[APPLICATION NOTE]

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DETERMINATION OF QUANTITATIVE PROTEIN SIGNATURES FOR DUCTAL CARCINOMA (BREAST CANCER) BY LC/MS PROTEOME ANALYSIS

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INTRODUCTION

With the availability of the human genome sequence, data-driven research for tackling the molecular grounds of multi-factorial, 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 is applied.

The low-energy functions contain all detectable peptide pseudo molecular ions. In a complementary fashion, the resulting high-energy data provides extensive multiplexed fragmentation information. The high-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 datasets and comparison of the peptide intensities across injections and between samples. Identification of peptides exhibiting a change in expression level is made using the peptide exact mass and the fragment ion information from the high-energy dataset.

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

EXPERIMENTAL

Sample preparation

75 µL of breast cancer and healthy tissue protein extract samples were taken up in 50 mM NH_4HCO_3, 0.1% RapiGest™ SF, 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 0.7 µg of protein digest on-column load.

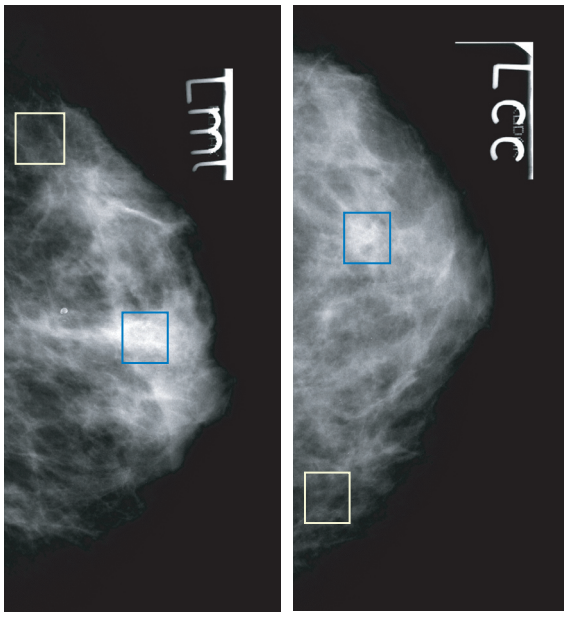

Figure 1. X-ray pictures (mammograms) illustrating the location of the cancer and healthy tissue; blue = cancer tissue; yellow = healthy, unaffected control tissue.

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 min) on the Waters® Expression^{E} High Definition Proteomics™ System, using as an inlet the nanoACQUITY UPLC® System and an Atlantis® 3µm C_{18} NanoEase™ 75 µm x 15 cm nanoscale LC column. Samples were run in triplicate.

The Expression^{E} System also included the Q-Tof Premier™ Mass Spectrometer, which was programmed to step between normal (5 eV) and elevated (25 to 40 eV) collision energies on the gas cell, using a scan time of 1.5 s per function over 50 to 1990 m/z. Protein identifications and quantitative information were generated by the use of dedicated algorithms, part of the Expression^{E} System informatics, and searching human-specific databases.

RESULTS

Protein and peptide replication

Figure 2 displays the peptide replication rate across both healthy and tumor tissue for patient A.

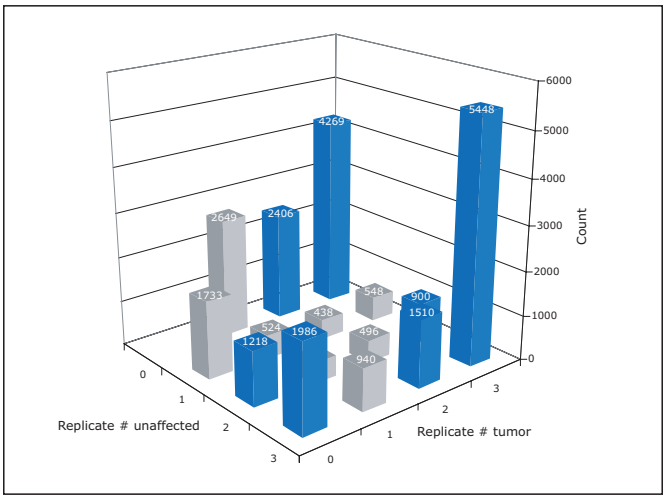

Figure 2. Accurate mass retention time pair replication rate (peptide count) vs. injection replicate # (tumor and unaffected conditions) for patient A; Grey = replication rate <2/3 injections. Blue = replication rate >2/3 injections (unique to tumor, 6675; unique to unaffected, 3204; common to both conditions, 7858).

