New protein biomarkers for histopathological classification of breast cancer

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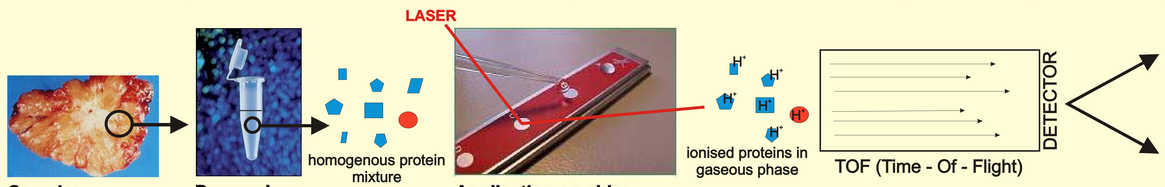
Breast cancer is one of the most common cancers in women in First World countries. In routine pathology, tissue samples examined by immunohistochemistry and fluorescent *in situ* hybridisation give us following information about breast cancer: grading, anatomical extent, presence/absence of lymph node metastases, steroid receptor and Her-2 overexpression/amplification status, and proliferative activity measured as Ki 67 index. We used Ciphergen technology based on SELDI TOF-MS (Surface Enhanced Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry) for analysis of protein expression profiles of 105 breast cancer tissue samples. The aim of this study was the identification of single proteins or protein patterns associated to commonly used diagnostic and prognostic characteristics, which could potentially be interesting as their complement.

Ciphergen method principle:

SELDI-TOF MS (Surface Enhanced Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry)

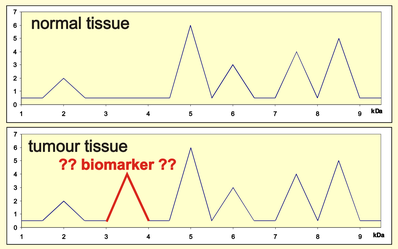


Sample:
- serum
- plasma
- tissue
- urine
- ...

Processing:
- denaturation
- fractionation
- lysis
- centrifugation
- ...

Application on chip:
- bounding the part of proteins from complex mixture onto the desired surface
- wash off of unbounded protein

Available surfaces:
- chemical (ion exchange, hydrofobic, hydrofilic, chelating)



Result:
spectra of protein profiles of individual samples

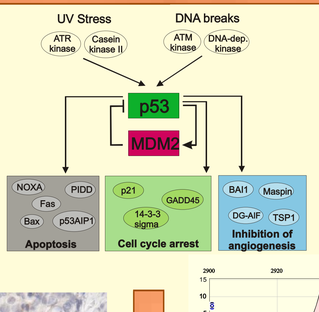
Experimental design:
- 105 female breast cancer patients treated at the Masaryk Memorial Cancer Institute (Brno, Czech Republic) in years 2004 and 2005 were chosen from consecutive sequence of operated women in clinical stage I or II without neoadjuvant therapy
- standard histopathological classification (morfology, grade, TNM classification, ER, PR, Her-2, Ki-67)
- chips IMAC 30 with Cu^{2+} chelated by nitriloacetic acid
- spectra measured in range 2-100 kDa
- laser intensity 190, sensitivity 5

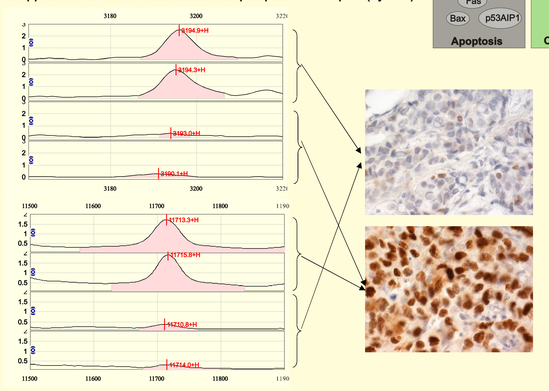
Specimens characterisation:
- pT2 tumours predominates (57 cases, 54 %) above pT1 (43 cases, 40 %) because of larger tumours are prefered to ensure availability of more tissue aliquots
- presence of one or more axillary lymph node metastasis is in 65 cases (63 %)
- the distribution in accordance with grade is uniform with a mild predominance of moderate and poor differentiates tumours (G1 26,6 %; G2 33,3 %; G3 37,1 %)
- the ensemble contains 72 ductal, 22 lobular carcinoma, 1 metaplastic, 3 mucinous, 2 papillar, and 5 mixed ductal lobular carcinoma
- oestrogen receptor expression was diagnosed in 86 cases (= 82 %) and progesteron receptor expression in 81 cases (= 78 %)
- overexpression of Her-2/neu receptor was detected by imunohistochemistry in 20 cases (= 19 %), gene amplification was present in14 (13 %) cases as evaluated by FISH
- expression of another biomarkers as p53, MDM2, cyclin D1, and oestrogen receptor beta was evaluated by imunohistochemistry in tissue microarrays

