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Research Article

Mitochondrial Encoded Targets of Breast Cancer Anti-Mitochondrial Antibodies Implications of Mitochondrial Autoimmunity for Breast Cancer Progression -

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INTRODUCTION

The association of auto-antibodies with the diagnosis of Breast Cancer [BC] and other cancers has been widely reported, but they have long been regarded as the expression of non-specific autoimmunity unrelated to cancer. Multiple studies have demonstrated that the majority of auto-antibodies in cancer sera target a vast group of cellular antigens called Tumor-Associated Antigens (TAAs) [1,2]. Recently, in a comprehensive study of auto-antibodies detected in patients with Infiltrating Ductal Carcinoma (IDC) and Ductal Carcinoma in Situ (DCIS) of the breast, we reported evidence that auto-antibodies in BC are not epiphenomena and suggested that they may participate in the carcinogenic process [3]. In that work we identified auto-antibodies in BC sera recognizing antigens located in mitochondria, centrosomes, nucleoli, centromeres, spindle apparatus, and the cytoskeleton, reminiscent of the autoimmune response characteristic of the Rheumatic Autoimmune Diseases (RADs) [4]. In particular, using immunofluorescence (IFA), we reported that high titre AMAs can be consistently detected in BC sera and in a small number of sera from healthy women [3]. AMAs are not known to occur in any disease other than the autoimmune diseases Primary Biliary Cholangitis (PBC) [5] and Pemphigus Vulgaris (PV) [6]. Since we have identified the punctate cytoplasmic auto-reactivity in a group of BC and non-cancer control sera as mitochondrial in origin (Figure 1) but could not determine the nature of the antigens using IFA [3], we proceeded to validate the serum mitochondrial auto-reactivity by identifying the mitochondrial gene products recognized as foreign by the immune system. Here, we report that BC sera contain AMAs targeting several enzyme components of the Electron Transport Chain (ETC) and other gene products encoded by mtDNA. We provide evidence that these AMAs found in BC sera reflect mitochondria autoimmunity, and discuss the implications of immune recognition of these mitochondrial antigens as foreign in the context of breast cancer progression. Part of these data has been reported in abstract form [7].

MATERIAL AND METHODS

Human subject

We used prospectively obtained sera prior to any treatment from a cohort of 100 cases of IDC of the breast, diagnosed by pathologic

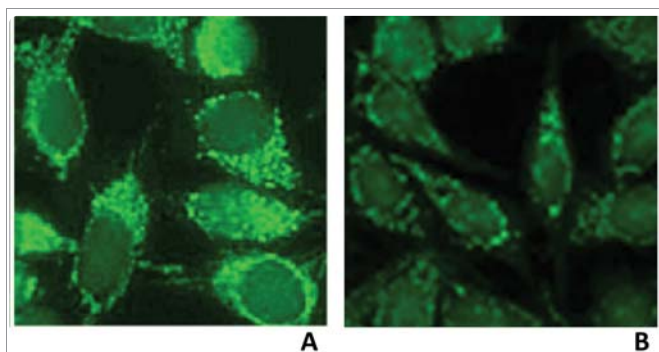


Figure 1: Mitochondrial auto-reactivity in BC and non-cancer sera IFA of a serum from a patient with IDC of the breast [A] showing cytoplasm studded with stained mitochondria. [B] Shows an occasional BBD control serum with mitochondrial staining on HEp-2 cells [dilution 1:500 in both A and B].

exam from a population of women ≥ 40 years of age undergoing annual screening mammography with Breast Imaging Reporting and Data System4 [BI-RADS4] assessment at Henry Ford Health System (HFHS). Prospectively obtained sera from 100 healthy women with biopsy-proven benign breast disease (BBD) and no cancer were used as controls. The demographic characteristics of cases and controls have been reported [3]. The study had approval from the HFHS and Wayne State University IRBs. Each of these women was invited to donate a 10 ml blood sample after signing an informed consent.