Figures 3 and 4 show the peptide and protein replication rates for the healthy tissue from a single patient.

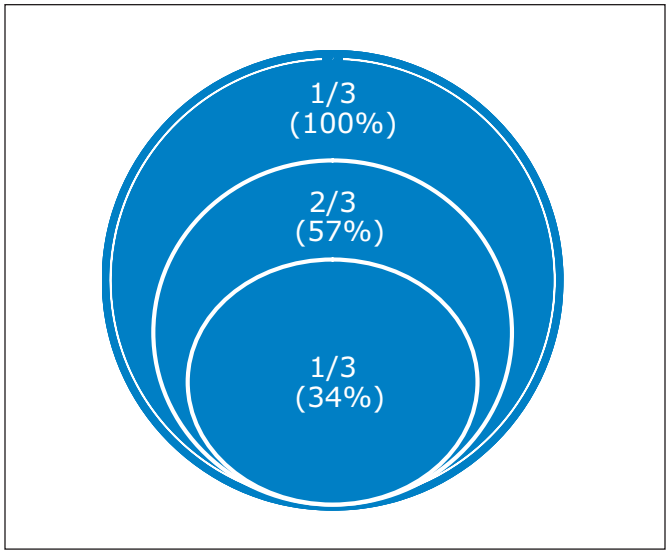

Figure 3. Peptide replication rate for condition G (unaffected control tissue), patient A; from 2 of 3 injections = 57%; from all 3 injections = 34%.

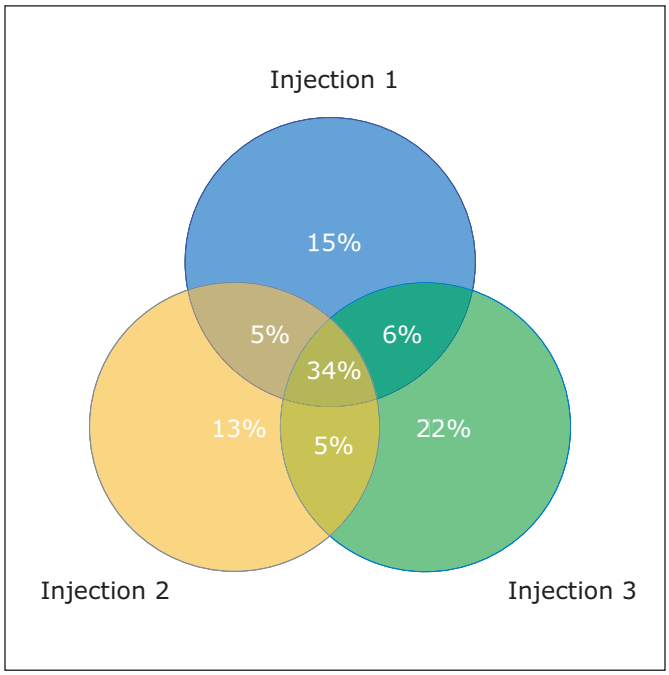

Figure 4. Protein replication rate condition G (unaffected control tissue), patient A; from 2 of 3 injections = 50%; from all 3 injections = 34%.

Clustering analysis

Figures 5, 6, and 7 display the PCA plots obtained from analysis of the low-energy precursor ion (peptide) information from the LC/MS experiments; retention time, mass, and intensity.

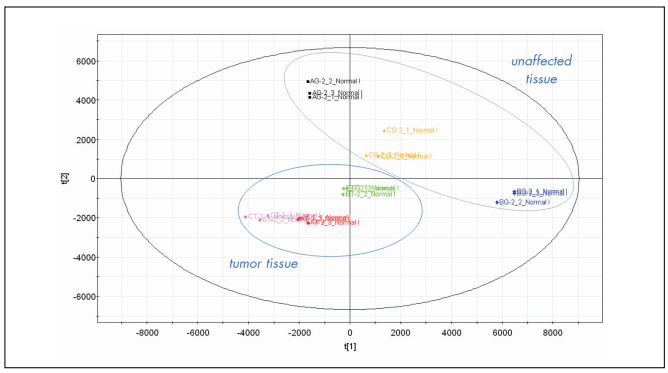

Figure 5. PCA scores plot of accurate mass retention time pairs (peptides) from all patients (A, B, and C) and conditions (tumor, T and unaffected, G); t[1] = 1st PCA component; t[2] = 2nd PCA component. The plot shows clear separation between unaffected and tumor tissue from each patient.