Evaluation procedure:
- spectra calibration by measured mass standard; normalisation by total ion current
- peak detection with signal/noise ratio > 3
- statistic analysis of peak intensities was done by non parametric Wilcoxon test (for 2 values of variables) or Kruskal-Wallis test (for 3 or more values of variables)

p53 level correlation

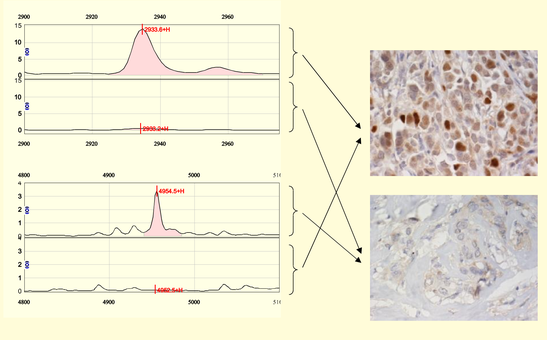
The p53 tumor supressor functions in many cellular processes: apoptosis, cell cycle arrest, DNA repair, recombination, cellular differentiation, and senescence.
After diverse stress stimuli, p53 level is risen due to phosphorylation stabilisation. Mutated p53 protein is predominantly accumulated in high levels.
We found two peaks correlating with p53 protein level on p<0,001. Peak of approx. ***m/z* 3194** correlates with wt p53 protein status and peak of approx. ***m/z* 11714** correlates with p53 positive samples (by IHC).





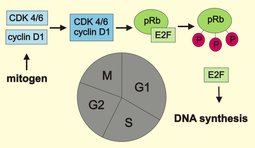
MDM2 level correlation

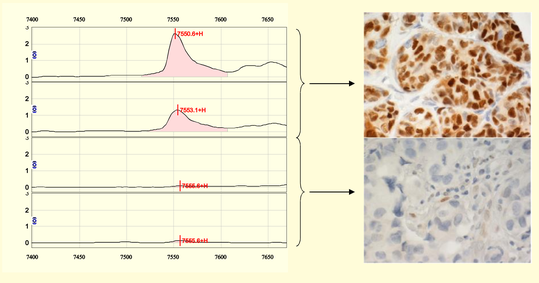
MDM2 is a p53-specific E3 ubiquitin ligase, that mediates the ubiquitin-dependent degradation of p53. MDM2 protein level is controled by wt p53. Together, they form a negative feedback loop controling low p53 level in non stressed cell. This loop is also affected by other regulatory proteins such as E2F1, pRb, and p107.
We found two peaks correlating with MDM2 protein level on p<0,001 of approx. **m/z 2933 and 4950**.



cyclin D1 level correlation

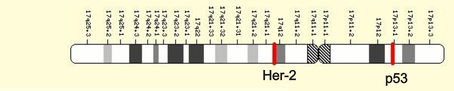
The passage of cell through the cell cycle is regulated by a series of enzymes (mainly cyclin-dependent kinases) and proteins (cyclins). Cyclin D1 falls into G1 cyclins, the expression of cyclin D1 is rapidly induced as breast cancer cells begin to divide, features a very short half-life, and is rapidly modulated in response to changes in extracellular environment.
We found two peaks correlating with cyclin D1 protein level on p<0,001 of approx.
***m/z* 7554 and 8707**

