# Autoantibodies in Breast Cancer Identify Proteins Involved in Self-Renewal and Epigenetic Chromatin Remodeling

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**Abstract:** Recent studies have shown that autoantibodies developing in cancer patients are tumor-associated and are promising biomarkers for the diagnosis and prognosis of cancer. Here we report a panel of signal transduction molecules with partial sequences identical to the oncogene Bmi-1, NIFK, a nucleolar protein interacting with the FHA domain of Ki-67, TAB182, a protein interacting with tankyrase-1, CNOT6L, a subunit of the CCR4-NOT complex and to Elp3, a subunit of the elongator complex that are recognized as autoantigens by breast cancer sera with the ability to discriminate between invasive breast cancer and normal sera with high sensitivity and specificity. The proteins bearing the epitopes recognized by these antibodies have conserved regions involved in protein-protein interactions participating in regulatory processes such as self renewal, cell proliferation and survival, chromatin modulation, transcriptional silencing and organ patterning, usually ascribed to stem cell function. In this work we demonstrate by autoantigen microarray analysis that autoantibodies in breast cancer sera have the potential to delineate pathway connectivity. Our data indicate that several pathways involved in maintaining telomere stability are the target of an autoimmune reaction in breast cancer. These findings suggest that autoantibodies with the ability to recognize autoantigens involved in self-renewal and epigenetic chromatin remodeling have the potential to predict an invasive tendency of breast cancer.

**Keywords:** Autoantibodies, Bmi-1, autoantigen microarray, epigenetics, biomarkers, breast cancer.

## INTRODUCTION

Recent studies have shown that antibodies to cancer-associated antigens appear early during carcinogenesis, that they are tumor relevant, and that they have potential as markers for the diagnosis and prognosis of cancer [1-8].

In our previous work on autoantibodies in the diagnosis of breast cancer [5,6] using a new antibody-based biomarker discovery approach [9], we reported a 12-phage breast cancer predictor group constructed with phage inserts recognized by sera from patients with breast cancer and not by non-cancer or autoimmune control sera with high sensitivity and specificity [5,6]. In that work we also identified several autoantigens including annexin XI-A, the KIAA1671 gene product, elongation factor-2, Grb2-associated protein 2, the p80 subunit of the Ku antigen, ribosomal protein S6, NIFK, a nucleolar protein interacting with the FHA domain of Ki-67 and other unknown autoantigens that could significantly discriminate between breast cancer and non-cancer control sera and could distinguish ductal carcinoma *in situ* (DCIS) from invasive carcinoma (INV) of the breast [5,6].

In this work we have further characterized by sequence determination of phage displayed antigens, autoantigen microarray reactivity of breast cancer and control sera and homology searches, a small group of expression sequence tags (ESTs) cloned by biopanning a T7 cDNA library of breast cancer proteins with identity to the oncogene Bmi-1 and other proteins recognized as autoantigens by breast cancer sera. This is the first report of these autoantibodies in breast cancer. Since the proteins bearing the epitopes recognized by these autoantibodies have in common their ability to participate in regulatory processes such as self renewal and epigenetic chromatin remodeling, the aim of this study was to demonstrate the association of this autoantibody panel with the diagnosis of breast cancer, DCIS and INV of the breast. In this work we demonstrate by autoantigen microarray analysis that autoantibodies in breast cancer sera have the potential to delineate pathway connectivity and to contribute to the diagnosis of invasive breast cancer.

## MATERIALS AND METHODOLOGY

### Selection of Sera for Immunoscreening and Biopanning of a T7 Phage cDNA Library

Sera from women with breast cancer with comprehensive outcome data collected by the Karmanos Cancer Institute Breast Cancer Prognostic Study (KCI- BCPS) at Wayne State University from 1975 to 1985, were used in this study. The characteristics of this cohort of patients have been reported [5]. The pathologic diagnoses of DCIS and INV of the breast in all patients were made when the tumor was first detected and sera from all women with breast cancer were obtained prior to any treatment. Nine sera from women with invasive breast carcinoma were chosen for immunoscreening a T7 phage cDNA display library of breast cancer proteins

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(Novagen), because they had strong, high titer IgG signals (1:500) on immunoblots, suggesting that the autoantibodies were abundant [5]. We screened plaques obtained from sequential rounds of immune-precipitation, and selected a subset of strongly recognized antigens to assemble a library of breast cancer autoantigens. After the final round of biopanning, phages were plated at low density on a lawn of E.coli, and plaque lifts were screened using patient sera to identify individual positive phages which were used to construct a breast autoantigen microarray as previously described [5].

