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Distribution analysis of the putative cancer marker S100A4 across invasive squamous cell carcinoma penile tissue

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ABSTRACT

MS-based proteomic methods were utilised for the first time in the discovery of novel penile cancer biomarkers. MALDI MS imaging was used to obtain the in situ biomolecular MS profile of squamous cell carcinoma of the penis which was then compared to benign epithelial MS profiles. Spectra from cancerous and benign tissue areas were examined to identify MS peaks that best distinguished normal epithelial cells from invasive squamous epithelial cells, providing crucial evidence to suggest S100A4 to be differentially expressed. Verification by immunohistochemistry resulted in positive staining for S100A4 in a sub-population of invasive but not benign epithelial cells.

1. Introduction

Penile cancer is a rare cancer in developed countries with approximately 500 cases diagnosed each year in the UK [1]. There is an increased incidence of penile cancer in South America and Western Africa, which has been putatively explained by the socioeconomic conditions and restricted access to health care in these countries [2]. Squamous cell carcinoma (SCC) is the most common subtype of penile cancer accounting for ~95% of new cases each year [3]. Other malignant tumour types include adenocarcinoma, lymphoma, melanoma, and various mesenchymal tumours such as Kaposi's sarcoma and leiomyosarcoma [4]. Premalignant conditions eponymously termed Bowen's disease, Erythroplasia de Queyrat and Bowenoid papulosis represent carcinoma in situ or penile intra-epithelial neoplasia (PeIN 3). These can develop into invasive SCC if left untreated [2]. The primary

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treatment for penile cancer has moved to more conservative penile preserving techniques in order to maintain function. These include glansectomy and reconstruction and glans resurfacing procedures which have a good cosmetic and functional outcome compared to radical surgery. [5]. The lymphatic dissemination of penile cancer occurs in a predictable step-wise fashion; firstly to the inguinal nodes and subsequently to the pelvic nodes and then the para-aortic nodes [6]. Clinically the best prognostic indicator is the presence of metastatic inguinal lymph nodes [7]. However, despite a number of potential molecular biomarkers and the development of nomograms, there is as yet no reproducible biomarker available which is a reliable indicator for prognosis and metastatic potential in the primary tumour [8].

In the 1990s, matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry imaging (MSI) was established [9,10]. MALDI MSI can be employed for the simultaneous in situ visualisation and spatial mapping of various classes of molecules, from drug metabolites [11] to large proteins [12,13]. The ability to detect signals in their native environment gives information on local production of biomolecules as well as their distribution across different disease states of the tissue. This information can be lost if a large-scale tissue digestion protocol is followed, such as in the more traditional bottom-up proteomics approach.

MALDI MSI provides an excellent platform upon which molecular pathology can be applied, giving the investigator a "first-view" of the global biomolecular profile of the tissue and the various micro-environments present [14]. It has successfully been applied to a number of cancers [15,16] to provide the basis for more targeted analysis either via the traditional immunohistochemistry route or more sophisticated shotgun-type mass spectrometry methods. In this study, we present MALDI MSI results showing differentially expressed biomolecules in penile cancer tissue. This initial primer experiment served as an excellent starting point to study the malignancy in its anatomical context. Furthermore, the immunoactivity of S100A4 protein in penile cancer tumour tissue was measured and its correlation to the clinical grading of the tumour was determined. From these measurements, it can be concluded that a sub-population of S100A4-positive epithelial cells exists in the tumour environment and is virtually absent in matched benign tissue.

2. Materials and methods

2.1. *Sample collection*

Fresh tissue specimens, superfluous to that required for diagnostic evaluation, were collected from patients undergoing surgery for the removal or biopsy of penile cancer at the Department of Urology, Royal Berkshire Hospital, Reading, UK. Formalin-fixed paraffin-embedded (FFPE) penile tissue was collected for immunohistochemistry at the Department of Urology, University College London Hospital, London, UK. All specimens were procured with the approval from the University of Reading and Berkshire Research Ethics committees (10/H0505/16) and full informed consent was granted by the participants.

2.2. *Tissue preparation and matrix application for MALDI MSI*

Two consecutive tissue slices with a thickness of 10 μm and 5 μm were cut from two different frozen tissue blocks (T1 and T2) from two different patients and labelled T1-10, T1-5, T2-10 and T2-5. The area of the T1-10 was approx. 16.5 mm × 15 mm and for T2-10 this was approx. 16.5 mm × 9.5 mm. A Leica CM3050 S cryostat microtome (Leica Biosystems, Peterborough, UK) was used to cut the slices from the fresh frozen tissue blocks, which were then thaw-mounted on ground steel MALDI target plates (Bruker Daltonics, Bremen, Germany) at −20 °C. The tissue slices were brought to room temperature under vacuum for 30 min. Tissue fixation and removal of salts and excess haemoglobin were done through a series of ethanol/water wash steps as described by Schwartz et al. [17]. Subsequently, the tissue slices were dried under vacuum for another 10 min. A matrix solution with a concentration of 10 mg/ml was made of sinapinic acid (Sigma–Aldrich Co. Ltd., Gillingham, UK) dissolved in a 60:39.8:0.2 solution of acetonitrile:water:trifluoroacetic acid, which were all MALDI grade reagents from Sigma–Aldrich. The tissue slices were spray-coated with the matrix solution using a TLC sprayer (Sigma–Aldrich) at an optimised distance of 30 cm. At this distance it was found that a light matrix coat was applied evenly and reproducibly across the slices. After spraying the tissue, it was allowed to dry for 5 min, before repeating the cycle. This process was repeated five times ensuring an even deposition of matrix was observed across the slice. A calibration spot consisting of a standard solution of proteins and peptides (Bruker Daltonics) was added beside the mounted tissue slice for calibration purposes.

2.3. *MS analysis and data processing*

Mass spectra were acquired over an *m/z* range of 2,000–18,000 in positive linear mode and a laser repetition rate of 50 Hz using an Ultraflex MALDI axial-TOF mass spectrometer (Bruker Daltonics) with the acquisition software FlexControl 3.0. Custom geometry files were created to specify the imaging area using the in-house software PIMSS [18]. Using AutoExecute in FlexControl, MS images were recorded with a 200-μm spatial resolution, averaging the data from 50 shots per image pixel. PIMSS was used to convert raw data files into Analyze 7.5 format for image visualisation in BioMap (Novartis, Basel, Switzerland; http://www.maldi-msi.org).

For further data analysis, a representative population of spectra from areas of different tissue pathology (70 spectra from each region of interest) were extracted from the data set of the T1-10 tissue slice. On these spectra, classification and statistical evaluation was performed using ClinProTools 2.1 (Bruker Daltonics). The spectra were first processed and peaks were detected using a signal-to-noise cut-off of 5. The peak intensity values used for subsequent analysis were based on peak height. A Mann–Whitney *U* test was used for pair-wise comparison of peak intensities from normal and cancerous regions.