

Introduction

Cervical carcinoma is the most frequent carcinoma in women in the developing countries where 83% of cases occur (1). The presently used cytomorphological assessment is the most reliable screening method, although it results in high numbers of false-positive and false-negative cervical smears (2), especially for detection of early stage cancer. Biomarker-based approaches (e.g. the detection of squamous cell carcinoma antigen-1) are less specific (3) and there is an urgent need to discover new specific biomarkers for the detection of cervical cancer. We used laser microdissection (LMD), which allows the capture of different cell types or well-defined regions of tissue (Figure 1a). We compared cancer tissue, healthy epithelium and stroma of the cervix from eight patients with cervical cancer by LMD of approximately 2500 cells. The isolated cells were digested with trypsin and peptides were analyzed by high-resolution mass spectrometry with or without prior chromatographic separation (nanoLC-Orbitrap-MS or MALDI-FT-ICR-MS, Figure 1b). Differentially expressed proteins in tissue were also measured in serum samples of cervical cancer patients at different stages of disease, pre-cancer patients [CIN: cervical intraepithelial neoplasia] and healthy subjects by western blot and ELISA.

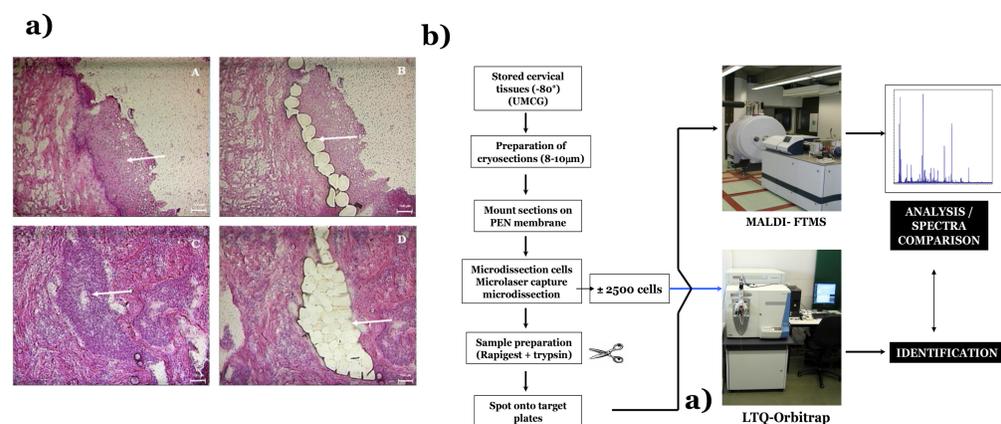


Figure 1. a) Laser microdissection from two tissue sections from a cervical cancer patient (patient #782, stage of cancer Ib1). Healthy (A, B) and cancer (C, D) before (A, C) and after (B, D) laser microdissection (LMD). Arrows depict areas from which cells were dissected for subsequent proteomics analysis. b) Sample preparation, measurement and analysis, routine protocol as described in Mustafa et al (4).

Material & Methods

Sample procurement (tissue). Tumor tissue was obtained from women undergoing either staging or surgery for having squamous cell cancer of the uterine cervix (Department of Gynecological Oncology, University Medical Center Groningen (UMCG), The Netherlands). Two biopsies were taken (one from the healthy cervical epithelium and one from the cancer). Only specimens with more than 70 % tumor were used.

Laser Microdissection. For laser microdissection (LMD) cryosections of 10 µm were prepared from each sample and mounted on to polyethylene naphthalate (PEN)-covered glass slides (P.A.L.M. Microlaser Technologies AG, Bernried, Germany). An area corresponding to approximately 2500 cells was collected in 10 µL 0.1% Rapigest SF detergent (Waters). Two-times 0.5 µL were used for peptide profiling by MALDI-FT-ICR-MS [Bruker Daltonics, USA; Fig. 1b] (replicate analyses) and 5 µL were used for protein identification by nanoLC-MS/MS (LTQ-Orbitrap) [Thermo Fisher Scientific, Germany; Fig. 1b].

We calculated the number of epithelial cells by assuming an average size of 10 x 10 x 10 µm for one cell.

Sample procurement (serum). Serum samples were obtained from the UMCG and stored at -80 °C in aliquots.

Results and discussion

By capturing ca. 2500 cells, we identified approximately 340 proteins according to HUPO criteria, with a significance threshold $p < 0.05$. Among them were a number of differentially expressed proteins of the heat shock protein family that were subsequently validated by western blotting and immunohistochemistry (see HsP90α below as an example). Western blot on 10,000 LMD-selected cells showed that HsP90α was only detectable in tumor cells (Figure 2).



Figure 2. Western blot for HsP90α of 10,000 cells in five cancer (C), five stroma (St) and five healthy (H) tissue samples. "PC" is positive control [ADI-LYC-HL101, Enzo].

