Molecular Risk Assessment for Breast Cancer Development in Patients with Ductal Hyperplasias

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Abstract

Purpose: It has been reported that approximately a million women are diagnosed with benign breast lesions that include ductal hyperplasias per year in the United States. Recent studies that followed women with benign lesions have established that about 8% to 9% of them will subsequently develop invasive breast cancer (IBC). However, currently, there are no means of identifying a subclass of "true precancerous tissues" in women with ductal hyperplasias who will subsequently develop cancer. The purpose of this study is to investigate whether expression of hyaluronoglucosaminidase 1 (HYAL1), a known tumor promoter, in hyperplastic tissues identifies a "true precancerous stage" and predicts subsequent IBC development.

Experimental Design: A retrospective study was conducted with archival benign tissues of various histologic types and clinical information on development/nondevelopment of IBC. The control group was hyperplastic tissues from women who had no prior history of IBC and did not develop cancer in 5 to 7 years after diagnosis (n = 81). The test group was hyperplastic tissues from patients who developed cancer (n = 82). HYAL1 expression was studied by immunohistochemistry, and the results were statistically analyzed for significant association to develop cancer (P value), specificity, sensitivity, positive predictive value, and negative predictive value.

Results: Statistical analysis of HYAL1 expression data showed very highly significant association between its expression and subsequent cancer development (P = 0) and very high sensitivity (0.83), specificity (0.84), positive predictive value (0.84), and negative predictive value (0.83).

Conclusions: The expression of HYAL1 in ductal hyperplastic tissues is a strong predictor of subsequent development of IBC; therefore, it can be applied as a diagnostic marker either singly or in combination with other marker(s) to screen benign tissues to predict subsequent development of IBC. Detection at the precancerous stage and treatment could drastically cut down breast cancer incidence and deaths from it.

Although a slight decline in breast cancer incidence has been reported in very recent years, it continues to be the most diagnosed cancer in the United States. It is estimated that ~180,000 women will be diagnosed with invasive breast cancer (IBC) in the year 2007 in the United States (1). In addition, reports show that more than half of all women develop some form of benign breast disease after the age of 20 years (2) and 1 million women are annually diagnosed in the United States with benign breast lesions that include usual ductal hyperplasia (UDH) with various histologic types and atypical ductal hyperplasia (ADH; ref. 3).

Epidemiologic studies with humans and animal studies have established that women with prior UDH have 2.0-fold higher risk of subsequently developing IBC in comparison with women who had none of these lesions and risk increased to 5.0-fold if the proliferation was associated with atypia (4–12). Two recent studies surveyed IBC incidence in 14,057 women with benign breast disease from a multiethnic population across the United States for a median of 13- to 15-year follow-up and found that about 8% to 9% of them subsequently developed IBC (13, 14). Based on the above studies, annually, a significant number of patients, about 80,000 to 90,000 (8-9% of 1 million), who are diagnosed with benign breast lesions will subsequently develop IBC in the United States. These women could be prevented from developing IBC if we can exactly identify patients with which subtype of benign lesions will subsequently develop IBC and treat them. However,

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currently, it has not been possible to precisely predict breast cancer development based on morphology/histology of the benign lesion. Molecular markers that can be applied to screen benign tissues and distinguish "true precancerous lesions" will be highly valuable for identifying and treating very high risk patients and preventing them from developing IBC.

With a goal of identifying the molecular markers that could be applied to screen benign tissues, we have been studying the molecular composition of benign lesions from patients with and without the history of developing breast cancer subsequently. We hypothesized that benign tissues from patients who developed cancer are the true precancerous lesions and those tissues have elevated expression of several cancer-promoting molecules. To test the above hypothesis and identify the elevated molecules, we analyzed global gene expression of limited number of benign lesions from patients who had subsequently or concurrently developed IBC in comparison with benign tissues from patients who had no prior history of IBC and did not develop cancer in 5 years after benign diagnosis. By the above approach, we identified elevated expression of several cancer-promoting molecules in benign tissues from patients who developed cancer (15). Some of the elevated molecules include those encoding for proteins that regulate cell cycle checkpoints, increase nucleic acid levels, degrade extracellular matrix, maintain cell polarity, and architecture and inhibit apoptosis (15). One of the highly elevated cancer-promoting molecules that degrade extracellular matrix was hyaluronoglucosaminidase 1 (HYAL1). It is an established tumor-promoting molecule for several cancers, including breast cancer. In the current study, we have explored whether its expression in benign tissues predicts subsequent breast cancer development.

