Utilising Quantitative Immunoproteomics To Reveal Differential Autoantibody Biomarker Panels In Serous Ovarian Cancer Patients

A thesis submitted for the degree of

Doctor of Philosophy

as a combination of conventional narrative and portfolio of publications by

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Abstract

Epithelial ovarian cancer accounts for 5% of all cancer deaths and greater than 50% of all gynaecological cancer deaths. It presents at a late clinical stage in more than 60% of patients, and is associated with a 5-year survival of only 30% in this group. In contrast, the 5-year survival for patients with organ-confined stage I ovarian cancer exceeds 90%, and most patients are cured of their disease. Thus, the detection of early stage ovarian cancer is the best way to improve survival. No clinically applicable method exists for the early detection of ovarian cancer. Hence, there is an unmet medical need for an accurate screening test.

Most scientific efforts towards early detection are focused on the discovery of tumour-associated antigens (TAA). Autologous antibodies against TAAs, however, may serve as more sensitive diagnostic markers. They circulate in the blood before TAAs and are usually more abundant than the TAAs themselves as a result of amplification through the humoral immune response. Accumulating evidence also suggests that a humoral response already exists during malignant transformation when aberrant gene expression is translated into premalignant cellular changes.

In this thesis, potential autoantibody biomarkers for ovarian cancer were discovered, verified and validated as an early detection test. A new immunoproteomic strategy was developed to identify novel autoantibodies that were elevated in serous ovarian cancer patients. Lysate extracted from the ovarian tissue of a patient was applied to an immunoaffinity column generated with autologous antibodies and a paired control immunoaffinity column. Relative quantification of captured autoantigens was performed using isotope coded protein label (ICPL) technology coupled with high resolution LC-MS. At a protein ratio cut-off of 1.45-fold, 148 autoantibodies were found to be enriched in ovarian cancer patients compared to the corresponding controls.
Upon bioinformatic prioritisation 50 autoantibody candidates were selected for verification. Protein microarray analysis of 98 samples revealed 9 autoantibody candidates to be significantly different in early stage cancer patients compared to healthy and benign controls. Biomarker candidates anti-ANXA1, anti-SAHH and anti-ARP3 showed the greatest potential where each marker achieved greater than 90% specificity at 83.3% sensitivity. As a 4-biomarker panel with the ‘gold standard’ for ovarian cancer detection, cancer antigen (CA)125, a sensitivity of 76.5% at 100% specificity was attained. These values of sensitivity and specificity for early stage ovarian cancer surpassed the minimum requirements for an implementable screening test and showed great promise as a diagnostic tool.

Validation of the top three autoantibody candidates using protein microarray revealed anti-ANXA1 to be the most robust and effective biomarker for stage I cancer detection. As a single biomarker anti-ANXA1 had 81.8% sensitivity and 71.9% specificity for stage I cancer compared to healthy and benign controls. Excitingly, in combination with CA125, a sensitivity of 71.4% at 100% specificity was achieved when differentiating stage I cancer patients from healthy individuals. For this level of effectiveness a positive and negative predictive value of 100% and 99.99% was achieved, respectively. Therefore, a biomarker panel containing anti-ANXA1 and CA125 may enable the development of a detection test that can be used to screen for stage I serous ovarian cancer in the general population. This promising discovery demands further investigation where continuing analysis in prospective samples is essential.

The discovery of a screening test is crucial to reduce the morbidity and mortality caused by ovarian cancer. This study investigates the presence of differential autoantibody signatures in serous ovarian cancer patients as potential biomarkers. Additionally, those identified TAAs that are functionally involved in carcinogenesis could also serve as therapeutic targets. Finally, the immunoproteomic approach developed here could be used in studies aiming to discover novel autoantibody biomarkers for other asymptomatic malignancies.
Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Karina Martin and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Karina Martin

....................................................
Acknowledgement of Help

A successful post-doctoral candidature is measured by the contribution of the work to the body of knowledge for scientists, clinicians and the broader community. However, it could not be achieved without learning, personal growth and support.

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Publications

Directly related to thesis:


Arising from thesis:

