Expression of Carcinoembryonic Antigen Cell Adhesion Molecule 6 Oncoprotein in Atypical Ductal Hyperplastic Tissues Is Associated with the Development of Invasive Breast Cancer

Indira Poola,[1] Babok Shokrani,[2] Rakesh Bhatnagar,[5] Robert L. DeWitty,[3] Qingqi Yue,[4] and George Bonney[4]

Abstract

Background: Epidemiologic studies have established that women with prior atypical ductal hyperplastic (ADH) lesions have a 5-fold increased risk of developing invasive breast cancer (IBC). However, there is currently no means of identifying a subclass of ADH from women who will most likely develop cancer. The purpose of this study is to investigate whether elevated expression of carcinoembryonic antigen cell adhesion molecule 6 (CEACAM6) in ADH tissues is associated with the development of IBC.

Methods: A retrospective study was conducted with archival ADH tissues and clinical information on the development/nondevelopment of IBC. The control group was ADH from patients who had no prior history of IBC and did not develop cancer within 5 years after the diagnosis of ADH (n = 44). The test group was ADH from patients who either developed cancer concurrently or subsequently after diagnosis (ADHC; n = 44). The expression of CEACAM6 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: Of the 44 control ADH tissues from patients with no history of cancer, 9 were positive for CEACAM6. Among the ADHC tissues, 40 of 44 samples were positive. Statistical analysis of CEACAM6 expression data showed a significant association between its expression and cancer development, high sensitivity, specificity, positive predictive value, and negative predictive value.

Conclusions: The expression of CEACAM6 in ADH lesions is strongly associated with the development of IBC, therefore, it can be applied as a diagnostic marker either singly or in combination with other marker(s) to predict IBC development in women with ADH lesions. It could also be a potential molecular therapeutic target for preventing IBC.

It is now well established that the majority of breast cancers arise in the milk ducts, and ductal hyperplasias and atypical ductal hyperplasias (ADH) are the earliest precancerous stages that progress to invasive breast cancer (IBC; reviewed in refs. 1, 2). A number of retrospective and prospective studies have established that the risk of developing carcinoma in a woman with prior benign proliferative changes without atypia was 2-fold higher, and the risk increased to 5-fold if the proliferation was associated with atypia in comparison to women who had none of these lesions (3–11). Because of the 5-fold increased risk of developing IBC, ADH lesions are considered to be advanced precancerous lesions. However, not every woman with an ADH lesion will develop cancer. There seem to be underlying biological abnormalities causing some to remain stable and others to progress to IBC. It is not possible to identify the biological abnormalities based on the morphologic appearance alone; therefore, we cannot predict which women with which subclass of ADH will subsequently develop cancer. Molecular markers that can distinguish the ADH that will progress to IBC from those that will not progress will be highly valuable in identifying women who are most likely to develop cancer.

Using global gene expression analysis, we recently identified >300 molecular markers that were differentially expressed in ADHC from patients who had cancer concurrently or developed subsequently in comparison with ADH from patients who had no prior history of breast cancer and did not develop cancer within 5 years following diagnosis (12). In the current

Authors' Affiliations: Departments of [1]Biochemistry and Molecular Biology, [2]Pathology, [3]Surgery, and [4]Statistical Genetics and Bioinformatics Unit, Howard University College of Medicine, Washington, DC, and [5]Biology Department, University of Alberta, Edmonton, Alberta, Canada

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Note: B. Shokrani is currently at the Pathology Division, Washington University School of Medicine, St. Louis, MO.

Requests for reprints: Indira Poola, Department of Biochemistry and Molecular Biology, Howard University School of Medicine, 520 W Street Northwest, Washington, D.C. 20059. Phone: 202-806-5554; Fax: 202-806-5553/5784; E-mail: ipoola@howard.edu.

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Table 1. Expression of MMP-1 and CEACAM6 proteins by immunohistochemistry in precancerous breast tissues

Histologic type of precancerous lesion	Cancer development (years prediagnosis of precancerous lesion)	Precancerous breast	Cancer breast	Age at which cancer developed
ADH	7 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	8 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	8 Free	NA	NA	NA
ADH	5 Free	NA	NA	NA
ADH	3 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	3 Free	NA	NA	NA
ADH	3 Free	NA	NA	NA
ADH	3 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	5 Free	NA	NA	NA
ADH	5 Free	NA	NA	NA
ADH	5 Free	NA	NA	NA
ADH	5 Free	NA	NA	NA
ADH	5 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	5 Free	NA	NA	NA
ADH	5 Free	NA	NA	NA
ADH	5 Free	NA	NA	NA
ADH	6 Free	NA	NA	NA
ADH	5 Free	NA	NA	NA
ADH	9 Free	NA	NA	NA
ADH	8 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	7 Free	NA	NA	NA
ADH	6 Free	NA	NA	NA
ADH	6 Free	NA	NA	NA
ADH	6 Free	NA	NA	NA
ADH	6 Free	NA	NA	NA
ADH	6 Free	NA	NA	NA
ADH	6 Free	NA	NA	NA
ADH	6 Free	NA	NA	NA
Fibrocystic change	1 Pre	Left	Left	52
Stromal fibrosis	1 Pre	Right	Right	72
Fibrocystic change	7 Pre	Left	Left	59
Lobular hyperplasia	3 Pre	Left	Left	41
Intraductal Hyperplasia	3 Pre	Right	Right	71
Intraductal papilloma	4 Pre	Right	Right	61
Fibrocystic change	2 Pre	Left	Left	80
Stromal fibrosis	3 Pre	Right	Right	64
Intraductal papilloma	3 Pre	Left	Left	62
Epithelial hyperplasia	2 Pre	Left	Left	70
Hyperplasia, fibrocystic change	5 Pre	Right	Right	71

