

Research Article

p16^{INK4a} Expression and Breast Cancer Risk in Women with Atypical HyperplasiaDerek C. Radisky¹, Marta Santisteban², Hal K. Berman³, Mona L. Gauthier⁴, Marlene H. Frost⁵, Carol A. Reynolds⁷, Robert A. Vierkant⁶, V. Shane Pankratz⁶, Daniel W. Visscher⁷, Thea D. Tlsty⁸, and Lynn C. Hartmann⁵

Abstract

p16, a nuclear protein encoded by the *p16^{INK4a}* gene, is a regulator of cell-cycle regulation. Previous studies have shown that expression of p16 in tissue biopsies of patients with ductal carcinoma *in situ* (DCIS) is associated with increased risk of breast cancer, particularly when considered in combination with other markers such as Ki-67 and COX-2. Here, we evaluated how expression of p16 in breast tissue biopsies of women with atypical hyperplasia (AH), a putative precursor lesion to DCIS, is associated with subsequent development of cancer. p16 expression was assessed by immunohistochemistry in archival sections from 233 women with AH diagnosed at the Mayo Clinic. p16 expression in the atypical lesions was scored by percentage of positive cells and intensity of staining. We also studied coexpression of p16, with Ki-67 and COX-2, biomarkers of progression in AH. Risk factor and follow-up data were obtained via study questionnaire and medical records. Forty-seven patients (20%) developed breast cancer with a median follow-up of 14.5 years. Staining of p16 was increased in older patients relative to younger patients ($P = 0.0025$). Although risk of developing breast cancer was not associated with increased p16 expression, joint overexpression of Ki-67 and COX-2 was found to convey stronger risk of breast cancer in the first 10 years after diagnosis as compared with one negative marker ($P < 0.01$). However, the addition of p16 levels did not strengthen this association. p16 overexpression, either alone or in combination with COX-2 and Ki-67, does not significantly stratify breast cancer risk in women with AH. *Cancer Prev Res*; 4(12); 1953–60. ©2011 AACR.

Introduction

Atypical hyperplasia (AH) of the breast is diagnosed in approximately 40,000 to 50,000 U.S. women per year, as it is found in about 4% to 5% of the 1,000,000 benign breast biopsies conducted annually (1, 2). Women diagnosed with AH have a substantial risk of subsequent development of breast cancer, with a cumulative incidence of 30% at 25 years (3). AH is thought to represent a precursor stage to *in situ* and invasive carcinoma: biopsies of breast tissue removed prophylactically from BRCA1/2 carriers contain various proliferative benign lesions including AH in more than 50% of cases (4, 5), and AH is frequently found in random periareolar fine needle aspirations from high-risk

women compared with normal risk women (6). AH is closely related to low-grade *in situ* carcinoma (7–9), with the distinction based mainly upon the extent of the lesion. We have previously investigated processes associated with breast cancer progression as biomarkers of breast cancer risk for patients with AH, finding that increased expression of the proliferation marker Ki-67 (10) or the inflammatory/invasive marker COX-2 (11) are each associated with a higher risk of developing cancer, and that expression of estrogen receptor is not associated with subsequent cancer development (12). Previous studies investigating ductal carcinoma *in situ* (DCIS) have found that expression of Ki-67 and/or COX-2 in combination with expression of p16^{INK4a} (p16) are associated with subsequent cancer incidence (13, 14); here, we assessed whether these markers had prognostic significance for patients with AH.

The p16 protein acts as a negative regulator of cell proliferation by inhibiting the phosphorylation of retinoblastoma (Rb) family members by cyclin-dependent kinases 4/6 (reviewed in ref. 15). Cells which have been stimulated to proliferate rapidly by oncogenic stimuli can activate an antiproliferative stress-associated senescence program, leading to increased expression of p16 and consequent maintenance of Rb in a hypophosphorylated state, inducing G₁ cell-cycle arrest (15). p16 expression is also increased in an age-associated fashion (i.e., replicative senescence) in tissues which have undergone repeated proliferative cycles over extended periods of time (16).

Authors' Affiliations: ¹Division of Biochemistry/Molecular Biology, Mayo Clinic in Jacksonville, Jacksonville, Florida; ²Departamento de Oncología, Clínica Universitaria de Navarra, Navarra, Spain; ³Department of Laboratory Medicine and Pathobiology, and ⁴Department of Medical Biophysics, Campbell Family Institute for Breast Cancer Research, University of Toronto, Toronto, Ontario, Canada; ⁵Division of ⁶Medical Oncology and ⁶Bio-medical Statistics and Informatics, ⁷Department of Anatomic Pathology, Mayo Clinic in Rochester, Rochester, Minnesota; and ⁸Department of Pathology, University of California San Francisco, San Francisco, California

Corresponding Author: Derek Radisky, Department of Cancer Biology, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224. Phone: 904-953-6913; Fax: 904-953-0277; E-mail: radisky.derek@mayo.edu

doi: 10.1158/1940-6207.CAPR-11-0282

©2011 American Association for Cancer Research.