Construction of a T7 cDNA-displayed multi-human BC cell line library

A random primer T7 cDNA library was constructed by directional cloning of randomly primed cDNA from 7 human breast carcinoma cell lines: SUM 44, SUM 102, SUM 149, SUM 159, MCF-7, SKBR, and T47D into a T7 phage display vector using T7 Select 10-3b vector and the Orient Express cDNA library construction system (Novagen, Billerica, MA, US) according to the manufacturer's instructions. Since the commercially obtained libraries are usually derived from a single malignant tumor, given the heterogeneity of BC [8], we constructed a multi-human BC cell line cDNA library. Total RNA from the BC cell lines was isolated using a RiboPure™ Kit (Ambion, Austin, Texas TX, USA) according to the manufacturer's instructions. For the construction of a T7 phage display cDNA library, poly(A)⁺ RNA was isolated from a pool of 1 mg total RNA using Straight A's mRNA Isolation System, and then the T7 Select * 10-3 Orient Express™ cDNA Cloning System. Random primers were used for the construction of the cDNA library and for the screening system as described (Novagen, Billerica, MA, USA). In brief, 4 μ g poly (A)⁺ RNA was reverse transcribed into double stranded cDNA. After flushing the cDNA ends, ligation of the cDNA with directional EcoR I/ Hind III linkers, digestion of the cDNA by EcoR I/ Hind III, and cDNA size fractionation, the prepared cDNA was inserted into the T7 Select 10-3 vector. The phage display cDNA library was constructed by packaging in vitro and plate proliferation. Plaque assay and PCR were used to evaluate the library. The resulting phage library contained 4.5×10^6 independent clones as determined by plaque assays. The library was amplified once by plate lysate amplification resulting in the phage titre of 4.3×10^{10} pfu/ml. After elution with Phage Extraction Buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 6 mM MgSO₄), the eluate was centrifuged and stored at -80°C . The insert size of individual clones of the complete library was analyzed by PCR with the forward primer 5'-GGAGCTGTCGTATTCCAGTC-3' and the reverse primer 5'-AACCCCTCAAGACCCGTTTA-3'.

Bio-panning of the cDNA library

A pool of sera from cases of BC [IDC and DCIS of the breast] and sera from healthy women displaying high titre [$\geq 1:320 - 640$] AMAs by IFA on HEp2-cells [3] was used for bio-panning the T7 cDNA library. A / G agarose beads (Santa Cruz Biotech, Santa Cruz, California CA, USA) were washed three times with PBS (50 mM sodium phosphate, pH 7.4, 0.15 M NaCl) and re-suspended in PBS plus 1% BSA before use. The A/G agarose beads were incubated with 100 μ l of a 1:50 dilution of the pool of cloning sera for 1 h at 4°C , washed with PBS, and incubated overnight at 4°C with the single T7 phage display library constructed from the seven BC cell lines. Beads were then washed with PBS plus 1% Tween 20 and used to infect *Escherichia coli* strain BLT5403. The culture was shaken until lysis, clarified by centrifugation, and the supernatant was subjected to up to 5 rounds of bio-panning.

Identification of phage-displayed tumor-associated proteins

Phage clones were isolated and the cDNA inserts were PCR-amplified using the T7 phage vector primer. Sequences of unique clones were checked for open reading frame status in the T7 expression vector. To identify potential auto-antigens that could help discriminate between cases and controls, the phages recognized by serum antibodies were amplified after up to 5 rounds of bio-panning, and 868 distinct clones recognized by BC sera were assembled as a microarray. Plaque-pure phages were grown to high titre in bacterial cultures that were incubated until complete lysis. Supernatants collected after a 10 min, 10,000 × g spin were arrayed in 384 well microtiter dishes. The entire 868 phage library was spotted in triplicate onto Epoxysilane-coated slides at a concentration of 1:2 phages: PATH buffer ratio, which produced an optimal print. Development and quantification of the auto-antigen microarray were performed as before [9]. Individual reactive phages were lifted onto a nitrocellulose membrane to detect differences in antibody reactivity among BC and non-cancer BBD sera. The plaque-lifted membranes were blocked with 2.5 % BSA in 1 × TBST for 1h at room temperature and incubated at 4°C overnight with serum from BC patients or pooled normal sera (1:100) that were not used in the immune screening procedure. The membranes were incubated with anti-human HRP-conjugated secondary Ab. The signals were detected with enhanced chemiluminescence as previously described [9].

Gene Ontology [GO] analysis

BLAST/ BLAT [10,11] output from the auto-antigen microarray analysis was used to generate a gene list used as input for GO analysis using R. An R package, called bio-mart, is a collection of databases implementing the Bio-Mart software suite (<http://www.biomart.org>). The use of this package in R enables retrieval of large amounts of data with a simple query. This package (Bio-conductor version: Release (3.2): Steffen Durinck biomartdev@gmail.com, Wolfgang Huber) was used for the GO analysis. The analysis created a text file of GO identifiers corresponding to the input gene list. Sequences with no significant similarity were not included in the analyses. From the 868 phage sequences recognized by BC sera on the auto-antigen microarray, 547 phage sequences showing single bands by PCR were successfully mapped to functional HGNC (Hugo Gene Nomenclature Committee) symbols [12].

