

Ning Qing Liu, René Braakman, and Arzu Umar

Prognostic protein markers for triple negative breast cancer

14 | NPC Highlights 12 | November 2010

Breast cancer is a mixture of biologically different disease entities. One of these subtypes, called triple negative, is associated with a poor prognosis and lack of effective treatment. No clinical biomarkers are available. The authors of this article were able to identify a set of prognostic proteins by comparative tissue proteomics using highly advanced technology. This may lead to better prediction of disease prognosis and the development of new therapies for triple negative patients.

Breast cancer is a very heterogeneous disease, consisting of different molecular subtypes. Women with the so-called 'triple negative' subtype of breast cancer have poor prognosis and survival compared to other subtypes due to the aggressive nature of these tumours and the current absence of suitable targets for therapy. Clearly, identification of appropriate prognostic protein targets for this group of patients is of vital importance. In the current project, we use a comparative tissue proteomics approach for the identification of prognostic protein markers for triple negative breast cancer.

Breast cancer affects 1:8 women throughout their lives and accounts for more than 3,000 deaths annually in the Netherlands. Tumour cells most commonly originate from epithelial cells lining the milk ducts or lobules (see Figure 1A). While histopathological parameters such as tumour grade, stage, and lymph node or distant metastasis have long been the golden standard

for predicting prognosis, molecular profiling has proven to be an important additional parameter [1]. Molecular subtypes of breast cancer as defined by gene expression profiling were initially described a decade ago as biologically distinct disease entities with different clinical outcomes [2,3].

Five subtypes The five main observed subtypes, luminal A, luminal B, HER2+, normal-like, and basal were named according to the expression of particular genes. The majority of breast tumours are of the luminal A subtype, which is characterised by, among other things, high expression of oestrogen receptor (ER) and progesterone receptor (PR), preferential metastasis to bone, and association with a relatively good prognosis (see Figure 1B). Luminal B type tumours have lower expression of ER and/or PR, HER2+ tumours have an amplification of the human epidermal growth factor receptor 2 (HER2) gene, and normal-like and basal type tumours have high expression of basal epithelial cell type keratins, such as keratin 5 and 17, and are mostly characterised by the absence of ER, PR, and HER2. For that reason the latter group is often referred to as 'triple negative'. More significantly, triple negative tumours preferentially

What this research is about:

Together with American colleagues: search for new biomarkers

Breast cancer is the most commonly diagnosed malignancy in women and affects one out of eight women throughout their lives. Breast cancer is not just one disease. Several subtypes are revealed and corresponding biomarkers could potentially be used for early detection, prognosis and therapy response prediction.

"For one subtype called triple negative, specific markers are lacking. Triple negative in general has a poor prognosis and poor patient survival," says Arzu Umar, researcher at the Department of Medical Oncology of the Erasmus Medical Centre in Rotterdam. A small subgroup of these patients accounts for this poor prognosis and group members will develop cancer in distant organs within a few years. The majority remain metastasis-free for longer periods of time. "We wanted to know what caused this difference and whether we could find clinical biomarkers to define the triple negative subtype."

This article describes the special technology and method the authors used to discover these biomarkers. "We obtained access to very advanced technology by collaboration with the American Pacific Northwest National Laboratory," Umar says. This laboratory is a user facility, which is ahead of any commercially available technology and therefore offers unique possibilities. The researchers were able to use a custom-made, ultra-thin and ultra-long separation column coupled to a high resolution mass spectrometer. Umar: "Since we were able to separate the proteins better, we could see more. If you can see more, chances are higher that you will identify differences and find interesting biomarkers. And so we did."

Umar and her colleagues detected a set of more than thirty proteins that can help to divide the group of the triple negative cancer into one with a good and one with a poor prognosis. Umar: "Our next step is to validate these proteins and unravel their function in the development of a triple negative type of breast cancer." In the end this may lead to better prediction of the prognosis and development of new therapies.

