

Chapter 19

Using Breast Milk to Assess Breast Cancer Risk: The Role of Mass Spectrometry-Based Proteomics

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Abstract Although mammography and treatment advances have led to declines in breast cancer mortality in the United States, breast cancer remains a major cause of morbidity and mortality. Breast cancer in young women is associated with increased mortality and current methods of detecting breast cancers in this group of women have known limitations. Tools for accurately assessing personal breast cancer risk in young women are needed to identify those women who would benefit the most from earlier intervention. Proteomic analysis of breast milk could identify biomarkers of breast cancer risk and provide a tool for identifying women at increased risk. A preliminary analysis of milk from four women provides a proof of concept for using breast milk to assess breast cancer risk.

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19.1 Introduction

19.1.1 Overview

Although mammography and treatment advances have led to declines in breast cancer mortality in the United States, breast cancer remains a major cause of morbidity and mortality, and racial disparities in the burden of this disease exist. Mammography has greatly increased the early detection of breast cancer, but has known limitations, especially in younger women with dense breasts. Breast milk provides access to breast tissue, in the form of exfoliated epithelial cells, that when combined with mass spectrometry-based proteomics may offer a novel way to identify young women at increased risk of developing breast cancer leading to earlier detection and improved outcomes.

19.1.2 Breast Cancer Incidence and Mortality

Nearly 1.4 million women across the world were diagnosed with breast cancer in 2008 and it is predicted that the number of cases will rise to 2.1 million by 2030. The highest age-adjusted incidence rates occur in westernized countries and are attributed to industrialization, urbanization, and access to mammography [1]. In the United States, about 232,000 new cases of invasive breast cancer were diagnosed in 2013. In the same year, approximately 39,620 US women were expected to die from breast cancer [2]. The current lifetime risk is 12.3 %, with 1 in 8 women expected to develop breast cancer in their lifetime.

In the United States, breast cancer mortality rates decreased by 34 % between 1990 and 2010 [2]. This decline in mortality is attributed to improvements in treatment and early detection. The benefits of these medical advances have not been observed equally among all groups of women. In 2010, breast cancer mortality among African American women was 41 % higher than in White women, despite the fact that the overall incidence of breast cancer is lower among Black women [2]. Lack of access to both mammographic screening and high quality care explain much of the racial differences in death rates, but differences in breast cancer tumor characteristics may also contribute to the higher mortality among Black women.

19.1.3 Breast Cancer in Young Women and Pregnancy-Associated Breast Cancer

Approximately 12 % of all breast cancers are diagnosed in women under 45 years of age [3]. Because of the density of the breast tissue in young women, mammography is less effective in detecting tumors in younger women. The breast tumors that develop

in young women tend to be aggressive, lack expression of the estrogen receptor, and are more likely to be associated with poorer survival compared to tumors that develop in older women. In addition, incidence rates among young Black women (less than 40 years old) are higher than those among similarly-aged White women [2]. Epidemiology studies show that giving birth at a young age, having multiple live births and prolonged breastfeeding provide protection against developing breast cancer [4, 5]. However, paradoxically, the hormonal changes associated with pregnancy result in a transient (about 5 years) increase in breast cancer risk [6, 7]. In summary, breast cancer in younger women remains a significant challenge for detection and treatment.

19.1.4 Limits of Mammography and Risk Assessment

Current guidelines of the U.S. Preventive Services Task Force recommend a mammogram every 2 years for all women between the ages of 50 and 74. Routine mammography screening is not recommended for younger women [8]. Despite its recognized value, mammography has limitations including false positives, false negatives, and over diagnosis. There is also a need for improved risk assessment. Personalized risk assessment would provide women the opportunity to make informed decisions regarding various interventions, such as Tamoxifen. In recent years, considerable effort has been directed towards identifying molecular markers of breast cancer risk and early disease; however, the collection of breast tissue for analysis remains a major obstacle. Methods of obtaining breast tissue from non-symptomatic women include collection of nipple aspirate, ductal lavage, and fine needle aspirate. While these methods have provided valuable information regarding breast biology, they have several limitations. Ductal lavage and fine needle aspirate are invasive and do not survey all of the ducts and lobes of a breast and therefore may not provide a sufficient screen of the breast limiting their value for predicting risk. Collection of nipple aspirate fluid is minimally invasive but about half the women sampled are determined to be non-secretors and half of the samples collected may not contain epithelial cells [9, 10] Access to the breast tissue present in breast milk of young women could likely provide a means of identifying molecular markers associated with breast cancer risk.