HsP90α appears to be promising as an early indicator of cervical carcinoma, since it was negative in healthy epithelium from prolapse patients (Figure 3c) and stained positive in tumor cells and in morphologically normal epithelium by immunohistochemistry (Figure 3a,b). Overexpression of HsP90α was confirmed on tissue microarrays (TMAs) from a set of different patients with cervical cancer in comparison to healthy controls.

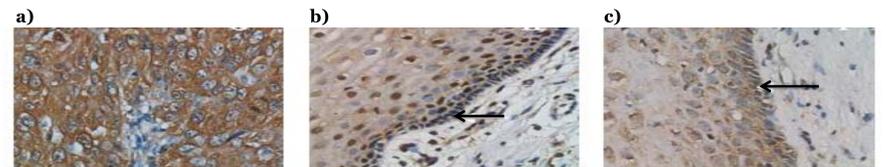


Figure 3. Immunohistochemistry for Hsp90α. a) tumor cells; b) morphologically normal epithelium; c) healthy epithelium from prolapse patient. Arrows indicate the staining of the basal epithelial layers (400-times magnification)

Up-regulation of HsP90 alpha was further confirmed by western blot in cancer tissue samples compared to healthy epithelium (Figure 4).

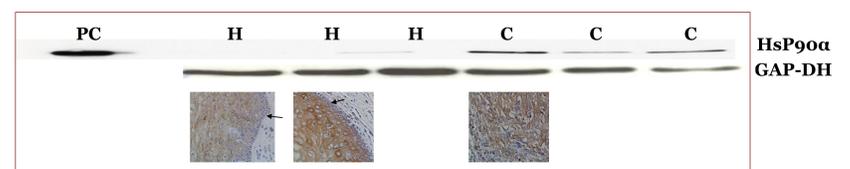


Figure 4. From top to bottom : western blot for HsP90α in three healthy controls (H) (morphologically normal epithelial tissue taken from the same biopsy as the tumor tissue) versus three cancer tissues (C) "PC" is positive control [ADI-LYC-HL101, Enzo]; GAP-DH was used as loading control; immunohistochemistry of HsP90α was positive (brown) for all tested tumor tissues. Arrows indicate the staining of the basal epithelial layers as negative, blue (400-times magnification).

Up-regulation of HsP90 alpha was translated from tissue into serum from cervical cancer patients at different stages of disease, cervical intraepithelial neoplasia (CIN) at low and high grades and healthy subjects by ELISA (Figure 5).

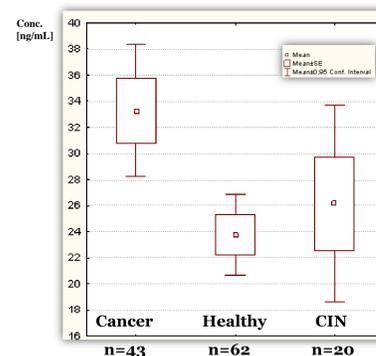


Figure 5. ELISA results for HsP90α in 62 healthy controls (H); 20 cervical intraepithelial neoplasia and 43 cancer serum samples. The difference between average values for cancer versus healthy was significant at 0.1% confidence level but not significant for cancer versus CIN ($p=0.11$) and healthy versus CIN ($p=0.48$) respectively.

A tendency towards increased concentrations of HsP90 alpha in serum from cervical cancer patients versus healthy subjects and patients with CIN stages was demonstrated (Figure 5).

Unfortunately, we were not able to assign all significantly different peptides fulfilling the criteria for tumor-specificity and discovered by comparison of cancer tissue, healthy epithelium and stroma of the cervix with LMD followed by MALDI-FT-ICR-MS and nanoLC-MS/MS (LTQ-Orbitrap). The developed SRM methods for HsP90α and other candidate-proteins will be used to screen a larger number of different serum samples from patients at various stages of disease with complete remission or recurrent disease using stable isotope labeled peptides as internal standards. This will allow to validate our initial findings and to obtain stronger statistical support for their validity as diagnostic markers for early cervical cancer or as prognostic markers for response to therapy.

Conclusion

We show on the example of HSP90 alpha that it is possible to translate a potential biomarker candidate that was discovered in tissue to serum of cervical cancer patients. Through a combination of LMD/tissue and serum proteomics, we discovered and confirmed a number of proteins as interesting biomarker candidates that should be further evaluated in conjunction with other diagnostic tests (e.g. cytology). In order to extend our findings in tissue and serum towards the development of a blood-based assay for cervical cancer to support cytological screening, we are currently analyzing sera from cervical cancer patients at different stages of disease versus healthy controls by LC-MS/MS in the selected reaction monitoring (SRM) mode.

References

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