# Over-representation of proteins identified as disease markers and their relation to post-mortem events

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## Background

Tissue sampling is a major traumatic event that can have drastic effects at the molecular level within a very short time frame resulting in loss of sample quality due to post-mortem changes. It has recently been reported that the same proteins, regardless of tissue or species, are often found expressed differentially in disease states, bringing into question the significance of these proteins as potential biomarkers. We report a remarkable overlap between proteins commonly identified as changing in 2D-GE proteomics studies [1, 2] and those found to change post-mortem. We recommend an alternative technique to prevent these post-mortem changes.

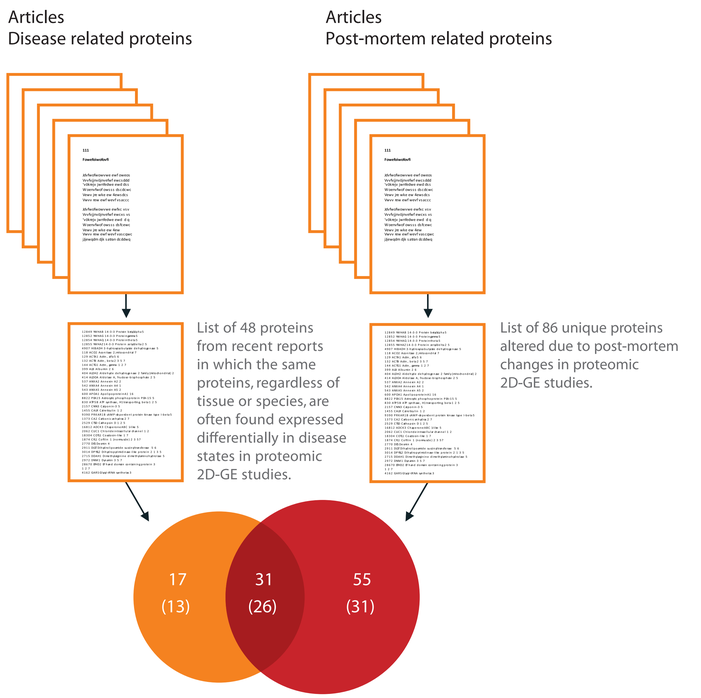


Figure 1. Comparison between the number of proteins that are frequently found as differentially expressed in the literature and the number of proteins that are found to be differentially expressed in studies of post-mortem changes. A remarkably high number (31) of the proteins identified in 2D-GE studies are the same. Numbers in parenthesis are the number of proteins containing phosphorylation sites.Venn diagram, left circle disease markers and right circle illustrates markers of post mortem change.

## Method

Data was drawn from studies investigating post-mortem changes in proteomic profiles [3, 4, 5, 6, 7, 8] to create a list of 86 unique proteins that appeared to be altered only due to sample handling. This list was compared with a list of 48 proteins often occurring in 2D-GE experiments, drawn from publications of Petrak et al. [1] and Wang et al. [2]. Various estimations were made to investigate an apparent overlap of proteins, for example, calculating the number of phosphorylations on target proteins (Figure 2-4).

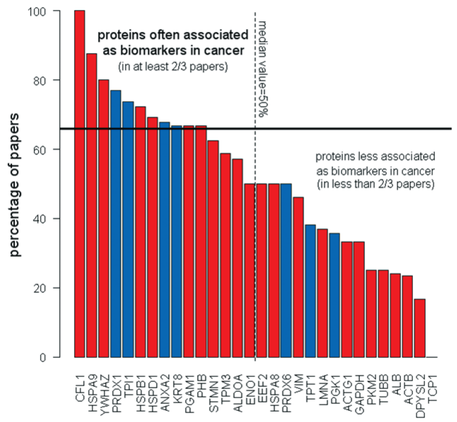


Figure 2. Number of papers found in PUBMED for each protein including the search term "biomarker" with or without cancer association.The median value was found to be 50%. Some proteins such as cofilin 1, HSP70KDa protein (mortalin), 14-3-3 protein zeta/delta and peroxiredoxin 1 are in at least two out of three papers associated with cancer. Red bars illustrates proteins also reported as markers of post mortem changes and blue bars are proteins not reported as markers of post-mortem changes.

