Comparative qualitative proteomic analysis of conditioned media from breast cancer cell lines

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ABSTRACT

Introduction:
One of the best ways to diagnose breast cancer early or to predict therapeutic response is to use serum or tissue biomarkers. Unfortunately, for breast cancer, we do not have effective biomarkers for either early detection or prediction of therapeutic response.

Hypothesis:
Novel candidate tumour markers for the early detection of breast cancer are secreted or shed proteins and can be detected in the tissue culture supernatants of human breast cancer cell lines.

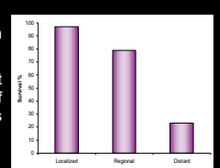
Methods:
A "bottom-up" proteomic approach and a two-dimensional liquid chromatography-tandem mass spectrometry (2D-LC-MS/MS) strategy was utilized to identify and compare levels of extracellular and membrane-bound proteins with low abundance in the conditioned media of breast cancer cell lines. Human breast epithelial cell lines from normal (MCF-10A), non-invasive (BT474) and metastatic (MDA-MB-468) origins were cultured in serum-free media. Proteins from the conditioned media were trypsin-digested and the resulting peptides fractionated using strong cation-exchange liquid chromatography (SCX). The SCX fractions were then analyzed using electrospray ionization tandem mass spectrometry (reverse phase LC-MS/MS) to identify the peptides present. Comparative analysis of the proteins identified in the normal, non-invasive and metastatic cell lines will provide candidate tumor markers for further investigation.

Results:
We have successfully identified over 400 proteins, using a 2-D linear ion-trap mass spectrometer (LTQ; Thermo Inc), from conditioned media of the breast cancer cell line MDA-MB-468. Two independent samples of the same cell line were prepared and over 250 secreted and/or membrane-bound proteins were identified in total. Among the identified proteins were proteases, receptors, protease inhibitors, cytokines and candidate breast cancer biomarkers such as human kallikrein 5, 6 and 10.

Conclusion:
Our strategy for delineating the secreted proteome of cancer cells will provide a large number of candidate molecules which can be examined in more detail for their ability to act as circulating breast cancer biomarkers.

INTRODUCTION

- Breast cancer is the leading cause of death among women with solid tumors in North America (1).
- Currently, mammography remains the cornerstone of breast cancer screening, despite its disadvantages such as a lack of specificity, high false positive and negative rates, hazardous exposure and discomfort experienced by patients.



- Alternatively, one of the best ways to diagnose cancer early or to predict therapeutic response is to use serum or tissue biomarkers.
- Carcinoembryonic antigen (CEA) and carbohydrate antigen 15.3 (CA 15.3) are the most commonly used tumor markers for breast cancer.
- However, these cancer biomarkers have proven to be ineffective in detecting the early stages of disease.
- Recent technological advances in the fields of genomics and proteomics have opened up new and exciting avenues for the discovery of biomarkers for the early detection of breast cancer.
- In particular, the use of mass spectrometers allow for the simultaneous examination of thousands of proteins in any given sample thus, making mass spectrometry (MS) ideal in identifying proteins and/or peptides as biomarkers, which may be present in abnormal amounts in patients with cancer.

OBJECTIVES

- Demonstrate that human breast cancer cell line (MDA-MB-468) can be cultured in large volumes in serum-free media
- Optimize cell culture techniques to minimize cell death by measuring intracellular protein lactate dehydrogenase (LDH) levels and to maximize secreted protein concentration
- Monitor the levels of our internal positive control secreted kallikrein proteins (eg. hK5, hK6, hK10) over time in culture
- Positively identify the internal control proteins and other proteins present in the conditioned media, via a "bottom-up" proteomic approach involving strong cation-exchange liquid chromatography (SCX) and mass spectrometry (LC-MS/MS)
- Classify identified proteins by cellular localization

METHODOLOGY

Cell Culture
- MDA-MB-468 cell line; grown in RPMI 1640 + 8% FCS
- $30x10^6$ cells seeded into two $175cm^2$ tissue culture flasks
- Changed to 30mL CDCHO serum-free media (Gibco)
- Cultured for 24 hours; conditioned media (CM) collected

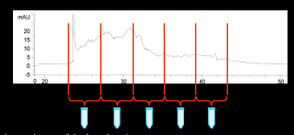
Sample Handling

Dialysis
- Molecular weight cut-off membrane of 3.5kDa
- 5L of 1mM ammonium bicarbonate overnight at 4°C

Lyophilization
- Samples lyophilized to dryness overnight

Trypsin Digestion
- Denatured, reduced, alkylated, desalted, lyophilized
- Trypsin digested overnight at 37°C