Table 1. Expression of MMP-1 and CEACAM6 proteins by immunohistochemistry in precancerous breast tissues (Cont'd)

Histologic type of cancer	Grade of cancer	Estrogen receptor/ progesterone receptor status	Nodal status	MMP-1 in stroma*	CEACAM6*
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	3
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	1	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	1
NA	NA	NA	NA	0	0
NA	NA	NA	NA	1	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	1	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0.5
NA	NA	NA	NA	0	1
NA	NA	NA	NA	0	0
NA	NA	NA	NA	1	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0.5
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	1	2
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0.5	1
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0.5	0
NA	NA	NA	NA	0	0
NA	NA	NA	NA	0	0.5
NA	NA	NA	NA	0	1
Intraductal carcinoma	2	ND	–	2	0
Invasive ductal carcinoma	2	ND	–	2	0
Metastatic adenocarcinoma	3	ND	+	1	0
Lobular carcinoma *in situ*	ND	ND	–	2	0
Invasive tubular ductal carcinoma	1	–/–	–	2	0
Invasive ductal and lobular carcinoma	3	ND	–	2	1
Invasive ductal carcinoma	3	+/–	–	1	2
Infiltrating ductal carcinoma	2	+/+	–	2	1
Invasive ductal carcinoma	3	+/–	+	2	1
Lobular cancer *in situ*	ND	ND	–	2	0.5
Invasive ductal carcinoma	2	ND	–	2	0

(*Continued on the following page*)

Table 1. Expression of MMP-1 and CEACAM6 proteins by immunohistochemistry in precancerous breast tissues (Cont'd)

Histologic type of precancerous lesion	Cancer development (years prediagnosis of precancerous lesion)	Precancerous breast	Cancer breast	Age at which cancer developed
Fibrocystic change	4 Pre	Left	Left	76
Fibrocystic change	9 Pre	Left	Left	70
Fibrocystic change	5 Pre	Right	Right	34
Intraductal hyperplasia	5 Pre	Right	Right	74
Intraductal papilloma	5 Pre	Left	Left	48
Fibrocystic change	1 Pre	Right	Right	35
Intraductal papilloma	5 Pre	Left	Left	48
Intraductal papilloma	3 Pre	Left	Left	62
Fibrocystic changes	3 Pre	Right	Right	66
ADH	2 Pre	Right	Right	70
ADH	1 Pre	Right	Right	69
ADH	2 Pre	Left	Left	51
ADH	7 Pre	Left	Right	79
ADH	3 Pre	Left	Left	70
ADH	3 Pre	Left	Left	31
ADH	1 Pre	Left	Left	63
ADH	4 Pre	Left	Left	70
ADH	5 Pre	Right	Left	41
ADH	3 Pre	Right	Right	45
ADH	2 Pre	Right	Left	49
ADH	5 Pre	Right	Right	61
ADH	2 Pre	Right	Right	64
ADH	4 Pre	Right	Left	49
ADH	Simultaneous	Same	Same	80
ADH	Simultaneous	Same	Same	48
ADH	Simultaneous	Same	Same	45
ADH	Simultaneous	Same	Same	62
ADH	Simultaneous	Same	Same	61
ADH	Simultaneous	Same	Same	55
ADH	Simultaneous	Same	Same	40
ADH	Simultaneous	Same	Same	61
ADH	Simultaneous	Same	Same	70
ADH	Simultaneous	Same	Same	37
ADH	Simultaneous	Same	Same	47
ADH	Simultaneous	Same	Same	50
ADH	Simultaneous	Same	Same	86
ADH	Simultaneous	Same	Same	55
ADH	Simultaneous	Same	Same	70
ADH	Simultaneous	Same	Same	47
ADH	Simultaneous	Same	Same	42
ADH	Simultaneous	Same	Same	45
ADH	Simultaneous	Same	Same	71
ADH	Simultaneous	Same	Same	51
ADH	Simultaneous	Same	Same	48
ADH	Simultaneous	Same	Same	46
ADH	Simultaneous	Same	Same	54
ADH	Simultaneous	Same	Same	59
ADH	Simultaneous	Same	Same	51
ADH	Simultaneous	Same	Same	50
ADH	Simultaneous	Same	Same	55
ADH	Simultaneous	Same	Same	75
ADH	Simultaneous	Same	Same	52
ADH	Simultaneous	Same	Same	69

NOTE: ND, not determined; NA, not applicable.
*The slides were scored in comparison with an arbitrary value of 5.0 assigned for IBC tissues.

