QUANTIFICATION OF DIAGNOSTIC PROTEIN SIGNATURES OF POLYGENIC DISEASES CHARACTERIZED BY MASS SPECTROMETRIC PROTEOME ANALYSIS: A STUDY ON MAMMA CARCINOMA

Waters

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OVERVIEW

- **Mammary Carcinoma is the most frequent malignant tumor in women. Proportion of mammary carcinoma in all reported new cancer cases is 24.4%World-wide the number of incidence exceeds one million per year (Germany ca. 47,500 per year). Raw incidence rate has been determined to 112.9 per 100,000 women. In Germany, one in ten women will suffer from mammary carcinoma during her lifetime. Five year survival rate is 76%**
- **Invasive ductal carcinoma is by far the most abundant breast cancer type with a proportion of up to 75%. At least 2/3 of all breast cancer cases occur in postmenopausal women**
- **In our investigation we studied tissue samples from three individuals that belong to the group of postmenopausal women suffering from invasive ductal carcinoma. The biological material has been extracted after full mammary gland removal**
- **The investigated tumor material consists of a mixture of tumor cells (ca. 50%) and so-called bystander cells (ca. 50%). The latter cells comprise connective tissue cells, stroma cells, endothelial cells, and infiltrating lymphocytes. The control material represents unaffected tissue consisting of connective tissue cells, stroma cells, endothelial cells, fat cells and to a lesser extend infiltrating lymphocytes. Due to inter-individual differences, the cellular composition of the control material is less well defined than that of the tumor tissue**

INTRODUCTION

With the availability of the human genome sequence, data driven research for tackling the molecular grounds of multifactorial, polygenic diseases can be considered a realistic challenge to the scientific community. In most recent research projects protein expression profiles are obtained using sophisticated MS based equipment, producing read-outs termed protein signatures rather than single protein markers.

In this study a comprehensive MS based discovery strategy is applied for a polygenic disease. The method employs the separation and detection of non-labelled tryptic fragments by means of an LC-MS acquisition. During the acquisition, the collision energy within the gas cell is continuously switched from low to elevated energy and no precursor isolation applied. The low energy functions contain all detectable peptide pseudo molecular ions. In a complementary fashion, the resulting elevated-energy data provides extensive multiplexed fragmentation information. The elevated energy fragment ions are aligned to their related precursor ions in chromatographic space by time and profile. Relative quantification is achieved via normalization of the MS data sets and comparison of the peptide intensities across injections and between samples. Identification of peptides exhibiting a change in expression level is using the peptide exact mass and the fragment ion information from the high-energy dataset.

An initial study was conducted on a relative small patient group and quantitative multivariance analysis performed. Initial results on samples from patients that suffer from ductal breast carcinoma indicate that expression levels of the newly found potential protein signatures might become useful in diagnosis and possibly prognosis.

METHODS

Sample Preparation

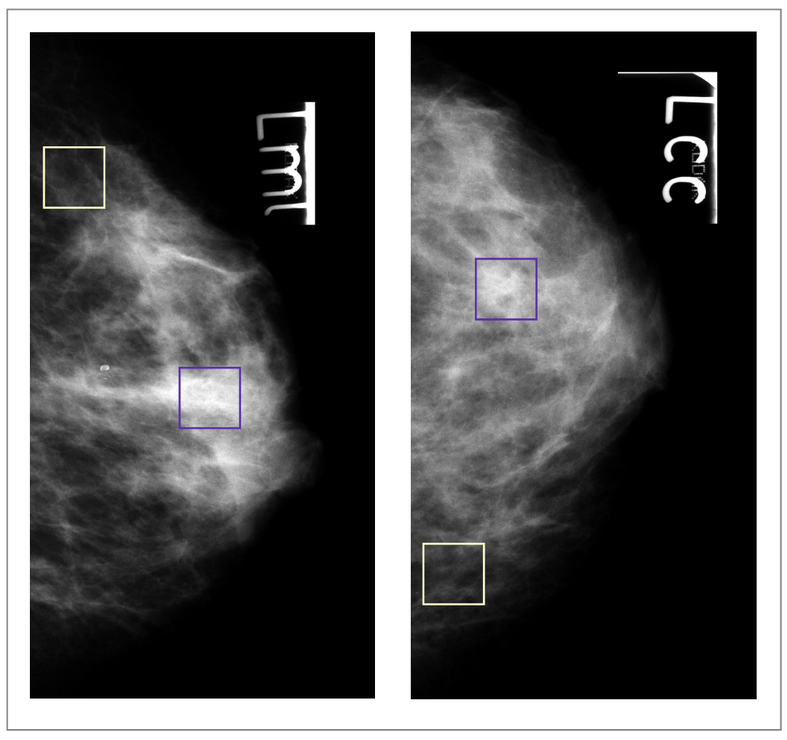

Figure 1. X-ray Pictures Illustrating The Location Of The Cancer And Healthy Tissue.
Blue = cancer tissue Yellow = healthy, unaffected tissue