Conversely, in the setting of deregulation of Rb, p16 expression can be elevated in proliferating cells as the growth-suppressive effects of p16 are abrogated downstream of Rb. p16 overexpression is, therefore, conditional on the functional state of Rb. p16 overexpression in the setting of Rb deregulation is indicative of cancer progression and, in mammary carcinoma, is a hallmark of the basal-like molecular subtype (13, 17). When assayed in premalignant lesions of the breast (i.e., DCIS), increased expression of p16 has been found to be associated with increased risk of subsequent development of cancer when expressed in combination with Ki-67 and/or COX-2 (13, 14).

As AH is thought to represent a precursor to DCIS (7, 18, 19), we hypothesized that combinatorial analysis of p16, Ki-67, and COX-2 expression in AH might stratify risk for subsequent development of DCIS or invasive breast cancer. To test this hypothesis, we first measured the expression of p16 in 233 women with AH and determined association with patient age, type of AH, and risk of subsequent development of cancer. We then studied the risk of subsequent tumor development with concurrent expression of p16, Ki-67, and COX-2 in AH.

Methods

Study population

Entry criteria for this study have been described previously (2, 3). Briefly, the samples used for this study were derived from a Mayo benign breast disease (BBD) cohort of 9,376 women ages from 18 to 85 who had excisional breast biopsies with benign findings at the Mayo Clinic in Rochester, MN between January 1, 1967, and December 31, 1991 (2). Women were excluded from the study if there was cancer found in any of the specimen. Of the 331 women within this cohort with AH, paraffin-embedded, formalin-fixed tissue for p16 staining was available for 233 of them. All protocol procedures and patient contact materials were reviewed and approved by the Institutional Review Board of the Mayo Clinic.

Follow-up and risk factor data

Follow-up for breast cancer events and risk factor information was obtained through Mayo medical records and a study questionnaire (2, 3). Family history was classified as negative, weak, or strong. Criteria for a strong family history were: at least one first-degree relative with breast cancer before the age of 50 years or 2 or more relatives with breast cancer, with at least one being a first-degree relative. Any lesser degree of family history was considered to be weak. Eligible cancer events include women with either DCIS or invasive cancer diagnoses.

Immunostaining

Immunostaining of COX-2 and Ki-67 was described previously (10, 11). p16 (16P07--NeoMarkers; MS1064-P) was assessed by immunohistochemistry using 5- μ m formalin-fixed, paraffin-embedded tissue sections. Samples were deparaffinized with 3 changes of xylene and rehy-

drated in a series of alcohols (100%, 95%, and then 70% EtOH) and rinsed well in running distilled water. Slides were then placed in a preheated 0.1 mmol/L EDTA, pH 8.0 retrieval buffer for 30 minutes and then cooled in the buffer for 5 minutes followed by a 5 minute rinse in running distilled water. After the heat-inactivated epitope retrieval step, slides were placed on the DAKO Autostainer for the following procedure (at room temperature). Sections were incubated with 3% H₂O₂ in ethanol for 5 minutes to inactivate the endogenous peroxidases. Sections were incubated in p16 antibody at 1:80 dilution for 30 minutes at room temperature. Sections were rinsed with TBS/Triton-X100 (TBST) wash buffer. Secondary incubation was with DAKO DUAL⁺, horseradish peroxidase for 15 minutes. The slides were rinsed with TBST wash buffer. Sections were then incubated in 3,3'-diaminobenzidine (DAB⁺; K3467, DAKO) for 5 minutes, counterstained with modified Schmidt's hematoxylin for 5 minutes, followed by a 3-minute tap water rinse to blue sections, dehydrated through graded alcohols and cleared in 3 changes of xylene and mounted with permanent mounting media. Using light microscopy, stained tissue sections were inspected by the study pathologist (H. Berman). Cytoplasmic immunopositivity with occasional nuclear staining was identified by the brown chromogen DAB. Colon cancer tissue was used as positive control, and no primary antibody was used as negative control. Representative p16 staining is illustrated in Fig. 1.

Statistical analysis

Data were summarized descriptively using frequencies and percentages for categorical variables and medians and interquartile ranges (IQR) for continuous variables. Among the 331 women with AH, we compared distributions of demographic and clinical variables by tissue availability for p16 staining using χ^2 tests of significance for categorical variables and Wilcoxon rank-sum tests for continuous variables.