19.1.5 Breast Milk

To date most of the research on the composition of human milk has been aimed at understanding the effects of breast milk on the growth and development of the infant, the biology of lactation, and the effects of mastitis [11]. Prior proteome analyses have focused on the proteins secreted into the milk, including growth factors, cytokines, and cleaved membrane proteins, and have documented changes in the

content of immune-related proteins with length of lactation; from colostrum through mature milk to weaning [12, 13]. A few studies have used ELISA methods to examine the relationship of a limited number of secreted proteins with breast cancer risk factors [14, 15]. However, the biomarkers most likely to be important for assessing breast cancer risk may be missed if only secreted proteins are examined, as the primary function of secreted proteins is to aid in the development of the infant. Therefore, it will be important to include intracellular proteins of the breast epithelial cells in proteome analyses of breast milk aimed at assessing breast cancer risk.

Milk droplets are produced in epithelial cells in the lobules of the gland and travel down the ductal system to the nipple, collecting exfoliated epithelial cells along the way. The intracellular proteins of the exfoliated epithelial cells could provide information on changes in protein expression related to risk. In addition to the relatively small percentage of epithelial cells, breast milk contains numerous leukocytes [16] with their own array of intracellular proteins. Thus breast milk provides an excellent sampling of intracellular, secreted, and vesicle cell-derived proteins from the entire mammary gland.

19.1.6 Epigenetic Markers of Risk in Breast Milk

To date, the use of breast milk for risk assessment and early diagnosis is limited to a few studies. For example we recently examined exfoliated epithelial cells isolated from breast milk for epigenetic signals associated with increased breast cancer risk [16, 17]. Comparisons of DNA methylation between women at average and increased risk revealed greater DNA methylation of several tumor suppressor genes in DNA from milk cells of women at increased risk [17]. These results provide proof of concept for the use of breast milk in an assay to assess risk, and are therefore encouraging, however, DNA methylation is only one way in which transcription and hence protein production is controlled. Therefore, an assay based directly on protein biomarkers could provide improved risk assessment.

19.1.7 Mass Spectrometry (MS)-Based Proteomics Analysis of Breast Milk for Assessing Breast Cancer Risk

Proteomics is an emerging approach that analyzes the proteome, i.e., the complete protein array or complement in a specific protein sample, such as the intracellular or extracellular protein complement, soluble, or membrane protein complement, or the cellular, tissue, organ or organism protein complement [18–22]. A variety of tissues such as biopsies of tumor and surrounding tissue or biological fluids such as blood, saliva, urine, tears, bronchial secretions, or breast milk can be used to conduct discovery-based proteomic research. Protein samples analyzed by proteomic approaches from different groups of individuals (for example blood from

Table 19.1 Demographics of the four African American women who provided breast milk samples

Sample	Current age (years)	Age at first birth	Pregnancies	Live births	Baby's age (days)	Family history of BC
1	32	32	1	1	44	No
2	34	34	1	1	150	No
3	36	22	6	5	136	No
4	29	27	2	2	356	No

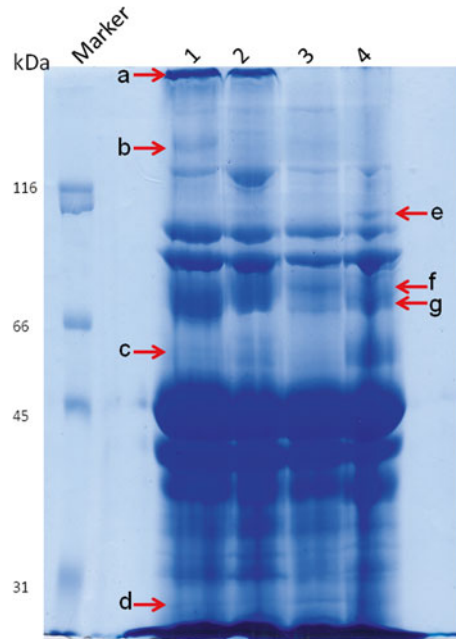
healthy controls and breast cancer cases), can be compared in quantitative terms through identification of the proteomes in each sample, and through protein quantification, protein post-translational modifications, and protein–protein interactions [21, 23–25]. Such comparisons can identify a single protein or a protein signature that is specific to one condition, which can be used to assess future risk of developing a disease. Ideally, this type of case–control analysis should be nested in a large prospective cohort study to allow discrimination of a proteome risk signature from a signature of the disease itself. The general proteomic approach could also be used for the assessment of treatment outcomes, as well as for the prognosis of disease progression.