NPC Research Theme T1: Cancer Proteomics

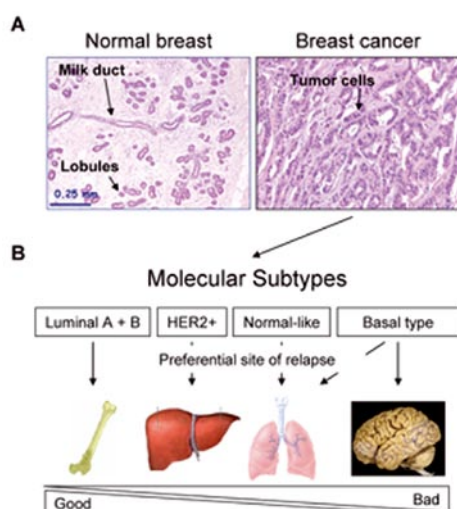


Figure 1 | Breast cancer development and progression.

A. Cross-section and haematoxylin/eosin stain of normal and cancerous breast tissue.

B. Different molecular subtypes of breast cancer, preferred sites of relapse and prognosis.

metastasize to lung and brain [4] and have the worst prognosis compared to other subtypes (see Figure 1B).

The majority of breast tumours (about 80 percent) are ER, PR, or HER2+ positive and can be effectively treated with targeted therapies directed against these proteins, such as hormonal therapies blocking production or function of oestrogens, and antibody or kinase inhibitor therapies blocking the HER2 pathway. However, about 15 percent of breast tumours are triple negative and effective treatment is not readily available. Identification of protein markers that are specifically expressed in triple negative tumours would not only benefit proper clinical classification, but would provide the basis for development of new, targeted therapies against this aggressive type of breast cancer [5].

Two kind of triple negative Despite a common triple negative phenotype, these tumours can clinically be defined as two separate groups based on disease prognosis. Within the triple negative subtype, 25 percent of the patients develop distant metastasis within three years, whereas 75 percent remain long-

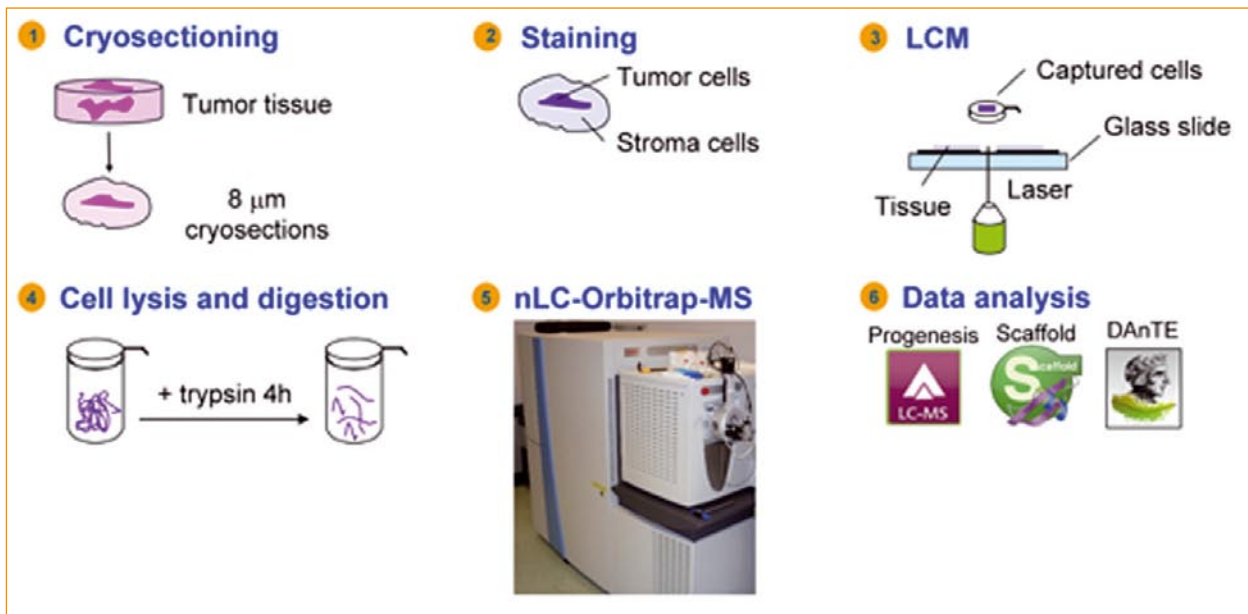


Figure 2 | Tissue proteomics work flow.

