

Identification of a New Panel of Serum Autoantibodies Associated with the Presence of *In situ* Carcinoma of the Breast in Younger Women

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Abstract Purpose: We examined the feasibility of using a panel of autoantibodies to multiple tumor-associated proteins as a method for early detection of breast cancer and, more particularly, carcinoma *in situ* (CIS).

Experimental Design: PPIA, PRDX2, and FKBP52 were identified as early-stage breast cancer autoantigens by proteomic approaches. The seroreactivity of a panel of antibodies consisting of these three antigens and two previously described autoantigens, HSP60 and MUC1, was tested on 235 samples (60 from primary breast cancer patients, 82 from CIS patients, and 93 from healthy controls) with the use of specific ELISAs. FKBP52, PPIA, and PRDX2 mRNA and protein expression levels were evaluated by reverse transcription-PCR and immunohistochemistry in early-stage breast tumors.

Results: Three of five autoantibodies, FKBP52, PPIA, and PRDX2, showed significantly increased reactivity in primary breast cancer and CIS compared with healthy controls. When combined, the five markers significantly discriminated primary breast cancer [receiver operating characteristic area under the curve, 0.73; 95% confidence interval (95% CI), 0.60-0.79] and CIS (receiver operating characteristic area under the curve, 0.80; 95% CI, 0.71-0.85) from healthy individuals. Importantly, the receiver operating characteristic – area under the curve value of the autoantibody panel was able to distinguish CIS, including high grades, from healthy controls in women under the age of 50 years (receiver operating characteristic area under the curve, 0.85; 95% CI, 0.61-0.92). Finally, only FKBP52 mRNA and protein levels were found to be increased in CIS and primary breast cancer compared with healthy breast tissue.

Conclusions: This autoantibody assay against a panel of five antigens allows for an accurate discrimination between early-stage breast cancer, especially CIS, and healthy individuals. These results could be of interest in detecting early breast cancer as an aid to mammography, especially in women under the age of 50 years with aggressive cancers.

The widespread use of screening mammography has resulted in increased detection of early-stage breast disease, particularly for carcinoma *in situ* (CIS) and early-stage breast cancer. However, incidences of stages I and II of the disease have not declined, suggesting a bias in the detection of indolent cancers rather than aggressive cancers. In addition, the predictive value of mammography declines in cohorts of patients with dense breast tissue as well as in premenopausal women (1). Although

magnetic resonance imaging is a very sensitive detection tool that has become standard for women at very high risk of developing breast cancer, it lacks sufficient specificity and cost-effectiveness for use as a general screening tool (2). Detection of breast cancer at the earliest stages results in a more favorable outcome (3). Therefore, there is an important need to improve the screening and diagnosis of early invasive and noninvasive tumors (i.e., CIS), especially in women under the age of 50 years that usually develop aggressive cancers.

The great opportunity for molecular tools to improve breast cancer outcomes based on early diagnosis has driven the search for diagnostic biomarkers. The identification of tumor-specific markers capable of eliciting an immune response early in tumor development seems to provide an effective approach for early diagnosis. Indeed, a humoral response to such antigens generates a remarkable biological amplification that has recently been reported to have potential for cancer detection (4). Interestingly, this response seems to appear months to years before the clinical diagnosis of a tumor, rendering serum autoantibody detection suitable for early-stage cancer diagnosis (5–7). Given that classical tumor biomarkers, such as CA15-3 and carcinoembryonic antigen (CEA), are only currently

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Received 12/23/08; revised 4/17/09; accepted 4/17/09; published OnlineFirst 7/7/09.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi:10.1158/1078-0432.CCR-08-3307

Translational Relevance

This study shows the clinical relevance of a combination of five antigens, identified by proteomic approach and validated in an independent cohort of 235 samples by ELISA, as a blood-screening test for early breast cancer detection. Of these five antigens, FKBP52 was found up-regulated in early-stage breast cancer, and its expression levels were correlated with cancer progression. Because mammographic screening declines in cohorts of patients with dense breast tissue and small lesions, as well as in premenopausal women, obtaining the status of this biomarker panel may guide the detection of early-stage cancer in women under the age of 50 years.

available for advanced-stage cancer detection or for monitoring treatment efficiency (8), dissecting humoral responses to cancer may provide an important tool for early diagnosis. In a previous study, we showed that heat shock protein 60 (HSP60) could be considered as a relevant tumor-associated antigen for early-stage and ductal *in situ* breast carcinoma detection (6). However, measurement of a single autoantibody will not provide the adequate sensitivity or specificity needed for early detection or screening (9). Here, we explore the diagnosis efficiency of a panel consisting of three new tumor-associated antigens identified with the use of a proteomic approach. To enlarge the set of potential autoantigens, we also included HSP60 along with Mucin 1 (MUC1), which has been shown to be an antigen highly immunogenic in breast cancer. We provide evidence that this seroreactivity pattern allows accurate detection of early breast cancer and, more particularly, for CIS and women under the age of 50 years with aggressive cancers. In addition, among the proteins of this panel, FKBP52 shows increased expression levels during breast cancer progression and thereby represents a potential immunotherapeutic target of early-stage breast cancer.

Patients and Methods

Sera and tissues. All human samples were collected prospectively between 2005 and 2007 at the CRLC Val d'Aurelle cancer institute, Montpellier, at the time of cancer diagnosis after obtaining written informed consent (RBM 03-63). Blood samples were centrifuged at 1,250g for 5 min, and sera were then stored at -80°C. For two-dimensional electrophoresis western blot experiments, a first set of sera was obtained from patients with breast cancer (clinical stage I, lymph node negative, and grade I; $n = 20$), other cancers (ovarian cancer, $n = 10$; prostate cancer, $n = 10$), autoimmune disease (rheumatoid arthritis, $n = 10$; systemic lupus erythematosus, $n = 10$), and healthy volunteers ($n = 20$). The mean age of subjects who donated sera for this study was 55.4 y (range, 35-71 y). For ELISA experiments, a second set of sera was obtained from 82 patients with CIS, 60 patients with early-stage primary breast cancer of different histotypes, and 93 matched healthy controls exempt of any breast and/or inflammatory disease (mean age, 55 y; range, 31-82 y). Detailed clinical and pathologic information for the *in situ* and invasive breast cancer patients are listed in Table 1. CIS and early-stage primary breast cancers were classified according to WHO classification of tumors and were graded according to Bloom-Richardson. No patients received neoadjuvant therapy; all had surgery within 4 wk of initial diagnosis of breast cancer. For two-dimensional gel electrophoresis experiments, two primary invasive breast tumor

tissues (T_3N_0) were obtained during surgical resection, and samples were immediately frozen at -80°C and stored until use. For reverse transcription-PCR experiments, early invasive (T_1N_0 , $n = 15$; T_1N_1 , $n = 14$), and healthy ($n = 29$) breast tissues were obtained during surgical resection. Samples were immediately frozen at -80°C and stored until use. For immunohistochemistry experiments, CIS ($n = 10$) and early invasive cancer tissues (stage I; $n = 10$) were obtained at the cancer institute CRLC Claudius Regaud, Toulouse, at the time of cancer diagnosis after obtaining written informed consent. Ten supplementary CIS and 10 supplementary primary breast cancer samples were analyzed for FKBP52 IHC.