HYAL1 is an endoglycosidase that cleaves extracellular matrix glycosaminoglycan, hyaluronic acid (HA), into small oligosaccharide units. HA is a polymer of repeating units of a disaccharide that consists of glucuronic acid and *N*-acetylglucosamine. In normal tissues, HA is known to keep tissues hydrated and maintain the osmotic balance (16). In addition, HA has been reported to regulate cell adhesion, migration, and proliferation by interacting with CD44 and RHAMM receptors on the cell surfaces (17). The levels of HA and its degrading enzyme, HYAL1, were reported to be intricately elevated in several cancer tissues. The stromal levels of HA were reported to be elevated in gastric, colon, breast, prostate, bladder, and lung cancers and elevated levels seem to promote tumor metastasis by interaction with receptors on the tumor cell surfaces and opening up spaces for tumor cell migration (18–21). The levels of HYAL1 were reported to be elevated in prostate, bladder, head and neck, laryngeal, and breast cancers and elevated levels correlate to tumor progression (22–36). The HYAL1-released small molecular weight HA oligosaccharides seem to bind RHAMM receptors on endothelial cells and induce adhesion, proliferation, and migration of these cells by activating focal adhesion kinase and mitogen-activated protein kinase pathways (37–40). In a recent study, Tan et al. (41) showed that silencing of HYAL1 gene expression by RNA interference in breast cancer cells induced cell cycle arrest and inhibition of cell proliferation *in vitro*. However, the expression of this marker has not been studied at precancerous stage of breast or any other tissue until now and its role in tumorigenesis is not known.

In the current study, we tested the HYAL1 protein expression in benign tissues by immunohistochemistry and present results to show that HYAL1 expression is highly predictive of subsequent breast cancer development irrespective of histologic diagnosis of the benign lesion.

Materials and Methods

Archival UDH/ADH tissues and follow-up clinical information on IBC development. Formalin-fixed, paraffin-embedded benign tissues that were stored in a humidity-controlled, air-conditioned facility at Howard University Pathology tissue archives were used for the current study. The paraffin blocks were prepared in Sakura Tissue-Tek automatic machine after fixing in 10% buffered formalin according to procedure recommended by machine manufacturer. The diagnosis was done on tissue slides cut from paraffin blocks. The clinical follow-up information on development/nondevelopment of cancer, in patients from whom benign tissues were derived, was obtained from tumor registry data banks, surgical pathology data banks, and patient visits to surgical oncologists.

The control samples were 32 UDH tissues of various histologic types and 49 ADH tissues. All control tissues were from patients who had no prior history of cancer and did not develop cancer in 5 to 7 years after benign diagnosis. The mean follow-up for ADH samples was 6.2 years (median, 6 years) with SD of 1.5, and for UDH samples, the mean follow-up was 7 years (median, 7 years) with SD of 0.9. The test samples were 32 UDH and 16 ADH tissues from patients who subsequently developed cancer (UDHC and ADHC, respectively) in 1 to 5 years after diagnosis (ADHC: mean, 2.9 years; median, 2.5 years, with SD of 1.7; UDHC: mean, 3.5 years; median, 3 years, with SD of 2.2). The histologic diagnosis of UDHC varied widely (Supplementary Table S1). In addition, 34 ADHC tissues from patients who had concurrent cancer, 26 negative control tissues from women undergoing reduction mammoplasty for cosmetic purposes, and 10 IBC tissues as positive tissues were also included in the study. Whenever ADHC tissues were derived from patients who had concurrent cancer, the slides