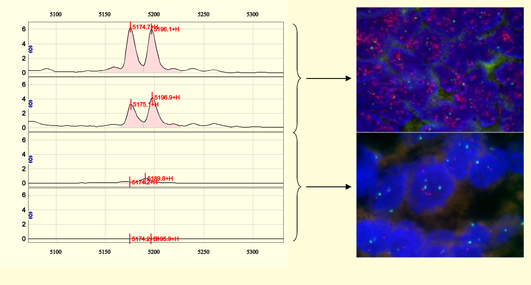




Her-2 amplification correlation

Amplification and overexpression of the Her-2/*neu* (c-*erb*B2) gene and protein have been identified in approx. 25% of invasive breast cancer. c-*erb*B2 gene encodes a transmembrane tyrosine kinase receptor protein that is a member of the HER family. After ligand binding, signal transduction including Ras/MAPK pathway, PI3K/Akt pathway, the JAK/STAT pathway, PLC-gamma pathway affect cell proliferation, survival, motility, and adhesion.
We found two peaks correlating with c-*erb*B2 gene amplification on p<0,001 of approx.
***m/z* 5175 and 5195**

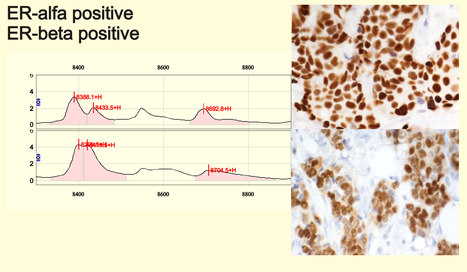




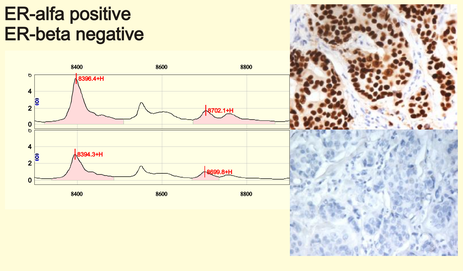
Oestrogen receptor correlation

The oestrogen receptor (ER) proteins, ER-alfa and ER-beta, are members of nuclear hormone receptor family. Upon ligand binding, ER dissociates from its inactive binding to HSP90, activates, undergoes a conformational change, dimerizes, and autophosphorylates through intrinsic tyrosine kinases. Activated ER dimers recruit coactivators and/or corepressors and eventually bind to recognition sequences termed oestrogen response elements, which regulate the promotor region of a variety of genes and can activate the mitogen activated protein kinase pathway, resulting in the activation of the AP-1 protein, fos and jun.
We found two peaks correlating with oestrogen receptor presence on p<0,001. Peaks of approx. ***m/z* 8395, 8426 and 8707** correlate with ER-alfa and peaks of approx. ***m/z* 8395 and 8707** correlate with ER-beta (by IHC).

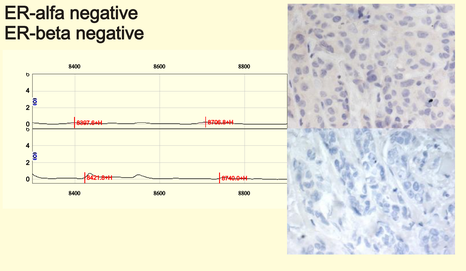
ER-alfa positive
ER-beta positive



ER-alfa positive
ER-beta negative



ER-alfa negative
ER-beta negative



Conclusion

In protein spectra we determined specific peaks significantly correlating with biologically important parameters of breast carcinoma (oestrogen receptor positivity, cyclin D1 overexpression, c-*erb*B2 gene amplification). Moreover, our data revealed peaks linked to p53 and MDM2 protein levels as well. Further, obtained data will be validated on independent set of samples and proteins of interest will be identified.

Future visions

- identication of concrete proteins and study of their function
- determination of characteristic signatures useful for breast carcinoma subclassification, analogical to RNA expression profiles

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