Two-dimensional gel electrophoresis and western blot analysis. All reagents and materials for two-dimensional gel electrophoresis experiments were purchased from GE Healthcare unless otherwise indicated. Twenty-micrometer-thick sections from two breast tumors were lysed, and proteins (150 μ g) were separated by two-dimensional gel electrophoresis as described (10). The gels were silver-stained according to the procedure of Shevchenko et al. (11). Separated proteins were transferred onto a Hybond-P polyvinylidene difluoride membrane (Millipore), and incubated with four pools of five sera from patients with early-stage breast cancer or with four pools of five sera from patients of a control group at a 1:200 dilution. Proteins were revealed with a horseradish peroxidase-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Laboratories) at a 1:3,000 dilution and with the use of a SuperSignal West Pico Chemiluminescent Substrate kit (Pierce). All pools of sera were assayed in triplicate.

A rabbit anti-PPIA polyclonal antibody (Biomol), a mouse anti-PRDX2 polyclonal antibody (Abnova), and a mouse anti-FKBP52 monoclonal antibody (Stressgen) were used at 1:10,000, 1:1,000, and 1:1,000 dilution for western blotting, respectively, and were processed as for incubations with patient sera. The secondary antibodies used were a goat anti-rabbit IgG horseradish peroxidase conjugated (Santa Cruz) or a goat anti-mouse IgG horseradish peroxidase conjugated (Jackson ImmunoResearch Laboratories) at a 1:5,000 dilution.

In-gel enzyme digestion and mass spectrometry. Spots from two-dimensional gel electrophoresis silver-stained gels were excised and in-gel digested with the use of trypsin (Gold; Promega) as previously described (12). Mass spectrometry analyses for protein identification were done by the proteomic platform of the Institute of Functional Genomics in Montpellier.

Autoantibody detection. HSP60 autoantibodies were detected in the sera of patients with the use of the anti-human HSP60 (total) ELISA kit from Stressgen (Assays Designs, Inc.) following the manufacturer's recommendations. Sera were diluted 1:500. PPIA, PRDX2, MUC1, and FKBP52 autoantibodies were detected with the use of MaxiSorp high-protein-binding plates (Nunc) coated with 100 ng of the correspondent recombinant protein (FKBP52, PRDX2, and MUC1 from Abnova Corporation; PPIA from Affinity BioReagents) overnight at room temperature: 4°C in 100 μ L of PBS. The plates were washed twice with PBS 0.1% Tween 20, and then blocked for 2 h at room temperature with PBS 0.1% Tween 20 and 2% bovine serum albumin. The plates were washed again twice and incubated with sera (diluted 1:500 for the ELISAs detecting HSP60, FKBP52, PPIA, and MUC1 autoantibodies, and 1:250 for the ELISA detecting PRDX2 autoantibodies) for 2 h at room temperature under rotation. Appropriate control antibodies specific for capture proteins [mouse anti-MUC1 monoclonal antibody (Abnova), rabbit anti-PPIA polyclonal antibody (Biomol), mouse anti-PRDX2 polyclonal antibody (Abnova), and mouse anti-FKBP52 monoclonal antibody (Stressgen)] were also included. After four washings, plates were incubated with a horseradish peroxidase-conjugated polyclonal antibody specific for human IgG, IgM, and IgA (Stressgen; dilutions per manufacturer recommendation) for 1 h at room temperature under rotation. After four washings, plates were incubated with 3,3',5,5'-tetramethylbenzidine (Stressgen) substrate solution for 15 min, and absorbance values were determined at 450 nm after the addition of H_2SO_4 to stop the reaction. Each serum was assayed in duplicate in at least three independent experiments.

Table 1. Clinicopathologic characteristics of breast carcinomas

Characteristics		Early-stage PBC (N = 60) N (%)		CIS (N = 82) N (%)
Age (y), median (range)		62 (43-84)		58 (40-82)
<50		9 (15)		14 (17)
50-59		15 (25)		29 (35)
60-69		20 (33)		22 (27)
≥70		16 (27)		17 (21)
Histology				
Ductal		55 (92)		71 (87)
Lobular		5 (8)		11 (13)
Histologic grade	1	18 (30)	Low	11 (14)
	2	28 (47)	Intermediate	30 (37)
	3	14 (23)	High	39 (49)
Tumor size	T ₁	60 (100)	<20 mm	37 (47)
			20-50 mm	37 (47)
			>50 mm	4 (6)
			Missing	4
Lymph node status				
Negative		60 (100)		82 (100)
ER α				
Negative		42 (70)		9 (29)
Positive		18 (30)		22 (71)
Missing				51
PgR				
Negative		26 (43)		18 (58)
Positive		34 (57)		13 (42)
Missing				51
Her-2 overexpression*				
Negative		23 (59)		4 (33)
Positive		16 (41)		8 (67)
Missing		21		70
Architecture				
Cribriform		NA		29 (45)
Comedocarcinoma		NA		20 (31)
Mixed, or other (papilloma)		NA		16 (24)
Missing		NA		6
Necrosis				
Absent		NA		58 (71)
Present		NA		24 (29)

Abbreviations: PBC, primary breast cancer; NA, not applicable; ER α , estrogen receptor alpha; PgR, progesterone receptor.

*Her-2 status was determined by immunohistochemistry according to the HercepTest scoring system.

RNA isolation, reverse transcription, and real-time quantitative PCR analysis. Total RNA from 29 breast tumors and 29 healthy breast frozen tissues were extracted from fifteen 20- μ m-thick sections with the use of the RNeasy mini kit (Qiagen). Complementary DNA was synthesized as previously described (6) with the use of Superscript III reverse transcriptase (Invitrogen). Real-time quantitative PCR was done with the use of the LightCycler 2.0 system (Roche). Data on *FKBP52*, *PPIA*, and *PRDX2* were normalized according to data from *TBP89* and *RS9* housekeeping genes. Sequences of forward and reverse primers are shown in supplementary data 1.

Immunohistochemistry staining. Immunohistochemical analyses were done on 4- μ m-thick sections of RCL2 fixed, paraffin-embedded breast tissue with the use of the Dako Autostainer Plus Link (Dako). Both deparaffinization and epitope retrieval were done in the PT Link system with the use of the provider procedure. All components used for IHC, except primary antibodies, are from the EnVision FLEX High pH kit (K8000; Dako). Tissue sections were treated for 45 min at 95°C with EnVision FLEX Target Retrieval Solution (DM812; Tris/EDTA buffer, pH9) for antigen retrieval. Tissue sections were then incubated with a rabbit anti-hFKBP52 polyclonal antibody (Proteintech), a rabbit anti-PPIA polyclonal antibody (Biomol), or a mouse anti-PRDX2 monoclonal antibody (Abnova) for 20 min at room temperature at 1:200, 1:2,500, and 1:1,000 dilutions, respectively. The blocking buffer used was the EnVision FLEX Peroxidase-Blocking Reagent (SM801). The

detection of the antibody binding was visualized with a peroxidase-conjugated polymer backbone (EnVision FLEX; SM802) with the use of diaminobenzidine as a chromogen. The sections were then counterstained with hematoxylin. Scores were obtained by estimating the average signal intensity (scale of 0-3) and the proportion of cells showing a positive signal (0%-100%). The intensity and proportion scores were then multiplied to give an overall IHC score.