Statistical Analysis

The statistical significance of the reactivity of the AMAs with BC diagnosis was calculated individually. PCR, sequence analyses, homology searches, hybridization of the printed page on glass slides, and development of the auto-antigen microarray and data pre-processing were performed as reported [9]. Briefly, the array background intensity was subtracted from the foreground intensity. Negative values were replaced by a value equal to half the minimum positive value. The within array median normalization was used on the log₂ ratios of red and green intensities. Replicated spots for the same marker were then summarized using median intensities. Student's T-test was used for identifying the markers that differentiate between the IDC and the control group. After identifying informative phages, the corresponding cDNA inserts were amplified by PCR using primers flanking the insertion site and sequenced by a commercial laboratory (Genewiz, South Plainfield, NJ). The sequences deduced from the cloned cDNA were searched for vector contamination

in the NCBI-Vee Screen and the vector related sequences were eliminated. Sequences were also screened on RepeatMasker.org to eliminate repetitive sequences. The GenBank database was searched for sequence homology to the identified cDNA sequences using the Basic Local Alignment Search Tool [BLAST] program [10]. Only the correct ORF-encoded proteins were identified by comparison with known sequences in the GenBank database. Homologies detected by BLASTn, BLASTx or tBLASTx were used and the smallest E values were recorded and conserved domains in the query sequences were determined. All the results from homology searches were confirmed by BLAST-like alignment tool [BLAT] [11].

RESULTS

We had previously validated the mitochondrial nature of the auto-reactivity to mitochondrial antigens detected by IFA by reacting BC sera on mitochondria-rich rodent tissue sections of stomach parietal cells and renal proximal tubuli [3]. To further validate our findings, we proceeded to identify the mitochondrial proteins targeted by AMAs by immune screening a T7 phage cDNA expression library constructed with mRNA obtained from seven established BC lines (see Methods). Our approach to identify mitochondria-related antigens in the cDNA library was to use BC sera containing high titre AMAs (≥ 1:320-640 dilution) detected by IFA for bio-panning the multi-human BC cell line cDNA library (Figure 1). Sera showing the presence of auto-antibodies of unknown specificity have been used successfully for immune screening cDNA expression libraries in order to isolate the putative TAAs [4,9]. This was followed by auto-antigen microarray construction, hybridization with sera from cases of BC and non-cancer controls, and development of the microarray as described [9]. From 547 Expressed Sequence Tags [ESTs] successfully mapped to Hugo Gene Nomenclature Committee [HGNC] symbols, 184 were unique gene products with significant immunochemical reactivity with BC sera. The putative tumor-associated clones were selected by plaque lift after immunodetection. From these unique gene products, 87 phages had sequences encoded by mtDNA, including 67 phages for ND5 [13], and one clone each of 16S rRNA (MTRNR2) [14], MT-ATP6 [15], and MT-COX1 [16]. The Gene Ontology [GO] analyses of the putative tumor associated clones targeted by auto-antibodies in BC sera showed that auto-reactivity was not restricted to mitochondrial specificities (Figure 2). Among the selected clones, a group of phages was found to express in-frame proteins encoded by mtDNA (Tables 1 and 2), whereas another group of phages expressed mitochondrial proteins encoded by nDNA, which will be the subject of a separate report. In this work we report the molecular characterization of the cloned ESTs identical to in-frame proteins encoded by mtDNA. The cloned phages are listed in table 1 and their respective reactive mitochondrial sequences encoded by mtDNA and targeted by AMAs are shown in table 2 [13,15-17]. Table 1 also shows that all the enzyme components of the complex I, IV and V targeted by AMAs in BC sera have evolutionary conserved domains. It is likely that the predominance of mitochondrial signatures in the repertoire identified by auto-antibodies in the cDNA library was influenced primarily by the selection of BC sera rich in AMAs for bio-panning the cDNA library. The sequences reported in table 2 as well as all sequences cloned from the cDNA library were linear stretches of nucleotides encoding for reactive amino acid sequences. A notable finding from bio-panning the cDNA library was the identification of 67 clones highly homologous to ND5. Immunodetection of ND5-expressing phages and immunoblots confirmed the antibody reactivity to ND5. IBs developed with a specific polyclonal ND5



antibody and with BC sera containing AMAs showed high binding capacity of ND5 antibodies and BC sera containing AMAs (Figure 3A and 3B). In contrast, ND5-expressing phages had negligible binding capacity with non-BC sera (Figure 3C).