Flash frozen tumour tissue is cryosectioned, stained, and subjected to laser capture microdissection to specifically isolate tumour cells. Proteins are extracted in surfactant and trypsin digested prior to nLC-MS analysis.

term metastasis-free. We hypothesise that activation of different molecular mechanisms must account for this difference in prognosis. It should thus be possible to identify markers that

we identified a putative protein profile that predicted therapy-resistance in breast cancer patients [7].

Prognosis proteins We have used the above described tissue proteomics strategy for comparative analysis of 62 triple negative breast tumours, of which 22 were from patients with poor prognosis and 40 were from patients with good prognosis. From each tumour tissue, tryptic digests derived from about 3,500 cells were analysed. On average, about 1,200 proteins were identified per sample, and in total 2,015 proteins were identified in all samples with two or more peptides. Initial data analysis and unsupervised hierarchical clustering revealed that a selection of peptides belonging to 34 proteins is able to separate triple negative tumours from patients with poor prognosis from those with good prognosis (see Figure 3) [8]. In line with our close collaboration with Dr. Ljiljana Paša-Tolić, Ning Qing

specifically associate with poor prognosis in patients with triple negative breast cancer. With NPC funding, we have performed comparative global proteome profiling on tumour tissues with the aim of identifying prognostic protein markers for triple negative breast cancer.

Pacific Northwest National Laboratory In collaboration with Dr. Ljiljana Paša-Tolić from the Pacific Northwest National Laboratory (PNNL), we have previously developed a tissue proteomics approach for the identification of protein markers (see Figure 2). Our strategy involves the use of fresh frozen breast cancer tissue, out of which cryosections are cut, which are subsequently stained in a mass spectrometry-friendly manner and subjected to laser capture microdissection (LCM). The LCM step is pivotal to enrich for epithelial tumour cells, since breast tissue is very heterogeneous and consists of a mixture of cells such as tumour cells and surrounding stromal cells. To enable large-scale cohort studies, we store microdissected samples at -80°C until all tissues have been subjected to LCM. Subsequently, proteins are extracted and trypsin digested prior to nLC-FTICR or Orbitrap MS analysis. Resulting MS data are analysed using Scaffold and Progenesis software, or free-ware developed at PNNL, such as DAnTE. The important part of our approach is the use of custom-made, ultra-long (50-80 cm), narrow diameter (30-75 μm) chromatography columns coupled to ultra-high pressure liquid chromatography. We have shown that this approach is sensitive enough to identify large numbers of proteins from just minute amounts of cells [6]. In addition,

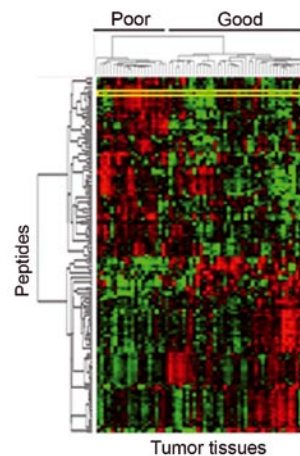


Figure 3 | Unsupervised hierarchical clustering of triple negative breast cancer tissues. Differentially abundant peptides distinguish tumours with good and poor prognosis.

Liu (PhD student supported by NPC) is currently visiting the mass spectrometry facility at PNNL for further, detailed analysis of his LCM samples.