### Autoantigen Microarray Construction, Hybridization, Quantification and Antigen Identification

Plaque-pure phages were grown to high titer in bacterial cultures that were incubated until complete lysis. Supernatants collected after a 10 minute x 10,000 g spin were arrayed in 384-well microtiter dishes. The entire phage-library was spotted in duplicate onto nitrocellulose-coated FAST Slides (Schleicher and Schuell) using a Flexys robot (Genomic Systems). Each slide was probed either with a serum from patients with DCIS, INV of the breast or with a control serum and with a mouse monoclonal antibody directed against a non-variable T7 phage coat protein (Novagen, Madison, WI) [5].

Probing the T7 display cDNA library with sera from women with breast cancer and normal controls were performed as previously reported [5]. Sera used for immunoscreening the T7 cDNA library were not used in the hybridization step. Sera from 15 women with DCIS and from 22 women with INV carcinoma of the breast consecutively enrolled in the KCI- BCPS with biopsy-proven diagnoses were chosen to hybridize the breast autoantigen microarray. Thirty three non-cancer, non-autoimmune control sera were obtained from asymptomatic women having no past or family history of breast or ovarian cancer. All sera were stored frozen at -80°C until use. Women chosen as controls were of similar age and race as the women with breast cancer (breast cancer mean age at diagnosis: 59.3 years; race, Caucasian, 95%, African American, 5%; control women, mean 56.4 years; race: Caucasian, 93%, African American, 7%). Patient reactivity was detected using CY3-labeled goat anti-human IgG+IgM secondary antibodies, and the amount of phages present in each spot was measured by binding of CY5-labeled goat anti-mouse IgG+IgM antibodies. Phage slides also contained 96 "blank" spots (buffer only) to measure background, and 32 "control" spots, which consisted of identical phage clones spotted in 32 equidistant positions to assess positional variability across each slide. CY3 and CY5 signals were read using a GenePix 4000, a slide array reader (Axon Instruments), and quantified using ImaGene software (ImaGene 5.1, BioDiscovery Inc). The CY3/CY5 ratios were calculated for each phage spot, and values for duplicate spots were averaged. We based the analysis on CY3/CY5 ratios for all signals corresponding to cancer and control sera. Log2-transformation of the Cy3/Cy5 ratios was applied [9]. For those ratios that were less and equal to 0, a threshold of 0.1 was used. Quantile normalization was performed. The goal of this between array normalization was to impose the same empirical distribution of intensities to each array, so that the intensities from each array could be comparable. As some phages have very small variations across the arrays, only phages whose variations were in the top 25% were used in the analysis. The bootstrap "out-of-bag" validation method was used to estimate the distribution of the sensitivities and the specificities from the KNN classification. A sensitivity analysis of number of neighbors was also performed.

We determined the identity of the autoantigens that were significantly recognized by multiple breast cancer sera on the microarray by sequencing the phage cDNA inserts. After identifying informative phages, the corresponding cDNA inserts were amplified by PCR using primers flanking the insertion site, and sequenced by the core facility of the Center for Molecular Medicine and Genetics at WSU. After eliminating phage-related sequences, the GeneBank database was searched for sequence similarities to our identified cDNA sequences using the BLAST program [10]. This study had the approval of the Human Investigation Committee of WSU.