75 µL of breast cancer and healthy tissue protein extract samples were taken up in 50 mM NH_4HCO_3, 0.1% RapiGest SF (Waters Corp.), pH 8.5 to a final concentration of ~ 1 µg/µL. Reduction and alkylation was with 2.5 µL 100 mM DTT and 2.5 µL 300 mM IAA, respectively. The proteins were digested with 1:25 (w/w) sequence grade trypsin overnight (16 hr). Trypsin was added immediately after the addition of DTT and IAA to limit endogenous protease activity.

RapiGest was removed by the addition of 2 µL conc HCl, followed by centrifugation, and the supernatant collected. Samples were diluted with 0.1% formic acid to an appropriate final working concentration prior to analysis—corresponding to an 0.5 µg of protein digest on-column load.

LC-MS CONDITIONS

LC-MS quantification experiments were conducted using a 1.5 hr reversed phase gradient at 250 nL/min(5 to 40% acetonitrile over 90 minutes) on a nanoACQUITY UPLC™ System (Waters Corp.) utilizing an Atlantis® 3 µm C18 NanoEase™ 75 µm x 15 cm nanoscale LC column (Waters Corp.). Samples were run in triplicate.

The Q-Tof Premier™ mass spectrometer (Waters Corp.) was programmed to step between normal (5 eV) and elevated (25-40 eV) collision energies on the gas cell, using a scan time of 1.5 s per function over 50-1990 m/z. Protein identifications and quantitative information were generated by the use of dedicated algorithms (Waters® Protein Expression Informatics), and searching various human specific databases.

RESULTS

Peptide/Protein Replication

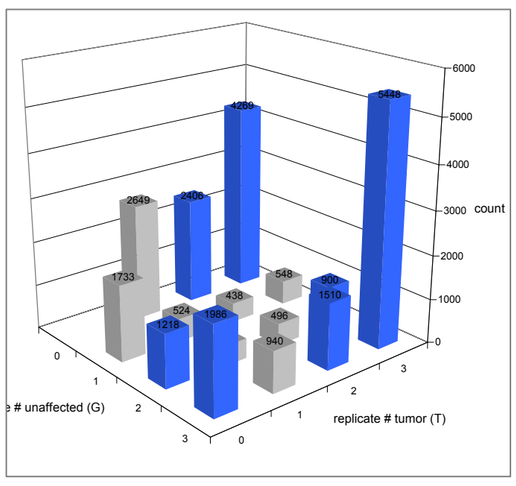

Figure 2. Replication Rate (count) vs. Injection # and Condition for Patient A. Blue = replication rate >= 2/3 Grey = replication rate < 2/3