DISCUSSION

In this work we report the molecular characterization of the ESTs cloned by bio-panning of a cDNA library with BC sera, identical to in-frame proteins encoded by mtDNA. The findings reported in Tables 1 and 2 validate the mitochondrial reactivity of AMAs in BC sera that was previously determined by IFA [3]. AMAs can be found sporadically in the sera from patients with some of the RADs but they have not been found consistently in any other disease [18] except for PBC and PV. AMAs are known to be present in the sera from patients with PBC, an autoimmune liver disease [5], and in the sera of patients with PV, an IgG autoantibody-mediated skin disease caused by a loss of epidermal cohesion and manifested by progressive blistering and non-healing erosions [6]. In PBC the diagnostic value of AMAs targeting the nuclear DNA-encoded 2-oxoglutarate dehydrogenase complex E2 is established as specific serologic markers of the disease [5]. In addition, there is evidence that auto-antibodies generated through somatic mutations are instrumental in producing loss of keratinocyte cell adhesion in PV [19]. It is important to note that the AMAs in BC sera target different mitochondrial specificities than those characteristic of PBC [5] or targeted by auto-antibodies in PV [6,19]. Our findings suggest that mitochondria autoimmunity

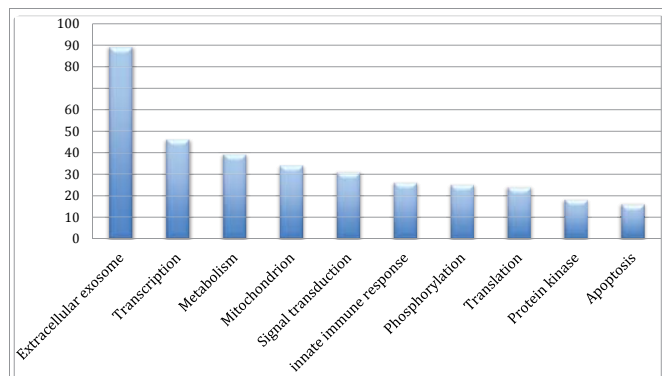


Figure 2: Gene Ontology analyses of sequences cloned by bio-panning a cDNA library of BC antigens with BC sera GO analysis using the unique sequences cloned by bio-panning the cDNA library with a pool of AMA-rich BC sera.

Table 1: Identity of the components of complexes I, IV and V of the ETC cloned from a T7 cDNA library.

| Antigen | Phage# | Homology [p] | BC Assoc [p] | GenBank ID [%] |
|---------------|--------|--------------|--------------|----------------------------|
| ND4 [13] | 249 | 8.00E-36 | 0.0007 | AIH15109.1 |
| NAD4L[17] | 547 | 0.001 | 0.0004 | ALS48800.1 |
| ND5[13] | 182 | 2.00E-43 | 0.0005 | AIH15110 |
| ND6[17] | 800 | 1.00E-11 | 0.001 | AEP2381.2 |
| COX1 [16] | 610 | 5.00E-11 | 1.00E - 03 | ADW26288.1 |
| MT - ATP6[15] | 194 | 9.00E-16 | 1.00E - 07 | AAZ02924.2 |

ND4, NADH dehydrogenase subunit 4; NAD4L, NADH dehydrogenase subunit 4L; ND5, NADH dehydrogenase subunit 5; ND6, NADH dehydrogenase subunit 6; COX1, Cytochrome c oxidase subunit 1; MT-ATP6, mtDNA-encoded ATP synthase 6; BC Assoc [p], Breast cancer association [p].

Table 2: mtDNA sequences of cloned phages encoding the enzyme components of mitochondrial complexes I, IV and V.