Table 1. Expression of MMP-1 and CEACAM6 proteins by immunohistochemistry in precancerous breast tissues (Cont'd)

Histologic type of cancer	Grade of cancer	Estrogen receptor/ progesterone receptor status	Nodal status	MMP-1 in stroma*	CEACAM6*
Invasive ductal carcinoma	3	−/−	+	2	1
Ductal carcinoma *in situ*	3	−/−	−	3	0
Invasive ductal carcinoma	3	−/−	+	2	0
Invasive adeosquamous carcinoma	2	−/−	+	2	1
Invasive papillary carcinoma	1	+/−	−	3	0
Invasive ductal carcinoma	3	ND	+	2	0
Invasive papillary carcinoma	1	ND	ND	1	1
Diffuse infiltrating lobular carcinoma	ND	ND	ND	0.5	0
Invasive ductal carcinoma	2	ND	+	1	1
Infiltrating ductal carcinoma	2	+/−	+	3	4
Invasive ductal carcinoma	3	ND	−	3	4
Intraductal carcinoma *in situ*	ND	+/−	−	2	2
Invasive ductal carcinoma	3	−/−	+	3	0.5
Lobular carcinoma *in situ*	ND	ND	−	0	3
Invasive ductal carcinoma	3	ND	−	2	2
Intraductal carcinoma *in situ*	ND	ND	−	2	2
Intraductal carcinoma *in situ*	ND	ND	−	3	2
Lobular carcinoma *in situ*	ND	ND	−	0	2
Invasive ductal carcinoma	ND	ND	−	3	1
Infiltrating ductal carcinoma	ND	ND	−	3	0
Invasive ductal carcinoma	ND	ND	−	3	2
Infiltrating ductal carcinoma	ND	ND	−	2	1
Invasive ductal carcinoma	3	−/−	+	3	2
Infiltrating ductal carcinoma	3	−/−	−	4	1
Intraductal carcinoma	2	ND	−	4	1
Invasive ductal carcinoma	3	ND	−	3	0
In situ lobular carcinoma	2	ND	−	3	2
In situ ductal carcinoma	2	ND	−	2	1
Invasive ductal carcinoma	3	−/−	+	4	2
In situ lobular carcinoma	ND	ND	−	3	3
Invasive lobular carcinoma	3	ND	−	3	1
Ductal carcinoma *in situ*	ND	ND	−	4	2
In situ ductal carcinoma	3	ND	−	3	1
Invasive lobular carcinoma	ND	+/−	−	4	3
Invasive ductal carcinoma	2	+/−	+	0	1
Invasive Ductal carcinoma	3	+/+	+	3	0
Ductal carcinoma *in situ*	ND	ND	−	2	1
Infiltrating ductal carcinoma	2	+/−	+	0	0
Ductal carcinoma *in situ*	ND	ND	−	4	2
In situ ductal carcinoma	3	ND	−	0	1
In situ ductal carcinoma	2	+/+	−	0	2
Invasive ductal carcinoma	1	+/+	+	4	4
Ductal carcinoma *In situ*	2	ND	−	0	1
Invasive ductal carcinoma	2	+/+	−	4	3
Infiltrating ductal carcinoma	1	ND	−	4	2
Invasive ductal carcinoma	2	+/+	−	4	3
Invasive ductal carcinoma	ND	−/−	−	3	2
Invasive ductal carcinoma	2	+/+	−	4	2.5
Lobular carcinoma *in situ*	ND	+/+	−	2	1
Ductal carcinoma *in situ*	2	ND	−	3	2
Medullary ductal carcinoma	3	+/+	−	3	1
Invasive ductal carcinoma	3	+/+	−	4	2.5
Intraductal carcinoma	ND	ND	−	3	3

study, using archival tissues, we retrospectively tested whether the expression of one of the most highly up-regulated molecules in ADHC, carcinoembryonic antigen cell adhesion molecule 6 (CEACAM6), is associated with the development of breast cancer. We report here that the CEACAM6 protein is highly expressed in ADH tissues from patients who either developed cancer concurrently or subsequently, and is highly predictive of developing invasive breast cancer.