## RESULTS

### Autoantibodies Recognizing Antigens Involved in Self-Renewal and Epigenetic Chromatin Remodeling Distinguish INV from DCIS of the Breast and from Normal Individuals

We have reported that a panel of autoantibodies in breast cancer sera is able to distinguish breast cancer from normal sera with high sensitivity and specificity, while some antigens could distinguish INV from DCIS of the breast [5,6]. In subsequent work reported here, we have identified by immunoscreening a T7 phage cDNA library of breast cancer proteins and autoantigen microarray analysis a group of breast cancer autoantigens involved in self renewal and epigenetic mechanisms with potential to contribute to the early diagnosis of invasive carcinoma of the breast. Table **1** shows a group of expression sequence tags (ESTs) with identity to proteins involved in self renewal and epigenetic mechanisms which reacted with high titer IgG autoantibodies present in breast cancer sera [11-15].

**Table 1. Breast Cancer Autoantigens Involved in Self-Renewal and Epigenetic Mechanisms**

| Autoantigen | GenBank Account | Homology | BLASTn | tBLASTx |
|---|---|---|---|---|
| Bmi-1 [11] | CB331930 | Bmi-1 | 8e-38 | 1e-9 |
| KIAA1671 [12] | CB331932 | TAB182 [13] | 0.0 | 1e-116 |
| RSAD1 [14] | CB334788 | Elp3 | 1e-115 | 6e-48 |
| CNOT6L [15] | CB331931 | CNOT6L | 2e-75 | 5e-25 |

The autoantigens identified by breast cancer sera by immunoscreening a cDNA library of breast cancer proteins, the account numbers of the cloned phage inserts (ESTs) in the GenBank, their homology revealed by searching databases and their corresponding e value using BLASTn and tBLASTx [10] are included. TB182, gi:116242814; RSAD1, radical S-adenosyl methionine containing domain, Elp3, NM_018346; CNOT6L, homo sapiens CCR4-NOT transcription complex, subunit 6-like, gi:115583678.

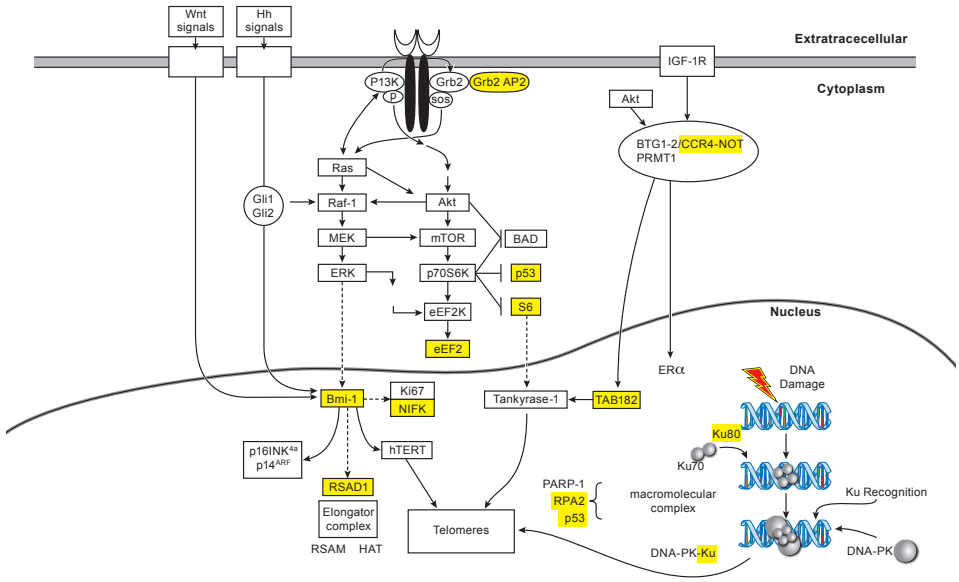


**Fig. (4). Autoantibodies identify signal tranduction molecules that may be activated in breast cancer.** Signal transduction molecules depicted in yellow elicit an autoimmune reaction in breast cancer. Interrupted arrows indicate possible pathway connectivity suggested by the autoantibody recognition of breast cancer-associated antigens. Abbreviations as in Table **1** and in the text.

rebounds in women with invasive cancer. It also possible that the molecular alterations which trigger an autoantibody response to the autoantigens reported here may be less prevalent in DCIS of the breast than in invasive carcinomas. Alternatively the observed effect may be due to unmeasured covariate differences between the groups of women.

