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Identifying Personalised and Shared Neoantigens in Breast Cancers

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Summary

A method has been developed for producing highly specific, polyclonal, or monoclonal antibodies, against breast cancer neoantigens. This technology could provide diagnostic and therapeutic antibodies against tumour neoantigens released by various solid tumours. The technology involves obtaining a portion of a lymph node that drains a breast cancer, dispersing its lymphocytes in culture medium to release a profile of individualised Antibody Secreting Cells (ASCs) that have responded to multiple neoantigens released by the cancer. These activated ASCs secrete antibodies that would normally be evoked by an in situ immune response. Collection of the secreted antibodies from the culture medium replaces the need to use blood serum in which antibodies may be bound to their antigens. Another feature of the technology is that it can reveal the nature of antigenic activity prevailing within progressing tumours. The method is, therefore, applicable to different types of cancer, to changing stages of the same cancer, as well as to ongoing levels of heterogeneity expressed in tumours with the passage of time. In brief, the value of the technology is based on its ability to detect the unique changes associated with evolving tumour antigenic expression in patients requiring effective immunotherapy related to the complexity of individual cancers.

INTRODUCTION

The identification of neoantigens in breast cancers, at various stages of their development, is described in this article. Antibodies developed from these neoantigens could be used for developing personalised treatments for all individuals who harbour a unique breast cancer. Our previous research has shown that breast and ovarian cancer cells may express shared and unique tumour-associated antigens within individual tumours [1-10].

In view of such personalised molecular makeup of various breast cancers, treatments that use a single therapeutic agent are unlikely to be effective in different individuals, even within the same cancer type. The current report shows that it would be feasible to establish personalised therapies for individual patients based on the specific neoantigens expressed at different stages of the same cancer or in different cancer types.

In some developing cancers, Tumour-Specific Antigens (TSAs) or Tumour-Associated Antigens (TAAs), are released into the extracellular fluid surrounding the growing tumour. The released neoantigens reach the local lymph nodes via lymphatic vessels. In response, B cells activated by the immunogenic tumour neoantigens are transformed into Antibody Secreting Cells (ASCs) that release antibodies against the tumour antigens. The antibodies, released into the blood stream, comprise an individualised immune response elicited by the cancer.

To replicate such antibody production *in vitro*, a tumour-draining lymph node is removed during surgery and its lymphocytes are released into culture medium. Activated lymph node cells establish the ASCs that are cultured for 5 days. During this incubation, specific antibodies, induced by the patient's tumour neoantigens, are released by the ASCs into the culture medium. The subsequent procedures for obtaining the antibodies that will be used for identifying the cancer's neoantigens are also described.

Several publications have discussed the presence of molecular heterogeneity of breast cancers and their impact on developing targeted therapy [3]. Another level of complexity, discussed in recent publications, is that within a single breast cancer, or ovarian cancer, there is a possibility that the tumour develops different cell types that express heterogeneous molecular fingerprints. In the technology described in the current paper, such changes could be detected in all evolving cancers, irrespective of whether they are due to genetic mutations, or other biological modifications. Overall, the technology described in the current paper can identify personalised neoantigens and corresponding autoantibodies for each patient. This enables the development of immunotherapies against unique tumour antigens in every individual harbouring a solid tumour.

Preparation of Antibodies for Identifying Personal Tumour Neoantigens

The method described in this section is based on procedures

for identifying the antibody response to Schistsome infections [3]. Sentinel tumour-draining lymph nodes, draining a cancer, are removed during surgery and a portion of a node is transferred into a bottle containing RPMI medium with 100 U/ml penicillin and 0.1 mg/ml streptomycin. The excised tissue is taken to the laboratory for immediate processing. Using sterile conditions, the node tissue is washed in RPMI with penicillin and streptomycin including 1% heat-inactivated foetal calf serum. Lymph node cells are dispersed into the medium using a scalpel. Cell suspensions are washed three times by centrifuging at 400 x g for 8 minutes. After counting, the lymphocytes are resuspended at 3 x 10^6 viable cells per ml in RPMI culture medium.