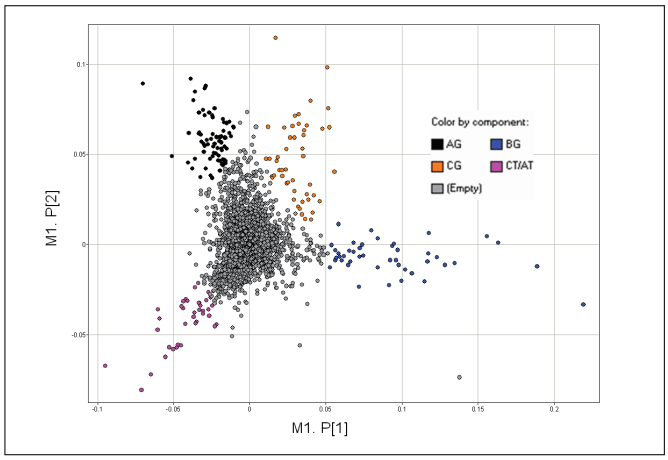

Figure 6. Loadings PCA plot of accurate mass retention time pairs (as in Figure 5); M1 p[1] = 1st PCA component; M1 p[2] = 2nd PCA component.

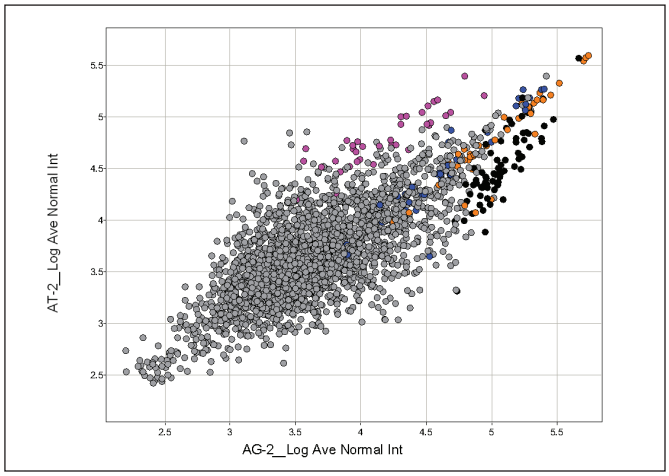

Figure 7. Log-log intensity visualization of accurate mass intensity pairs from Figure 5 and 6 (condition G (x-axis) vs. condition T (y-axis) for patient A). Cluster annotations as for PCA in Figure 6. Interestingly, the peptides (unique to patient and condition shown above) visualized in this plot are of relative high abundance.

Relative quantification

Presented in Figure 8 is a log-log plot of the peptide precursor ion intensity between the unaffected and tumor tissue for a single patient. Displayed are those ions that are statistically up- or down-regulated ($p<0.05$ and $p>0.95$). The peptides were subsequently searched utilizing both the peptide mass and fragment ion information, of which an example is shown in Figure 9.