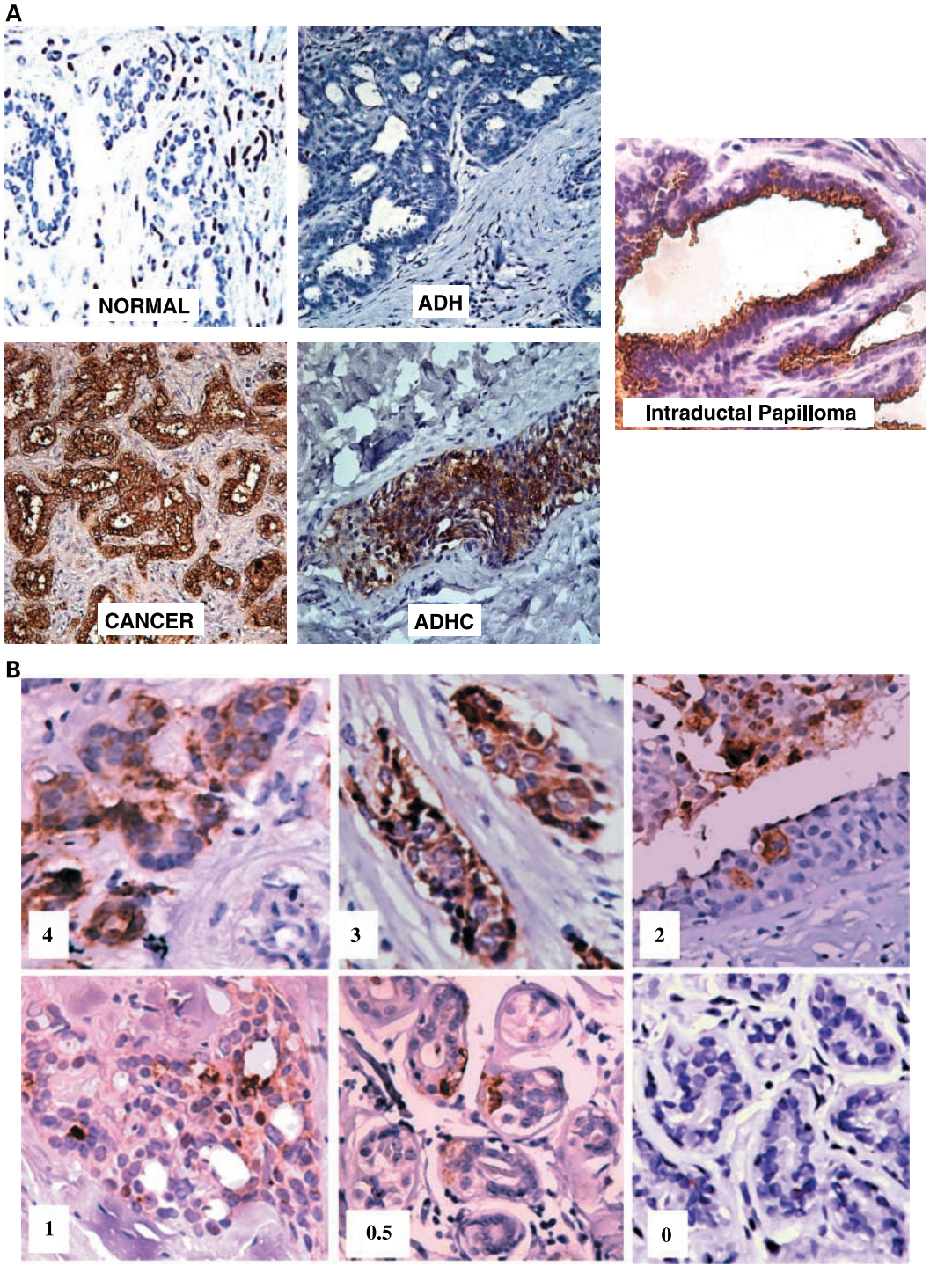

Fig. 1. *A,* CEACAM6 expression in representative normal, non-ADH benign (intraductal papilloma), ADH, ADHC, and IBC tissues by immunohistochemistry. Formalin fixed paraffin-embedded archival tissues were immunostained with antibodies against CEACAM6 as described in Materials and Methods. Representative tissues from each category (magnification, ×40). Strong staining was observed in IBC, ADHC tissues, and CEACAM6-positive non-ADH benign tissue (intraductal papilloma). CEACAM6 staining could be seen both in the cytosol and outer cell membranes of ductal epithelial cells in all the positive tissues. *B,* representative samples of five intermediate grading scores, 0.5, 1.0, 2.0, 3.0, and 4.0.