| |
|---|
| <i>P#</i> 249: ND4 Sequence [Conserved Domain Oxidored_q1 superfamily] |
| AATATGACTAGCTTACACAATAGCTTTTATAGTAAAGATACCTCTTTACGG ACTCCACTTATGACTCCCTAAAGCCCATGTCGAAGCCCCCATCGTGGGT CAATAGTACTTGCCGCAGTACTCTTAAAAC TAGGCGGCTATGGTATAATAC GCCTCACACTATTCTCAACCCCTGACAAAACACATAGCCTACCCCTTC CTTGTAATCCCTATGAGGCATAATTATAACAAGCTCCATCTGCCTACGA CAAACAGACCTAAAAGCTTGGCGCCGCACTCGAGTAACTAGTTA |
| <i>P#</i> 547: NAD4L Sequence [Conserved Domain Oxidored_q2 superfamily] |
| CTATNATGAACAGANATAGNATTATTCCTTCTAGGCATATGACGGAGGNT AGGNNNGTGTAGCGATATACTAGTATTCTTANAAGTGAGATGTACCATAA CCGNTCCCT |
| <i>P#</i> 182: ND5 [Conserved Domain NADH5_c superfamily] |
| CGATTGAGCGTTTTGGCTCGTAAGAAGCCCTAGATAGGGGATTGTGCGG TGTGTGATGCTAGGGTAGAATCCGAGTATGTTGGAGAAATAAATGTGCA TAGTGGGGATTTATTTAAGTTTGTGGTTAGGTAGTTGAGGTCTAGGGC TGTTAGAAGTCTAGGAAAGTGACAGCGAGGGCTGTGAGTTTTAGGTAGA GGGGGATTGTTGTTGGAAGGGGGATGCAGGGGAAAGCT |
| <i>P#</i> 800: ND6 [Conserved Domain PRK06433 superfamily] |
| ACCTCAACCCCTGACCCCATGCCTCAGGATACTCCTCAATAGCCATCG CTGTAGTATATCCAAGACAACCATCATTCCCCTAAATAAATAAAAAAA CTATTAACGCTTGAAT |
| <i>P#</i> 610: COX [Conserved Domain Heme-Cu_oxidase_I super family] |
| GGAGGTGAAATATGCTCGTGTGCTACGTCTATTCCTACTGTAAATATAT NGGTGTGCTCACACGATAAACCTTAGGAAGCCAATTGATATCATAGCTCA GACCATACCTATGTATCCAAGCTTGGCGCCGCACTCGAGTAACTAGTTA ACCCCTTGGGGCCTCTAAAC |
| <i>P#</i> 194 MT-ATP6 [Conserved Domain] ATP synth_8 superfamily |
| CTTTAGTGTGTGTATGGTTATCATTTGTTTTGAGGTTAGTTTGATTAGCAT TGTTGGGTGGTGATTAGTCGGTTGT |
| Mitochondrial antigens as in Table 1; <i>P#</i> , phage number |

targeting key components of the ETC and other mitochondrial gene products are a feature of BC not found in the diseases reported to be associated with AMAs. These findings raise important questions related both to the possible causes of the immunogenicity of mitochondrial proteins and to whether mitochondrial autoimmunity participates in BC progression.

Why is mitochondrial autoimmunity found in breast cancer?

AMAs have been found in the serum of women with BC that are not present in the serum of women who do not have BC, and that are present at lower levels if at all in the serum of women with BBD. We propose two possible explanations for these findings that are not mutually exclusive. The first is that mitochondrial auto-reactivity is the reflection of autoimmunity triggered by mitochondrial TAAs. It is notable that immune-screening of a cDNA library of potential BC antigens with BC sera containing AMAs led to the cloning of ESTs with identity to 6 of the 13 subunits of the ETC encoded by mtDNA, including four of the 7 subunits in complex I. The high evolutionary conservation of the enzyme components of the ETC and of the complex V genes listed in tables 1 and 2 supports the autoimmune nature of this process. Bearing on this suggestion, an *in silico* study of large antigen sets by Backes, et al. [20]. provided evidence that proteins that are evolutionary conserved show specific sequence motifs and are more likely to become immunogenic In addition, in the RADs there

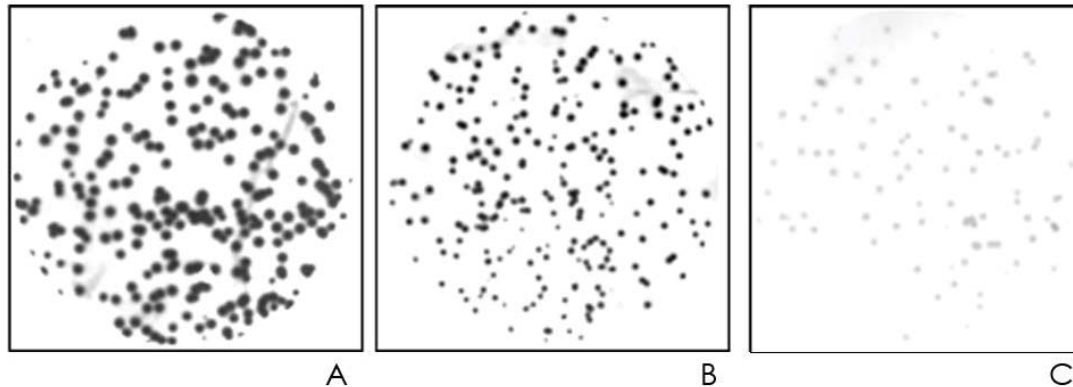


Figure 3: Gene Ontology analyses of sequences cloned by bio-panning a cDNA library of BC antigens with BC sera GO analysis using the unique sequences cloned by bio-panning the cDNA library with a pool of AMA-rich BC sera.