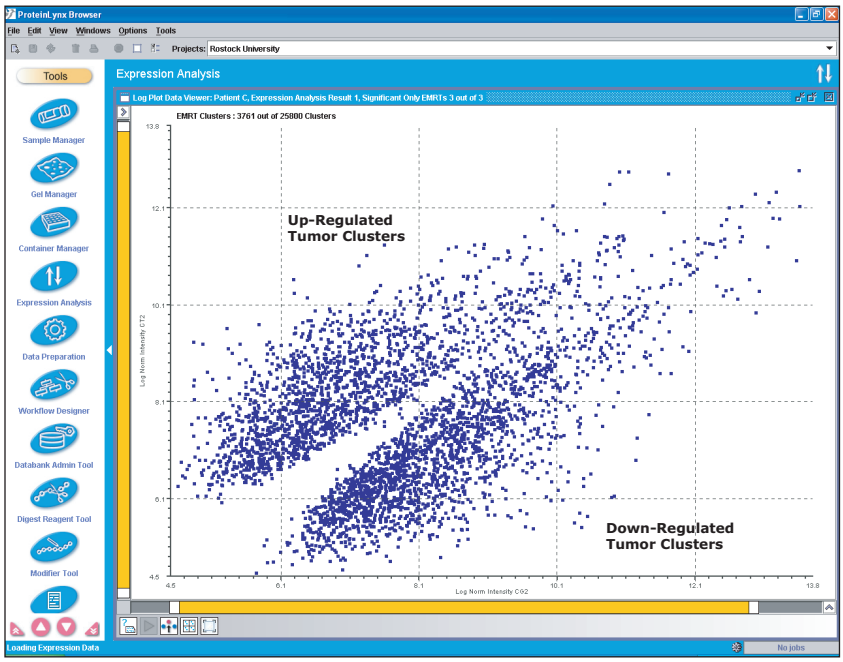

Figure 8. Significantly up- and down-regulated peptides shown here were selected for protein identification by database search. Log-log intensity condition T vs. G of patient C.

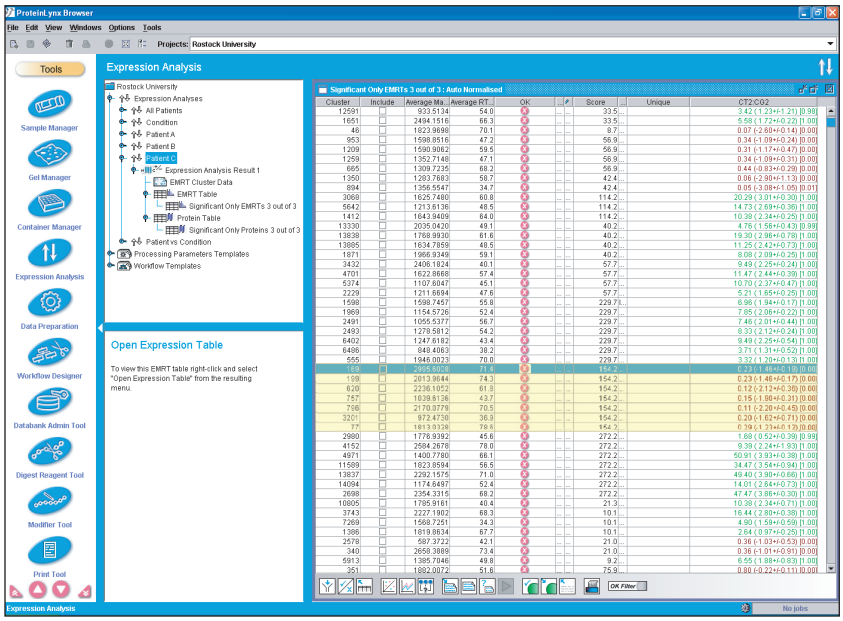

Figure 9. Protein identification results with peptides from each protein listed (accurate mass retention time pair search results of high-energy fragmentation data). Peptides identified from down-regulated osteoinductive factor protein in condition T of patient C are highlighted.

In Figure 10, peptide-level information is displayed in further detail for the osteoinductive factor protein highlighted in Figure 9. Of particular note is the consistency of the intensity profile across the peptides (blue bars). These peptides are then annotated on a log-log precursor ion distribution plot, which is shown in Figure 11.

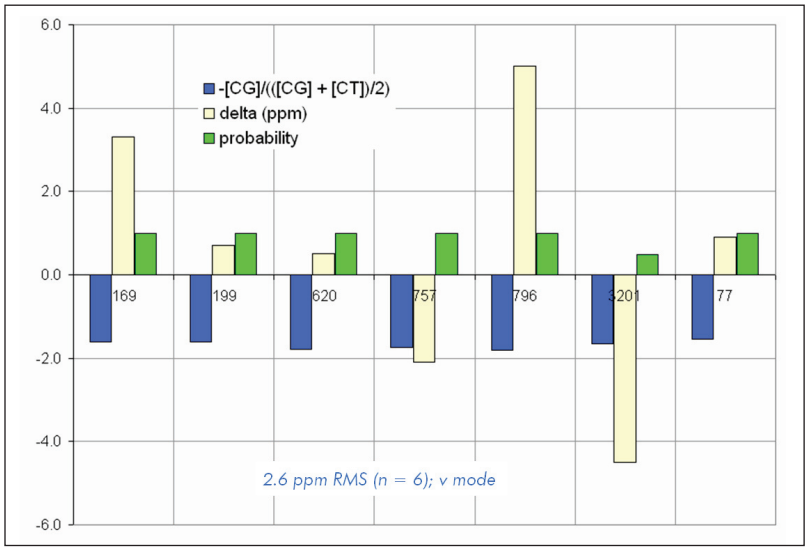

Figure 10. Measure of the consistency in relative abundance (blue), mass measurement error (yellow), and protein identification probability (green) for the highlighted peptides (shown here by cluster number) in Figure 9. The results display a high consistency for all peptides matched to osteoinductive factor protein.