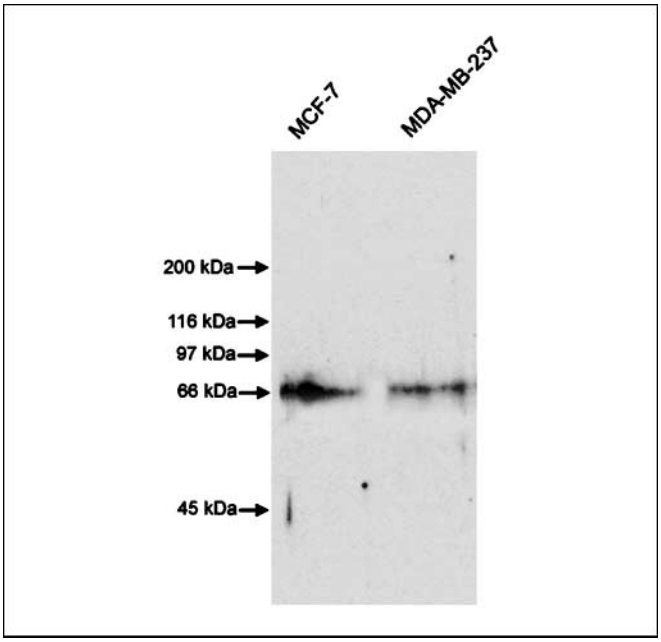

Fig. 1. Western blotting of MCF-7 and MDA-MB-237 breast cancer cell line extracts with affinity-purified anti-HYAL1 peptide antibody. The antibody detected a single protein band of an expected M_r of about 60,000 to 65,000.

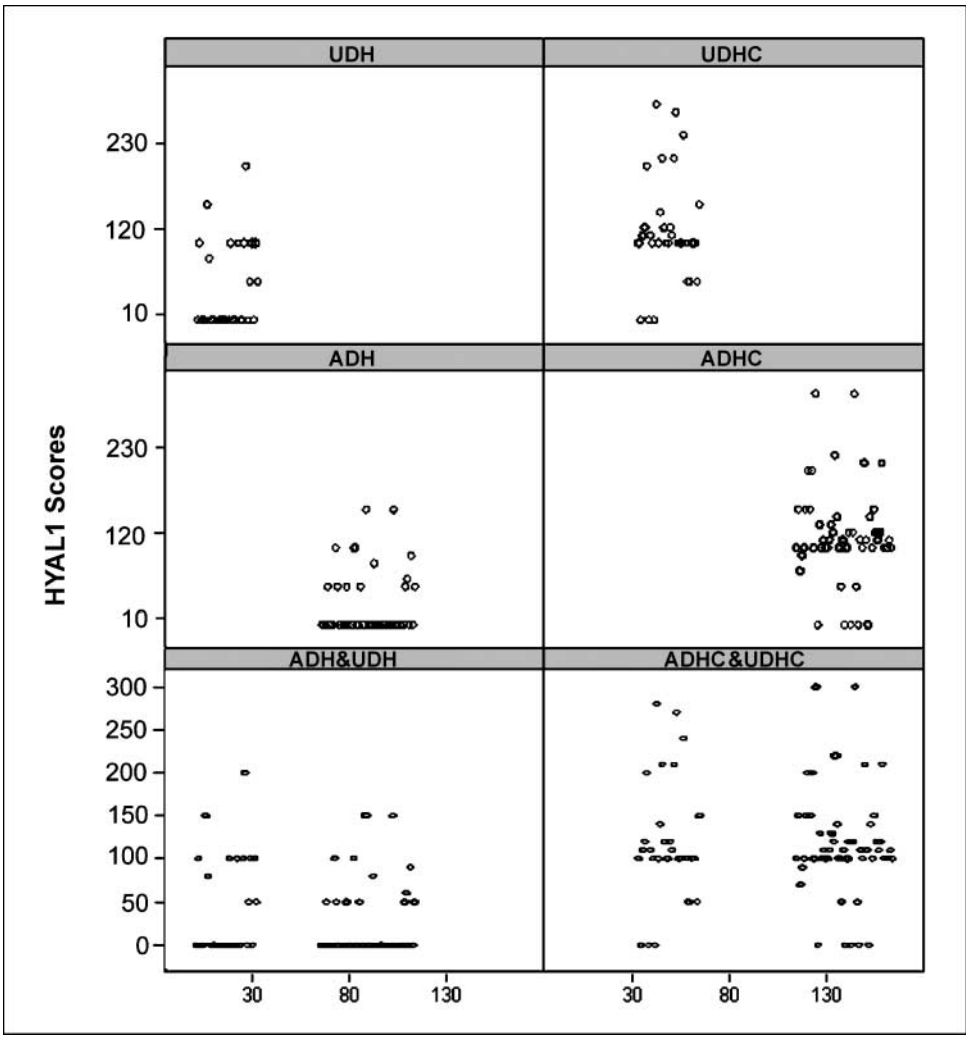

Fig. 2. Scatter plots of HYAL1 protein expression data in UDH, UDHC, ADH, and ADHC tissues are shown. Scatter plots are shown for UDH and UDHC combination; ADH and ADHC combination; and UDH, ADH, UDHC, and ADHC combination.

were cut from blocks that were prepared from regions far away from the cancer site. To ascertain that all the sections cut from each block had the benign tissues, the first and the last section cut from each paraffin block was stained with H&E and examined for histology, and only those containing the desired tissues were used.