are many examples of auto-antibodies targeting conserved domains included the active sites of enzymes [4,21]. The seminal work of Eng Tan suggested that conserved epitopes recognized by human auto-antibodies might also be included in the active sites of intracellular proteins [4,21]. His work also predicted that autoantibody signatures might provide insight into critical pathways deregulated in cancer. The second possibility to explain our findings stems from the fact that mitochondrial components could arise from cell breakage caused by the inflammation typically associated with primary tumors and also with trauma. Both mtDNA and the N-formylated peptides present in mitochondria but not elsewhere in the cell contain patterns similar to bacterial components and thus can bind to specific receptors and activate the innate immune system [22,23] Indeed, mtDNA and mitochondrial auto-reactivity have been observed developing in both the sera of victims of physical trauma and in sera from patients with cancer [24, 25], including this report.

There is no doubt that somatic mitochondrial mutations frequently occur in breast and other cancers [26-30]; what is not clear in the case of cancer is whether either the mutated mtDNA or the proteins containing somatic mutations are part of the disease etiology and/or pathogenesis. The presence of mtDNA containing heteroplasmies in the serum of cancer patients has long been known, but considerations such as the high level of silent changes in protein coding genes has provoked doubt about the causative role of these mutations [26, 27]. More recently, mutations present in cancer patients were shown to have two features not present in the variations that are found in cancer-free individuals [27,30,31] If present in functional mitochondria, such mutations would produce a level of mutant mitochondrial proteins. One feature is that a higher proportion of the mutations are found in coding regions, and the other is that a higher proportion of mutations found in cancer patients are absent from mtDNA databases that represent normal geographical variations.

Mitochondrial Autoimmunity May be the Link Between Mitochondrial Somatic Mutations and BC

A unique finding in our work was the cloning of multiple copies of ND5. The recognition of the 16S mitochondrial ribosomal RNA by auto-antibodies in BC sera is of interest because humanin encoded by this gene, and other mitochondrial-derived peptides are thought to be involved in the regulation of apoptosis, insulin sensitivity, and

inflammatory markers, all processes that may participate in cancer progression [14]. The bio-panning of numerous identical clones from cDNA libraries has been reported [32] but the cause of cloning redundancy here remains unclear. ND5 is a key component of complex I targeted by auto-antibodies in BC (Table 1). Since variations in the abundance of certain cDNA sequences in libraries are known to occur, cloning redundancy could be attributed to the relative abundance of ND5 mRNA responsible for generating AMAs targeting this molecule in BC tissue. Why would ND5 be especially targeted by autoimmunity in BC? Somatic mtDNA mutations have long been associated with breast and other cancers, particularly involving complex I [26-31]. Santidrian, et al. [33] presented evidence in support that mitochondrial complex I activity and NAD⁺/NADH balance regulate BC progression in MDA-MB-435 and in MDA-MB-231 cancer cells. They studied the contribution of complex I activity to BC progression, based on expression of the yeast NADH dehydrogenase Ndi1 in human tumor cells, which can be functionally expressed in mammalian cells, and can restore complex I function in diseased cells. In addition, Sharma et al. showed that Ndi1 expression in tumor cells deficient in complex I can reduce soft agar colony formation and suggested that complex I dysfunction promotes tumorigenesis through ROS alteration and AKT activation [34]. These studies found that interference with tumor cell complex I function enhances metastatic aggressiveness and provided evidence that specific modulation of complex I function can significantly alter tumor growth and metastatic activity [33,34]. They concluded that complex I mutations found in primary tumors of BC patients may play a key role in disease progression. A next generation study [NGS] of the mutational patterns in BC by McMahon and LaFramboise provided evidence that a substantial majority of BC harbor somatic mtDNA mutations and also reported significant enrichment of complex I among somatic mutations [28], although roughly in proportion to gene size. The study of Backes et al found evidence that the probability of the occurrence of somatic mutation is increased in genes with long exons [20]. The ND5 gene, however, is exceptional in having the most somatic mutations after the control region. Furthermore, compared to germ line mutations, all ETC genes have a higher ratio of non-synonymous to synonymous mutations, predicting the production of altered proteins. The study of Park et al. reported the association of heteroplasmic mutations of ND5 and aggressive BC [29]. Other studies have also detected germ line as well