In a pilot study we explored proteomic analysis of whole milk using MS with a focus on identifying proteins that could be associated with breast cancer risk. For this preliminary study we selected milk from four young African American women (see Table 19.1). Participants were between the ages of 29–34 years at the time of milk collection. Age at first birth ranged from 22 to 34 years old. Two women were nursing their first child, one woman was nursing her second, and the fourth woman was nursing her fifth child. Baby's age, a surrogate for length of lactation, ranged from 44 to 356 days. None of the women had a family history of breast cancer.

Details of the biochemical fractionation of the breast milk samples used in the present study are described elsewhere [13]. Briefly, 2 mL of whole milk from each participant was directly subjected to ultracentrifugation, and 150 μ L of supernatant was run on an SDS-PAGE gel. Because the ultracentrifugation step was not preceded by a slow centrifugation step, which is often used to deplete milk samples of cellular content, it is likely that the current analysis included a large number of intracellular proteins released from both the leukocytes and epithelial cells during the ultracentrifugation, as well as the more abundant secreted proteins.

Figure 19.1 presents the SDS-PAGE gel for the four samples. As can be seen in the gel, the overall pattern of most of the major bands is similar among all four samples and bands that include some of the high abundance proteins (e.g., lactotransferrin at 78 kDa) are clearly present. Sample specific-bands are also present. The red arrows in Fig. 19.1 mark bands that differ among the four samples. For example, samples 1 and 2 have a high molecular weight band (arrow a) that is missing from the other two samples, and sample 3 is lacking or has reduced levels at several bands present in the other samples (arrows e, f, and g).

Fig. 19.1 SDS-PAGE gel showing protein band profiles from four human milk samples demonstrate differences among women. *Red arrows* indicate differences in protein bands among the samples



To identify proteins, the bands for each sample were cut into ten pieces, and enzymatically digested and run on nanoliquid chromatography-tandem mass spectrometry according to a published procedure [24]. Scaffold 4 was used to identify proteins using the stringent setting of 99 % probability of correctly identifying a protein from a minimum of two peptides. A relatively small number of proteins, about 100, were identified in each sample and there was considerable similarity among samples.

To gain an initial understanding of the role of the identified proteins, we merged the protein datasets and used pathway software to provide a descriptive analysis. Thus, the data in Figs. 19.2 and 19.3 are based on a compilation of proteins from all four samples and are presented to demonstrate the range of biological processes represented by the identified proteins (Fig. 19.2), and the cellular component to which the proteins belong (Fig. 19.3). With respect to biological processes, the general categories of proteins in Fig. 19.2 are very similar to the proteome signature of milk from five Italian women that was previously reported by Picariello and colleagues [13] suggesting that this method produces similar results and in general the milk proteome is qualitatively similar. However, the inclusion of the cells resulted in an increased intracellular and nuclear component. Twenty-three of the identified proteins locate to the extracellular region, 23 to the intracellular organelle, and 14 to the nucleus (Fig. 19.3), suggesting that both proteins secreted into the milk and proteins from the cells in the milk were detected with the mass spectrometry analysis.

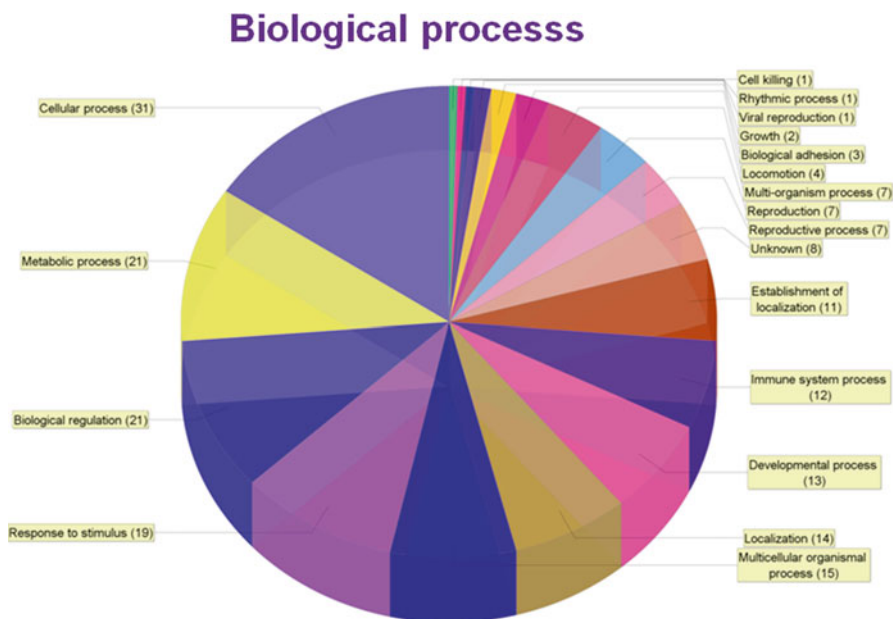


Fig. 19.2 Pie chart showing the distribution of proteins detected in the four milk samples based on biological process