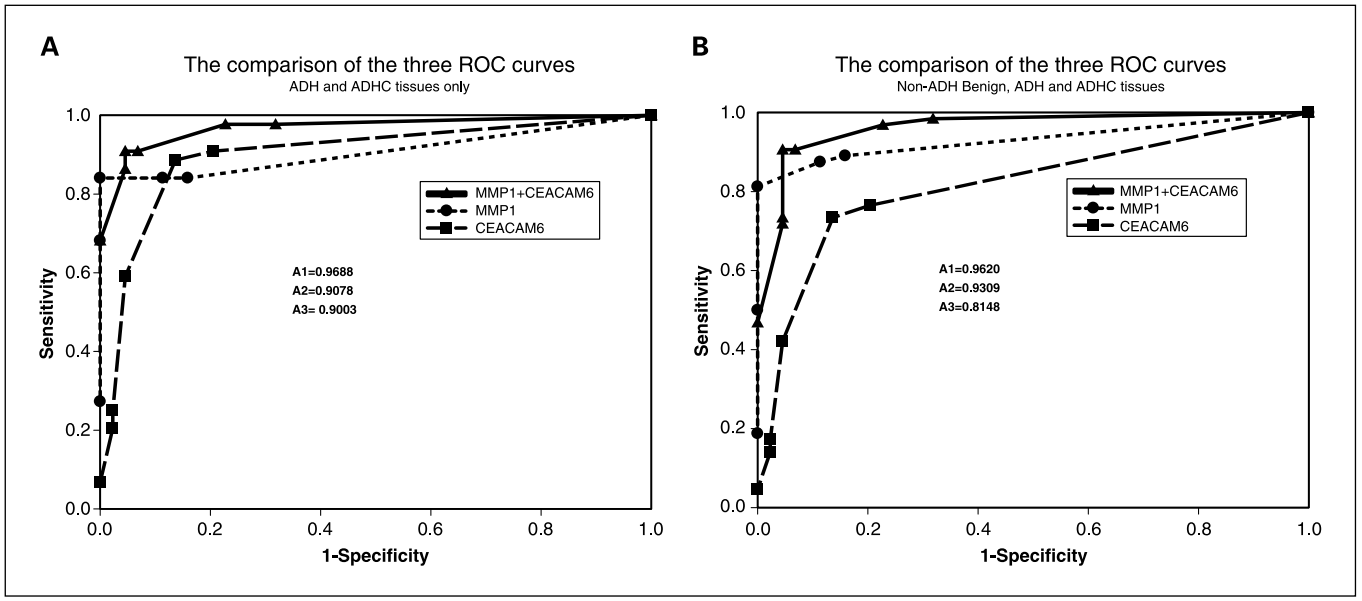

Fig. 2. ROC curves for MMP-1, CEACAM6, and MMP-1 plus CEACAM6. ROC curves for the markers MMP-1 and CEACAM6 individually and in combination. *A,* data generated with ADH and ADHC samples only; *B,* data including the non-ADH benign along with ADH and ADHC. In both (*A*) and (*B*), A1, A2, and A3 are the areas under the three ROC curves of MMP-1 and CEACAM1 together, MMP-1, and CEACAM6, respectively.

Materials and Methods

Archival ADH tissues and follow-up clinical information on IBC development. Formalin-fixed paraffin-embedded precancerous tissues that were stored in the Howard University Pathology tissue archives were used for the current study. The clinical follow-up information on the development/nondevelopment of cancer, in patients from whom precancerous tissues were derived, was obtained from tumor registry data banks, surgical pathology data banks, and patient visits to surgical oncologists and clinical oncologists. A total of 44 samples from each of the ADH and ADHC groups were used. Among the 44 samples in the ADHC group, 30 were derived from patients who had both cancer and atypical lesions concurrently, and 14 were from patients who first had ADH and subsequently developed cancer in 1 to 5 years. All 44 control ADH tissues were from patients who had no prior history of breast cancer and did not develop cancer within 5 years after the diagnosis. Whenever ADHC tissues were derived from patients who had concurrent cancer, the slides were cut from blocks which were prepared from regions far away from the cancer site. In addition to ADH and ADHC cases, 20 non-ADH benign tissues from patients who subsequently developed cancer were also included in the study. To ascertain that all the sections cut from each block had the precancerous 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. Paraffin-embedded formalin-fixed invasive breast cancer and normal breast tissue slides were also obtained from Howard University Pathology tissue archives for positive and negative controls, respectively.

Immunohistochemical staining. The presence of CEACAM6 protein was studied in formalin-fixed, paraffin-embedded precancerous tissues by immunohistochemistry using mouse monoclonal antibodies against CEACAM6 and Super Sensitive Polymer-HRP IHC detection system, both obtained from BioGenex (San Ramon, CA). The slides with tissue sections were processed and immunostained, and blinded to any knowledge regarding the development/nondevelopment of cancer, as previously described (12, 13). Briefly, slides were deparaffinized and antigens retrieved as described previously (13) and blocked with 3% H_2O_2 in methanol for 20 minutes and washed with PBS. The slides were treated with protein block (0.1% fish gelatin, 1% bovine serum albumin, 0.1% Triton X-100, and 0.05% Tween 20 in PBS) and incubated with primary antibody for 45 minutes at room temperature. The slides were washed four times for 3 minutes each with PBS and incubated with super enhancer (supplied with the kit) for 15 minutes at room temperature. The slides were drained and incubated with horseradish peroxidase – conjugated secondary antibody for 30 minutes and then with substrate (3,3′-diaminobenzidine liquid chromogen, from DakoCytomation, Carpinteria, CA) for 5 minutes. Finally, the slides were washed and stained with hematoxylin, mounted with DPX, and visualized under Leica DMRXA microscope. The tissues were immunostained for the presence of matrix metalloproteinase 1 (MMP-1) protein as previously described (12). Briefly, after antigen retrieval and blocking with 3% H_2O_2, the slides were incubated with the above protein block without 0.1% fish gelatin and then incubated with mouse anti-MMP-1 antibodies (1:100 dilution) for 30 minutes. The slides were washed and incubated with EnVision peroxidase conjugated secondary antibody (DakoCytomation) for 30 minutes. The slides were washed and incubated with peroxidase substrate as above. All the stained slides were scored for the presence of CEACAM6 and MMP-1 by two pathologists for staining intensity [scale, 0-5 in comparison with an arbitrary number (5) assigned to invasive breast cancer slides as positive controls and (0) to normal breast tissue slides as negative controls that were similarly stained] and the percentage of positive cells (0-100%).

Statistical analysis. The significance of the association of CEACAM6 protein expression and cancer development was evaluated using a χ^2 test. The sensitivity (percentage of ADHC samples that were positive for the marker) and specificity (percentage of control ADH 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. The *P* values, sensitivity, specificity, PPV, and NPV were determined individually for CEACAM6, and in combination with MMP-1, which we have previously shown to be expressed in precancerous lesions from patients who subsequently or concurrently developed cancer. All analyses were done for ADH and ADHC combination and for non-ADH benign, ADH, and ADHC combination of tissues. The receiver operating characteristic

Table 2. ROC statistics for MMP-1, CEACAM6 and MMP-1 plus CEACAM6 in precancerous breast tissues

Precancerous tissues	Sensitivity			Specificity		
	MMP-1	CEACAM6	MMP-1 Plus CEACAM6	MMP-1	CEACAM6	MMP-1 Plus CEACAM6
ADH and ADHC only	0.84	0.87	0.98	0.87	0.86	0.77
Non-ADH benign, ADH and ADHC	0.88	0.73	0.97	0.89	0.86	0.77

NOTE: All the calculations were done using the expression levels (grading scores) at 0.5 to 1.0 for both markers.