Immunohistochemical staining. For the current study, we used affinity-purified rabbit polyclonal antibody against 18–amino acid HYAL1 peptide (amino acids 321-338; refs. 22, 42), which was extensively characterized for sensitivity and specificity using a variety of cell lines and cancer tissues (22–31). We further characterized it by testing in two breast cancer cell lines, MCF-7 and MDA-MB-237, on Western blots and found to be highly specific to HYAL1 (Fig. 1). Immunohistochemistry was done randomly blinded to the knowledge on development/nondevelopment of cancer using standard procedures with a single batch of antibody preparation within 1 week on all of the samples. Briefly, slides were deparaffinized and antigens were retrieved in target retrieval solution (Dako) by heating for 25 min in a steamer as previously described (15, 43, 44) and blocked with 3% H_2O_2 in methanol for 20 min. The slides were washed with PBS and incubated with avidin block (Vector Laboratories) for 10 min at room temperature followed by washing with PBS and incubating in biotin block (Vector Laboratories) for 10 min at room temperature. The slides were washed with PBS and incubated with rabbit anti-HYAL1 (1:750 dilution in PBS containing 0.1% bovine serum albumin, 0.01% Triton X-100, and 0.005% Tween 20) overnight at 4°C. A control set of tissues was incubated with normal rabbit serum at the above dilution. After washing away the excess primary antibody with PBS, the slides were incubated with link solution (Dako) for 25 min at room temperature. The slides were washed and incubated with streptavidin-horseradish peroxidase (Dako) for 25 min at room temperature. The slides were washed and incubated with substrate (3,3′-diaminobenzidine liquid chromogen from DakoCytomation) solution for 5 min. Finally, the slides were washed and counterstained with hematoxylin, mounted with DPX, and visualized under Leica DMRXA microscope. The immunohistochemical procedure was repeated thrice for all the samples. All the slides were scored independently by two pathologists for staining intensity qualitatively based on the degree of stain and the number of cells stained in the ductal epithelial cells and in comparison with the negative control (reduction mammoplasty) tissues (score, 0) and positive control (cancer) tissues (score, 300). The final grading scores ranged from 50 to 300. To ascertain that HYAL1 protein was not deteriorated with storage, we evaluated two benign tissues in the test group that were stored for 7 years and found no deterioration of the marker.

Statistical analysis. The significance of the association of HYAL1 protein expression and cancer development was evaluated using three tests: Kruskal-Wallis, *t* test, and Wilcoxon rank-sum test. To visually see the difference of HYAL1 protein expression in test and control groups, scatter plots are drawn for different data combinations. The sensitivity (percentage of test benign samples that were positive for the marker), specificity (percentage of control benign samples that were negative for the marker), positive predictive value (PPV; correctly predicting cancer

development in patients who were positive for the marker), and negative predictive value (NPV; correctly predicting nondevelopment of cancer in patients who were negative for the marker) were determined using S-PLUS software. All analyses were done for UDH and UDHC combination, ADH and ADHC combination, and for all tissues combined. The receiver operating characteristic (ROC) curves were generated as follows. For each threshold value (using all marker expression grading scores as threshold values), if the measured value is greater or equal to the threshold value, then it is considered as a positive test; otherwise, it is a negative test. Thus, each threshold value determines a point with coordinates (1-specificity, sensitivity). The threshold values ranged from 50 to 300. The ROC curves were generated by connecting all the points determined by all the threshold values in an increasing order.

Results

HYAL1 protein is highly expressed in benign tissues from patients who subsequently developed cancer. In the UDHC category, 29 of 32 test samples were strongly positive for HYAL1. In the control UDH samples, 11 showed some level of expression. In the ADHC category, 46 of 50 tissues were strongly positive, and of the 49 ADH tissues, 13 showed some level of HYAL1 protein expression (Supplementary Table S1). These results were highly reproducible when repeated. From the HYAL distribution data in the samples, the mean HYAL expression in ADH, ADHC, UDH, and UDHC is 0.26, 1.08, 0.42, and 1.11 with SDs of 0.5, 0.66, 0.66, and 0.70, respectively; the power can reach to 90% with an α level of 0.01 if the sample in each subset is $\geq$30. Therefore, the sample sizes in ADH (n = 49) and ADHC (n = 50) as well as in UDH (n = 32) and UDHC (n = 32) categories are large enough for the study.

