Hypothesis/Commentary

Role of Physical Activity in Modulating Breast Cancer Risk as Defined by *APC* and *RASSF1A* Promoter Hypermethylation in Nonmalignant Breast Tissue

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Abstract

Physical activity reduces breast cancer risk. Promoter hypermethylation of the tumor suppressor genes *APC* and *RASSF1A*, which is potentially reversible, is associated with breast cancer risk. We conducted a cross-sectional study in 45 women without breast cancer to determine the association of physical activity with promoter hypermethylation of *APC* and *RASSF1A* in breast tissue. We used quantitative methylation-specific PCR to test the methylation status of *APC* and *RASSF1A*, and questionnaires to assess study covariates and physical activity (measured in metabolic equivalent hours per week). In univariate analyses, the study covariate, benign breast biopsy number, was positively associated with promoter hypermethylation of *APC* ($P = 0.01$) but not *RASSF1A*. Mulitvariate logistic regression indicated that, although not significant, physical activities for a lifetime [odds ratio (OR), 0.57; 95% confidence interval (95% CI), 0.22-1.45; $P = 0.24$], previous 5 years (OR, 0.62; 95% CI, 0.34-1.12; $P = 0.11$), and previous year (OR, 0.72; 95% CI, 0.43-1.22; $P = 0.22$) were inversely related to promoter hypermethylation of *APC* but not *RASSF1A* for all physical activity measures. Univariate logistic regression indicated that physical activities for a lifetime, previous 5 years, and previous year were inversely associated with benign breast biopsy number, and these results were approaching significance for lifetime physical activity (OR, 0.41; 95% CI, 0.16-1.01; $P = 0.05$) and significant for physical activity in the previous 5 years (OR, 0.57; 95% CI, 0.34-0.94; $P = 0.03$). The study provides indirect evidence supporting the hypothesis that physical activity is inversely associated with promoter hypermethylation of tumor suppressor genes, such as *APC*, in nonmalignant breast tissue. (Cancer Epidemiol Biomarkers Prev 2007;16(2):192–6)

Introduction

Past observational studies have shown that physical activity reduces breast cancer risk among premenopausal and postmenopausal women and women of diverse races and ethnicities (1-4). Several of these studies showed that women who engage in 3 to 4 h per week of moderate to vigorous levels of exercise have a 30% to 40% lowered risk for breast cancer, ranging up to 70% for the most active women. In one study, physical activity was associated with a decreased risk for receptor-positive (estrogen receptor/progesterone receptor–positive) and receptor-negative (estrogen receptor/progesterone receptor–negative) breast cancers in premenopausal and postmenopausal women (3). Physical activity at adolescence was also found to be associated with a delayed age at breast cancer onset among women with *BRCA1* and *BRCA2* mutations, which are inherited risk factors for breast cancer (4).

It is thought that the relationship between physical activity and breast cancer risk may have a hormonal mechanism. Exercise interventions have been shown to decrease circulating estrogen levels in premenopausal and postmenopausal women (5, 6). High endogenous estrogen levels have been shown to be positively associated with breast cancer recurrence in postmenopausal women (7) and are linked to an increased risk for primary breast cancer (8, 9).

There is also growing evidence that estrogens play a dual role in the etiology of breast cancer: by stimulating cell proliferation (10) and by silencing genes implicated in breast carcinogenesis (11-13). DNA methylation in the promoter regions of tumor suppressor genes is a frequent mechanism of transcriptional silencing in breast cancer, as well as other cancers. This process has also been referred to as ''epigenetic'' and is potentially reversible (14). Cells that have accumulated these epigenetic alterations are prone to becoming tumor cells (14). Although the relationship between estrogen and the methylation of genes is unknown, there is some evidence that estrogen alters the methylation patterns of genes. Studies in mice have shown that diethylstilbestrol (15) and estradiol (16) elicit genetic methylation changes that result in heavier uteri (15) and uterine tumors (16). Recently, it was shown that estradiol and diethylstilbestrol induced promoter hypermethylation of the putative tumor suppressor genes *E-cadherin* and *p16* in nontumor human breast cells (12).

There is direct evidence that promoter hypermethylation of several tumor suppressor genes is associated with breast carcinogenesis (14). Studies have shown that promoter hypermethylation of the putative tumor suppressor genes *APC, RASSF1A, RARβ2, H-cadherin*, and *HIN1* occurs more frequently in breast cancer than in nonmalignant breast tissue adjacent to the breast cancer, and, although less frequent, these changes have been noted in the breast tissues of women without breast cancer (17-24). In a study, among women

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without breast cancer, it was shown that promoter hypermethylation of *APC* and *RASSF1A* was positively associated with the number of benign breast biopsies, classifying age as <50 or ≥50 years (24), as defined by the Gail mathematical model for breast cancer risk (25). Two or more benign breast biopsies before the age of 50 years has been shown to be strongly associated with breast cancer risk in several large prospective studies (26-29).