The length of follow-up for each woman in the study was defined as the number of days from her benign biopsy to the date of her breast cancer diagnosis, death, or last contact. In addition, women with prophylactic mastectomy, or with a diagnosis of lobular carcinoma *in situ* (LCIS) or DCIS, were censored at the corresponding date of occurrence. We estimated relative risks, overall and by strata of p16, Ki-67, and COX-2 staining levels, with standardized incidence ratios (SIR) by dividing the observed numbers of incident breast cancer by population-based expected values. The approach allowed us to compare rates of breast cancer in our cohort with that of the general population rather than an internal referent group, recognizing that all women in our cohort were at some increased risk of breast cancer from their diagnosis of AH. Expected values were calculated by apportioning person-years of follow-up for each woman into 5-year age and calendar-period categories, thereby accounting for any age or cohort period effects on the risk of cancer, and multiplying these by the corresponding breast cancer incidence rates from the Iowa Surveillance

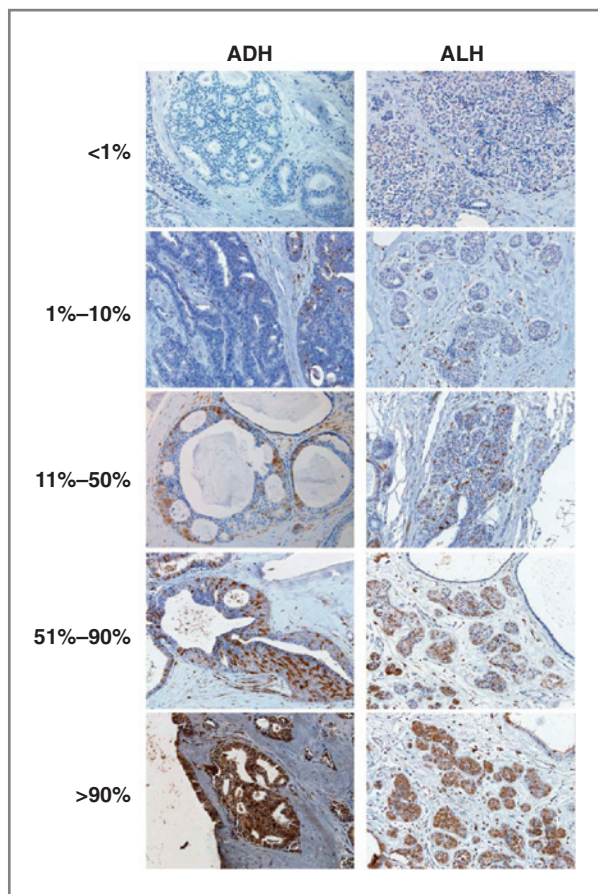


Figure 1. Staining levels of p16 in AH. Samples of atypical ductal hyperplasia (ADH, left column) and atypical lobular hyperplasia (ALH, right column), showing fractional staining levels of less than 1% cells staining positively for p16 (top row), 1% to 10% p16⁺ cells (second row), 11% to 50% p16⁺ cells (third row), 51% to 90% p16⁺ cells (fourth row), and more than 90% p16⁺ cells (bottom row).

Epidemiology and End Results (SEER) registry. This reference population was chosen because of its demographic similarities to the Mayo Clinic population (80% of cohort members reside in the top Midwest). Separate analyses were carried out for percentage of staining and intensity of staining. Potential heterogeneity in SIRs across p16, Ki-67, and COX-2 staining levels, as well as combinations of the 2 or 3 variables, was assessed by use of Poisson regression analysis, with the log-transformed expected event rate for each individual modeled as the offset term. We visually displayed observed and expected event rates using cumulative incidence curves, although accounting for the effects of death as a competing risk (20). Expected events were calculated for each 1-year follow-up interval in a manner similar to that used for determining SIRs. A modified Kaplan–Meier approach was used to accumulate expected incidence over these intervals. The expected curve was then smoothed by linear interpolation. All statistical tests were 2-sided, and all analyses were conducted with the SAS (SAS Institute, Inc.) software system.