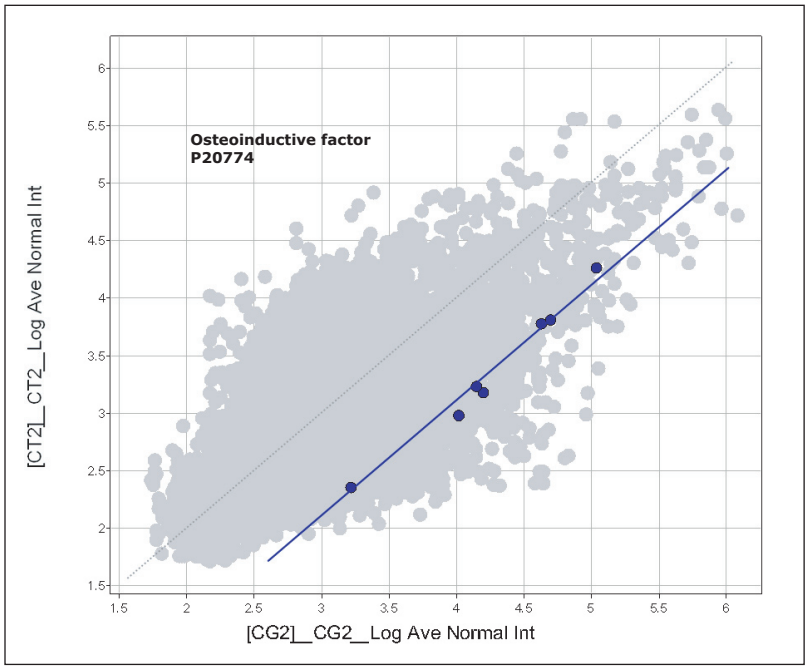

Figure 11. Log-log intensity condition T vs. G of patient C. Annotations of peptides from osteoinductive factor (identified in Figures 8 and 9) is shown to be down-regulated in tumor tissue of all patients.

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. These condition signatures are displayed in Figure 12, where it can be clearly seen that these profiles are consistent across patients A and C. These signatures do not require comparative analysis and therefore could easily be extended for larger scale studies.

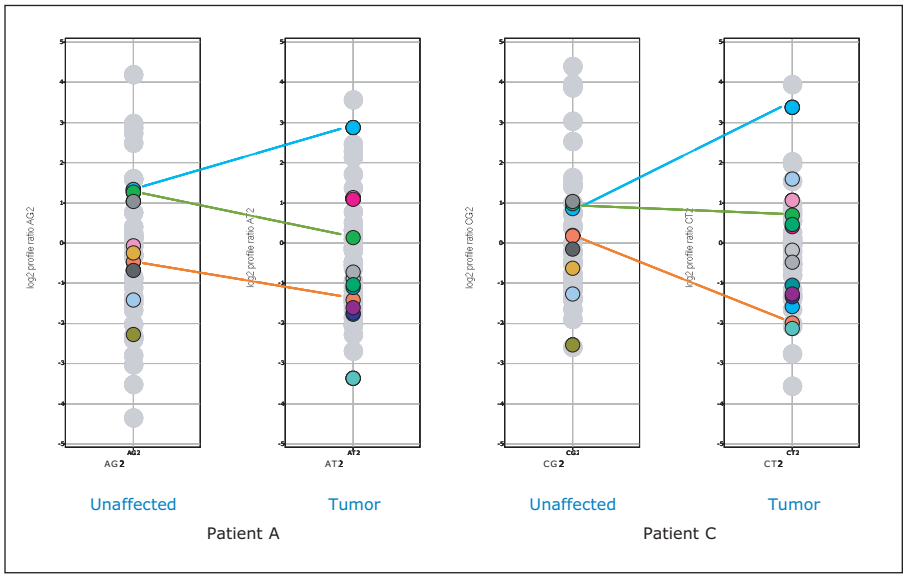

Figure 12. Protein signatures from unaffected and tumor tissue for patients A and C. Colors represent different proteins identified across all patients and conditions and their absolute concentration.

CONCLUSIONS

The presented 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

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 us to identify proteomics analysis technique independent signatures.

Acknowledgements

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