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# Annexin A2 and alpha actinin 4 expression correlates with metastatic potential of primary endometrial cancer☆

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ABSTRACT

The prediction of lymph node metastasis using clinic-pathological data and molecular information from endometrial cancers lacks accuracy and is therefore currently not routinely used in patient management. Consequently, although only a small percentage of patients with endometrial cancers suffer from metastasis, the majority undergo radical surgery including removal of pelvic lymph nodes. Upon analysis of publically available data and published research, we compiled a list of 60 proteins having the potential to display differential abundance between primary endometrial cancers with versus those without lymph node metastasis. Using data dependent acquisition LC-ESI-MS/MS we were able to detect 23 of these proteins in endometrial cancers, and using data independent LC-ESI-MS/MS the differential abundance of five of those proteins was observed. The localization of the differentially expressed proteins, was visualized using peptide MALDI MSI in whole tissue sections as well as tissue microarrays of 43 patients. The proteins identified were further validated by immunohistochemistry. Our data indicate that annexin A2 protein level is upregulated, whereas annexin A1 and α actinin 4 expression are downregulated in tumours with lymph node metastasis compared to those without lymphatic spread. Moreover, our analysis confirmed the potential of these markers, to be included in a statistical model for prediction of lymph node metastasis. The predictive model using highly ranked *m/z* values identified by MALDI MSI showed significantly higher predictive accuracy than the model using immunohistochemistry data. In summary, using publicly available data and complementary proteomics approaches, we were able to improve the prediction model for lymph node metastasis in EC.



## 1. Introduction

Endometrial cancer (EC) is the second most common gynaecological cancer and a significant contributor to gynaecological related mortality in the world [1]. Despite the disease being diagnosed at an early stage, clinicians are faced with the challenge of predicting the stage of the disease so that the best surgical approach can be selected. Consequently, although only a small percentage of patients with EC suffer from metastasis the majority will undergo pelvic lymphadenectomy for diagnostic, prognostic and therapeutic reasons. This procedure is associated with

*Abbreviations:* EC, Endometrial cancer; LNM, Lymph Node Metastasis; FFPE, Formalin Fixed Paraffin Embedded; MALDI MSI, Matrix Assisted Laser Desorption/Ionisation Mass Spectrometry Imaging; LC-MS/MS, Liquid Chromatography Mass Spectrometry; IHC, Immunohistochemistry; ANXA2, Annexin A2; ANXA1, Annexin A1; ACTN4, α actinin 4; ERBB2, Receptor tyrosine protein kinase erb-2; EGFR, Epidermal growth factor receptor.

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significant morbidity including lower extremity lymphoedema [2]. Predictive tissue markers for LNM could prevent unnecessary surgery, thereby directly reducing EC morbidity. Primary pathological characteristics such as histological type, myometrial invasion, cervical stromal invasion, lympho-vascular space invasion (LVSI), tumour size, and tumour grade have been investigated for their association with LNM [3]. However, only lympho-vascular space invasion has been shown to correlate with LNM, but is unable to reliably predict metastatic lymph node spread [4].

Various models aiming to predict lymph node metastasis in EC have been presented. A recent study combined 10 individually published models for predicting LNM in EC to an independent cohort of 519 patients in order to evaluate and compare the predictive capacity of each model [5]. Of the models analysed, one preoperative model [6] and three postoperative models [7–9] performed well with AUC values of ≥0.75 and false negative rates of <5%. In general, the study found postoperative models based on pathological characteristics outperformed preoperative models based on clinical imaging techniques such as MRI. Advances in technology are also starting to result in the identification of biomarkers with better sensitivity and specificity. Recently, we used a MALDI MSI approach to analyse primary endometrial tumours and were able to correctly classify 88% of 43 patients [10].

Besides pathological characteristics, other parameters can be used as part of a predictive model, for example a model combing serum CA125 levels with the analysis of progesterone receptor (PR) and proliferation marker Ki67 by immunohistochemistry achieved an AUC of 0.82 [11].

Moreover, genetic markers of LNM, including p53, oestrogen and progesterone receptors, and DNA ploidy, show potential to form part of a prediction model [12]. Biomarkers identified by analysis of mRNA abundance either by microarray or RNA sequencing show great promise, but do not necessarily correlate with protein expression levels [13]. This discrepancy is relevant given many common pathology procedures such as immunohistochemistry (IHC) rely on antibodies which specifically measure the abundance of a protein within a sample. Mass spectrometry (MS) based proteomic profiling is therefore a promising alternative for biomarker discovery. Complementary MS techniques, such as liquid chromatography coupled electrospray ionisation MS/MS (LC-ESI-MS/MS) used with MALDI mass spectrometry imaging (MALDI MSI), can not only identify and quantify relative changes in protein abundance, they can also determine the spatial distribution of proteins and peptides of interest, which may correlate with disease severity.