Statistical analysis. The comparison of the different markers between sera of healthy controls, early invasive primary breast cancer, and CIS was done with the use of a nonparametric Kruskal-Wallis test. Differences were considered statistically significant when $P < 0.05$. Multivariate analysis is based on receiver operating characteristic curves, which allow the characterization of the discrimination between two well-defined populations. The generalized receiver operating characteristic criterion (13, 14) finds the best linear combination (virtual marker) of the tumor markers such that the area under the curve is maximized. Sensitivity, which represents its ability to detect the diseased population, and specificity, which represents its ability to detect the nondiseased population, for individual and combined marker performance, were evaluated with the use of the optimal threshold value calculated to maximize the Youden's index. This index is defined as the sum of sensitivity and specificity minus 1. Statistical analysis was done with the use of Stata 10.0 software (StataCorp. 2007; Release 10) and mROC software (15).

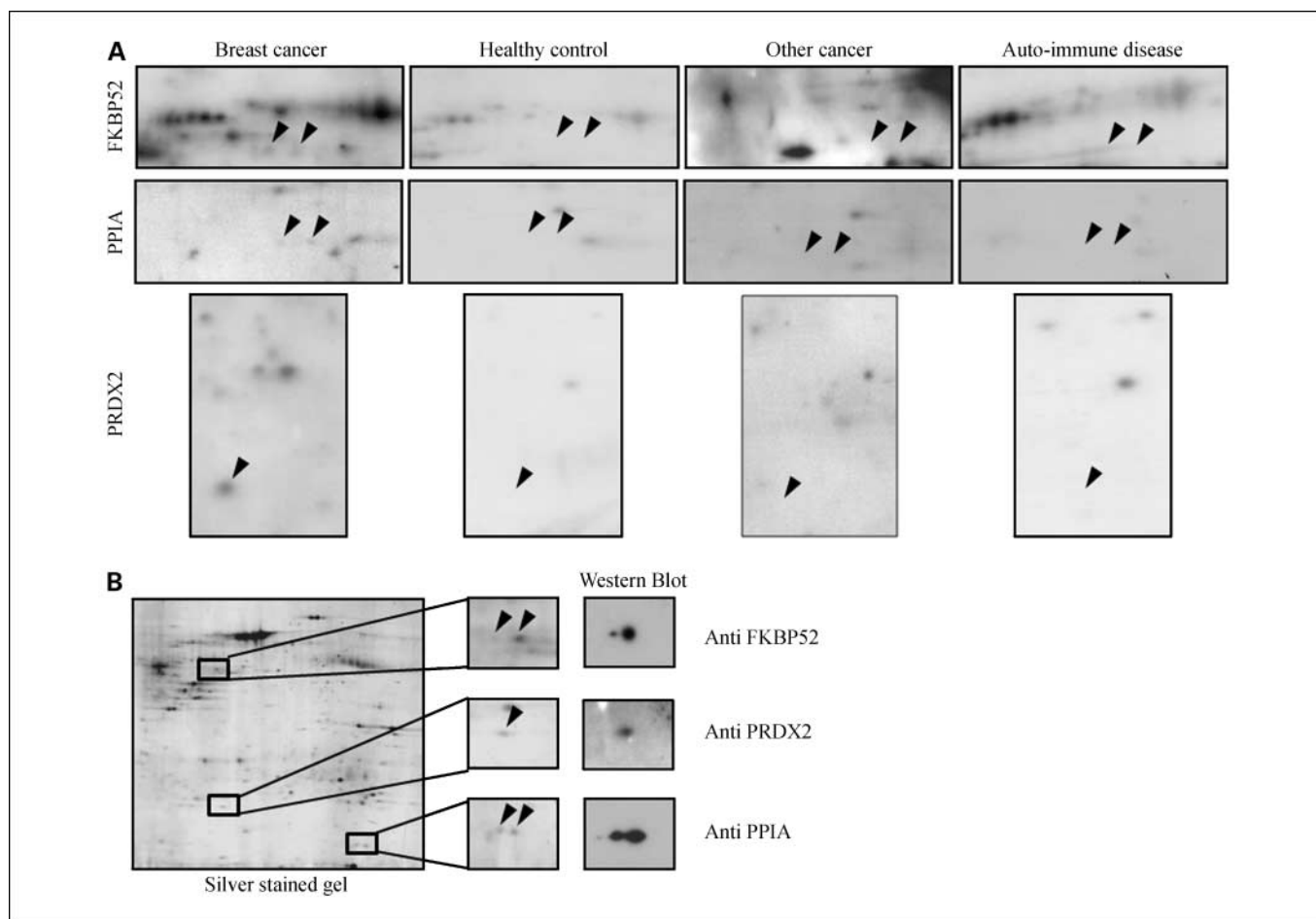


Fig. 1. Detection of FKBP52, PPIA, and PRDX2 as autoantigens in early-stage breast cancer. *A*, specific detection of the three tumor-associated antigens in the sera of patients. Enlargement of the region containing each protein revealed with early-stage breast cancer, other cancers, autoimmune disease, and HC sera is shown. *B*, detection of FKBP52, PPIA, and PRDX2 with the use of specific antibodies. Black arrows, spots corresponding to each protein. HC, healthy controls.

Quantitative PCR data were analyzed with the unpaired Mann-Whitney test. The level of statistical significance was set at the value of $P < 0.05$.

Results

Autoantibody reactivity targeted at FKBP52, PPIA, and PRDX2 in sera from newly diagnosed patients with early breast cancer. Sera obtained from 20 newly diagnosed patients with early-

stage breast cancer, 20 patients with autoimmune disease, 20 patients with prostate or ovarian cancers, and 20 age-matched healthy subjects were investigated for the presence of autoantibodies against early invasive breast tumor tissue proteins, separated by two-dimensional gel electrophoresis. Several spots exhibited a remarkably high reactivity with all breast cancer sera pools although not with controls. Among the protein spots identified with high confidence by matrix-assisted laser

Table 2. Area under the receiver operating characteristic curves and median comparison done for individual markers

Marker	Median			HC/cancer	
	HC	PBC	CIS	P	AUC (95% CI)
FKBP52	0.32 (0.12-0.70)	0.39 (0.11-3.13)	0.51 (0.10-2.71)	<0.0001*	0.685 (0.619-0.745)
PPIA	0.24 (0.08-0.53)	0.34 (0.10-2.48)	0.35 (0.10-2.46)	<0.0001*	0.694 (0.628-0.754)
PRDX2	0.20 (0.05-0.43)	0.27 (0.07-2.32)	0.26 (0.08-1.92)	0.008*	0.656 (0.589-0.719)
HSP60	0.21 (0.08-0.56)	0.23 (0.08-2.07)	0.18 (0.07-2.68)	0.544	0.568 (0.5-0.635)
MUC1	0.31 (0.06-0.69)	0.40 (0.09-2.35)	0.30 (0.08-2.09)	0.151	0.588 (0.519-0.655)

NOTE: Values are expressed as medians, and populations were compared through a Kruskal-Wallis test.