Strong Cation Exchange Liquid Chromatography
- High performance liquid chromatography (HPLC)
- Digested peptides were loaded onto a SCX column
- Linear gradient, eluded with 1M ammonium formate
- 20 fractions were collected and pooled into 5 fractions



Mass Spectrometry (LC-MS/MS)
- Fractions loaded on a C-18 reversed-phase chromatography column (Varian Inc.)
- Coupled online to a 2-D Linear Ion Trap (LTQ, Thermo Inc) electrospray ionization (ESI) mass spectrometer
- Mass spectra from both samples #1 and 2 (independent samples) were searched using the MASCOT search engine

Data Analysis
- Each protein identified was tabulated and its cellular localization determined
- Gene Ontology (GO), NCBI, Human Protein Reference Database (HPRD)

RESULTS

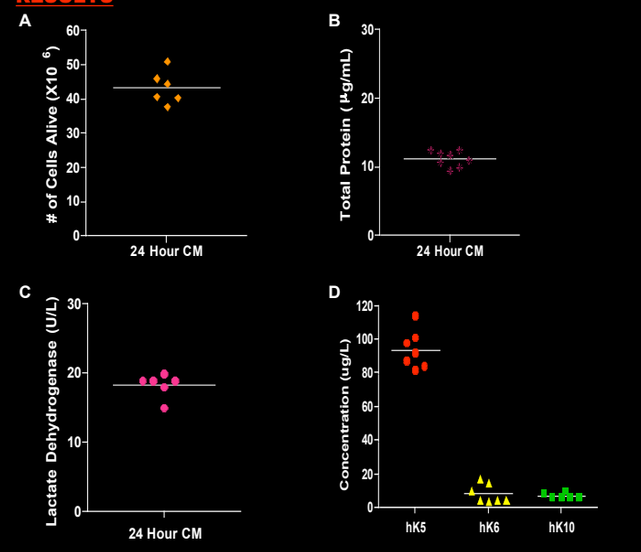
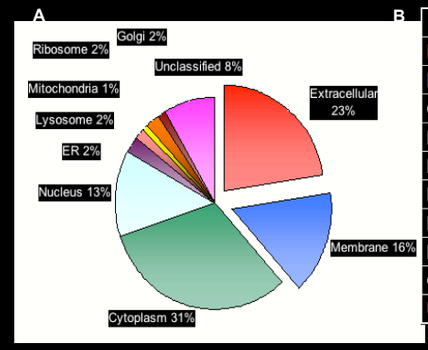


Figure 1: Cell number, protein, LDH and kallikrein levels in 24 hour MDA-MB-468 conditioned media. (A) Total number of cells alive during harvest of the CM; (B) Total protein concentration observed (C) LDH levels indicating 2% cell death; (D) hK5, hK6 and hK10 levels. This procedure was repeated several times for reproducibility.

RESULTS



Localization	# of Proteins
Extracellular	118
Membrane	83
Cytoplasm	165
Nucleus	69
ER	12
Lysosome	9
Mitochondrion	5
Ribosome	11
Golgi	8
Unclassified	43

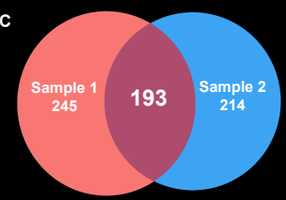


Figure 2: Classification of proteins by cellular localization from MDA-MB-468 and the overlap of proteins identified by MS between sample #1 and #2. (A) Sample #1: A total of 437 proteins were identified with ~40% being classified as extracellular and membrane-bound. (B) Table illustrating the breakdown of the cellular localization for sample #1. (C) Venn diagram: 193 proteins (~50%) are common between to the two samples.

SUMMARY & CONCLUSION

- Successfully identified over 400 proteins, using a 2-D linear ion-trap mass spectrometer in the CM of a breast cancer cell line.
- Internal control proteins hK5, hK6 and hK10 were identified by MS (validating sample preparation)
- 250 secreted and membrane-bound proteins were identified in the CM.
- Other interesting proteins identified include ones that have established roles in breast cancer development (cell growth, differentiation, metastasis), are linked to early onset breast cancer (E-cadherin) or are potentially useful to investigate further as novel candidate markers.
- These proteins will need to be compared to other cell lines to be further narrowed down.
- Our strategy for delineating the secreted proteome of cancer cells will provide a large number of candidate molecules which can be examined in more detail for their ability to act as circulating breast cancer biomarkers.

REFERENCES

1) van Diest PJ, van der Wall E, Baak, JPA. Prognostic value of proliferation in invasive breast cancer: a review. **J Clin Pathol** 2004;57:675-681.

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