Results

Patient characteristics

Within our AH cohort of 331 women, samples from 233 patients were available to be stained for p16. We compared these 233 subjects with the remaining 98 and saw no significant differences in terms of distributions of case status, age at AH, family history of breast cancer, and for breast cancer patients, time to diagnosis ($P > 0.05$ for each attribute). The characteristics of the subjects included in this study are presented in Table 1. Median postbiopsy follow-up was 14.5 years for the 233 women, 47 of whom (20.2%) have developed breast cancer. Of the 47 women in this study who developed breast cancer, 34 had documented invasive cancer, 10 had DCIS, and 3 had confirmed breast cancer but with unknown tumor stage.

p16 immunostaining of atypical hyperplasia samples

In the 233 samples, we analyzed both percentage of cells positive for p16 and intensity of staining. For extent of p16 positivity, we used the following categories: 0, 1% to 10%, 11% to 50%, 51% to 90%, and more than 90% atypical positive cells (Fig. 1). Intensity was categorized as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong) staining. The correlation between these 2 measures was 0.76 ($P = 0.001$). Because of this strong correlation, and because results using intensity of staining were similar to those using percentages, all subsequent results focus on percentage of positive cells. For comparisons with clinicopathologic features, we combined the p16-expression categories into 2 groups as follows: p16 low-expressing (0% to 10% cell positive) and p16 high-expressing (11+% cell positive), as using an 11%+ threshold separates individuals into groups of approximately two thirds with less than 11% positive cells and one third with 11% or more. The positive effects of this categorization are 2-fold. First, substratifying women who already have a relatively uncommon condition in the population (women with AH compose only 3% to 4% of the total number of women with clinically-confirmed BBD) into categories smaller than one third of all AH results in subgroups representing less than 1% of all women with BBD, which limits our ability to assess public health significance. Second, because of sample size restrictions, substratifying P16 into smaller categories prohibited the examination of combinations of P16 with Ki-67 and COX-2 shown in Table 3. Using these measures, we found a significant ($P = 0.0025$) association of greater p16 expression with increasing age at BBD diagnosis (Table 1). We also examined the data categorizing women into 0% positive cells and 1%+, which also separated women into groups of approximately two thirds and one third. This threshold yielded similar risk estimate results as with the 11% threshold, but attenuated the association with age at BBD, which was no longer statistically significant. We saw no association between level of p16 expression and year of benign biopsy, indication for biopsy, number of atypical foci, lobular involution status, family history, type of AH, and time of follow-up (Table 1).

Table 1. Association of p16 expression (%positive cells) with demographic and clinical variables among women with atypia

	p16 percentage of cells/positive		<i>P</i> ^a
	0%–10% (<i>N</i> = 158)	11+% (<i>N</i> = 75)	
Age at BBD diagnosis			0.0025
<30	3 (100%)	0 (%)	
30–39	8 (88.9%)	1 (11.1%)	
40–49	47 (82.5%)	10 (17.5%)	
50–59	40 (58%)	29 (42%)	
60–69	40 (72.7%)	15 (27.3%)	
70+	20 (50%)	20 (50%)	
Year of benign biopsy			0.3588
1967–1971	8 (88.9%)	1 (11.1%)	
1972–1976	18 (78.3%)	5 (21.7%)	
1977–1981	18 (62.1%)	11 (37.9%)	
1982–1986	45 (70.3%)	19 (29.7%)	
1987–1991	69 (63.9%)	39 (36.1%)	
Indication for biopsy ^b			0.1738
Lump	70 (72.9%)	26 (27.1%)	
Mammogram	87 (64.4%)	48 (35.6%)	
Number of atypical foci			0.6472
1 focus	90 (70.3%)	38 (29.7%)	
2 foci	42 (65.6%)	22 (34.4%)	
3+ foci	26 (63.4%)	15 (36.6%)	
Involution ^c			0.2132
None	12 (85.7%)	2 (14.3%)	
Partial	124 (67.8%)	59 (32.2%)	
Complete	19 (59.4%)	13 (40.6%)	
Family history ^d			0.5063
No family history	91 (68.4%)	42 (31.6%)	
Weak family history	22 (59.5%)	15 (40.5%)	
Strong family history	36 (70.6%)	15 (29.4%)	
Type of AH			0.4596
ADH	63 (65.6%)	33 (34.4%)	
ALH	71 (67%)	35 (33%)	
Both ADH and ALH	24 (77.4%)	7 (22.6%)	
Follow-up, y			0.5163
Median (IQR)	14.5 (9.0–18.1)	14.7 (9.7–18.6)	

^aWilcoxon rank-sum test for follow-up in years, χ^2 tests of statistical significance for all other variables.

^bIndication for biopsy was available for 231 of 233 women.

^cInvolution status was available for 229 of 233 women.

^dFamily history of breast cancer was available for 221 of 233 women.

Association of p16 expression with breast cancer risk

We found no association between extent of p16 expression and risk of subsequent breast cancer (Table 2, Fig. 2). The overall risk of cancer in the p16 low-expressing group was 3.89 [95% confidence interval (CI), 2.72–5.56] and relative risk (RR) = 3.91 (95% CI, 2.43–6.28) in the high-expressing group. Similar results were found for cells with low-intensity staining (0–1+) versus 2–3+ staining (data not shown). We also examined time to breast cancer from AH biopsy and found no association with p16 expression

(Fig. 2). The median time to breast cancer in the low p16 group was 12.9 years versus 13.5 years in the high p16 group.