as somatic mutations in mitochondrial protein coding genes such as ATP6, ND1, ND2, COX1, COX3, ND4, ND5 and CytB [Reviewed in refs 26,27]. D-J Tan et al reported somatic mutations in three quarters of the patients with BC, with the majority of the mutations restricted to the control regions (D-loop) of mtDNA, but mutations were also detected in the 16SrRNA, ND2, and ATP6 genes [30]. Relevant to the immunogenicity of the enzymes of the ETC found in our study, an independent line of investigation using trans-mitochondrial cybrids also incriminated the involvement of mtDNA mutations resulting in dysfunction of the mitochondrial respiratory chain and tumorigenesis [Reviewed in ref 35]. Collectively, this method unraveled a major tumorigenic route linking mtDNA mutations to the decrease of the activity of one or more of the respiratory chain complexes, an impaired electron flux along the respiratory chain, associated with increased electron leakage and increased basal ROS production with alteration of mtDNA, resistance to apoptosis, enhanced cell proliferation and growth leading to tumor formation. Here we propose that the immunogenicity of the enzyme components of complex I (Table I) may be related directly or indirectly to their relative enrichment in somatic mutations [28]. In agreement with this proposal, a direct involvement was suggested by several studies reporting an association between mitochondrial somatic mutations and autoimmunity [36,37]. It has been demonstrated that peptides of mutated or aberrantly expressed mitochondrial proteins can be recognized by the immune system. Chen et al. tested the hypothesis that altered self-proteins derived from mtDNA somatic mutations may play a role in the development of autoimmunity, raising the possibility that mtDNA somatic mutations trigger immune responses [36]. This group provided proof of principle that the immune system can recognize peptides arising from spontaneous somatic mutations. Gu et al reported that damaged mtDNA is associated with increased expression of class I MHC, providing evidence that mutated peptides derived from somatic mutations in mtDNA can be recognized by the immune system [37]. However, the reports of conflicting data [Reviewed in 26, 27] suggest that the relationship between mitochondrial somatic mutations and carcinogenesis and / or cancer progression, although compelling, is not straightforward. Indeed, although mitochondrial somatic mutations are prevalent in BC and other cancers, the complexity of carcinogenesis has been again recognized [38]. Although mutated proteins can be recognized by the immune system as non-self and to generate autoantibody responses [36,37], structural changes in mitochondrial antigens as a consequence of mutations may not cause most of the immunogenicity of the gene products reported here. The autoantibody response observed in cancer sera as well as in the sera of patients with RADs targets both mutated and wild type proteins [2,4,20]. In addition, the autoantibody response observed in BC sera is not limited to mitochondrial auto-reactivity as indicated by the GO analysis of all the sequences targeted by auto-antibodies in BC (Figure 2). Moreover, it is possible that AMAs in cancer sera may not be restricted to BC, since mitochondrial mutations have been reported in other cancers [26, 27] Still, our findings using IFA in BC sera resembling features typically found in the RADs [3] and the present report showing that auto-reactivity of the enzymes of the ETC is a feature of BC are highly indicative of mitochondrial autoimmunity and together with the reports of a strong association between somatic mitochondrial mutations and BC progression [26, 27, 29], suggest a link between mitochondrial autoimmunity and somatic mutations. If not directly, how could somatic mutations be causally related to mitochondrial auto-reactivity? A clue can be provided by the reports of an association