The data on all the control and test samples are summarized as scatter plots in Fig. 2. Staining was observed in the cytosols of ductal epithelial cells in all the positive tissues. Control slides that were incubated only with secondary antibodies in the absence of primary antibody did not show any staining (data not shown). Among the 26 reduction mammoplasty tissues, only 3 showed some positivity (data not shown) and all the 10 cancer tissues were strongly positive. A representative tissue

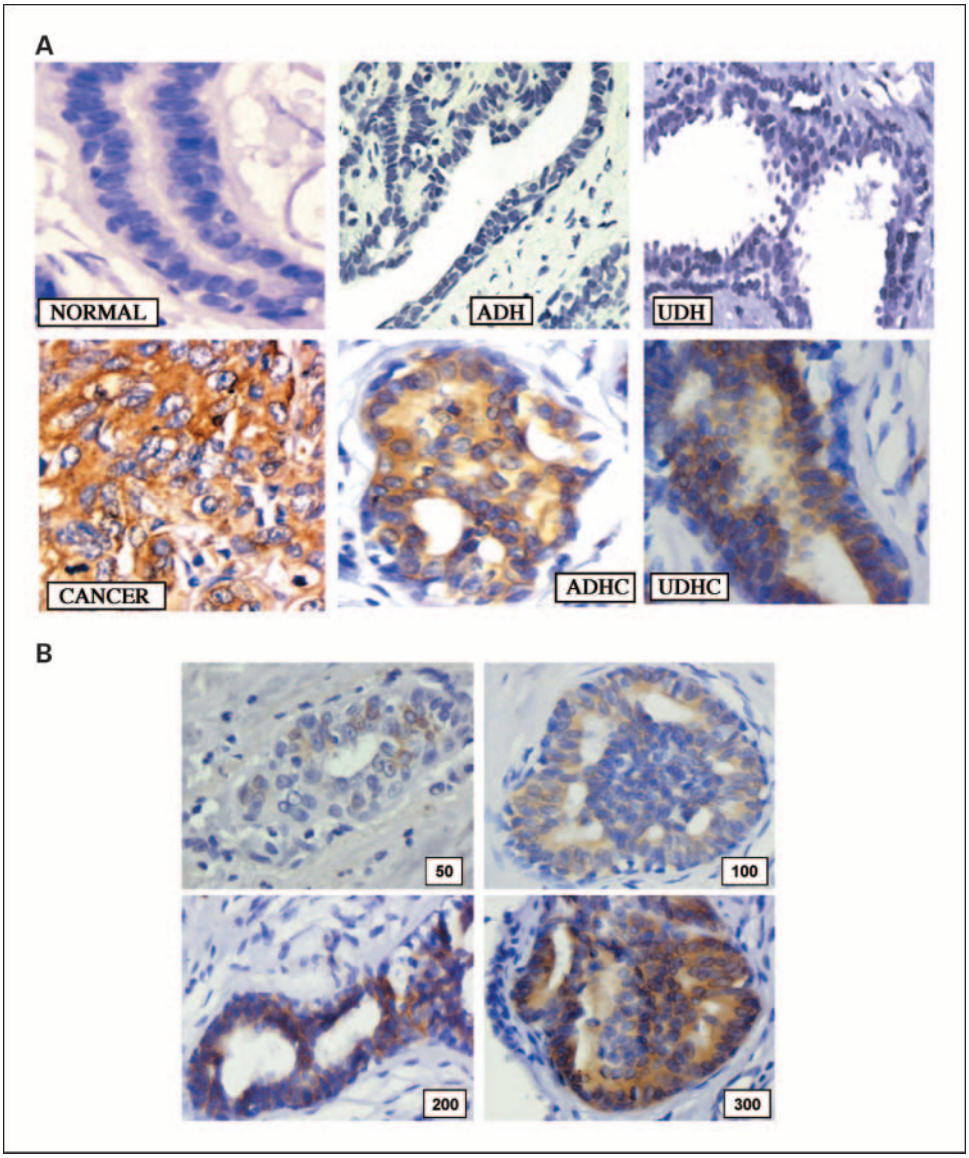

Fig. 3. HYAL1 protein expression in representative breast tissues by immunohistochemistry. Formalin-fixed, paraffin-embedded archival tissues were immunostained with antibody against HYAL1 as described in Materials and Methods. *A,* representative tissues from each category of normal, UDH, ADH, ADHC, UDHC, and IBC tissues. Magnification, ×40. Strong staining was observed in IBC, UDHC, and ADHC tissues. HYAL1 staining could be seen in cytosols of ductal epithelial cells in all the positive tissues. *B,* representative tissues of four intermediate grading scores: 50, 100, 200, and 300.