Coexpression of p16 with Ki-67 and COX-2

We evaluated the effect of coexpression of p16 with proliferative (Ki-67) and protumorigenic (COX-2) markers. We have previously evaluated Ki-67 staining for 172 (10) and COX-2 for 158 (11) of these 233 women. Staining of p16, Ki-67, and COX-2 on sequential slides from a single

Table 2. Risk of breast cancer by p16 expression in AH

Percentage of cells positive for p16	N	Events	Expected events	SIR (95% CI) ^a	P ^b
0	80	18	4.2	4.24 (2.67–6.73)	0.88
1–10	78	12	3.5	3.45 (1.96–6.08)	
11–50	45	11	2.6	4.15 (2.30–7.50)	
51+	30	6	1.7	3.52 (1.58–7.84)	

^aSIRs and corresponding 95% CI, comparing the observed number of breast cancer events to those expected on the basis of incidence rates from Iowa SEER data. Analyses account for the effects of age and calendar period.

^bTest for heterogeneity of the SIR across staining levels.

block has been carried out in 158 women. We did not find that addition of Ki-67 or COX-2 expression levels modified the association of p16 with cancer risk (Table 3). We previously showed that higher Ki-67 levels in AH are associated with breast cancer risk (10). Adding p16 to this analysis did not strengthen the association (not shown). Overexpression of both Ki-67 plus COX-2 showed an increased breast cancer risk among patients in the first 10 years (SIR, 6.70; 95% CI, 3.19–14.1) as compared with one negative marker. Further substratification into women with high expression levels of all 3 biomarkers ($N = 13$) resulted in an SIR of 9.38 (95% CI, 3.52–25.0). Although seemingly higher, the addition of P16 did not result in a statistically significant increase in risk over and above the contribution of just Ki-67 and COX-2, perhaps in part due to the limited sample size in this group of women. Thus, results for this triple high expression group should be interpreted with caution.

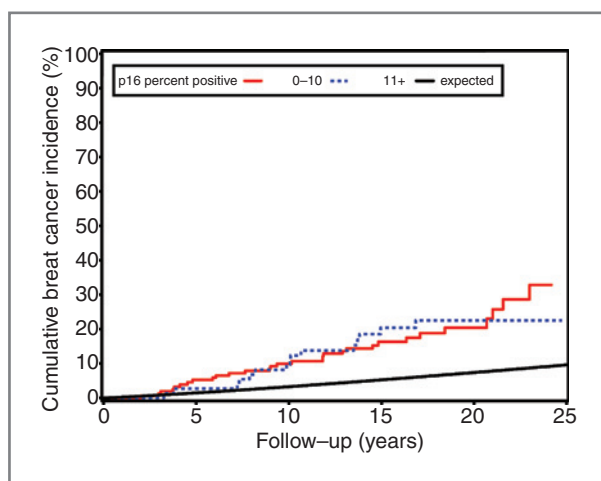


Figure 2. Expression of p16 is not related to cumulative breast cancer incidence or time to breast cancer for women with AH. Observed and expected events are cumulated after accounting for death as a competing risk and are plotted as a function of follow-up interval and stratified by p16 expression levels. Red line, 0% to 10% cells staining for p16; blue line, 11+% cells staining for p16; black line, expected breast cancer incidence according to Iowa SEER survey.

Discussion

We quantified p16 expression in a well-characterized cohort of 233 women with AH in which 47 patients had developed breast cancer with a median follow-up of 14.5 years. We found that 32% of the biopsies showed more than 10% atypical cells staining positive for p16. We found that p16 expression levels correlated with age at biopsy ($P = 0.0025$) but not with other demographic and clinical features. We found that patients showing increased expression of p16 in AH had no significant risk of subsequent development of breast cancer, even when considered in combination with expression of Ki-67 and COX-2.

p16 is a biomarker of 2 opposing phenotypes—overexpression of p16 can indicate either activation of a senescence program or loss of growth control through deregulation of Rb signaling. Studies in cultured primary human breast epithelial cells suggest that these phenotypes can be distinguished through combined measurement of p16 and proliferation rate (21). In invasive breast cancer, combined overexpression of p16 and increased proliferation rate is unique to a subset of tumors of the basal-like molecular subtype (13). It has also been observed that a subset of DCIS lesions show combined increase in p16 and proliferation (assessed by Ki-67). This appears biologically significant as DCIS lesions with increased p16/Ki-67 show increased risk for subsequent tumor development (13, 14). In these DCIS studies, the threshold of Ki-67 positivity was set at 10%. The median Ki-67 level in the series of DCIS is higher than in the series of AH; in the AH cohort, the median Ki-67 is 1% and the a cutoff of 2% cells positive for Ki-67 was used to dichotomize groups of individuals with higher or lower degrees of staining (10). The relative lower proliferation rate in AH may suggest that the high-risk p16 phenotype is uncommon in AH. Alternatively, deregulation of Rb in a subset of AH may not correspond to high proliferation, and expression of other biomarkers (i.e., E2F targets) may be more informative when combined with p16. Finally, to the extent that p16 expression in AH reflects activation of cellular senescence, prevalence of p16 in AH suggests that a substantial percentage of these lesions maintain intact growth regulation.