Antibody secretion from ASCs is promoted by supplementing the culture medium with 2 mmol/L glutamine, 10% heat-inactivated foetal calf serum and 0.5 mmol/L 2-mercaptoethanol. Antibody secretion is further enhanced by adding 0.1 ng/ml recombinant IL-6 and 2.5 ug/ml pokeweed mitogen. The release of tumour-specific or tumour-associated antibodies into the culture medium continues for 5 days. Subsequently, culture supernatants containing antibodies (ASC-probes) are collected by centrifuging the medium at 1,800 rpm for 10 min to remove the cells. The ASC-probes are stored in 3-5 ml aliquots at -20°C until use.

When possible, we also embed a part of the lymph node in paraffin and examine sections to determine whether the lymph node contained tumour metastases.

Purification and Labelling of the Released Antibodies

The antibodies released into the culture medium are purified by affinity chromatography using a Protein-G column. The cell-free culture medium is applied to the column and 10 volumes of loading buffer are used to remove unbound proteins. Subsequently, the proteins that are bound to the column are collected using elution buffer (0.1 M glycine in hydrochloric acid, pH 2.7). The eluate containing antibodies is neutralised using 100 ul 5 M tris and hydrochloric acid, pH 8.0. The antibody samples are then dialysed thoroughly with phosphate buffered saline (PBS) and stored at -20°C.

Control antibodies are purified from the serum of patients who did not have breast cancer. The method is the same as that used for purifying ASC supernatants.

In preparation for labelling, the purified antibodies are incubated with biotin (0.45 mg/ml concentration) in DMSO for 2 hours at room temperature. The labelling is ended by overnight dialysis with PBS. The biotinylated antibodies are then stored at -20°C.

WESTERN BLOTTING

A detailed description of the methods used is available in the following publication [10].

Identifying Neoantigens in a Solid Tumour

Recent publications have discussed the influence of molecular heterogeneity in the cancer cells of different subtypes of breast malignancies [1,7,8,]. As mentioned earlier, the present article deals with procedures for developing personalised therapy in individuals who have cancers that contain distinct kinds of molecular fingerprints. The approach that is discussed involves the development of personalised antibodies against the evolving tumour-specific neoantigen composition of the patient's tumour cells.

In planning methods for personalised treatments of heterogeneous solid tumours, the following procedures need to be established.

Identification of Neoantigens in Breast Cancers and the Development of Personalised Antibodies

As indicated in the introduction, the antibodies against an individual's tumour neoantigens were obtained from cultures of ASCs collected from a tumour-draining lymph node. These antibodies were labelled with biotin and used to identify the neoantigens released by the patient's cancer cells. The procedure involved lysis of the breast cancer cells, followed by separation of their proteins using two-dimensional Western blots [10] and detection of cancer proteins on the blots using the prepared biotinylated antibodies. Every tumour neoantigen, released by the cancer cells, that induced antibody secretion from ASCs in the draining lymph node, can be identified using this procedure.

In applying this method to breast cancer, two distinct protein spots, detected on Western blots shown in [Figure 1], were selected for further analysis. The two protein spots were isolated and sent to the Monash University Protein Analysis Facility for determining the amino acid sequence of each tumour antigen. The results indicated that the larger protein could be a "Transcriptional Factor" and its amino acids composition is shown in [Figure 2]. This protein was given the name "CP-1" (cancer protein -1). The second protein was described as a "Hypothetical Protein" and its amino acid composition is shown in [Figure 3]. This protein was given the name "CP-2", and at this stage it is considered a breast cancer-associated protein of unknown function.

ELISA TESTS BASED ON PEPTIDES DERIVED FROM CP-1 AND CP-2 BREAST CANCER PROTEINS

Monoclonal antibodies were generated against two peptides derived from CP-1 and against four peptides derived from CP-2. These monoclonal antibodies were used to establish an ELISA assay for detecting breast cancer antigens in the serum of 15 women who had breast cancer and 15 controls with healthy breast tissue. The results are shown in [Figure 4].