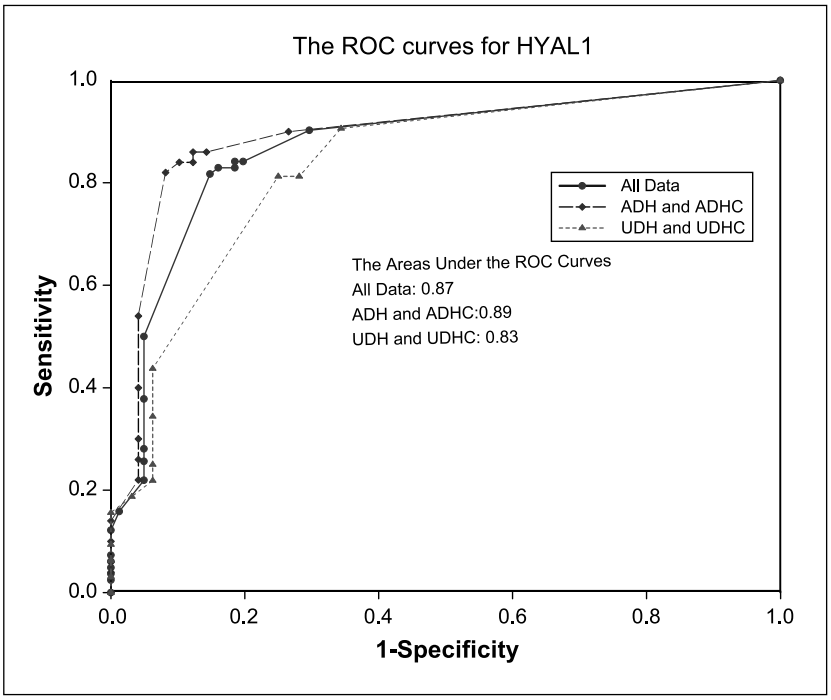

Fig. 4. ROC curves for HYAL1 in UDH and UDHC; ADH and ADHC; and UDH, UDHC, ADH, and ADHC. The areas under the three ROC curves of HYAL1 were 0.83 for UDH and UDHC combination of tissues, 0.89 for ADH and ADHC tissues, and 0.87 for all the tissues combined.

from each of normal, UDH, ADH, UDHC, ADHC, and IBC is shown in Fig. 3A and images of representative samples of four intermediate grading scores are shown in Fig. 3B.

To test if there is a significant difference in the expression of HYAL1 protein in the test and control samples, we analyzed the data in three different ways: (*a*) UDH and UDHC combination; (*b*) ADH and ADHC combination; and (*c*) all tissues combined by *t* test, Wilcoxon rank-sum test, and Kruskal-Wallis tests. The results consistently indicated that both UDHC and ADHC samples were significantly different from UDH and ADH samples, respectively. All the *P* values from the above three tests are summarized in Table 1. We also applied the same statistical methods to test if there were differences between concurrent and simultaneous ADHC tissues and found that they were not significantly different from each other ($P = 0.44$ and 0.8, *t* test and Wilcoxon rank-sum tests, respectively). We further analyzed the data to see if there is any possible relationship between HYAL1 protein expression levels and other variables, such as age of cancer development, grade, estrogen receptor/progesterone receptor status, and nodal status, using regression and ANOVA. Our results (not shown here) indicated that none of the above variables was significantly associated with HYAL1 protein expression levels.