Table 3. Risk of breast cancer by p16 expression with Ki-67 and COX-2

Staining category ^a	N	Events	Expected events	SIR (95% CI) ^e	P ^f
p16 ^b					0.99
0%–10%	158	30	7.7	3.89 (2.72–5.56)	
11+%	75	17	4.4	3.91 (2.43–6.28)	
p16/Ki-67 ^c					0.92
0%–10%/2+	32	3	1.6	1.86 (0.60–5.78)	
0%–10%/<2	84	14	4.4	3.20 (1.90–5.41)	
11+%/2+	19	5	1.1	4.47 (1.86–10.7)	
11+%/<2	37	6	2.3	2.66 (1.20–5.93)	
p16/COX-2 ^d					0.03
0%–10%/0 or 1+	73	8	3.9	2.08 (1.04–4.15)	
0%–10%/2+ or 3+	60	12	3	4.01 (2.28–7.07)	
11+%/0 or 1+	37	4	2.2	1.78 (0.67–4.74)	
11+%/2+ or 3+	32	9	1.8	5.03 (2.62–9.66)	

^ap16 results based on 233 subjects with p16 results; p16/Ki-67 combinations based 172 subjects with both p16 and Ki-67 results; p16/COX-2 combinations based on 202 subjects with both p16 and COX-2 results.

^bp16 staining is dichotomized as percentage of cells stained, 0% to 10% versus 11+%.

^cKi-67 staining is dichotomized as percentage of cells stained, 0 or 1 versus 2 or higher (10).

^dCOX-2 staining is dichotomized by general staining intensity, 0 or 1+ versus 2+ or 3+ (11).

^eSIR and corresponding 95% CIs, comparing the observed number of breast cancer events to those expected on the basis of incidence rates from Iowa SEER data. Analyses account for the effects of age and calendar period.

^fTest for heterogeneity of the SIR across staining levels.

Women diagnosed with AH are at significantly elevated risk for subsequent development of cancer, but methods for estimating risk for individual women with AH are lacking. We have found that risk models commonly used for women with atypia, which rely primarily on clinical and epidemiologic features, carry out no better than chance alone for individual women with atypia (22, 23). Instead, we have found that direct examination of pathologic characteristics of the breast tissue biopsy can be used to better stratify cancer risk for women with AH. For example, we have shown that delayed or reduced lobular involution (24), the presence of multiple atypical foci (3), and elevated expression of COX-2 (11) and Ki-67 (10) are all associated with increased risk for subsequent development of cancer. We predict that a risk model that integrates morphologic and molecular features with clinical and epidemiologic information will improve breast cancer risk assessment.

We found a significant association between p16 expression in the atypical lesions and increasing age at biopsy ($P = 0.0025$). p16 levels have been shown to increase with age in many different tissue types (16, 25–27), although association of p16 with age in breast tissue has not been described previously to our knowledge. The consequences of p16 expression in aging tissue are not known. Some evidence suggests that increased expression of p16 directly leads to tissue degeneration associated with aging (28): deletion of p16 in a genetic model of premature aging results in attenuated phenotype and extended lifespan (29), and caloric restriction that retards aging phenotypes in experimental models also attenuates the accumulation of p16-

positive cells (16). Activation of p16 in aging tissues has been proposed to be a response to extended replication and potentially to serve a tumor suppressive function; however, cells in which p16 expression is maintained for extended periods of time are known to secrete proteins that alter tissue structure and function and promote tumor development (30, 31). In this cohort of women with atypia, we saw no evidence of reduced or increased risk of breast cancer with higher p16 expression. Investigations with larger cohorts and further analysis of senescence-associated biomarkers may help to determine whether increased p16 expression in older women reflects activation of a tumor-suppressive senescence program or a tumor-potentiating phenotype.

