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Complement C3f serum levels may predict breast cancer risk in women with gross cystic disease of the breast

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ABSTRACT

Gross cystic disease (GCDB) is a breast benign condition predisposing to breast cancer. Cryopreserved sera from GCDB patients, some of whom later developed a cancer (cases), were studied to identify potential risk markers. A MALDI-TOF mass spectrometry analysis found several complement C3f fragments having a significant increased abundance in cases compared to controls. After multivariate analysis, the full-length form of C3f maintained a predictive value of breast cancer risk. Higher levels of C3f in the serum of women affected by a benign condition like GCDB thus appears to be correlated to the development of breast cancer even 20 years later.

Biological significance
Increased complement system activation has been found in the sera of women affected by GCDB who developed a breast cancer, even twenty or more years later. C3f may predict an increased breast cancer risk in the healthy population and in women affected by predisposing conditions.

1. Introduction

One of the most relevant problems in breast cancer prevention is how to identify the women who might be at higher risk of developing this disease and, therefore, who might gain the greatest benefit from periodic surveillance with new imaging technologies or from chemoprevention measures. Gross cystic disease of the breast (GCDB) is a common benign disease of the mammary gland, affecting some 7% of women in Western countries [1]. It is associated with a 2–4 fold increased risk of developing breast cancer, probably as a consequence of the evolution of the proliferative epithelial changes which are commonly associated with this benign condition [2]. Though breast cancer risk appears to be associated with cyst type and the intracystic level of steroids or growth factors [3–5], so far no specific serum risk marker has been reported yet in the women affected by GCDB. To this end, proteomic studies could be quite informative, especially when nanotechnologies are applied.

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In particular, mass spectrometry (MS) is well suited for the detection of low molecular weight proteins or peptides that might not be detectable with traditional techniques, and that might well represent a dynamic reflection of tissue function. A few studies have addressed the possibility of applying a proteomic approach to the analysis of cyst fluid or nipple aspirates in the diagnosis of breast cancer [6–8]; more encouraging results have been achieved by applying these techniques to the study of the serum peptidome of women diagnosed with breast cancer or to follow disease outcome of patients after breast surgery [9–11]. In particular, Villanueva et al. [12] identified specific degradation patterns associated with breast cancer and able to generate proteomic signatures potentially useful to diagnose this disease. However, so far just a few studies used biological fluids, collected before breast cancer diagnosis, in proteomic profiling approaches to this disease. Namely, Opstal-van Winden et al. [13] performed a serum surface-enhanced laser desorption/ionization (SELDI)-time of flight (TOF) analysis at the peptidome level and identified two signals, of *m/z* 3323 and *m/z* 8938 respectively, able to identify the women subsequently developing a breast cancer up to three years before cancer was diagnosed. These findings, though preliminary, prompted us to investigate the possible association of serum peptidome profiles with breast cancer risk in a group of women diagnosed with GCDB but followed for a much longer period of time (up to 20 or more years since blood collection). On the basis of preliminary work on the matrix assisted laser desorption/ionization (MALDI)-TOF MS analysis of cryopreserved sera of a group of healthy donors [14], we have begun by analyzing the sera of a group of women diagnosed with GCDB even twenty or more years before developing breast cancer and those of an appropriately matched group of comparable women who were still breast cancer-free by the same date limit. The results of this analysis form the object of the present article.

2. Materials and methods

2.1. Study design and ethical aspects

Serum samples were obtained from 600 patients affected by GCDB at the time of first cyst aspiration. Samples were collected between 1985 and 1993, and stored at −80 °C. A proportion of these women were followed up at our institution and a few of them have been found to have developed a breast cancer. Many more were lost to follow-up and were diagnosed with breast cancer at other hospitals. Information about the destiny of these women in relation to the development of a breast cancer was obtained by consulting the Genoa Tumor registry.

At the date limit of December 31th 2010, fifty out of 600 women were found to have developed a breast cancer but for only thirty women a number of serum aliquots adequate to be processed for the purposes of the present research were available. In fact a large subgroup of the original cohort had been enrolled in previous sera-epidemiological studies and no residual serum aliquots were available any longer for them. Data concerning women reproductive history and family history of breast cancer had been recorded at the time of clinical examination. The thirty women developing a breast cancer following first cyst aspiration and for whom an adequate number of serum aliquots was actually available served as cases. These women were individually matched (according to a 1:2 ratio) with 60 women who, by the same date limit, were still breast cancer free and who served as controls. Matching was done on the basis of age at first cyst aspiration (cases: median age 43.5 years, range 34–54; controls: median age 44 years, range 35–54) and family history of breast cancer (positive in 24% of both cases and controls). Moreover, as the duration of cryopreservation could potentially affect the protein profile, as it was previously evidenced by us [14], cases and controls were also rigorously matched for the length of cryopreservation period (cases: median length 22.5 years, range 19–24; controls: median length 22.5 years, range 20–24). All the women had provided their consent to blood drawing and cryopreservation; moreover the present study project was approved by the Ethics Committee of the IRCCS AOU San Martino Hospital and National Cancer Research Institute of Genoa, Italy. The permission from the Italian Data Protection Authority has been also obtained to get the information relative to the women health condition for the women lost to follow up.

2.2. Analytical procedures

Acetonitrile, methanol and water were LiChroSolv from Merck (Darmstadt, Germany), trifluoroacetic acid (TFA) and α-cyano-4-hydroxycinnamic acid (CHCA) were from Fluka (St. Louis, MO). Serum samples preparation, MALDI-TOF/MS and MS/MS analysis procedures were described in a previous paper [14]. In order to avoid procedural bias, both case and control samples were randomly distributed during processing and analysis. All spectra were acquired in quadruplicate in a mass range from 800 to 3000 *m/z*.

2.2.1. Solid phase extraction

As functionalized superparamagnetic beads appear to be best suited in preparation of low molecular weight (LMW) fraction from complex matrices, samples were processed using Dynabeads® RPC 18 (Invitrogen Dynal, Irvine, CA) with C18 alkyl-modified surface, which are intended for peptide concentration in samples.

Some modifications were made to the Dynabeads® RPC 18 manufacturer's protocol. Briefly, 40 μl of beads suspension were washed one time with water and three times with 100 μl of 200 mM NaCl, 0.1 % TFA. The beads were re-suspended in 20 μl of water, mixed with 50 μl of serum and left at room temperature for 5 min. After incubation, the tube was placed in the manual magnetic particle concentrator (Dynal MPC®, Invitrogen Dynal) and the supernatant was discarded. The peptides-enriched beads were washed three times with 300 μl of 0.1 % TFA in water and the bound fraction was eluted by incubation, at room temperature for 2 min, with 12 μl of a 1:1 acetonitrile:water solution, to which 3.5 pmoles/μl of an internal standard peptide (MW 1419.76) were added. The standard peptide, used to normalize the data, was directly spiked in the eluting solution to avoid any interference during the binding reaction between analytes and the magnetic beads. In Supplementary Fig. S1 we report the analysis by Tricine–SDS–urea–PAGE of the fraction obtained by solid phase extraction using Dynabeads® RPC 18 with C18 alkyl-modified surface. As can be seen, the extracted serum sample (lane1) appears to