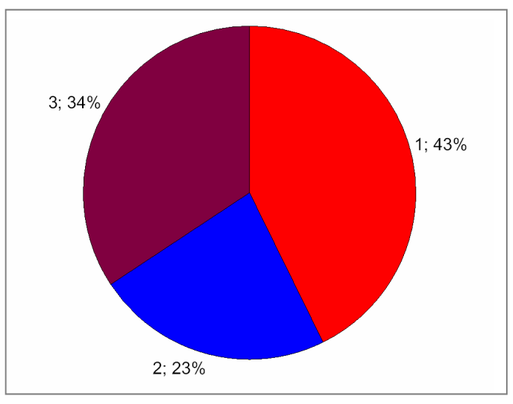

Figure 3. Peptide Replication Rate Condition G, patient A. 2/3 = 57% 3/3 = 34%

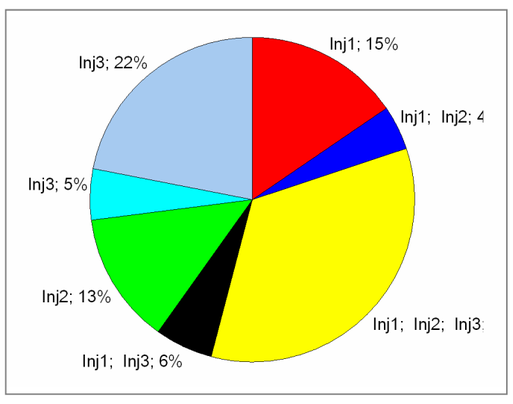

Figure 4. Protein Replication Rate Condition G, patient A. 2/3 = 45% 3/3 = 34%

PCA CLUSTERING

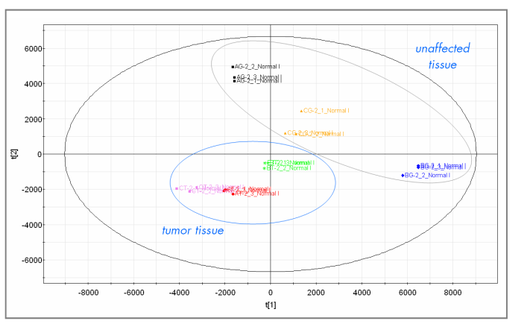

Figure 5. Scores Plot PCA Accurate Mass Retention Time Pairs All Patients. t[1] = 1st PCA component; t[2] = 2nd PCA component.

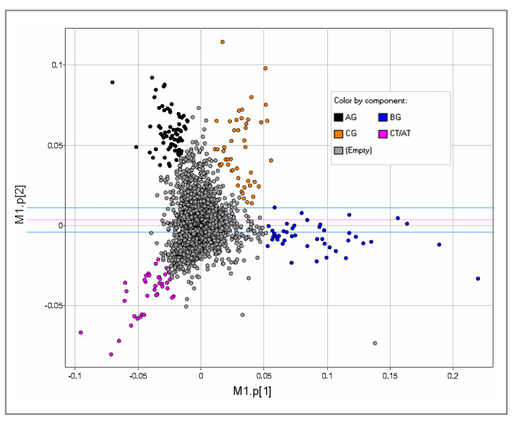

Figure 6. Loadings Plot PCA Accurate Mass Retention Time Pairs All Patients. M1 p(1) = 1st PCA component M1 p(2) = 2nd PCA component.

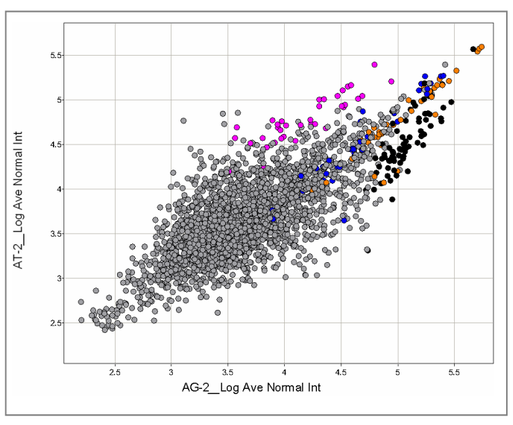

Figure 7. Log-Log Intensity Condition G vs. T for Patient A— Cluster Annotations as for PCA in Figure 6.

RELATIVE QUANTIFICATION

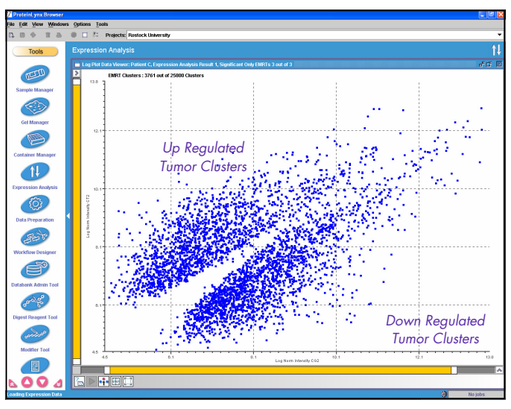

Figure 8. Significantly Regulated Peptides. Log-Log Intensity Condition T vs. G of Patient C.

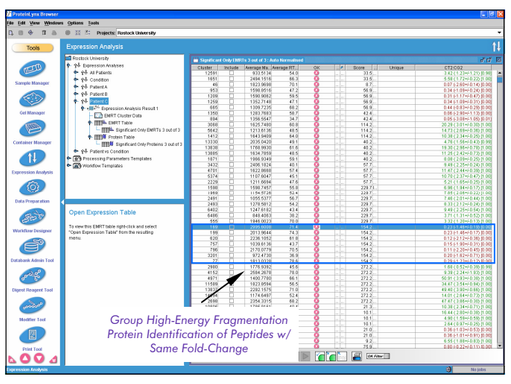

Figure 9. Accurate Mass Retention Time Pair Search Results High Energy Fragmentation Data of a Down-Regulated Protein in Condition T of Patient C Shown in Figure 8.