(ROC) curves were generated as follows. For each threshold value, if the measured value (marker grading score, or sum of two grading scores for the two markers combined in our situations) 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). For MMP-1 and CEACAM6 separately, the threshold values were the five grading scores (0.5, 1, 2, 3, and 4). For MMP-1 and CEACAM6 combined, the threshold values were 0.5, 1, 1.5, 2, 2.5, 3, and 3.5, which give 100% specificity. All the ROC curves were generated by connecting all the points determined by all the threshold values in an increasing order. To determine the best way to combine the two markers, we first analyzed the data by logistic regression analyses and obtained the coefficients 1.8 and 1.6 for MMP-1 and CEACAM6, respectively. Based on these, we combined the markers by two ways: (*a*) because the coefficients are almost the same, the first way we combined the markers was by simply adding the grading scores, and (*b*) the second way we combined the markers was by first multiplying the marker grading scores with their respective coefficients and then adding those values. We compared the results of the two combinations by using their corresponding ROC curves.

Results

CEACAM6 is a glycosylphosphatidylinositol-anchored cell surface protein that functions as a homotypic intercellular adhesion molecule and can block anoikis (apoptotic response induced in normal cells by inadequate or inappropriate adhesion to substrate) of several different cell types. Elevated levels of CEACAM6 were shown to play an instrumental role in tumorigenesis by disrupting the functions of integrins, which in turn, affect cell-ECM interactions, cell polarity, and architecture, and inhibition of cell differentiation (14, 15). It is over-expressed in a number of human malignancies including breast cancers (16–18), and increased levels of CEACAM6 are inversely correlated to the differentiation state of cancer cells. CEACAM6 was extensively investigated in gastrointestinal cancers. Duxbury et al. reported that CEACAM6 gene silencing impairs anoikis and the *in vivo* metastatic ability of pancreatic adenocarcinoma cells (17). It was reported to be up-regulated at the early stages of colorectal cancers such as early adenomas and hyperplastic polyps (19). An increased level of CEACAM6 was also shown to be an independent prognostic factor in colorectal cancers (15, 16).

By comparing the global gene expression profiles of ADH and ADHC tissues, we previously identified CEACAM6 as the third most highly up-regulated molecule (ratio, 37; *P* value, 9.5×10^{-6}) among the genes that showed significantly increased expression in ADHC tissues (12). Here, we conducted a retrospective study to test whether CEACAM6 protein expression in precancerous lesions is associated with the development of invasive breast cancer.

CEACAM6 protein is highly expressed in precancerous tissues from patients who developed cancer either concurrently or subsequently. Using immunohistochemistry, we tested CEACAM6 protein expression in 44 samples each of ADH and ADHC tissues, and 20 samples of non-ADH benign tissues from patients who subsequently developed cancer in 1 to 5 years. The histologic diagnosis of non-ADH benign lesions varied from hyperplasia, fibrocystic change, to intraductal papilloma (Table 1). The results for CEACAM6 protein in all the precancerous tissues are presented in Table 1. Of the 44 ADH samples, 9 tissues showed some level of CEACAM6 expression. The remaining 35 samples were negative for the presence of CEACAM6. Among the ADHC tissues from patients with a history of cancer, 40 of the 44 samples tested showed significant levels of expression. However, among the 20 non-ADH benign tissues tested, only 9 showed the presence of CEACAM6 protein. Staining was observed in both cytosol and cell membranes of ductal epithelial cells in all the positive tissues. None of the cells in the stroma were positive. All the positive slides were scored qualitatively based on the intensity/degree of stain and the number of cells stained in the ductal epithelial cells and in comparison with cancer tissues. Among the positive tissues, the level of expression varied from 0.5 to 4 as seen in Table 1. A representative tissue from each of normal, non-ADH benign (intraductal papilloma), ADH, ADHC, and invasive breast cancer are shown in Fig. 1A, and images of representative samples of all five intermediate grading scores are shown in Fig. 1B.

We compared the expression of CEACAM6 protein with another marker, MMP-1. In a previous study, using a sample size of 30 ADH, 17 non-ADH benign, and 44 ADHC tissues, we showed that it was highly expressed in ADHC and non-ADH benign tissues from patients who subsequently developed cancer (12). In the current study, we increased the control ADH tissues from 30 to 44 and non-ADH benign tissues from 17 to 20 for staining with MMP-1. For comparative purposes, here, we present the previous and current data on MMP-1 expression along with CEACAM6 expression data (Table 1). As seen in Table 1, the CEACAM6 expression pattern in ADH and ADHC tissues is comparable with MMP-1. However, CEACAM6 is not as widely expressed as MMP-1 in non-ADH benign tissues from patients who subsequently developed cancer (Table 1).