Abbreviation: HC, healthy controls.

*The test is considered significant ($P < 0.05$). The area under the receiver operating curve indicates the diagnostic accuracy of biomarkers.

desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry, we focused on three, PPIA, PRDX2, and FKBP52, which are likely to play a role in breast carcinogenesis (Fig. 1A). Identifications were confirmed with two-dimensional gel electrophoresis western blotting with the use of specific monoclonal antibodies (Fig. 1B).

Discriminatory power of individual and combination of biomarkers. To assess the diagnostic accuracy of single antibody versus combined antibody measurements, the average levels of autoantibodies directed against PPIA, PRDX2, FKBP52, and two other previously described tumor-associated antigens, HSP60 (6) and MUC1 (16), were quantified by ELISA in sera from 60 early-stage primary breast cancers, 82 CIS, and 93 healthy controls (Table 1). All five markers showed individual significant differences between early-stage primary breast cancer and healthy controls, whereas only FKBP52, PPIA, and PRDX2 autoantibodies allowed significant discrimination between CIS and healthy controls (Table 2; Supplementary Data 2). The receiver operating characteristic curves that discriminate the cancer groups from healthy controls are shown in Fig. 2. We evaluated the diagnostic accuracy of the panel and determined the receiver operating characteristic area under the curve to be 0.74 [95% confidence interval (95% CI), 0.67-0.79] when we compared both cancer groups with healthy controls (Fig. 2A). When the optimal threshold was defined, the test achieved a sensitivity of 60.5%, a specificity of 77.2%, and a diagnosis accuracy of 67.1%. Discrimination between primary breast cancer and healthy controls gave an area under the curve of 0.73 (95% CI, 0.60-0.79) with a sensitivity of 55.2%, a specificity of 87.9%, and a diagnostic accuracy of 75.1% (Fig. 2B). Interestingly, the combination was able to discriminate CIS from healthy controls with the highest area under the curve (0.80; 95% CI, 0.71-0.85; Fig. 2C), yielding a sensitivity of 72.2%, a specificity of 72.6%, and a diagnostic accuracy of 72.4%. To further validate our results, a leave-one-out cross-validation algorithm was done with the same data used above. Interestingly, the predictive accuracies were similar to the values calculated with the use of the mROC criterion, proving the robustness of the seroreactivity pattern (Supplementary data 3).

Correlation with hormonal, histologic, and clinical variables. We next evaluated the correlation of our predictor with classic biological, histologic, and clinical variables in both cancer groups (for a list of these variables, see Table 1). We did

not find a correlation with any of the variables except for age of CIS patients. Indeed, the medians of four of the five markers (all except HSP60) were significantly different between women under the age of 50 years and women above the age of 50 years (Table 3). In younger, premenopausal women, CIS patients are at high risk of developing aggressive tumors with a high potential for invasiveness. We therefore determined the detection accuracy of the panel in these patients. The area under the curve remained high, with a value of 0.85 (95% CI, 0.61-0.92), and the autoantibody panel exhibited a specificity of 81.6%, a sensitivity of 73.6%, a diagnostic accuracy of 73.4%, a predictive positive value of 67.3%, and a negative predictive value of 85.7%.

Expression and localization of FKBP52, PPIA, and PRDX2 in early-stage breast cancer compared with healthy breast tissue. Because autoantibody reactivity against HSP60 and MUC1 were associated with the overexpression of the corresponding proteins in breast cancer compared with healthy breast tissue, we examined the expression levels of FKBP52, PPIA, and PRDX2 by real-time quantitative PCR on 29 early breast tumors and 29 healthy breast tissue samples. We used two different genes, RS9 and TBP89, as internal controls and calculated the ratio between each gene expression level and each internal control to normalize data. We compared the normalized ratios between noncancerous and breast tumors in three separate experiments. We found that expression levels of PPIA and PRDX2 mRNAs were not modified between the two groups (1.13-fold $\pm$ 0.30-fold and 1.50-fold $\pm$ 0.15-fold changes, respectively; Fig. 3B and C). Interestingly, FKBP52 mRNA expression in early breast tumors was significantly higher ($P < 0.0001$), with a 6.03-fold $\pm$ 1.47-fold increase compared with noncancerous breast tissue (Fig. 3A). To confirm these differences at the protein level, we did immunohistochemistry (IHC) on early breast tumors (stage I) and CIS samples. FKBP52 immunostaining was strong and localized predominantly in the cytoplasm and nucleus of normal, *in situ*, and invasive ductal cells (Fig. 3D; supplementary data 4). The expression of FKBP52 was strongly increased in CIS and invasive carcinoma compared with normal breast epithelium. PPIA and PRDX2 were homogeneously expressed in the cytoplasm of normal, *in situ*, and invasive cells, and showed no differences in protein expression between healthy and either CIS or primary breast cancer (Fig. 3E and F), confirming reverse transcription-PCR results.

Table 2. Area under the receiver operating characteristic curves and median comparison done for individual markers (Cont'd)

HC/PBC		HC/CIS	
P	AUC (95% CI)	P	AUC (95% CI)
0.049*	0.632 (0.533-0.721)	<0.0001*	0.728 (0.648-0.798)
0.003*	0.681 (0.585-0.766)	<0.0001*	0.704 (0.622-0.776)
0.006*	0.664 (0.568-0.75)	0.003*	0.649 (0.565-0.726)
0.031*	0.643 (0.546-0.73)	0.423	0.514 (0.5-0.595)
0.040*	0.628 (0.53-0.717)	0.586	0.558 (0.5-0.64)