between mitochondrial somatic mutations and the unfolded protein response [39,40]. A central function of the endoplasmic reticulum (ER) is to coordinate protein biosynthesis and cellular secretory activity. Alterations in ER homeostasis cause accumulation of misfolded / unfolded proteins in the ER and in the mitochondrial matrix [39, 40]. To maintain ER / mitochondrial matrix homeostasis, eukaryotic cells have evolved highly specific signalling pathways to ensure that its protein folding capacity is not overwhelmed. The Unfolded Protein Response (UPR), an essential adaptive intracellular mechanism is required if the cell is to survive the ER stress. Proteins that are misfolded in the ER are retained until they reach their native conformation or are retro-translocated back into the cytosol for degradation by the 26S proteasome. However, it is possible that this homeostatic process may be impaired in BC, since the 26S proteasome and other proteins involved in targeting misfolded proteins for degradation are also breast TAAs [41 and unpublished data]. The UPR has been implicated in a variety of diseases including metabolic, neurodegenerative, inflammatory diseases, and is known to be activated in cancer including BC [39,40. 42-46]. We propose that the link between somatic mutations and BC progression may reside in ER and mitochondrial matrix stress caused by somatic mutations leading to the accumulation of unfolded or misfolded proteins. There are multiple reports of somatic mutations causing ER stress triggering the UPR [39, 40]. Since mitochondria have their own protein synthesizing machinery [47], accumulation of misfolded proteins in the mitochondrial matrix could occur as a consequence of stress caused by mitochondrial somatic mutations; in addition, ROS are increased in solid tumors [48-50], and it is conceivable that exposure to excessive oxidative insult is an additional factor in producing ER and mitochondrial matrix stress. Todd et al [51] have discussed the growing evidence that dysregulation of the UPR may participate in the development of autoimmunity. The linearity of the reactive sequences cloned from the cDNA library in table 2 is compatible with the immune recognition of unfolded or misfolded proteins. Although it is also possible that direct ROS damage to mtDNA and mitochondrial proteins which undoubtedly occurs [48-50], may contribute to the immunogenicity of mitochondrial proteins in cancer cells, we do not have evidence in support of this possibility. We propose that the link between somatic mitochondrial mutations and BC may be in part indirect, through the generation of AMAs. A causal relationship between mitochondrial somatic mutations via ER and mitochondrial stress and mitochondrial autoimmunity may exist; this hypothesis needs to be examined further. We suggest that an in-depth study of mitochondrial autoimmunity in patients with BC as well as in pre-malignant breast tissue may result in the identification of needed diagnostic and prognostic biomarkers for early aggressive BC.

Relevant to our proposal, there is an established link between chronic inflammation and the generation of solid tumors [52,53]. Chronic inflammation fueled by autoimmune breast tissue damage may provide the rationale for the reported paradoxical association of B-cell hyperactivity and BC progression [54,55]. The proposed model of cancer progression based on mitochondrial autoimmunity implies a vicious cycle of mitochondria/ER stress, immune recognition of accumulated unfolded or misfolded proteins by auto-reactive immune cells, autoimmune damage of the target organ, and chronic inflammation with generation of pro-tumoral signals. Misfolded proteins could be recognized as non-self by auto-reactive immune cells in the context of MHC class II molecules, but other explanations linking the UPR and autoimmunity [51] could explain the generation of mitochondrial autoimmunity. Future studies based on this new

paradigm of cancer progression may allow the recognition of the pro-tumorigenic effect of autoimmune breast tissue damage resulting in chronic inflammation that may herald the dawn of a new era in the treatment of BC. We suggest that treatment modalities such as protecting the integrity of mitochondria, the induction of tolerance to antigens critically targeted by the autoantibody response, combined with selective suppression of B-cell hyperactivity and anti-inflammatory treatment, may contribute to improve the prognosis and decrease the mortality of aggressive BC.

SUMMARY

This is the first report of AMAs targeting the mtDNA-encoded enzyme components of complexes I, IV and V and other gene products encoded by mtDNA in BC sera. We interpret these results as the expression of mitochondrial autoimmunity targeting breast TAAs. We discuss the implications of mitochondrial autoimmunity in BC and propose the hypothesis that there is a causal relationship between the concerted effect of mitochondrial somatic mutations and ROS insult producing ER and mitochondrial matrix stress leading to the accumulation of unfolded or misfolded proteins and to activation of the UPR. We further hypothesize that targeting of multiple breast TAAs by AMAs and other auto-antibodies in concert with inflammatory cytokines may cause autoimmune breast tissue damage resulting in chronic inflammation. In the RADs multiple auto-antibodies targeting TAAs in target organs are pathogenic and responsible for autoimmune tissue damage [56-59]. The importance of the B cell response, and the pathogenic action of auto-antibodies and inflammatory cytokines as effectors of autoimmune tissue damage in the RADs has been widely recognized and we suggest that autoimmune damage leading to chronic inflammation and a pro-tumorigenic effect in BC may also be B cell-dependent. This proposal implies autoantibody internalization allowing the access of auto-antibodies to their intracellular targets. Penetration of autoantibodies into cells has been demonstrated in multiple studies since the report of Reichlin, et al. [60] [reviewed in ref 61]. Following the model of the RADs such as rheumatoid arthritis and systemic lupus erythematosus in which autoantibody biomarkers can be demonstrated in the sera of patients many years before the clinical diagnosis of the disease [4,62,63], we suggest that the pro-tumorigenic effect of autoimmune breast damage is a forerunner of BC and this could be detectable in the pre-clinical phase of the disease.

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