Presentations


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## Abbreviations

<table>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>µl</td>
<td>Microlitre</td>
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<tr>
<td>1-DE</td>
<td>One-dimensional poly-acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>2-DE</td>
<td>Two-dimensional poly-acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>AA</td>
<td>Amino acid</td>
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<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AU</td>
<td>Absorbance units</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3[(3-Cholamidopropyl)dimethylammonio]-propanesulfonate</td>
</tr>
<tr>
<td>CID</td>
<td>Collision induced dissociation</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DIGE</td>
<td>Difference gel electrophoresis</td>
</tr>
<tr>
<td>DMP</td>
<td>Dimethyl pimelimidate dihydrochloride</td>
</tr>
<tr>
<td>DSS</td>
<td>Disuccinimidyl suberate</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
</tr>
<tr>
<td>ETD</td>
<td>Electron transfer dissociation</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>Fab</td>
<td>Fragment antigen binding</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>Fc</td>
<td>Crystallisable fragment</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Association</td>
</tr>
<tr>
<td>FIGO</td>
<td>International Federation of Gynecology and Obstetrics</td>
</tr>
<tr>
<td>fmol</td>
<td>Femtomole</td>
</tr>
<tr>
<td>G-250</td>
<td>Colloidal Coomassie</td>
</tr>
<tr>
<td>HCCA</td>
<td>α-cyano-4-hydroxy cinnamic acid</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEPES</td>
<td>2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HRAM</td>
<td>High resolution, accurate mass</td>
</tr>
<tr>
<td>I.D.</td>
<td>Inner diameter</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>ICAT</td>
<td>Isotope coded affinity tags</td>
</tr>
<tr>
<td>ICPL</td>
<td>Isotope coded protein labels</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IPA</td>
<td>Ingenuity pathway analysis</td>
</tr>
<tr>
<td>IT</td>
<td>Ion trap</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>Isotope tagging for relative and absolute quatitation</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LIT</td>
<td>Linear ion trap</td>
</tr>
<tr>
<td>m</td>
<td>Mass</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption/ionization</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>Mr</td>
<td>Molecular Weight</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem MS</td>
</tr>
<tr>
<td>Nd:YAG</td>
<td>Neodymium-doped yttrium aluminium garnet</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>nLC</td>
<td>Nano-LC</td>
</tr>
<tr>
<td>OCS</td>
<td>Ovarian cancer screening</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphoric acid</td>
</tr>
<tr>
<td>PAGE</td>
<td>Poly-acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>pmol</td>
<td>Picomole</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonylfluoride</td>
</tr>
<tr>
<td>PMT</td>
<td>Photon multiplier tube</td>
</tr>
<tr>
<td>PTMs</td>
<td>Post-translational modifications</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>Q</td>
<td>Quadrupole</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised controlled trial</td>
</tr>
<tr>
<td>rf</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>roc</td>
<td>Risk of ovarian cancer</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver operating characteristic</td>
</tr>
<tr>
<td>RP</td>
<td>Reverse-phase</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sec</td>
<td>second</td>
</tr>
<tr>
<td>SILAC</td>
<td>Stable isotope labeling by amino acids in cell culture</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal-to-noise</td>
</tr>
<tr>
<td>t</td>
<td>Time</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumour associated antigen</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline Tween-20</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion chromatogram</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
</tr>
<tr>
<td>$T_R$</td>
<td>Retention time</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)-aminomethane</td>
</tr>
<tr>
<td>VSN</td>
<td>Variance stabilisation and normalisation</td>
</tr>
<tr>
<td>XIC</td>
<td>Extracted ion chromatogram</td>
</tr>
<tr>
<td>z</td>
<td>charge</td>
</tr>
</tbody>
</table>
Chapter 1 Manuscript Context

A deep understanding of ovarian cancer, the immune system, biomarkers as well as proteomics and mass spectrometry was essential to perform the quality of research presented in this thesis. To this end a comprehensive literature review on ovarian cancer autoantibody biomarkers and discovery techniques was performed. Furthermore, an appreciation of the current 'gold standard' biomarker for ovarian cancer, cancer antigen (CA)125 was critical in order to understand the current challenges, requirements and need for a screening test for ovarian cancer. In joint authorship, mining of the literature was performed to produce an extensive review on the structure and function of CA125. These two reviews, which form parts of chapter 1, summarise the current knowledge and challenges faced in the field of ovarian cancer detection and screening.
Chapter 1 Manuscript 1

Exploring the Immunoproteome for Ovarian Cancer Biomarker Discovery

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International Journal of Molecular Sciences ISSN 1422-0067
www.mdpi.com/journal/ijms

Review

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Keywords: ovarian cancer; autoantibodies; immunoproteomics
Statement of authorship for manuscript 1

Karina Martin (Candidate)

Statement of contribution (in terms of the conceptualisation of the work, its realisation and its documentation)

Performed the literature review and wrote the manuscript.

Certification that the statement of contribution is accurate

Signed……………………………………………………………………Date……………………

Carmela Ricciardelli (co-author)

Statement of contribution (in terms of the conceptualisation of the work, its realisation and its documentation)

Manuscript evaluation.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed……………………………………………………………………Date……………………

XXX
Peter Hoffmann (co-author)

Statement of contribution (in terms of the conceptualisation of the work, its realisation and its documentation)

Manuscript evaluation.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed…………………………………………………………Date……………………

Martin K Oehler (co-author)

Statement of contribution (in terms of the conceptualisation of the work, its realisation and its documentation)

Manuscript evaluation and acted as corresponding author.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed…………………………………………………………Date……………………
Chapter 1 Manuscript 2

Deciphering the Molecular Nature of Ovarian Cancer Biomarker CA125


International Journal of Molecular Sciences ISSN 1422-0067
www.mdpi.com/journal/ijms

Review

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Keywords: CA125; MUC16; ovarian cancer; biomarker; mass spectrometry
Statement of authorship for manuscript 2

Karina Martin (Candidate)

Statement of contribution (in terms of the conceptualisation of the work, its realisation and its documentation)

Performed the literature review and wrote the manuscript.

Certification that the statement of contribution is accurate

Signed………………………………………………………….Date……………………

Florian Weiland (co-author)

Statement of contribution (in terms of the conceptualisation of the work, its realisation and its documentation)

Performed the literature review and wrote the manuscript.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed………………………………………………………….Date……………………

XXXIII
Mcn

Martin K Oehler (co-author)

Statement of contribution (in terms of the conceptualisation of the work, its realisation and its documentation)

Manuscript evaluation.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed…………………………………………………..Date……………………

Peter Hoffmann (co-author)

Statement of contribution (in terms of the conceptualisation of the work, its realisation and its documentation)

Manuscript evaluation and acted as corresponding author.

Certification that the statement of contribution is accurate and permission is given for the inclusion of the paper in the thesis

Signed…………………………………………………..Date……………………