Strengths of our study include central pathologic review of atypia samples and long term postbiopsy follow-up. Our access to extensive medical record information permits assessment of a number of potential covariates; in combination with our previous characterization of this cohort, we could examine associations within subsets of concomitant pathologic features. Limitations of the study include the semiquantitative nature of tissue-based biomarker studies, which is further complicated by the generally weak character of p16 immunoreactivity in AH. Furthermore, although to our knowledge this is the largest examination of biomarker information in women with AH to date, we had relatively low statistical power to detect heterogeneity in SIRs, particularly when including Ki-67 and COX-2 as biomarkers. Investigation of larger or additional cohorts may be required to determine whether p16 expression levels

can provide additional discriminatory power when combined with other biomarkers.

Conclusions

p16 has emerged as a marker and mediator of cellular senescence, a key step in the process of preventing tumor progression. Previous studies have found that patients with DCIS with increased p16 expression, in combination with Ki-67 (indicating active proliferation) or with COX-2 (indicating activation of inflammatory and invasive phenotypes), show significantly higher likelihood of progression to breast cancer, and that the sustained activation of p16 in these cases could be due to bypass of normal senescence responses. Here, we find that expression of p16 in patients with AH is not associated with future cancer incidence, even when combined with expression of Ki-67 and/or COX-2, which may indicate that the p16-associated senescence pathway remains intact in AH. Comparison of p16 expression with other patient characteristics revealed a significant correlation only for the increased proportion of p16-expressing lesions in older patients ($P = 0.0025$), consistent with previous studies showing age-dependent increases in p16

expression in other tissues. Determining whether the increased abundance of p16-positive cells in AH of older women is associated with tumor protective or promoting effects of senescence activation will require further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Linda Murphy for immunostaining and Patricia Haugen for her perspective as a patient advocate.

Grant Support

This work is supported by NCI grants CA122086, 132789 and by the Mayo Clinic Breast SPORE CA116201.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 31, 2011; revised July 22, 2011; accepted August 29, 2011; published OnlineFirst September 15, 2011.

References

- Ghosh K, Melton LJ III, Suman VJ, Grant CS, Sterioff S, Brandt KR, et al. Breast biopsy utilization: a population-based study. *Arch Intern Med* 2005;165:1593-8.
- Hartmann LC, Sellers TA, Frost MH, Lingle WL, Degnim AC, Ghosh K, et al. Benign breast disease and the risk of breast cancer. *N Engl J Med* 2005;353:229-37.
- Degnim AC, Visscher DW, Berman HK, Frost MH, Sellers TA, Vierkant RA, et al. Stratification of breast cancer risk in women with atypia: a Mayo cohort study. *J Clin Oncol* 2007;25:2671-7.
- Hoogerbrugge N, Bult P, de Widt-Levert LM, Beex LV, Kiemeny LA, Ligtenberg MJL, et al. High prevalence of premalignant lesions in prophylactically removed breasts from women at hereditary risk for breast cancer. *J Clin Oncol* 2003;21:41-5.
- Kauff ND, Brogi E, Scheuer L, Pathak DR, Borgen PI, Hudis CA, et al. Epithelial lesions in prophylactic mastectomy specimens from women with BRCA mutations. *Cancer* 2003;97:1601-8.
- Fabian CJ, Kamel S, Zalles C, Kimler BF. Identification of a chemoprevention cohort from a population of women at high risk for breast cancer. *J Cell Biochem Suppl* 1996;25:112-22.
- Allred DC, Mohsin SK, Fuqua SA. Histological and biological evolution of human premalignant breast disease. *Endocr Relat Cancer* 2001;8:47-61.
- Arpino G, Laucirica R, Elledge RM. Premalignant and *in situ* breast disease: biology and clinical implications. *Ann Intern Med* 2005;143:446-57.
- Guray M, Sahin AA. Benign breast diseases: classification, diagnosis, and management. *Oncologist* 2006;11:435-49.
- Santisteban M, Reynolds C, Barr Fritcher EG, Frost MH, Vierkant RA, Anderson SS, et al. Ki67: a time-varying biomarker of risk of breast cancer in atypical hyperplasia. *Breast Cancer Res Treat* 2010;121:431-7.
- Visscher DW, Pankratz VS, Santisteban M, Reynolds C, Ristimaki A, Vierkant RA, et al. Association between cyclooxygenase-2 expression in atypical hyperplasia and risk of breast cancer. *J Natl Cancer Inst* 2008;100:421-7.
- Barr Fritcher EG, Degnim AC, Hartmann LC, Radisky DC, Boughey J, Anderson S, et al. Estrogen receptor expression in atypical hyperplasia: lack of association with breast cancer. *Cancer Prev Res* 2011;4:435-44.
- Gauthier ML, Berman HK, Miller C, Kozakeiwicz K, Chew K, Moore D, et al. Abrogated response to cellular stress identifies DCIS associated with subsequent tumor events and defines basal-like breast tumors. *Cancer Cell* 2007;12:479-91.
- Kerlikowske K, Molinaro AM, Gauthier ML, Berman HK, Waldman F, Bennington J, et al. Biomarker expression and risk of subsequent tumors after initial ductal carcinoma *in situ* diagnosis. *J Natl Cancer Inst* 2010;102:627-37.
- Kim WY, Sharpless NE. VHL inactivation: a new road to senescence. *Cancer Cell* 2008;13:295-7.
- Krishnamurthy J, Torrice C, Ramsey MR, Kovalev GI, Al-Regaiey K, Su L, et al. Ink4a/Arf expression is a biomarker of aging. *J Clin Invest* 2004;114:1299-307.
- Herschkowitz JI, He X, Fan C, Perou CM. The functional loss of the retinoblastoma tumour suppressor is a common event in basal-like and luminal B breast carcinomas. *Breast Cancer Res* 2008;10:R75.
- Lopez-Garcia MA, Geyer FC, Lacroix-Triki M, Marchio C, Reis-Filho JS. Breast cancer precursors revisited: molecular features and progression pathways. *Histopathology* 2010;57:171-92.
- Santen RJ, Mansel R. Benign breast disorders. *N Engl J Med* 2005;353:275-85.
- Gooley TA, Leisenring W, Crowley J, Storer BE. Estimation of failure probabilities in the presence of competing risks: new representations of old estimators. *Stat Med* 1999;18:695-706.
- Campisi J. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell* 2005;120:513-22.
- Boughey JC, Hartmann LC, Anderson SS, Degnim AC, Vierkant RA, Reynolds CA, et al. Evaluation of the Tyrer-Cuzick (International Breast Cancer Intervention Study) model for breast cancer risk prediction in women with atypical hyperplasia. *J Clin Oncol* 2010;28:3591-6.
- Pankratz VS, Hartmann LC, Degnim AC, Vierkant RA, Ghosh K, Vachon CM, et al. Assessment of the accuracy of the Gail model in women with atypical hyperplasia. *J Clin Oncol* 2008;26:5374-9.
- Milanese TR, Hartmann LC, Sellers TA, Frost MH, Vierkant RA, Maloney SD, et al. Age-related lobular involution and risk of breast cancer. *J Natl Cancer Inst* 2006;98:1600-7.