From the group of phage-coded ESTs listed in Table **1**, the Bmi-1 oncogene has attracted a great deal of interest because of its participation in functions attributable to stem cells [11,17,18,38]. Bmi-1 is a transcriptional repressor belonging to the polycomb group (PcG) that plays a role in gene regulation in both normal and cancer stem cell proliferation through epigenetic mechanisms, such as methylation or acetylation of chromatin, initially reported as an oncogene collaborating with c-Myc in the induction of B-cell lymphomas [11]. The regulation of self-renewal of specific stem cells by several PcG genes, including Bmi-1, suggests a link between cell homeostasis and carcinogenesis [39,40]. The association of Bmi-1 overexpression with breast cancer has been reported [41,42] and it has been suggested that Bmi-1 regulates telomerase activity in MECs and plays a role in the development of human breast cancer [42].

The proliferation marker Ki-67 is widely used to detect proliferating cells, as it is only expressed during cell cycle progression [24]. Ki-67 contains an FHA domain and it has been suggested that this protein could interact with phosphorylated proteins through this domain [43]. Ki-67 is a component of an 11-gene signature associated with a conserved Bmi-1-driven pathway, suggested by Glinsky *et al.* to represent a subset of highly malignant cancers with poor prognosis, including lung, prostate and breast cancer [22]. Autoantibodies to Bmi-1 and to Ki-67 have also been reported in patients with lung cancer [44], consistent with the possibility that the presence of these antibodies may indicate an invasive tendency of malignancy. The Bmi-1-driven pathway reported by Glinsky *et al*, the association between the antibodies to Bmi-1 and NIFK in breast cancer sera (Fig. **3**), and the finding that Bmi-1 and NIFK are recognized as breast cancer autoantigens by the sera of some women with breast cancer (Table **1**) suggest that Bmi-1 may influence cell proliferation through Ki-67 (Fig. **4**).

A number of developmental signaling pathways, such as Wnt, Notch, and Hedgehog (Hh), play a role in regulating the self-renewal of normal stem cells in the hematopoietic system, the skin, the nervous system, and the breast [18,21]. The effects of the Wnt and the Hh signaling pathways on growth and patterning during embryonic development are thought to be mediated by polycomb gene Bmi-1 [18,21]. It has also been demonstrated that Bmi-1 expression can be up-regulated by Hh signaling in human mammary stem/progenitor cells [18]. We have reported that the Grb-2 AP2, the binding partner of Grb-2 [28] is a breast cancer autoantigen [5]. Effector molecules bind to phosphorylated tyrosine residues on the RTK's *via* the adaptor protein, Grb2/SOS [28]. Our finding that the same cloning serum led to the to the identification of both Grb2-AP2 and Bmi-1, and the recognition of this pair of antigens by multiple breast cancer sera on the microarray suggest the possibility that in some patients with breast cancer, signals transduced through the ras signaling cascade may activate Bmi-1 (Fig. **4**). Recently, Datta *et al.* examined the oncogenic potential of Bmi-1 in MCF10A cells [45]. Although Bmi-1 overexpression alone did not result in oncogenic transformation in MCF10A cells, Bmi-1 co-overexpression with activated H-ras resulted in efficient transformation of MCF10A cells *in vitro*.

We have reported that the KIAA1671 protein is a breast cancer autoantigen [5,6]. Table **1** shows the results of a homology search indicating the identity of this protein with TAB182, the tankyrase-1 binding protein [13]. We have reported that partial sequences identical with the KIAA1671/tankyrase 1 antigen and the ribosomal protein S6 were significantly associated with the diagnosis of breast cancer [5,6]. This association may suggest that signals proceeding from mTOR may reach the telomeres *via* ribosomal