In each of the first three panels in [Figure 4], monoclonal antibodies against two peptides were used to detect tumour antigens in sera of women with breast cancer and in control sera. The best results were obtained in panel two, using monoclonal antibodies against peptides 3 and 4 derived from CP-2. In this panel, the pair of monoclonal antibodies detected 12(80%) breast

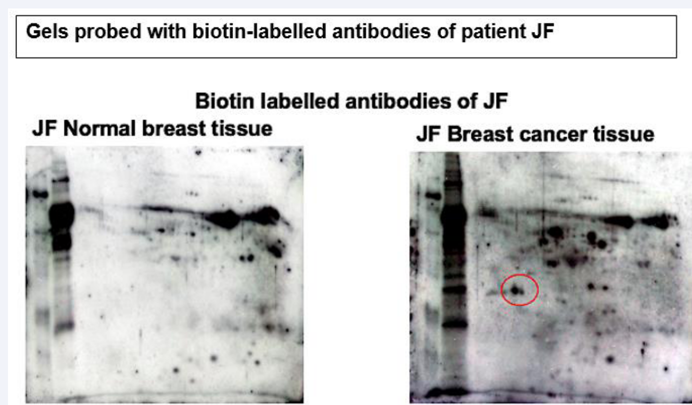

Figure 1 Two-dimensional Western blots, showing protein spots in normal breast tissue and in breast cancer tissue (both from patient JF), after being probed with biotin labelled ASC-probes. In the breast cancer blots, the antibodies detected the two cancer protein spots that are circled in red. These two cancer proteins were isolated and sent for analysis of their amino acid sequences. Other protein spots, visible in the cancer tissue, were not analysed in these initial studies.

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Protein Spot 1 P49711 Transcriptional Factor
  Zinc Finger Domains C-2-C-12-H-3-H
1   MEGDAVEAIV EESETFIKGK ERKTYQRRRE GGQEEDACHL PQNQTDGGEV
51  VQDVNSSVQM VMMEQLDPTL LQMKTEVMEG TVAPEAEAAV DDTQIITLQV
101 VNMEEQPINI GELQLVQVPV PVTVPVATTS VEELQGAYEN EVSKEGLAES
151 EPMICHTLPL PEGFQVVKVG ANGEVETLEQ GELPPQEDPS WQKDPDYQPP
201 AKKTKKTKKS KLRYTEEGKD VDVSVYDFEE EQQEGLLSEV NAEKVVGNMK
251 PPKPTKIKKK GVKKTFQCEL CSYTCPRRSN LDRHMKSHTD ERPHKCHLCG
301 RAFRTVTLLR NHLNTHTGTR PHKCPDCDMA FVTSGELVRH RRYEHTHEKP
351 FKCSMCDYAS VEVSKLKRHI RSHTGERPFQ CSLCSYASRD TYKLKRHMRT
401 HSGEKPYECY ICHARFTQSG TMKMHILQKH TENVAKFHCP HCDTVIARKS
451 DLGVHLRKQH SYIEQGKKCR YCDAVFHERY ALIQHQKSHK NEKRFKCDQC
501 DYACRQERHM IMHKRTHTGE KPYACSHCDK TFRQKQLLDM HFKRYHDPNF
551 VPAAFVCSKC GKTFTRRNTM ARHADNCAGP DGVEGENGGE TKKSKRGRKR
601 KMRSKKEDSS DSENAEPDLD DNEDEEEPAV EIEPEPEPQP VTPAPPPAKK
651 RRGRPPGRTN QPKQNQPTAI IQVEDQNTGA IENIIVEVKK EPDAEPAEGE
701 EEEAQPAATD APNGDLTPEM ILSMMDR
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Figure 2 Amino acid sequence of breast cancer protein CP-1.

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Protein Spot 8 Hypothetical Protein
  Zinc Finger Domains C-2-C-12-H-3-H
1   MALPQGLLTF RDVAIEFSQE EWKCLDPAQR TLYRDVMLEN YRNLVSLDTS
51  SKCMMKMFSS TGQGNTEVVH TGTLQIHASH HIGDTCFQEI EKDIHDFVFQ
101 WQENETNGHE ALMTKIKKLM SSTERHDQRH AGNKPIKNEL GSSFHSHLPE
151 VHIFHPEGKI GNQVEKAIND AFSVSASQRI SCRPKTRISN KYRNNFLQSS
201 LLTQKREVHT REKSFQRNES GKAFNGSSLL KKHQIIHLGD KQYKCDVCGK
251 DFHQKRYLAC HRCHTGENPY KCNECGKTFS HNSALLVHKA IHTGEKPYKC
301 NECGKVFNQQ SNLARHHRVH TGEKPYKCEE CDKVFSRKSH LERHRRIHTG
351 EKPYKCKVCD KAFRRDSHLA QHTVIHTGEK PYKCNECGKT FVQNSSLVMH
401 KVIHTGEKRY KCNECGKVFN HKSNLACHHR LHTGEKPYKC NECGKVFNRK
451 SNLERHHRLH TGKKS
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Figure 3 Amino acid sequence of breast cancer protein CP-2.