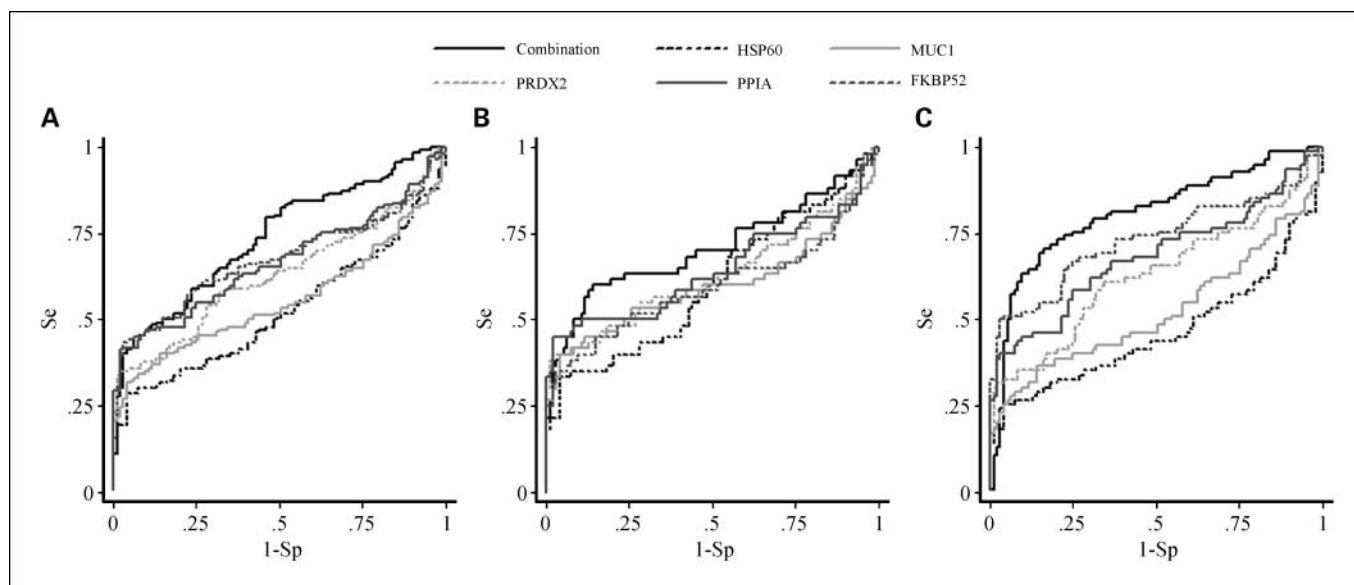


Fig. 2. Receiver operating characteristic curve analysis of FKBP52, PPIA, PRDX2, HSP60, and MUC1 to discriminate patients with PBC ($n = 60$) and CIS ($n = 82$) from HC ($n = 93$). A, comparisons between both cancer groups and HC, (B) PBC and HC, and (C) CIS and HC. PBC, primary breast cancer.

Discussion

We report here a proteomics-based analysis to identify proteins that elicit a humoral response in early-stage breast cancers, including CIS. In contrast to many studies that use cell cultures as an antigenic source (17–22), breast tumor–induced immune responses were analyzed with the use of two-dimensional gel electrophoresis western blot analysis from human tissue, in which proteins occur in their natural state and environment. This should increase the number of correct conformational epitopes necessary for relevant autoantibody recognition and binding in a seroreactivity-based approach. From several candidate tumor autoantigens identified, we observed for the first time that PPIA, PRDX2, and FKBP52 induced a specific reactivity with serum from early invasive breast cancer patients compared with controls. Finally, we focused on these three autoantigens in combination with HSP60 and MUC1. HSP60, which belongs to the family of chaperone proteins, induces a humoral reaction in colorectal cancer (23) and hepatocellular carcinoma (24), and has recently been shown to elicit a serologic reaction in primary breast cancer (6, 25) and ductal CIS (6). HSP60 immunogenicity seems to be caused by the protein overexpression in several cancers (6). MUC1 is also a well-known autoantigen in cancer (5, 26–28). Anti-MUC1 antibodies were shown to be present in 8% to 26% of early-stage breast cancer patients (9) and in 23% of ductal CIS patients (5). The presence of autoantibodies to HSP60 and MUC1 has also been correlated with an improved disease-specific survival in invasive breast cancer (29). FKBP52 is a chaperone protein implicated in regulating steroid hormone receptor activity (i.e., progesterone, estrogen, and glucocorticoid receptors; ref. 30). Particularly, FKBP52 is associated with the hormone-bound glucocorticoid receptor complex and allows its translocation to the nucleus, where the glucocorticoid receptor can exert its transcriptional activity. Because glucocorticoid-induced resistance occurs in breast cancer, FKBP52 overexpression could be implicated in

glucocorticoid receptor–mediated resistance to chemotherapy and, further, in breast carcinogenesis. These functions remain to be elucidated. PPIA has been recently shown to be involved in the progression of human breast cancer. Loss of PPIA expression or activity results in inhibition of breast cancer cell growth, motility, and invasion *in vitro*, as well as inhibition of metastasis in mouse models with xenografts (31). In addition, knockdown of PPIA in a mouse model of non–small cell lung cancer xenografts was correlated with slower tumor growth and an increase in tumor apoptosis (32). Peroxiredoxins represent a family of antioxidant enzymes that use cysteine residues to decompose peroxides and mediate signal transduction in mammalian cells (33). Autoantibodies elicited against peroxiredoxins have been detected in several types of cancers (18, 34), including breast cancer (25), showing their potential utility in cancer diagnosis. To our knowledge, FKBP52, PPIA, and PRDX2 seroreactivities have never been investigated in pre-invasive breast cancer.

When we evaluated the diagnostic accuracy of each autoantibody marker in our validation population, we found that three of the five biomarkers allowed us to significantly

Table 3. Median comparison done for individual markers in women with CIS aged under 50 y and aged above 50 y

Marker	<50, N = 14	≥50, N = 68	P*
FKBP52	0.83 (0.29-2.49)	0.43 (0.10-2.71)	0.010
PPIA	0.54 (0.16-2.46)	0.33 (0.10-1.71)	0.006
PRDX2	0.40 (0.12-1.92)	0.24 (0.08-1.26)	0.003
HSP60	0.24 (0.09-1.83)	0.18 (0.07-2.68)	0.204
MUC1	0.53 (0.15-2.09)	0.29 (0.08-2.07)	0.009

NOTE: Values are expressed as medians (range). Groups were compared through a Kruskal-Wallis test.

*The test is considered significant when $P < 0.05$.