protein S6 (Fig. **4**). Tankyrases are poly (ADP-ribose) polymerases that enhance telomerase access to telomeres. Autoantibodies to the tankyrase 1 binding protein TAB182 are likely to reflect telomere dysfunction in breast cancer. Multiple evidence suggest that telomere-associated events are relevant to stem cell function, to senescence and to carcinogenesis [46]. It is relevant that other molecules located at the telomere ends are also targeted by autoantibodies in breast cancer. We have reported that a partial sequence identical to Ku80 is recognized by multiple breast cancer sera and contributes to distinguish DCIS from invasive carcinoma of the breast [5,6]. Ku80 is an important component of the NHEJ DNA repair pathway involved in the repair of double strand breaks in mammalian cells [31]. We have shown that the 32 kDa subunit of RPA is the target of autoantibodies in breast cancer [3]. The autoimmune response triggered by multiple components of the DNA repair machinery, by Bmi-1 and by the tankyrase 1 binding protein TAB182 in breast cancer sera suggests the involvement of the telomeres in breast carcinogenesis (Fig. **4**).

We have identified CNOT6L, a subunit component of the CCR4-NOT complex as a breast cancer autoantigen (Table **1**). The CCR4-NOT complex is involved in protein-protein interactions and its activation may reflect the involvement of the PI3/Akt kinase and MAPK kinases (ERK1/2 and p38) pathways as well as the co-regulatory activity of the IGF-I (Fig. **4**) [15]. It has been reported that depletion of endogenous CCR4-NOT subunits in breast cancer cells results in deregulation of ER alpha target genes [15]. Autoantibodies targeting the CCR4-NOT complex may indicate the activation of this pathway in certain patients with breast cancer.

The identification of the RSAD1 domain of Elp-3 as a breast cancer autoantigen (Table **1**) is another example of the participation of antibody-recognized epitopes involved in protein-protein interactions, instrumental in epigenetic chromatin remodeling. Identical sequences have been cloned from colon carcinoma, chondrosarcoma, islets of Langerhans, lung cancer as well as from embryonic tissues [14]. We speculate that the association of Bmi-1 and RSAD1 on the autoantigen microarray (Fig. **3**) could be a clue indicating that Bmi-1 might influence the set of reactions catalised by RSAD1 (Fig. **4**). Purification of chromatin-associated hyperphophorylated RNAPII from yeast led to the isolation of the elongator multisubunit complex [47]. The histone acetyltransferases and methyltransferases are part of a class of chromatin remodeling complexes that catalyze post-translational histone acetylation and methylation reactions [48]. The Elp3 subunit of the elongator complex in humans contains a well characterized highly conserved C-terminal acetyltransferase (HAT) domain and its central region shares significant sequence homology to the radical SAM superfamily [49,50]. Human holo-elongator complex has robust histone acetyltransferase activity directed against histone H3 and H4 [50].

Whether the cells harboring the molecular changes responsible for eliciting an autoimmune reaction in breast cancer sera reported here, conform to the current definition of cancer stem cells or progenitor cells is unknown. A prospective validation study using high throughput microarray analyses could establish the value of these autoantibodies for the early diagnosis of invasive breast cancer. The identification of patients with tumors with invasive tendency at the time of diagnosis could have important implications for the choice of therapy of patients with newly diagnosed breast cancer.

The finding that some pairs of breast cancer autoantigens are associated on the microarray is reminiscent of the linked antibodies characteristic of systemic lupus erythematosus [51]. Although the significance of these findings is unknown, their presence in cancer sera merits further investigation.

The cancer stem cell hypothesis [19-21] has important implications in the field of biomarker discovery. Since the non-tumorigenic cells forming the bulk of the tumor are probably many cell cycles removed from the cells responsible for the cancer-initiating events and are thus heterogeneous and antigen diverse, a large portion of the tumor-associated antigens identified by currently used methods, although indeed tumor-associated, have probably originated in the bulk of the non-tumorigenic but yet antigenic cells. It is likely that a biomarker discovery approach targeting the cancer stem cell compartment may in the future, yield diagnostic and prognostic panels with the highest accuracy.

## CONCLUSION

In summary, this is the first report of autoantibodies to Bmi-1 and other autoantibodies recognizing epitopes developing in proteins known to be involved in key molecular reactions participating in self renewal, cell differentiation, patterning, chromatin modifications and on the regulation of telomerase. A prospective study involving a larger number of patients and non-cancer controls should determine whether these autoantibodies can discriminate between patients with early forms of invasive breast cancer and normal women with high accuracy.

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