25. Janzen V, Forkert R, Fleming HE, Saito Y, Waring MT, Dombkowski DM, et al. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* 2006;443:421–6.
26. Krishnamurthy J, Ramsey MR, Ligon KL, Torrice C, Koh A, Bonner-Weir S, et al. p16INK4a induces an age-dependent decline in islet regenerative potential. *Nature* 2006;443:453–7.
27. Ressler S, Bartkova J, Niederegger H, Bartek J, Scharffetter-Kochanek K, Jansen-Durr P, et al. p16INK4A is a robust *in vivo* biomarker of cellular aging in human skin. *Aging Cell* 2006;5:379–89.
28. Rodier F, Campisi J. Four faces of cellular senescence. *J Cell Biol* 2011;192:547–56.
29. Baker DJ, Perez-Terzic C, Jin F, Pitel K, Niederlander NJ, Jegannathan K, et al. Opposing roles for p16Ink4a and p19Arf in senescence and ageing caused by BubR1 insufficiency. *Nat Cell Biol* 2008;10:825–36.
30. Coppe JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* 2010;5:99–118.
31. Coppe JP, Patil CK, Rodier F, Sun Y, Munoz DP, Goldstein J, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol* 2008;6:2853–68.

Cancer Prevention Research

p16^{INK4a} Expression and Breast Cancer Risk in Women with Atypical Hyperplasia

Derek C. Radisky, Marta Santisteban, Hal K. Berman, et al.

Cancer Prev Res 2011;4:1953-1960. Published OnlineFirst September 15, 2011.

Updated version	Access the most recent version of this article at: doi:10.1158/1940-6207.CAPR-11-0282
Supplementary Material	Access the most recent supplemental material at: http://cancerpreventionresearch.aacrjournals.org/content/suppl/2011/11/28/1940-6207.CAPR-11-0282.DC1

Cited articles	This article cites 31 articles, 8 of which you can access for free at: http://cancerpreventionresearch.aacrjournals.org/content/4/12/1953.full#ref-list-1
-----------------------	---

Citing articles	This article has been cited by 3 HighWire-hosted articles. Access the articles at: http://cancerpreventionresearch.aacrjournals.org/content/4/12/1953.full#related-urls
------------------------	---

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
----------------------	--

Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
-----------------------------------	--

Permissions	To request permission to re-use all or part of this article, use this link http://cancerpreventionresearch.aacrjournals.org/content/4/12/1953 . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.
--------------------	--