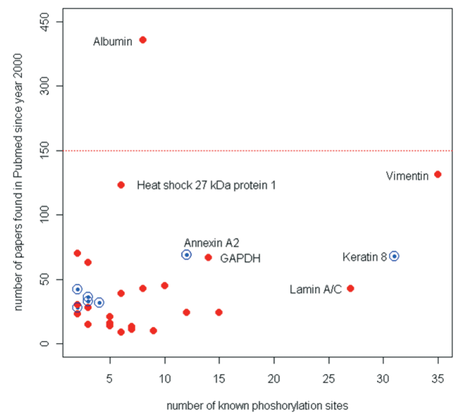


Figure 3. Comparing number of papers found in PUBMED including search terms biomarker/marker or cancer versus number of phosphorylation sites revealed that 30 out of 48 proteins contain at least two sites. If albumin is excluded there is a statistical significant relationship (Pearson correlation coefficient=0.49, r221=0.24, p<0.01) between number of papers found and number of phosphorylation sites. Red dots illustrate proteins that have been reported as potential protein markers of post-mortem changes and blue dots are proteins not reported as markers of post-mortem changes.

## Result

Comparing these two protein subsets we found 31 (65%) between the lists. To study this in respect of proteins frequently found to be differentially expressed, we examined how many of these proteins had known phosphorylation sites. We found that 39 (81%) of them contain at least one phosphorylation site and, with respect to post-mortem stability, 26 of the 31 proteins (84%) contain at least one known phosphorylation site (Figure 1). This clear overrepresentation of phosphorylation sites, compared to an average of approximately 30% of the total proteins in a cell, indicates that proteins and their post-translational modifications change substantially and rapidly post-mortem.

It should be noted that, since phosphatase inhibitors are often used to prevent loss of phosphorylations, their use in the absence of kinase inhibitors can result in hyper-phosphorylation of specific sites [9].

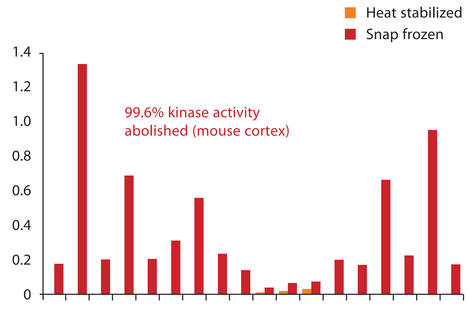


Figure 4. Graph shows the 18 most fluorescent peptides from a 144 peptide array in a kinase activity assay of brain homogenates that had been previously snap frozen or heat stabilized. Only three out of 144 peptides produced measurable fluorescence in heat-stabilized samples. Of the remaining 126 peptides not shown, 73 additional peptide substrates produced measurable fluorescence in homogenates that had been snap-frozen.

Data extracted from:Thermal stabilization of tissues and the preservation of protein phosphorylation states for two-dimensional gel electrophoresis, Smejkal et al., Harvard Medical School , Electrophoresis 2011.

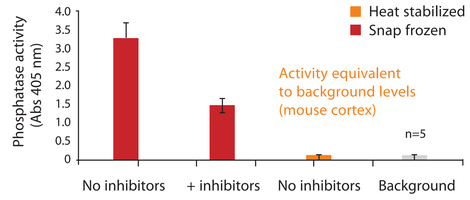


Figure 5. Heat stabilization abolishes phosphatase activity (10).Tissue samples of mouse cortex were snap frozen or heat stabilized. Samples were solubilized in buffers using microtip sonication. Snap-frozen samples were solubilized with and without phosphatase inhibitors. Phosphatase activity compared in snap-frozen and heat-stabilized samples. Activity was measured using colorimetric pNpp phosphatase kit (AnaSpec).

Data from: Heat stabilization of the tissue proteome: a new technology for improved proteomics. Svensson et al., J. Proteome Res. 2009.

## Conclusion

We conclude that post-mortem changes, particularly in phosphorylation states, may distort our view of in vivo proteomic profiles. Conventional snap-freezing of tissue samples, usually with subsequent addition of enzyme inhibitors to prevent proteases and phosphatase activity, is not sufficient to prevent sample degradation during subsequent analysis. Figure 4 and 5 show the level of enzymatic activity still present in such samples in comparison with enzymatic activity present in samples that have been rapidly heat stabilized (Stabilizor™ system, Denator, Sweden). There is little or no enzymatic activity in the heat-stabilized samples.

We believe that the use of rapid heat stabilization, as an alternative to conventional snap-freezing, instantly and permanently stops enzymatic activity thereby preventing post-mortem changes to reveal the actual in vivo profile. This approach may help us differentiate true biomarkers from those proteins found in any situation where cells are under stress.

Figure 6.
Stabilizor system containing Stabilizor T1 instrument and Maintainor Tissue cards

## References:

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