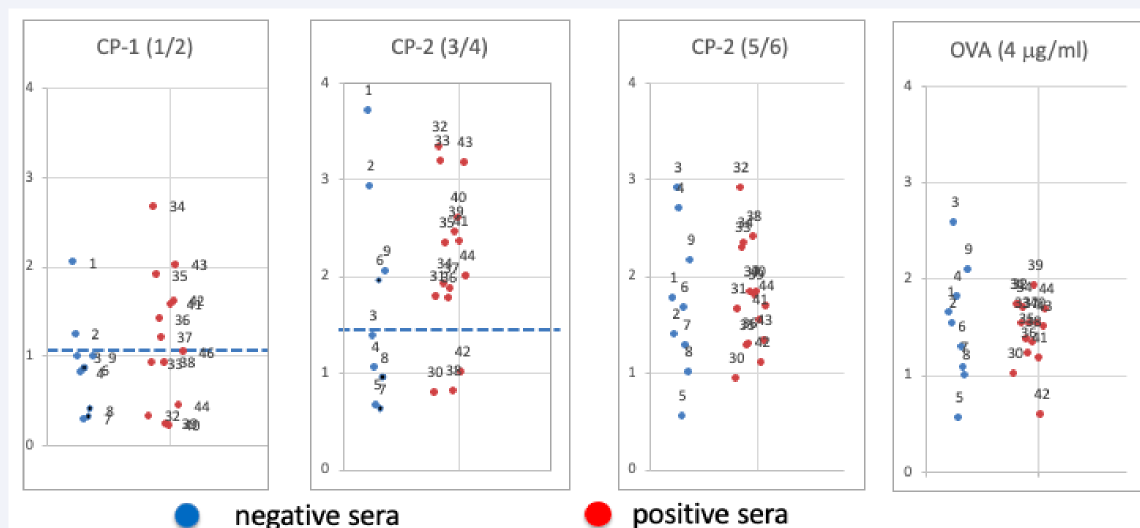

Figure 4 Results of ELISA assays, using monoclonal antibodies derived from peptides of CP-1 and CP-2. The ELISA tests were applied to sera obtained from breast cancer patients (represented by red dots) and sera from control women (blue dots). Monoclonal antibodies against the two peptides of CP-1 (CP-1.1 and CP-1.2), and four peptides of CP-2 (CP-2.3, CP-2.4, CP-2.5, CP-2.6) were tested for their reliability in detecting breast cancer sera versus control sera.

cancers in 15 positive sera that were tested. The monoclonal antibodies against the two peptides of CP-1 detected 53% of the positive cases.

If a diagnostic ELISA for detecting breast cancer were to be established for further testing, it is likely that antibodies against the peptides of CP-2 (CP-2.3 and CP-2.4) would produce the most reliable results. It is also possible that the combination of antibodies against the two peptides of CP-2 with the two peptides of CP-1, may improve the detection of breast cancers in developing a diagnostic ELISA blood assay.

In addition, a technology that identifies the molecular structure of tumour-specific or tumour-associated antigens, within individual heterogeneous tumours, could become a tool to assist in the development of personalised immunotherapy. When using the technology described the identification of unique proteins (or unique modifications to the protein) that accompany tumour growth, invasion and metastasis is possible, the application of the method for identifying cancer neoantigens, and their antibodies, may make it feasible to establish personalised immunotherapies for every individual harbouring the cancer. This proposal is based on the knowledge that a personal protein fingerprint can now be discovered for every woman diagnosed to have breast or ovarian cancers [2,10]. Although the development of such personalised immunotherapy is an attractive concept, its application and effectiveness for managing individual cancers would need to be validated in clinical trials.