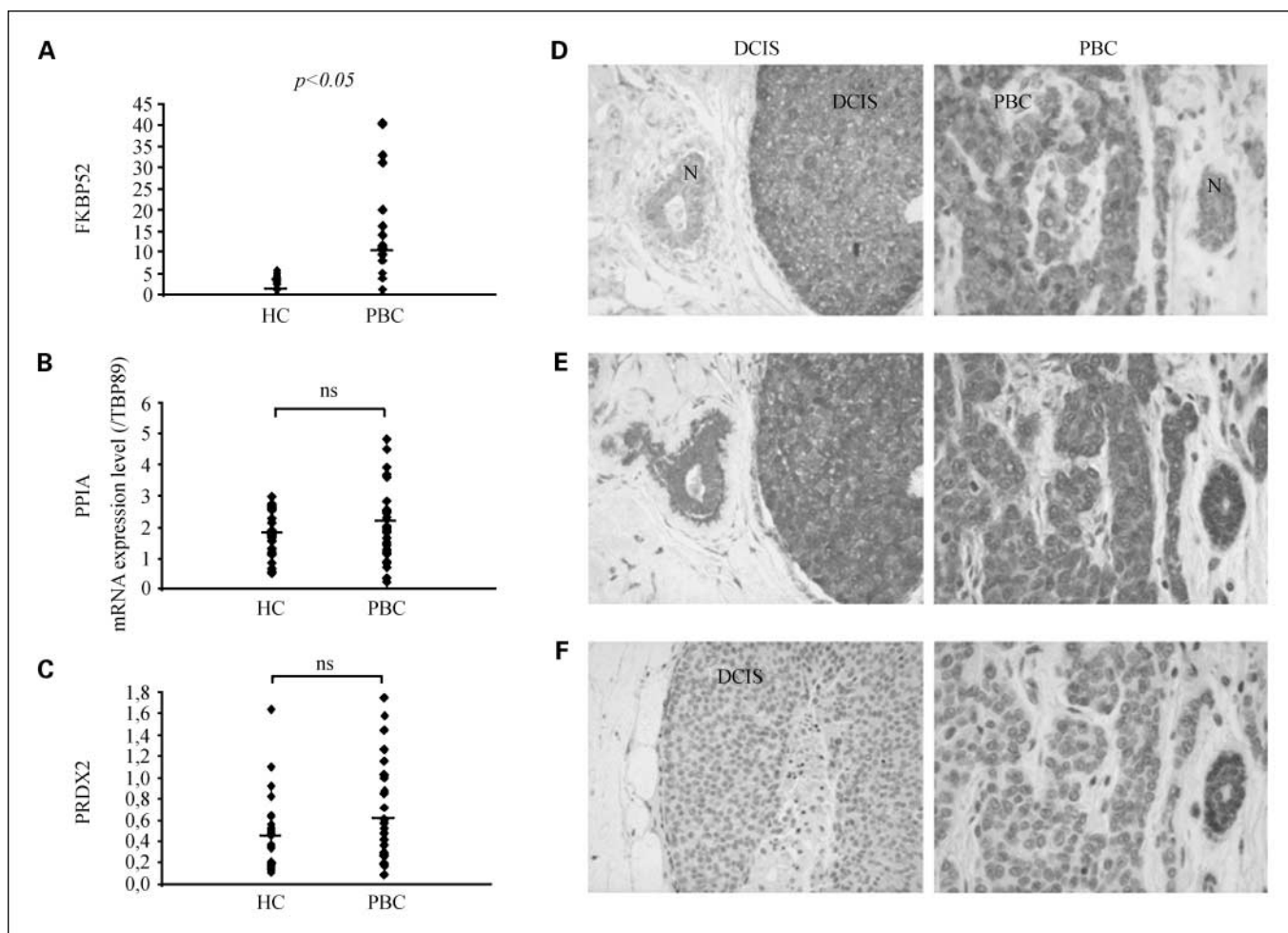


Fig. 3. FKBP52, PPIA, and PRDX2 mRNA and protein levels in CIS and PBC. Total mRNA from PBC ($n = 29$; stage I) and healthy breast tissues ($n = 29$) were extracted and submitted to RT followed by qPCR to measure FKBP52 (A), PPIA (B), and PRDX2 (C) expression levels. Results, means of three experiments. The quantity of mRNA levels is expressed as arbitrary units of normalized TBP89 expression level. The significance of the mRNA expression differences was assessed with the unpaired Mann-Whitney test. NS, not significant. Representative immunohistochemical staining of FKBP52 (D), PPIA (E), and PRDX2 (F) in CIS and PBC. Normal ducts (N), CIS, and PBC are indicated. Original magnification, $\times 200$.

distinguish breast cancer from noncancer patients in a univariate analysis. We then estimated the diagnostic accuracy of the combination of these five biomarkers with the use of a generalized receiver operating characteristic criterion (15). We observed diagnostic accuracies of 67.1%, 75.1%, and 72.4% for detecting both cancer groups, primary breast cancer, and CIS, respectively. Importantly, these values remained high in the leave-one-out cross-validation, proving the robustness of our classifier. More remarkably, our autoantibody panel allows a diagnosis improvement in CIS samples with a better accuracy than other biomarkers (i.e., CA15-3 and CEA) currently used in clinical practice (35). Recently, Chapman et al. showed that, taken separately, each of the seven markers (p53, c-myc, HER2, NY-ESO-1, BRCA1, BRCA2, and MUC1) was present in a maximum of 23% of patients with ductal CIS, whereas the combination of the seven markers enabled detection of this cancer in 45% of the affected patients (5). Similarly, Casiano et al. showed that a panel of seven autoantibodies (IMP1, p62, Koc, p53, c-MYC, cyclin B1, or survivin) was able to detect 43.8% of undefined-stage breast cancer patients against 8% for only one of them (4). Lu et al. found that p53 autoantibodies

alone could not discriminate cancers from controls (area under the curve, 0.48), whereas adding another marker (HER2/Neu) resulted in an area under the curve of 0.61, and combining four antigens increased the area under the curve to 0.63 (9). Our present data are in agreement with published data for panel autoantibody assays and underline the importance of selecting the right combinations of markers, with rigorous criteria that are still to be defined, to achieve maximal sensitivity and specificity.

When we intended to find a correlation between detection of autoantibodies and clinical, histologic, and hormonal variables, we found that women with CIS under the age of 50 years expressed significantly higher levels of four of the five markers compared with older women. This subgroup of women could be discriminated from healthy controls with an area under the curve of 0.85. These results are of major importance because these younger women are at high risk of developing invasive and aggressive tumors that are difficult to detect. Indeed, existing imaging techniques fail to diagnose most precancerous lesions of the breast, especially in women under the age of 50 years (2, 36, 37). CIS should always be suspected in the

presence of nonpalpable breast lesions, particularly in women who frequently show dense breasts (36). In such cases, diagnosis can only be made by histologic examination and, for this reason, all suspicious nonpalpable lesions should be biopsied. Biological tests could then be done as complementary tests to mammography or magnetic resonance imaging in younger women, as proposed elsewhere (5). However, because the number of patients under the age of 50 years was limited ($n = 14$) in our study, this assay needs to be validated on larger patient cohorts. Finally, based on our approach, prospective studies can address the question of whether seroreactivity profiling may enhance CIS detection or pathologic changes of previously identified benign proliferative lesions in high-risk breast cancer populations bearing the *BRCA1/BRCA2* mutation.

Autoimmune responses are found in a wide range of tumors (34, 38–49). However, the mechanism by which immunogenicity appears remains unknown. A prerequisite for an immune response against a cellular protein is its presentation as an antigen. It is not clear why only a subset of patients with a specific tumor type develops a humoral response to a particular antigen. At least four main categories of tumor-associated antigens can be proposed: (a) those resulting from point mutations (such as p53), (b) those encoded by genes with tumor-specific expression, (c) differentiation proteins, and (d) antigens encoded by genes that are overexpressed in tumors. FKBP52 protein and mRNA have previously been shown to be overexpressed in breast cancer cell lines and human tumors. In the present study, we show for the first time that this protein is overexpressed in early-stage breast tissues at the mRNA and protein levels compared with healthy breast tissue. Accumulation may be an important component in the development of this humoral response, but we cannot exclude the fact that mutations of the protein or other mechanisms, which can modify its processing, its function, or its routing, can also lead to such a response. As PPIA and PRDX2 remained unchanged at the mRNA and protein levels, the autoantibodies elicited against these proteins may occur for other reasons. Thus, further studies will be needed to investigate more specifically the origin of PPIA and PRDX2, but also FKBP52-mediated humoral responses.

