to be used as a novel toxin-carrier in the study of cancer targeting therapy [46].

Where as, 35.58KDa GST fusion protein MRPL46 is a short N terminal fragment of mitochondrial ribosomal proteins **L46** (31.705KDa). Mammalian mitochondrial ribosomal proteins are encoded by nuclear genes and help in protein synthesis within the mitochondrion. Mitochondrial ribosomes (mitoribosomes) consist of a small 28S subunit and a large 39S subunit. This gene encodes a 39S subunit protein. Their expression is enriched in testis.

It is evident from our whole cell ELISA, immunofluorescence assay and cross inhibition assay that ascite antibody specifically from primary ovarian patients getting internalised in OVCAR cells. The possible mechanisms for antibody targeting intracellular antigens has to be further investigated. We observed an enhanced antibody uptake by OVCAR cells in culture upon serum starvation. In 2003, Stephen cooper demonstrated serum starvation led to arrest cells at G1 and G0 phases [47]. Thus suggesting these particular stages of the cell cycle contribute to the ability of cells to take up the antibodies. Further, the cell surface staining performed with same set of antibodies on OVCAR cell treated with endocytosis inhibitor and incubated on ice. The result we obtain implies the presence of cell surface receptor. Antibodies binding to these specific receptor gets internalised by endocytosis or pinocytocis. Another possibility could be some of the intracellular antigens or proteolytic fragments of intracellular target might get externalised and displayed on the surface of cancer cells by unconventional secretion [48], enabling the antibodies to bind and trigger immune responses.

Moreover, the occurrence of specific antibodies present in cancerous and non cancerous control sample targeted against intracellular TAA's present in OVCAR cell was well demonstrated with our cell lysate ELISA. These intracellular tumor-associated antigens may elicit a very early host immune response in course of tumor development. This feature of antibody targeting intracellular antigen could apparently be used for cancer vaccines as well as to develop biomarkers that are capable of measuring varying level of autoantibodies which are stable and abundant even at the early stage of tumor in reasonably short time [5]. During tumor progression, cancer cells tend to shed extracellular protein thus making intracellular proteins a

better choice for antigen-induced antibody therapies. A reduction in cross reactivity and minimal side effects can be achieved by using epitope based peptide vaccine, thereby increasing the specificity [49].

The data presented in our study demonstrates the capability of our approach in biomarker discovery and has a great potential to develop diagnostic marker for the early detection of ovarian cancer. With this approach we have identified and validated six different tumour associated antigens, which includes CREB3, MRPL46, BCOR, HMGN2, HIP1R, EXOSC10 with a key role in tumor regulation. We believe that identification of more TAAs could help in better understanding of the molecular pathology involved in cancer development and progression.

Thus, our future work includes generation of a new phage library derived from ovarian cancer tissue, ORF filtering and validation by massive sequencing. The identified antigen will be validation on a larger number of serum samples, including various stages of ovarian cancer; samples form other cancerous condition and controls. The prospective marker interacting with tumor specific IgG present in ascitic fluid should be capable to determine the state of ovarian cancer and distinguish ovarian cancer from any others type. The clinical correlation and disease association of these identified antigens should be studied. A Comparative study of individual Vs panal biomarker will be done with the intention of improvising both their specificity and sensitivity, to develop an efficient method for early detection. Biomarker panel are capable of detecting multiple autoantibody present in same sample is promising approach which should be carefully validated. Understanding the physiological activators, interactors and targets of these identifies TAA's will derive new knowledge in transcriptional regulation and new strategies in the intervention of human diseases. We conclude that the proposed approach represents a valid strategy to high-throughput profiling ovarian cancer.