Two different types of cancer treatments could be evaluated in the proposed clinical trials. Firstly, based on the studies described above, specific immunotherapy could be developed using peptides derived from the breast cancer neoantigens. The efficacy of this personal therapeutics would need to be tested in such clinical trials. Alternatively, hybridism-based monoclonal antibodies, or recombinant antibodies, raised against the tumour neoantigens, or against their specific peptides, could also be evaluated as therapeutic agents. This new approach to breast cancer treatments could yield beneficial outcomes in cancers that currently do not have effective therapies. The goal of such novel immunotherapy applications would be to eventually replace breast cancer chemotherapies that are poorly tolerated by many patients.

Identification of Tumour Neoantigens that are Shared by Breast Cancers of Different Patients

As shown in [Figure 1], biotin labelled ASC-probes, derived form a lymph node that drained the breast cancer of patient JF, detected two distinct antigens on a two-dimensional Western blot. The same antibodies were used in two-dimensional Western blots of breast cancers from four different patients and the results are shown in [Figure 5]. As may be seen, the two protein spots that were identified as CP-1 and CP-2 in patient JF were also expressed in each of the breast cancers from four different patients.

Lysates of the breast cancer cell line MCF-7, a metastatic cancer that originated from luminal breast epithelium, were also examined using two-dimensional Western blots. As before, the blots were probed with biotin labelled antibodies derived from the breast cancer patient JF [Figure 6]. Shows that the same two protein spots that were detected in breast cancer tissues shown in [Figure 1,6,7] were also found in the cancer cell line. These

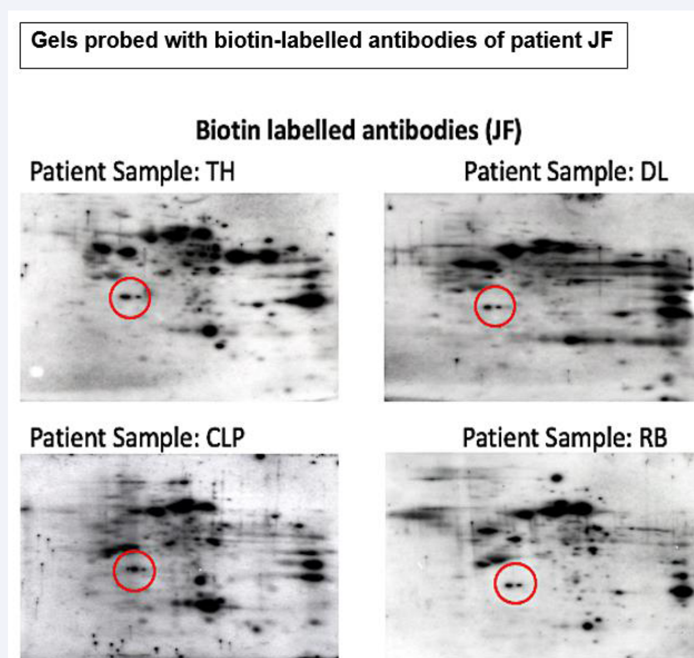

Figure 5 Two-dimensional Western blots, showing protein spots detected in breast cancer tissues from four different patients. All these Western blots were probed with antibodies released from the lymph node that drained the breast cancer of patient JF shown in Figure 1.