Although serum immunoglobulin reactivity against human proteins has been reported to occur in cancer sera some 10 years ago, our knowledge about the nature of the immune response in this pathology is still incomplete. To further characterize the immunoglobulin subclasses implicated in the humoral response to early breast carcinoma, we did heavy-chain specific ELISAs. For the five autoantigens tested, the humoral reaction in sera mainly consisted of IgG and IgM

responses. For HSP60, an IgE autoantibody reactivity was also observed (supplementary data 5). Our results confirm that the humoral response observed in our samples depends on the nature of the autoantigen and underline the complexity of humoral response to cancer. Therefore, the detection of all subclasses of autoantibodies should be done to develop diagnostic tests of early-stage breast cancer.

CIS are complex clinical situations. Even if they do not metastasize to the axillary lymph nodes or elsewhere, CIS, especially of the ductal type, should be considered as early-stage cancers. Indeed, for the time being, it is not possible to identify with absolute certainty the lesions that will progress to invasive carcinoma or the ones that will remain quiescent. Therefore, surgeons balance the risk of resecting too much tissue, causing unnecessary cosmetic damage, or resecting too little and leaving an increased risk of recurrence. Genetic and proteomic alterations occur quite early in the development of tumors. Although knowledge of the biological features of premalignant lesions remains limited, mainly because of the difficulty of obtaining such samples, humoral response studies could represent an alternative approach for identification of early-stage biomarkers.

In this study, we identified a new panel of sera autoantibodies that is associated with the presence of CIS. Given the heterogeneity of breast tumors and the complete absence of available early-stage diagnostic breast tumor markers, our results are extremely encouraging for the future development of seroreactivity assays for the early detection of primary breast cancer and CIS, especially in women under the age of 50 years. However, new studies with larger cohorts need to be done across multiple populations before clinical development to show that CIS could be prospectively detected with the use of our five-autoantibody panel. Interestingly, Hanash et al. has recently shown that autoantibody-signature cancer diagnosis is possible months before cancer reaches a size that can be picked up by imaging technology (7, 50). Such investigations should be done for breast cancer. Finally, and perhaps most importantly, biomarkers of susceptibility and short-term risk are likely to provide insight into the biology of tumors that develop, leading to new interventions to support prevention. PPIA, PRDX2, and FKBP52 should be explored for their potential role in the first steps of breast carcinogenesis and should be considered novel potential targets for drug therapies and immunotherapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplementary data 1: Primer sequences used in qPCR experiments

Name	Sequence	Annealing temperature
FKBP52 forward	5'-TGGTAGGGATGAGGTCTGATAAGA-3'	60°C
FKBP52 reverse	5'-AGGGAAAGGATGAGGAAAACG-3'	60°C
PPIA forward	5'-CCTGGTGGTGCATGCCTAGT-3'	60°C
PPIA reverse	5'-CTCACTCTAGGCTCAAGCAATCC-3'	60°C
PRDX2 forward	5'-TGTGATCGTCCGTGCGTCTA-3'	60°C
PRDX2 reverse	5'-CGATGCGCGCGTTAC-3'	60°C
TBP89 forward	5'-TTTTCTTGCTGCCAGTCTGGAC-3'	65°C
TBP89 reverse	5'-CACGAACCACGGCACTGATT-3'	65°C
RS9 forward	5'-AAGGCCGCCCCGGGAAGTCTGAC-3'	62°C
RS9 reverse	5'-ACCACCTGCTTGCGGACCCTGATA-3'	62°C

Supplementary data 2: Sensitivity, specificity, predictive values and diagnosis accuracy of the 5 markers in cancer, PBC and CIS.

Marker	FKBP52			PPIA			PRDX2			HSP60			MUC1		
Comparison	HC/cancer	HC/PBC	HC/CIS	HC/cancer	HC/PBC	HC/CIS	HC/cancer	HC/PBC	HC/CIS	HC/cancer	HC/PBC	HC/CIS	HC/cancer	HC/PBC	HC/CIS
Se	50.1	43.1	54.8	49.6	48.2	51.3	45.2	45.4	45.1	35.0	43.7	27.3	36.5	42.5	31.9
Sp	87.2	89.5	86.7	87.3	88.8	85.7	85.5	87.4	84.0	87.1	86.1	89.5	87.9	86.9	88.6
PPV	85.7	72.6	78.4	85.6	73.5	76	82.6	69.9	71.3	80.5	67.0	69.7	82.1	67.7	71.2
NPV	53.4	70.9	68.5	53.2	72.7	66.6	50.6	71.3	63.4	46.8	70.3	58.2	47.6	70.1	59.6
Diagnostic accuracy	64.8	71.3	71.8	64.5	72.9	69.6	61.1	70.9	65.8	55.6	69.5	60.4	56.8	69.5	62.0

Values are represented in %.

Se (sensibility) = number of true positive / number of cases

Sp (specificity) = number of true negative / number of controls

PPV (positive predictive value) = $\frac{\text{prv} \times \text{Se}}{\text{prv} \times \text{Se} + (1 - \text{prv}) \times (1 - \text{Sp})}$ where “prv” corresponds to the Prevalence of the disease, estimated by the proportion of diseased subjects in the population.

NPV (negative predictive value) = $1 - \frac{\text{prv} \times (1 - \text{Se})}{\text{prv} \times (1 - \text{Se}) + (1 - \text{prv}) \times \text{Sp}}$

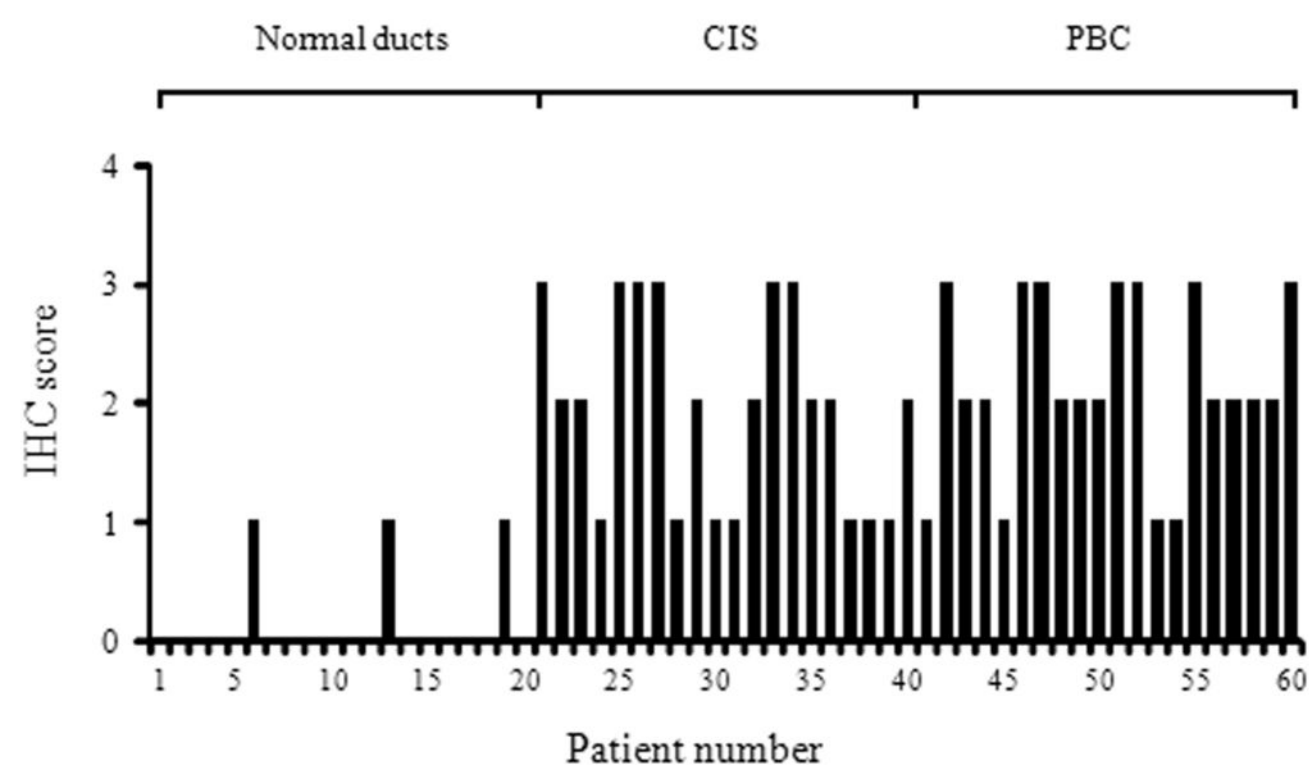
Diagnostic accuracy = (number of true positive + number of true negative) / total number of samples.