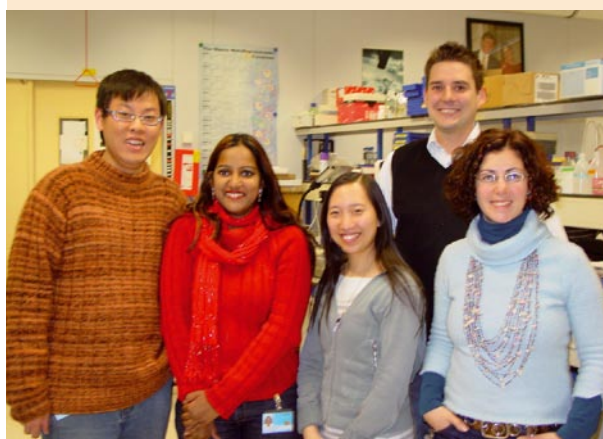
Next validation In conclusion, a fruitful and long-standing collaboration with Dr. Paša-Tolić from PNNL has provided a basis for establishing sound protocols that enable in-depth comparative proteomics studies on clinical cohort samples. In the current, NPC funded, project a triple negative breast cancer cohort was analysed by nLC-Orbitrap MS and comparative proteome analysis was performed using different software tools. Preliminary data showed that patients with poor prognosis could be separated from patients with good prognosis based on 34 differentially abundant proteins. The next step is to investigate whether the abundance level of these proteins associates with clinical parameters such as patient survival. Most importantly, these data will be validated in an independent patient cohort before any translation towards the clinic can be made. Once validated, our data will provide an additional toolbox for clinicians to better predict patient prognosis, pathway analysis of key proteins will provide insight into the biology of triple negative breast cancer, and protein targets will have been identified for the development of new and targeted therapies.

References

- 1 Wang, Y. *et al.* (2005) Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* 365, 671-9.
- 2 Perou, C. M. *et al.* (2000) Molecular portraits of human breast tumours. *Nature* 406, 747-52.
- 3 Sorlie, T. *et al.* (2003) Repeated observation of breast tumour subtypes in independent gene expression data sets. *Proc Natl Acad Sci U S A* 100, 8418-23.
- 4 Smid, M. *et al.* (2008) Subtypes of breast cancer show preferential site of relapse. *Cancer Res* 68, 3108-14.
- 5 Cleator, S. *et al.* (2007) Triple-negative breast cancer: therapeutic options. *Lancet Oncol* 8, 235-44.
- 6 Umar, A. *et al.* (2007) NanoLC-FT-ICR MS improves proteome coverage attainable for approximately 3000 laser-microdissected breast carcinoma cells. *Proteomics* 7, 323-9.

- 7 Umar, A. *et al.* (2009) Identification of a putative protein profile associated with tamoxifen therapy resistance in breast cancer. *Mol Cell Proteomics* 8, 1278-94.
- 8 Liu, N. Q. *et al.* (2010) Identification of prognostic protein markers for triple negative breast cancer. *Manuscript in preparation.*

Research team



Laboratory of Breast Cancer Genomics and Proteomics:
From left to right: Ning Qing Liu, ?, ?, René Braakman and Arzu Umar.

Contact

Arzu Umar PhD
Erasmus MC Rotterdam
Department of Medical Oncology
Laboratory of Breast Cancer Genomics and Proteomics
Josephine Nefkens Institute, Be401
P.O. Box 2040
3000 CA Rotterdam
The Netherlands
T +31-10-7044373
a.umar@erasmusmc.nl
www.erasmusmc.nl/interne_oncologie/research/ResearchPrograms/cancer_genomics_and_proteomics/

Summary

Breast cancer is the most commonly diagnosed malignancy in women in the Western world, with 13,000 new patients each year in the Netherlands alone. Extensive research on gene expression profiling has shown that breast cancer is a mixture of biologically different disease entities, referred to as molecular subtypes. Of all molecular subtypes, the triple negative phenotype in particular associates with poor prognosis and poor patient survival. Intriguingly, only a small subgroup of triple negative tumours (25 percent), which metastasize to distant organs within three years, accounts for this poor prognosis. Currently no clinical markers are available to identify triple negative tumours based on positive expression, to predict

summary

disease prognosis, and against which to target therapy. The aim of our project was to identify prognostic protein markers for triple negative breast cancer using a comparative tissue proteomics approach. We have subjected frozen breast cancer tissue sections to LCM and prepared tryptic digests for nLC-MS analysis. Peptide abundance levels from poor prognosis samples were compared to good prognosis samples to identify differentially abundant peptides and their corresponding proteins. A selection of 34 differentially abundant proteins appeared to significantly differentiate between the two groups. Careful validation of these proteins may lead to better prediction of disease prognosis of triple negative breast cancer patients. Furthermore, functional analysis of key proteins may help unravel the biology of triple negative breast cancer and may lead to the development of new therapies against target proteins.