To test if there is a significant difference in the expression of these two markers between concurrent and nonconcurrent ADHC tissues, we compared their expression data individually with ADH samples as well as with each other. We applied

Table 2. ROC statistics for MMP-1, CEACAM6 and MMP-1 plus CEACAM6 in precancerous breast tissues (Cont'd)

PPV			NPV			*P*		
MMP-1	CEACAM6	MMP-1 Plus CEACAM6	MMP-1	CEACAM6	MMP-1 Plus CEACAM6	MMP-1	CEACAM6	MMP-1 Plus CEACAM6
0.88	0.87	0.81	0.85	0.88	0.97	4×10^{-11}	9×10^{-12}	3×10^{-12}
0.92	0.89	0.86	0.83	0.69	0.94	2×10^{-14}	3×10^{-9}	5×10^{-15}

standard t test and χ^2 tests by categorizing the grading scores to ≥ 0.5 as detected and <0.5 as not detected. The two tests consistently indicated that both nonconcurrent and concurrent ADHC samples are significantly different from ADH samples (for ADH and nonconcurrent ADHC combination, P values for MMP-1 and CEACAM6 were 1.1×10^{-16} and 8.0×10^{-10} by t test, and 6.1×10^{-6} and 5.4×10^{-6} by χ^2 test, respectively; for ADH and concurrent ADHC combination, the P values for MMP-1 and CEACAM6 were 0 and 3.5×10^{-11} by t test, and 3.6×10^{-8} and 1.7×10^{-8} by χ^2 test, respectively). However, the concurrent and nonconcurrent ADHC are not significantly different from each other (P values for MMP-1 and CEACAM6 by t test were 0.23 and 0.44, and by χ^2 test they were 0.8 and 0.79, respectively).

CEACAM6 expression in ADH tissues is highly predictive of developing breast cancer. The CEACAM6 and MMP-1 expression data in Table 1 were statistically analyzed for the significance of association with developing cancer (P values), sensitivity, specificity, PPV, and NPV in six ways: (a) CEACAM6 expression data in ADH and ADHC tissues, (b) MMP-1 expression data in ADH and ADHC tissues, (c) combination of CEACAM6 and MMP-1 expression in ADH and ADHC tissues, (d) CEACAM6 expression data in non-ADH benign, ADH, and ADHC tissues, (e) MMP-1 expression data in non-ADH benign, ADH, and ADHC tissues, and (f) combination of CEACAM6 and MMP-1 expression in non-ADH benign, ADH, and ADHC tissues. We combined MMP-1 and CEACAM6 expression data by two ways as described in Materials and Methods. The results generated by adding the grading scores of both markers as the combined value are presented in Fig. 2A and B and Table 2. Figure 2A shows the ROC curves for MMP-1, CEACAM6 individually, and both markers together for ADH and ADHC samples only. Figure 2B shows the ROC curves for MMP-1 and CEACAM6 individually and both markers together with the inclusion of non-ADH benign tissues along with ADH and ADHC samples. Both CEACAM6 and MMP-1 showed very high sensitivity and specificity as individual markers, either in ADH and ADHC tissues only, or with the inclusion of non-ADH benign tissue (Table 2). Both markers have comparable sensitivity, specificity, PPV, NPV, and P values. The sensitivity, NPV, and P values were improved when both markers are taken together compared with individual markers. The specificity and PPV were only slightly reduced when both markers were taken together compared with individual markers. The inclusion of non-ADH benign samples decreased the sensitivity and NPV for CEACAM6, presumably because only $\sim$50% of the samples included showed its expression. However, if both markers are taken together, the inclusion of non-ADH benign tissues only has a slight effect on overall ROC values.

We also analyzed the expression data by combining MMP-1 and CEACAM6 in a second way: by first multiplying the grading scores with their respective coefficients and then adding those values together. The results of this method of combination were compared with the above results using their corresponding ROC curves. We found that there was negligible difference between the ROC curves generated by the above two different ways of combining the markers (data not shown).

Discussion

One of the risk factors that predict invasive breast cancer development, according to the Gail model, is the previous history of ADH lesions. Epidemiologic, clinical, and animal studies have established that women with ADH lesions have a 5-fold increased risk of developing invasive breast cancer subsequently. Because of this increased risk, it is recommended that women who are diagnosed with ADH should receive tamoxifen as a prophylactic therapy to arrest the progression of ADH lesions to invasive carcinoma (20–22). However, not every woman who is diagnosed with ADH will subsequently develop breast cancer. Currently, it is not possible to identify a subclass of ADH from women who will subsequently develop IBC based on morphologic appearance. Because of the lack of this knowledge, women with ADH lesions are either over-treated indiscriminately with tamoxifen or not treated at all. As a result, women who have no risk of developing IBC are unnecessarily subjected to the serious side effects of tamoxifen (23), such as pulmonary embolism, deep vein thrombosis, stroke, and endometrial cancers, and less serious side effects such as cataracts, vasomotor instability, nausea, and vaginal bleeding. On the other hand, women who are at risk of developing IBC, but choose not to receive tamoxifen treatment to avoid side effects because of the lack of knowledge on the subsequent development of IBC, will not get the benefit of prophylactic therapy. Molecular markers that can identify a subclass of ADH from women who will subsequently develop IBC will be highly valuable for selecting women for prophylactic therapy and preventing the development of IBC.

To understand the molecular processes during transformation from ADH to cancer, and to predict which ADH lesions will likely progress to cancer, a number of molecular markers have been previously investigated (reviewed in refs. 1, 2). Most important among these include, estrogen receptor-α, p53, Ki67, and Her2/neu. Although elevated expression of estrogen receptor-α (24) and Ki67 (25) was observed in ADH by immunohistochemistry, correlations to subsequent development of cancer were not conclusively established. p53

expression studies in ADH have also not conclusively established the association with cancer development (26). The oncogene Her2/neu was also studied in precancerous tissues as a possible predictor of cancer development and the results showed that although a low level of expression was associated with increased risk, only a small percentage (9.5%) of patients who subsequently developed cancer expressed this gene (27).