Supplementary data 3: Leave-one-out cross-validation*

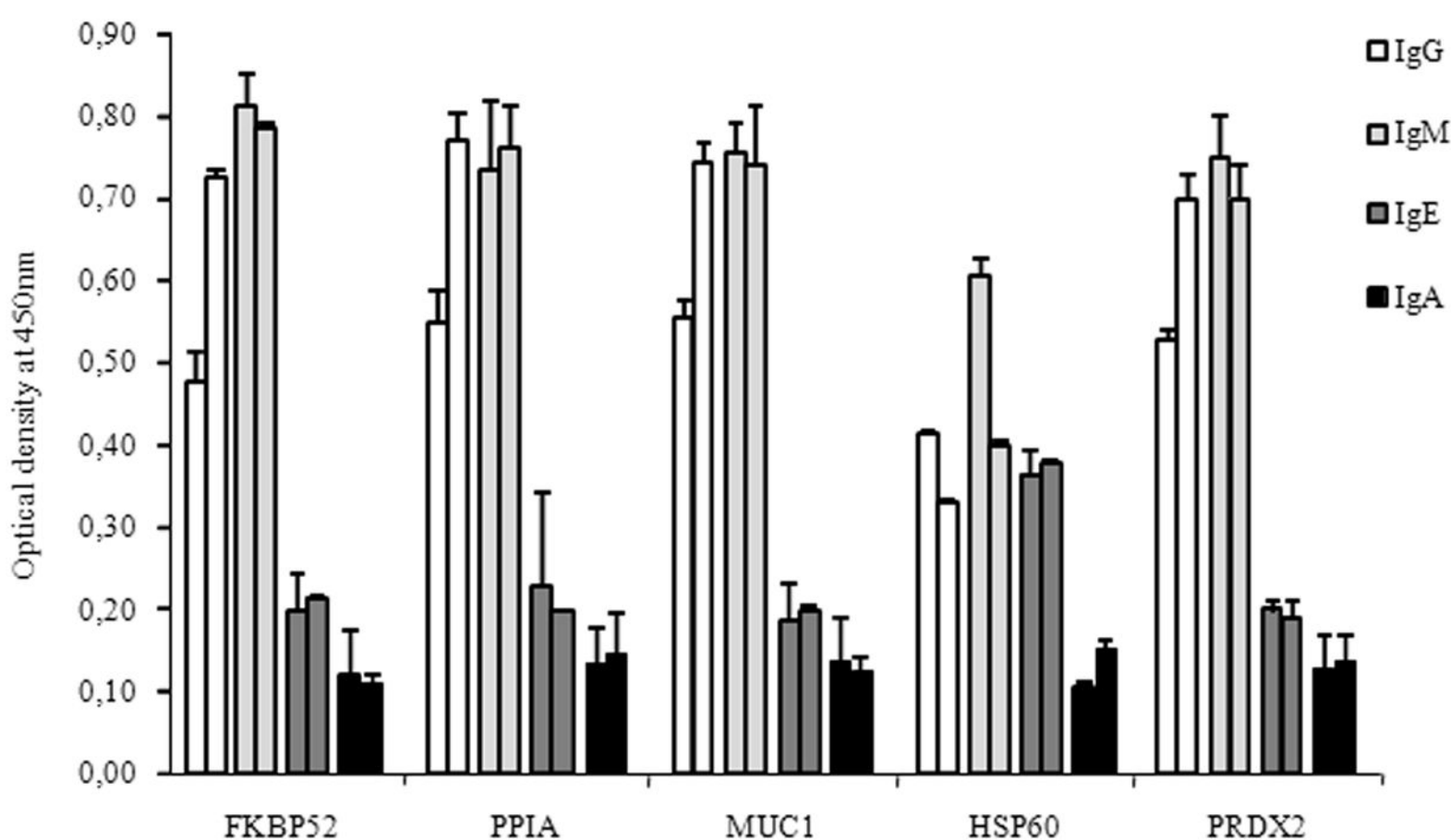
Comparison	Sensitivity (%)	Specificity (%)	Diagnostic accuracy** (%)
Cancer vs Healthy	52.1	77.4	62.1
PBC vs Healthy	53.3	87.1	73.8
CIS vs Healthy	70.7	76.3	73.7

*One sample was removed from the statistical model containing a total of 235 samples (respectively 153 and 175) and a classifier was generated to predict the status, healthy or cancer (respectively PBC and CIS), of the removed sample using the rest of the samples. This procedure was repeated for all samples.

**Diagnostic accuracy = (number of true positive + number of true negative)/total number of samples.



Supplementary data 4: Variations in FKBP52 protein expression in normal ducts, CIS and PBC by IHC. IHC scores were determined as described in 'patients and methods' section.



Supplementary data 5: Characterisation of human Ig subclasses of autoantibodies. Two pools of 5 sera positive for each autoantibody were subjected to ELISA assays to determine Ig subclass of autoantibodies, according to the procedure described in material and methods. As secondary antibodies, we used an anti-human IgG, γ chain specific (#A6029, Sigma), an anti-human IgM, $\text{Fc}_{5\mu}$ fragment specific (#109-035-043, Jackson ImmunoResearch Laboratories), an anti-human IgE, ϵ chain specific (#A9667, Sigma), or an anti-human IgA, α chain specific (#A0295, Sigma). Each pool was assayed in duplicate in 2 separate experiments. Results are presented as the mean of $\text{OD} \pm \text{SD}$ for each pool.