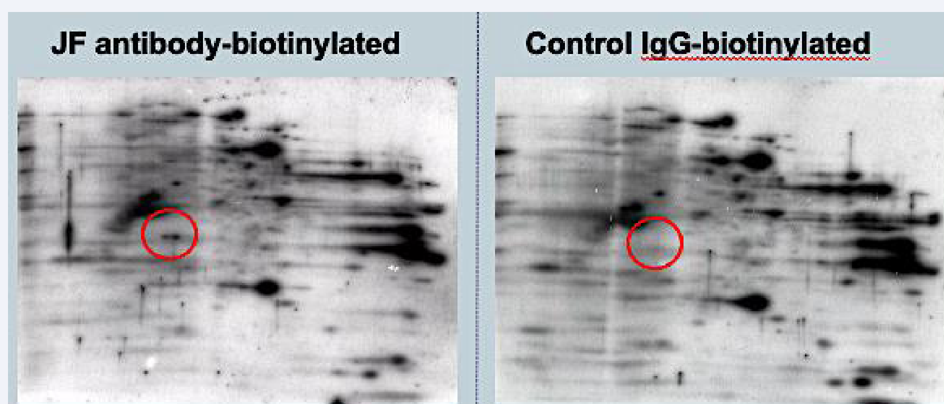

Figure 6 Two-dimensional Western blots, showing the previously detected two protein spots (circled in red in the left panel), that were also observed in a breast cancer cell line MCF-7. As described above, the separated proteins on these gels were probed with biotinylated antibodies derived from a lymph node draining the breast cancer of patient JF.

results suggest that CP-1 and CP-2 are expressed in a range of different breast cancer tissues.

Proposals for Developing Personalised Immunotherapy for Breast Cancers in Different Patients

The technology, described in the present article, could assist in furthering the understanding required to enable the introduction of personal immunotherapy for patients who are developing early breast cancers, as well as patients in whom the cancers are more advanced. The reliability of this concept would need to be developed further and then evaluated in special clinical trials in which the therapy is developed individually for each cancer patient.

Such specific and innovative treatment may be found to be effective in cancers that are currently difficult to manage due to a poor response or toxicity induced by current therapies. Unlike in other clinical trials, the therapeutic agents will not comprise

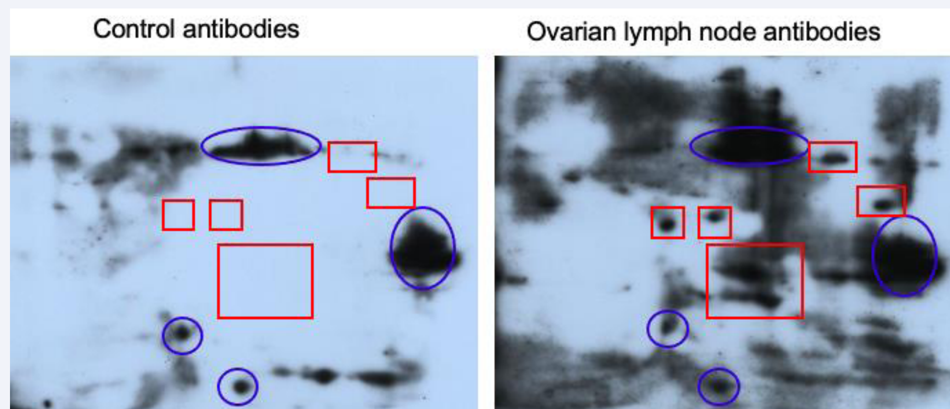

Figure 7 Two-dimensional Western blots of lysates of an ovarian cancer cell line. When these were treated with control antibodies, such as IgG, dark areas were detected which represent landmark proteins that are present in each blot. These dark areas are circled in blue in each panel. The red rectangles enclose cancer proteins (antigens) detected by antibodies released by a lymph node that drained an ovarian cancer. Each of these antigens can be isolated and their molecular structure determined for developing several new immunotherapies. As may be seen, such antigens were not detected in equivalent positions in the control panel.

one, or two, identical medications that require to be tested in standard trials. Instead, the previously used single treatment will be replaced with personal immunotherapy that is established for each patient during the clinical trial.

Another approach, particularly in breast cancers, is to establish a clinical trial using immunotherapy based on the protein structure of CP-1 and CP-2 with further understanding of the immune response identified in this article. This type of trial would test antibodies raised against previously unknown breast cancer antigens that are widely shared by different types and stages of breast tumours. As the molecular structure of these antigens has been identified in the present studies [Figure 2,3], unique antibodies, produced against each protein, could be used as novel therapies, that are developed at the beginning of each clinical trial.

Detection of Neoantigens in Ovarian Cancer Cells Using the Method Described in this Paper

Lysates of an ovarian cancer cell line (NIH: OVCAR-3) were studied on 2D Western blots and probed with biotin-labelled antibodies against ovarian cancer antigens. The results are shown in [Figure 5]. As may be seen, several distinct proteins were clearly identified in the ovarian cancer cell line. As the cell line was derived from an advanced metastatic ovarian adenocarcinoma, the identified proteins may not represent cellular proteins in earlier stages of the cancer. However, it is worth noting that the cancer antigens expressed in the ovarian cell line, were identified by antibodies derived from ASCs of lymph nodes associated with earlier stages of ovarian cancers.