HYAL1 protein expression is highly predictive of developing breast cancer in patients with ductal hyperplasia. The HYAL1 expression data (Supplementary Table S1) were statistically analyzed for sensitivity, specificity, PPV, and NPV in three ways: (*a*) UDH and UDHC tissues; (*b*) ADH and ADHC tissues; and (*c*) combination of UDH and UDHC and ADH and ADHC. The results are presented in Table 1 and Fig. 4. HYAL1 showed very high sensitivity, specificity, PPV, and NPV for UDH and UDHC combination and ADH and ADHC combination or all tissues combined (Table 1). Figure 4 shows ROC curves for three different combinations of tissues. The areas under the ROC curves were 0.83 for UDH and UDHC combination of tissues, 0.89 for ADH and ADHC tissues, and 0.87 for all the tissues combined (Fig. 4).

Discussion

In the current study, we have tested HYAL1 expression for potential application to screen benign tissues and predict subsequent cancer development. The results presented here (Supplementary Table S1; Table 1; Figs. 2–4) establish that expression of HYAL1 in benign tissues is highly associated with development of IBC irrespective of histologic diagnosis of

Table 1. ROC statistics for HYAL1 in benign breast tissues

Benign tissues	Sensitivity	Specificity	PPV	NPV	*P*		
					Kruskal-Wallis	*t* test	Wilcoxon rank-sum test
UDH and UDHC + ADH and ADHC	0.83	0.84	0.84	0.83	0	0	2.2e-17
ADH and ADHC	0.84	0.90	0.89	0.87	2.5e-12	3.5e-14	2.5e-12
UDH and UDHC	0.81	0.75	0.76	0.80	1.9e-6	2.3e-6	1.9e-6

NOTE: All the calculations were done using the expression levels (grading scores) at 50 to 100.

benign tissues. It has very high sensitivity, specificity, PPV, and NPV and very low *P* values (Table 1), showing that it is an excellent predictive marker of breast cancer development in women with UDH or ADH type of benign lesions. Our results presented here show that HYAL1 is as widely expressed in UDHC as ADHC from women who subsequently developed IBC. In our study of 32 UDHC and 50 ADHC test samples, it was detected in most of the cases (Supplementary Table S1; Fig. 2). The areas under the ROC curves are very high for ADH or UDH type of tissues or combinations of UDH and ADH type of tissues (Fig. 4). Although the area under the ROC curves (Fig. 4) and ROC statistical values (Table 1) seem slightly higher for ADH tissues than UDH type of benign tissues, the values for UDH type of tissues are statistically highly significant. When data on both types of tissues were combined, HYAL1 showed very high sensitivity, specificity, PPV, and NPV and very low *P* values (Table 1; Fig. 4).

The data presented here also show that HYAL1 is far superior in comparison with the previously studied marker, carcinoembryonic antigen cell adhesion molecule 6, for predicting IBC development in patients who have UDH type of benign lesions (44). Because a large majority of benign tissues are UDH type, HYAL1 will be very highly valuable for screening these tissues to identify a "true precancerous UDHC lesion" and predict subsequent development of IBC. HYAL1 has comparable sensitivity, specificity, PPV, NPV, and *P* values with another previously studied marker, matrix metalloproteinase-1 (15, 44), although we have previously tested its presence only in 20 UDHC tissues and none of UDH control tissues (15, 44). HYAL1 has comparable sensitivity, specificity, PPV, NPV, and *P* values to carcinoembryonic antigen cell adhesion molecule 6 and matrix metalloproteinase-1 in distinguishing ADHC from ADH type of lesions (44). Therefore, HYAL1 could also be applied to predict IBC development in patients with ADH type of lesions either singly or in combination with matrix metalloproteinase-1 and carcinoembryonic antigen cell adhesion molecule 6.

The results presented here also show, for the first time, that HYAL1 is expressed at the precancerous stage in the breast tissue; therefore, it seems to contribute to tumorigenesis in this tissue. It is possible that HYAL1 is expressed at the precancerous stages of several other tissues.

In summary, HYAL1 is an excellent diagnostic marker that could be applied either singly or in combination with matrix metalloproteinase-1 and carcinoembryonic antigen cell adhesion molecule 6 to screen histologically diverse types of benign tissues and detect a "true precancerous UDHC as well as ADHC benign tissues" and identify patients who are most likely to develop IBC subsequently. It could also be a potential molecular marker for screening women who have no lesions by mammography or magnetic resonance imaging using samples of ductal cells obtained by procedures such as ductal lavage collection and random periareolar fine-needle aspiration procedures and identifying those who are at very high risk for developing breast cancer. Identification and treatment of patients with "true precancerous lesions/cells" who are most likely to develop IBC could significantly reduce the breast cancer incidence and deaths from it.

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