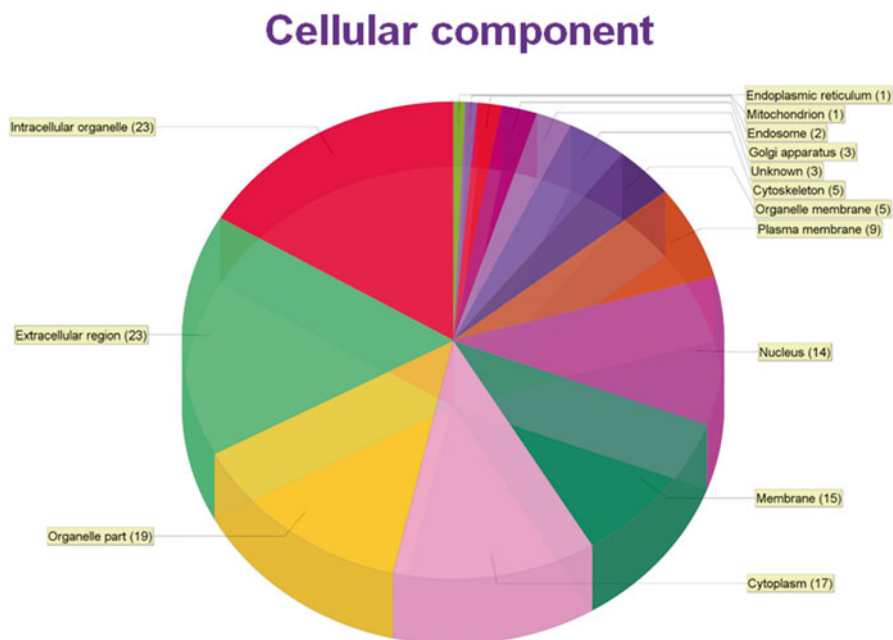


Fig. 19.3 Pie chart showing the distribution of proteins detected in the four milk samples based on cellular component

Comparison of the lower abundance proteins from the individual MS analyses for each of the four milk samples revealed some interesting preliminary differences that require further verification. In particular, the protein spectra of milk from participant 2 included proteins absent from the other milk samples and not previously identified in other milk proteome studies [13] that could be biomarkers of risk. Mucin 16 (previously known as CA125), a membrane-associated protein and known cancer biomarker [26] was identified by two peptides in sample 2 and not in the other three samples. Mucin 16 is involved in regulating cell adhesion which has led to its use as a biomarker for resectability in epithelial ovarian cancer [27]. The milk from subject 2 also contained detectable Axin2, an intracellular protein, and a marker of activated Wnt/ β -catenin signaling. This finding is of particular interest because our previous analysis of epithelial cells from breast milk revealed DNA promoter methylation of a critical Wnt pathway antagonist, SFRP1, in women at increased risk of developing breast cancer [17]. Promoter methylation of SFRP1 leads to decreased SFRP1 protein levels which we have observed to result in enhanced Axin2 expression (S. Schneider, personal communication), increased proliferation, and decreased sensitivity to cell death in an immortalized human mammary epithelial cell line [28]. While these results are preliminary they support the hypothesis that analysis of proteins in breast milk may provide information regarding breast cancer risk.

19.2 Conclusion

This preliminary proteomic analysis of breast milk focused on detecting proteins associated with breast cancer risk and therefore both the secreted proteins and cellular proteins were included, as we did not remove the cellular fraction. Proteins previously identified with breast cancer were detected in one sample supporting the potential of a proteomic analysis of breast milk for detecting increased breast cancer risk. While encouraging, significant improvements in methodology are required as the number of proteins detected was low. Methods that deplete the sample of high abundance proteins without removing the low abundance proteins must be optimized. Other challenges for exploiting breast milk and proteomics for early detection and risk estimate relate to the establishment of a large prospective cohort of young women with stored milk specimens for a nested case-control study to robustly examine the usefulness of the proteomic approach. Special attention should be given to Black women who are at increased risk of developing breast cancer at a young age and to those with a family history of the disease.

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