MALDI MSI is a label free MS technique which can visualize the spatial intensity distribution of hundreds of peptides in a single tissue section whilst maintaining the sample morphology. Previously, we have shown the capacity of this technique in discriminating regions of normal endometrium from cancer [14] and moreover were able to predict the LNM status of EC patients from primary tumour cores with an accuracy of 88% [15]. Additionally, we were able to identify and validate α-Actin-2 and plectin as potential discriminators for LNM in EC [10].

For over 100 years clinical pathology has relied upon the description of tissue morphology by trained specialists for tumour classification and grading. The use of specific antibodies and immunohistochemistry (IHC) has provided targeted molecular information of the primary tumour, such as ErbB2 status for breast cancer treatment. In contrast, MALDI MSI provides non-targeted molecular information of any tissue. Previous studies in several different cancers have shown that MALDI MSI can acquire a more comprehensive proteomic picture than immunohistochemistry [16–18], and as such, MALDI MSI is slowly being introduced into commercial pathology laboratories around the world. The images generated by the technique allow new insights into tumour tissues as MALDI MSI allows for the detection and characterisation of tumour cells and their environment in a spatial context. The introduction of MALDI MSI equipment into pathology laboratories has the potential to extend current diagnostic and prognostic capabilities, revolutionising clinical pathology. However, the full extent of this potential is yet to be demonstrated.

Given the multitude of studies that have identified potential biomarkers of EC metastasis, the aim of this study was to generate a predictive model of EC metastasis using previously identified biomarkers following their validation at the protein level, and compare the performance of the biomarker model to a predictive model generated from MALDI MSI data. To achieve this, a list of target proteins was compiled from a combination of literature searches and analysis of EC data extracted from The Cancer Genome Atlas (TCGA). Overall, a list of 60 potential biomarkers was compiled for comparison in EC with metastasis compared to those without metastasis. From this list, the biomarkers detectable at the protein level were characterised using the techniques of data dependent acquisition (DDA) LC-ESI-MS/MS, data independent acquisition (DIA) LC-ESI-MS, and MALDI MSI. Following this analysis 3 proteins were selected for validation by IHC: annexin A2, annexin A1, and α actinin 4. The results of this IHC analysis were used to generate a predictive model of EC metastasis. IHC was chosen as the method for validation and model generation given its routine use in clinical pathology laboratories. Separately, a predictive model of metastasis was generated from the MALDI MSI analysis of tissue microarrays containing tumours from patients with or without metastasis. When compared the MALDI MSI model showed a higher prognostic accuracy than the IHC model.

## 2. Material and Methods

### 2.1. Sample collection and Tissue specimens

Fifty-three tissue EC samples were retrieved from the archives of the Institute of Medical and Veterinary Science, Adelaide, South Australia, Royal Prince Alfred Hospital, Sydney, New South Wales, John Hunter Hospital, Newcastle, New South Wales and King Edward Memorial Hospital, Perth, Western Australia. All specimens were formalin fixed and paraffin embedded (FFPE), and tumours were classified according to the World Health Organisation, and staged according to the International Federation of Gynaecology and Obstetrics (FIGO). The study was approved by the ethics committees of the respective hospitals. None of the patients had received pre-operative chemotherapy and/or hormone therapy. The detailed histo-morphological and clinical characteristics were described in our previous study [10]. Haematoxylin and Eosin (H&E) stained tissue sections were annotated by a pathologist. EC patient samples in which tumour metastasized to pelvic lymph nodes were categorized as “with LNM” and the primary tumours which did not metastasize to lymph nodes were categorized as “without LNM”. Of the 53 patient samples used in the study, 43 were used in the construction and analysis of the Tissue Microarrays (TMAs), and 10 were used for LC-ESI-MS/MS and in whole tissue MALDI MSI analysis.

### 2.2. Tissue microarray design and construction

Forty-three EC samples were randomly divided into two equal groups for assembly of tissue microarrays (TMAs); a training set used to identify molecular discriminators (referred to as TMA1), and a test set for validation (referred to as TMA2). Of the 43 samples, 16 were positive, with the remaining 27 negative for LNM, respectively.

For TMA assembly, 1.5 mm tumour tissue cores were excised and arranged vertically in an empty paraffin block using a tissue arrayer (3DHistech TMA Master, SciTech, Victoria, Australia). Two tissue cores from tumour dense areas were taken from each patient. The layouts of both TMAs were randomized to avoid bias caused by core location within the TMAs (data not shown). Six μm thick tissue sections were cut from both TMA's and mounted onto indium tin oxide (ITO) (Bruker Daltonics, Bremen, Germany) conductive glass slides for MALDI MSI analysis. Sections were also placed on plain glass slides