In an effort to precisely identify patients with which type of precancerous lesions will subsequently develop cancer, and to understand the complex biological process that lead to the progression from precancerous stage to cancer, we previously analyzed the global gene expression of ADH from patients with (n = 6) and without a history of cancer (n = 10), and identified a number of molecules that were significantly differentially expressed in ADH from patients with a history of cancer (12). Based on the genes that were differentially expressed in ADH from patients with a history of cancer, it seems that the progression from the ADH stage to cancer is highly complex. It involved alterations in the expression of genes that regulate both epithelial cells and stroma. Based on the changes in the expression of genes, some of the processes significantly deregulated were cell cycle check points, nucleic acid biosynthesis, degradation of extracellular matrix, maintenance of cell polarity and architecture, cell proliferation, apoptosis, and epidermal growth factor receptor signaling (12). Among the differentially expressed genes, we previously tested the expression of one of the most highly up-regulated genes in ADH tissues, MMP-1, in archival precancerous tissues by immunohistochemistry. Our results indicated that MMP-1 protein expression was significantly associated with the development of breast cancer ($P = 2.7 \times 10^{-9}$; ref. 12).

Because of the complexity of breast carcinogenesis, and the heterogeneity of precancerous lesions, more than one molecular marker would be needed for making definitive predictions of subsequent cancer development. Screening and prediction of subsequent development of cancer based on multiple molecular markers, both individually and in combination, will be more reliable and will have higher patient acceptance in a clinical situation. The sensitivity will also be higher when multiple markers are considered together compared with a single marker. In addition, establishing multiple marker expression in precancerous tissues that are highly likely to progress to cancer could lead to the design of novel targeted prophylactic molecular therapies for treating premalignant lesions and preventing the development of IBC.

Keeping the above in mind, we investigated the expression of one of the most highly up-regulated genes, CEACAM6, at its protein level retrospectively by immunohistochemistry in archival precancerous breast tissues in the current study. Our rationale for studying the expression of CEACAM6 is 2-fold: (a) to validate it as a predictive marker for breast cancer development both individually and in combination with the previously studied marker, MMP-1, so that predictions could be made based on at least two markers in a clinical situation for patient acceptability and reliability, and (b) establishing CEACAM6 in patients with precancerous tissues could have therapeutic implications. A number of recent studies have established that blocking cell-cell adhesion with CEACAM6-targeted antibodies are excellent blockers of cancer progression (28–34) and vaccines based on CEACAM6 in clinical trials for preventing the progression of breast as well as colon cancers have been highly promising (35–37). Thus, establishing the expression of CEACAM6 in precancerous tissues that are highly likely to develop into cancer could become the basis for treating the patients with CEACAM6-targeted therapies and/or vaccination preventing them from developing cancer.

The results presented here (Tables 1 and 2; Figs. 1 and 2) establish that expression of CEACAM6 in ADH tissues is significantly associated with the development of IBC. It has very high sensitivity, specificity, PPV, and NPV, and very low P values (Table 2), demonstrating that it is an excellent predictive marker of breast cancer development in women with ADH lesions. Our results also show that CEACAM6 has significantly higher sensitivity than the oncogene, Her2/Neu (0.87 versus 0.095; ref. 27) for screening precancerous lesions to predict cancer development. The data presented here also show that CEACAM6 has similar sensitivity, specificity, PPV, NPV, and P values with the previously studied marker, MMP-1 (Table 2) in ADH tissues. The data presented here also show that the predictive potential considerably increased when CEACAM6 and MMP-1 expression data were combined. As seen in Table 2, the sensitivity and NPV are considerably increased when both CEACAM6 and MMP-1 expressions are combined compared with individual markers. Specificity and PPV are also very high when both markers are combined, although they are slightly lower than individual markers, presumably due to differences in the individual variation in the expression of each of these two markers in different tissues.

In addition to ADH, we also evaluated the expression of CEACAM6 in non-ADH benign tissues to test if it could be applied to predict cancer development, both individually and in combination with MMP-1. Our results presented here show that CEACAM6 is not as widely expressed as MMP-1 in non-ADH benign tissues from women who subsequently developed IBC. In our study of 20 non-ADH benign tissues as MMP-1, only ~50% of samples showed its expression (Table 1). However, the data presented in Table 2 on CEACAM6 in combination with MMP-1 shows that it could be applied together with MMP-1 to predict cancer development in patients with non-ADH benign lesions.

In summary, CEACAM6 is an excellent diagnostic marker that could be applied to screen ADH and identify patients who are most likely to develop IBC subsequently. In addition to being an excellent predictive marker, CEACAM6 could also be a potential molecular target for treating patients who express this molecule in their precancerous lesions and preventing them from developing invasive breast cancer. The identification and treatment of patients with ADH and non-ADH lesions who are most likely to develop IBC could significantly reduce the number of deaths from breast cancer. CEACAM6 together with MMP-1 may also prove useful for screening women who have no lesions by mammography 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 of developing cancer.

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