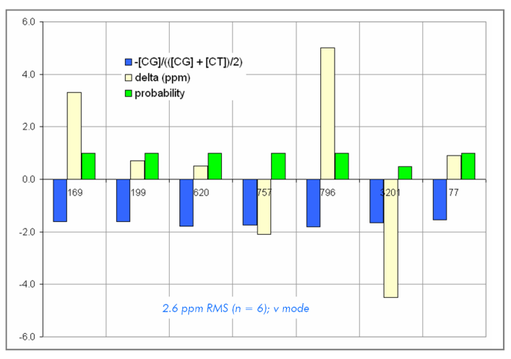

Figure 10. Quantitative Search Query Summary for the Highlighted Peptides in Figure 9. Relative Profile (blue), Delta Search Query (yellow), and Probability (green) vs. Cluster #.

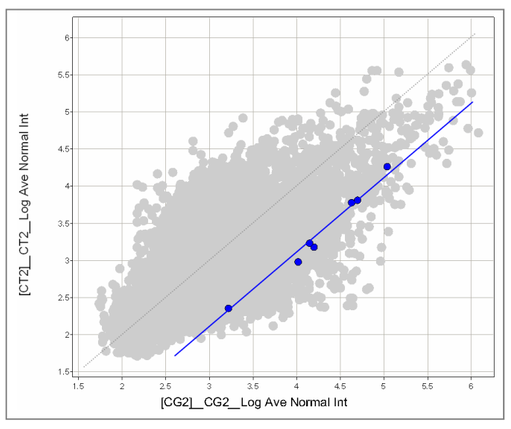

Figure 11.Log-Log Intensity Condition T vs. G of Patient C. Peptide Annotations of the 5-Fold Down-Regulated Protein Identified in Figure 9.

Protein Signatures

Absolute protein amounts were estimated and expressed as the ^{2}log ratio vs. a protein spike at the 150 fmol level providing both an instrument specific absolute concentration response factor and condition signatures.

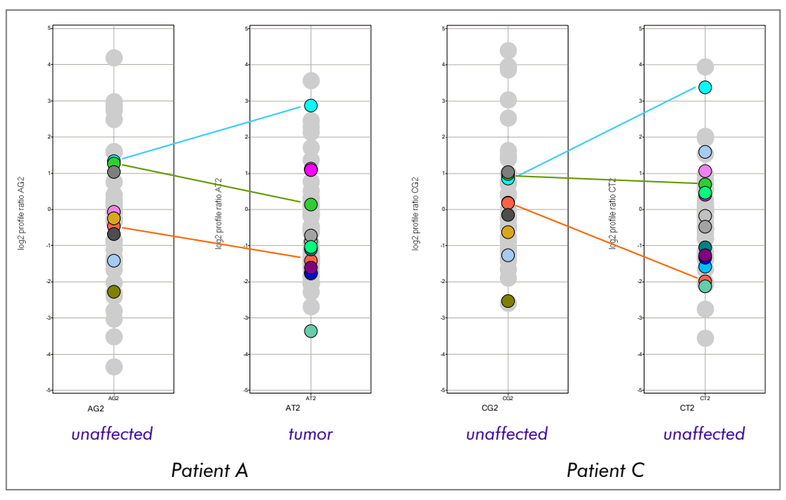

Figure 12. Protein Signatures for Conditions A and T of Patients A and C, Respectively.

FUTURE WORK

Future works will focus on the complementary analysis of invasive ductal carcinoma samples by means of 2D PAGE analysis followed by MALDI-TOF PMF analysis. The same selection criteria will be applied as within the approach presented in this study allowing to identify proteomics analysis technique independent signatures.

CONCLUSIONS

- **Our approach takes into consideration that both, tumor and control materials are from the same persons**
- **Inter-individual protein abundance differences are excluded by a stringent selection procedure in which only those proteins are considered as disease-related that occur in all three investigated patients only those proteins are considered that are regulated synchronously in all three comparisons**

Acknowledgement

Kieran Neeson and Scott Geromanos are kindly acknowledged for their assistance and contributions.

References

Quantitative Proteomic Analysis by Accurate Mass Retention Time Pairs. J.C. Silva, R. Denny, C.A. Dorschel, M.V. Gorenstein, I.J. Kass, G.-Z. Li, T. McKenna, M.J. Nold, K. Richardson, P. Young, and S.J. Geromanos. Anal. Chem. 2005, 77, 2187-2200.
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