DISCUSSION

For the first time, in the management of various cancers, it has become possible to identify the molecular structures of most of the intracellular proteins that drive cell division, invasion and metastasis at various stages of cancer development. The tumour-draining lymph nodes comprise the essential components for identifying the composition of a tumour's intracellular protein landscape. The neoantigens that are released from the developing tumour evoke antibody production by ASCs in the local lymph nodes and these are released into the blood stream. In our laboratory, however, the lymph node cells are dispersed in a culture medium into which they release specific antibodies. The antibodies that are secreted into the culture medium (ASC-probes) are collected, labelled, and used to identify the neoantigens in the cancer that generated them in situ. This sequence of steps is an effective method for detecting the molecular structure, and heterogeneity, of a range of cancer proteins in early and advanced tumours. It is also the method that could provide the basis for personalised immunotherapies for individuals harbouring breast or ovarian cancers.

In a recent review dealing with molecular profiling of tumours for improving cancer therapies [6], the focus appeared to be the identification and interpretation of new gene discoveries. Also, the authors expressed optimism about the role of next-generation sequencing in developing precision cancer therapies. Yet the accurate discovery of cancer cell genes would also require studies that demonstrate clear links between the new genes and their expressed proteins being the direct drivers of oncogenesis. In our current paper, we have proposed that the specific proteins that are involved in inducing tumour cell division, invasion and spread, can be identified directly using antibodies derived from the lymph nodes that drain progressing stages of the cancer. It is also important to note, that only the tumour neoantigens that are released from the malignant cells, trigger the immune response in the draining lymph nodes.

Our discovery of the molecular structure of two proteins

in breast cancer, which are expressed across several different patients, as well as within a cell line derived from breast cancer metastases, are valuable findings. Studies will need to be designed to determine whether these two proteins, their peptides, or post-translational modifications to these proteins, can be used for developing monoclonal antibodies or vaccines against a range of breast cancers.

Studies designed to evaluate the effects of CP-1 and CP-2 on cell growth in vitro, and the interpretation of the results, would need to take into consideration, that both proteins exert their effects within the nuclear-cytoplasmic compartment of the cancer cells. From their action on local lymph nodes, we also know that both proteins are released from the cancer cells. Thus, in addition to inducing naïve B cell maturation into ASCs and downstream antibody production in lymph nodes, the proteins that are released by the cancer directly into the blood stream, may also promote metastasis. Based on such information, if one, or both, of these cancer proteins promote the growth and spread of tumour cells, it is reasonable to assume that antibodies raised against each protein, could provide effective immunotherapy in breast cancer patients. It is also worth noting, that the ubiquity of each protein, in different types of breast cancers, and their presence in associated cell lines, may be related to their influence on cell growth. Additionally, the released CP-1 and CP-2, that enter the blood stream, may inhibit the innate and adaptive immune system, and promote the growth of the primary cancer and its metastases.

A prominent feature of the dialogue between the local lymph nodes in breast cancers, and probably ovarian cancers, is that each of these cancers releases several specific antigens, which stimulate the B cells in local nodes. This primary immune response, results in the release of many specific antibodies into the blood stream, in each of the cancers. In contrast, the clinical approach to managing such cancers is to conduct trials that evaluate a single promising immunotherapeutic antibody that has been shown to be sufficiently effective in a specific type of breast or ovarian cancer. As far as we are aware, none of these single agents have been reported to supress the cancers in early, advanced, or metastatic stages. Moreover, the known heterogeneity of breast [7] and other cancers, diminish the likelihood of successful treatment when single therapeutic agents are used against malignant tumours that contain diverse and evolving cell populations.