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B.5b Participation at courses or summer schools organized by others than the PhD program

Course title and location	Organizer(s) and affiliation(s)	Number of hours	
Joint National Ph.D. Meeting 2011, Gubbio (PG)	Associazione di Biologia Cellulare e del Differenziamento and Societa Italiana di Biofisica e Biologia Molecolare.	27 hrs/ 3 days	20–22 October 2011
BD-Flow cytometry Training Program, Buccinasco (MI)	Becton Dickinson	16hrs/ 3days	2-3 & 28 May 2012
8th International Congress on Autoimmunity, Granada, Andalucia, Spain	Autoimmunity network	50hrs/5 days	9-13 May 2012
Genetics and Molecular Medicine, Universita' del Piemonte Orientale "A. Avogadro", (NO)	Prof. Steve Ellis (Louisville University), COREP, Universita' del Piemonte Orientale "A. Avogadro"	20 hrs/ 10 days	21 May- 1 June 2012
Joint National Ph.D. Meeting 2012, Rimini (RN)	Associazione di Biologia Cellulare e del Differenziamento and Societa Italiana di Biofisica e Biologia Molecolare.	27 hrs/3 days	To be held on 11–13 October 2012

B.8b

Periods (months) in foreign institutions and laboratories (address)	
Publications (articles, posters, abstracts)	**Posters** **Identification of novel tumor-associated antigens (TAAs) in ovarian cancer,** Joint National Ph.D. Meeting 2011, Gubbio **A Novel Platform For Autoantigen Discovery,** 8th International Congress on Autoimmunity, Granada

B.XX Attended seminars

Speaker	Title
"Neuronal Death: Recruitment of programmed necrosis and autophagy"	**Philip BEART** Florey Neuroscience Institutes, University of Melbourne, Parkville, Australia
"Next generation sequencing in T-ALL"	**Dr. Kim De Keersmaecker** VIB Center for the Biology of Disease, Center for Human Genetics KU Leuven (Belgium)
"Recent Advances in Hematopoietic Stem Cell Gene Therapy: from microRNA Regulation to Targeted Gene Transfer"	**Prof. Luigi Naldini** San Raffaele, Telethon Institute for Gene Therapy (HSR-TIGET)
"Molecular control of human fetal globin expression: towards a potential cure for B-thalassemia and sickle cell anemia"	**Prof. Sjaak Philipsen** Erasmus MC-Cell Biology, Dr. Molewaterplein 50, 3015 GE Rotterdam, The Netherlands
"High-throughput Biochemical Target Investigation Unveils a Novel Function of miR-21 as a Negative Modulator of Signal Transduction in T-lymphocytes"	**Prof. J. N. Bouwes BAVINCK** Department of Dermatology, Leiden University, Olanda
"Linfomi cutanei primitivi"	**Prof. Pino Macino** Dipartimento di Biotecnologie Cellulari ed Ematologia

	Università degli Studi di Roma "La Sapienza"
"Numerical simulations as virtual microscope at the nanoscale: some examples with dendritic molecules"	**Prof. Andrea Danani** Chief of the Laboratory of Applied Mathematics and Physics-LAMFI Department of Innovative Technologies-DTI, University of Applied Science of Southern Switzerland-SUPSI, Lugano, CH
"Microparticles as novel effectors in Inflammation"	**Prof. Mauro PERRETTI**, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London London, UK
"Resolvins and Omega-3 in Inflammation"	**Prof. Mauro PERRETTI**, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London London, UK
"Role of Diacylglycerol kinases in the control of T cell activation and differentiation programs"	**Prof.ssa Isabel MERIDA** Centro Nacional de Biotecnologia, Madrid
"Signalling pathways controlling integrin trafficking during invasion"	**Dott.ssa Elena RAINERO** Beatson Insitute for Cancer Research, Glasgow, UK
"Exosomes Shuttle RNA"	**Prof. Lötvall J.**, Department of Internal Medicine, Sahlgrenska Academy, University of Gothenburg
"Next-generation DNA sequencing and target arrays in the clinics"	**Dr. Paolo Fortina** Department of Cancer Biology, Jefferson Genomics Laboratory, Kimmel Cancer Center, Thomas Jefferson University Jefferson Medical College, Philadelphia, PA, USA
"Alpha-MSH and the melanocortin system in inflammation"	Prof. **Mauro PERRETTI**, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London London, UK
"Galectins-carbohydrate binding protein: sweet or sour?"	Prof. **Mauro PERRETTI**, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London London, UK
"The resolution of inflammation: players and targets"	Prof. **Mauro PERRETTI**, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London London, UK
"New trends in allergy and immunology"	**Prof. Joseph A.Bellanti**, Professor of Pediatrics and Microbiology-Immunology Director, International Center for Interdisciplinary Studies of Immunology Georgetown University School of Medicine