Our technology identifies the secreted antigenic signatures of cancer cells that have developed, or are emerging, within heterogeneous tumours. In such tumours, diverse neoantigens (proteins) drives cell growth, motility, invasion, and adhesion to extracellular matrices, enabling the cancer cells to spread and survive at various metastatic sites. It is unlikely that any single, or even a combination of two, immunotherapeutic agents could control all mechanisms that drive cancer progression. As mentioned above, the adaptive immune system launches an anti-tumour offensive by releasing a large range of antibodies from lymph node B cells that have matured and developed into ASCs. In our view, clinical immunotherapy employing a range of effective antibodies would emulate the natural immunological response against each cancer. The technology presented in our paper, makes it possible to produce, and clinically evaluate, a range of therapeutic antibodies that are required for blocking many of the harmful effects of cancers. A new clinical approach, that requires the use of multiple anticancer antibodies, may prevent repeated failures related to the use of single immunotherapeutic agents. Nevertheless, we are aware that new treatments, which advocate the use of multiple personalised antibodies in different patients, would be difficult to implement in the current regimens of established cancer therapy.

Recently, the use of a cocktail of therapeutic monoclonal antibodies has been approved by the FDA for the treatment of Ebola virus infections in humans [4,5]. In one study the viral antigen was targeted by a mixture of three monoclonal antibodies, one of which blocked the entry of the Ebola virus into susceptible cells [9]. In these clinical trials it was also found that the monoclonal antibodies worked as a mutually beneficial group, since withdrawal of one reduced the effectiveness of the treatment. These findings are relevant to the present work because they show that multiple monoclonal antibodies can be used effectively in human immunotherapy. When further work identifies the major antigens that are responsible for cancer growth and its spread, a group of personalised therapeutic antibodies can be generated to block the tumour's further growth and spread.

Immunotherapy, in which a single antibody is used against a single cancer protein, is unlikely to be effective against many cancers that are driven, without exception, by multiple proteins. Clinical trials that involve the testing of a single immunotherapy against different types and stages of breast or ovarian cancers are not unlikely to achieve a cure, nor will a monotherapy prevent the progression of complex and diversified malignancies. A new approach, including the use of several effective antibodies in each patient, is required to overcome the slow immune-based progress in the treatment of cancers. The technology described in the present paper provides a method for identifying a group of antibodies for individualised therapies. In the following proposed clinical trials, treatment outcomes will need to be judged by preventing cancer progress, or by achieving a cancer cure, using highly specific antibodies in individual patients. We propose that this would be preferable to the repeated testing of possible success or failure of a single immunotherapeutic agent in patients harbouring similar or different breast cancers. The use of immune monotherapy has been on trial long enough, we now need to test individualised cocktails of antibodies, in a range of patients who harbour a range of heterogenic cancers.

The lack of success in treating heterogeneous cancers using a single antibody was clearly pointed [3]. The authors used the following wording in their paper: "a single drug may not be adequate to treat a genetically heterogeneous tumour, since pre-treatment cancer cell population harbouring resistance mutations, even if present in a low frequency, can contribute

to therapeutic failure and poor outcome". Yet it is only recently that it has become possible to identify, in each patient's tumour, specific tumour neoantigens that enable us to generate a range of therapeutic antibodies for every cancer.

In the clinical testing of a cocktail of monoclonal antibodies, for treating breast cancers, we propose the establishment of two groups, with 100 patients in each group. During the first week in both groups, the trial would begin with administration of monoclonal antibodies against CP-2. In our recent studies, this protein has been shown to be expressed in different patients with different types of breast cancers. If after two weeks of treatment, adverse effects are not present, monoclonal antibodies against CP-1, which is also expressed in various breast cancers, will be added to the treatment protocol of patients comprising group 1. However, patients in group 2 (control group), will continue to receive the monoclonal antibody against CP-2 throughout the trial, as this will comprise monotherapy that will be compared with the build-up of an antibody cocktail in group 1. In the proposed treatment group, each patient's unique cancer proteins will be identified within about 6 weeks after surgical resection. Monoclonal antibodies will be raised against three selected cancer proteins (say anti-CP3, anti-CP4 and anti-CP5). Thus, as treatment continues in group 1, an additional monoclonal antibody will be added weekly, so that after about two months a cocktail of 5 immunotherapeutic agents will be administered to each patient, provided unforeseen complications have not occurred. The outcomes of breast cancer treatments using a cocktail of individualised monoclonal antibodies, that attempts to mimic an adaptive immune response, could subsequently be compared with the monotherapy in the control group, after 6 months and 12 months of therapy.

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