Several studies have shown that when breast cancer cells with promoter hypermethylation of *APC, RASSF1A*, and *RARβ2* are treated with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine, this leads to their demethylation and reexpression (18, 19, 30). Alternatively, lifestyle changes may reverse promoter methylation of tumor suppressor genes. A recent study found that two common catechol-containing polyphenols, caffeic acid and chlorogenic acid, partially inhibited methylation of the promoter region of the *RARβ2* gene in cultured MCF-7 and MDA-MB-231 human breast cancer cells (31).

Thus, because physical activity is associated with reduced breast cancer risk and lower circulating estrogen levels among premenopausal and postmenopausal women, and that estrogen may induce promoter hypermethylation of *APC* and *RASSF1A* in breast tissue, which are epigenetic markers of breast cancer risk, it is possible that physical activity decreases or reverses promoter hypermethylation of these tumor suppressor genes in nonmalignant breast tissue. Using this rationale, we conducted a cross-sectional study of premenopausal and postmenopausal women to investigate the a priori hypothesis that physical activity is inversely associated with promoter hypermethylation of tumor suppressor genes, such as *APC* and *RASSF1A*, in nonmalignant breast tissue. A related a priori hypothesis was that promoter hypermethylation of tumor suppressor genes, such as *APC* and *RASSF1A*, in nonmalignant breast tissue is positively associated with circulating estrogen levels. We assessed physical activity over a lifetime, as well as in the previous 5 years and the previous year, because gene promoter hypermethylation is potentially reversible over shorter intervals of time.

Materials and Methods

Study Design. The cross-sectional study involved premenopausal and postmenopausal women without breast cancer. The purpose of the study was to determine the association of (*a*) lifetime physical activity, (*b*) physical activity over the previous 5 years, and (*c*) physical activity over the previous year with the study outcome, promoter hypermethylation of *APC* and *RASSF1A* in nonmalignant breast tissue.

Study Population. The women that participated in this study were patients from The University of Texas Southwestern Mary L. Brown Genetics and Risk Assessment Center. One hundred six women without breast cancer underwent bilateral random periareolar fine-needle aspirate biopsies from 2000 through 2004 to determine the methylation status of *APC* and *RASSF1A* in their breast tissues. The University of Texas Southwestern Medical Center at Dallas institutional review board approved the study.

Assessment of Physical Activity and Factors Associated with Breast Cancer Risk. Lifetime physical activity was determined in these women up until the time of the breast fine-needle aspirate biopsies using an interviewer-administered questionnaire. This questionnaire uses cognitive recall and is reliable in terms of recall, including all types of physical activity (occupation, household, and exercise and sports activities) and measurement variables (frequency, intensity, and duration; ref. 32). Based on a compendium of physical activities and their associated metabolic equivalents (MET; ref. 33), we used MET-hours per week as the measure of physical activity during a lifetime, as well as in the previous 5 years and the previous year. One MET is defined as the energy expenditure for sitting quietly, which for the average adult is ~3.5 mL of oxygen × kg of body weight^{-1} × min^{-1} or kcal × kg of body weight^{-1} × height^{-1} (33).

Selected factors associated with breast cancer risk [age, age at menarche, parity, body mass index (BMI), number of biopsies for benign breast disease, family history of breast cancer in a first-degree relative, and menopausal status; refs. 34-36] were covariates for the study and were obtained with an interviewer-administered questionnaire.

Seventy-four women had up-to-date contact information (phone and address). The response rate for the study questionnaires was 61% (n = 45).

Assessment of Gene Methylation Status. We obtained breast epithelial cells using bilateral random periareolar fine-needle aspirate biopsies (37). Breast epithelial cells derived from the breast fine-needle aspirate biopsies were examined by cytology to exclude breast cancer cases. Genomic DNA from breast epithelial cells was used to run quantitative methylation-specific PCR (23) to determine whether the methylation status of *APC* and *RASSF1A* was positive or negative. The methylation status of a gene was scored as positive if >1% of the gene copies had promoter hypermethylation in one or both breasts. Using the cutoff of >1% methylated gene copies for *APC* and *RASSF1A*, we observed a significant difference in the incidence of promoter hypermethylation positivity between breast carcinoma (n = 40) and nonmalignant breast tissues (n = 106) obtained by fine-needle aspirate biopsy, with P = 0.0002 for *APC* and $P < 0.0001$ for *RASSF1A*.[4]

Statistical Analysis. We used Microsoft Access to create the study database. We used Fisher's exact test and χ^2 test to determine the association of the covariates [age, age at menarche, parity, body mass index (BMI), number of biopsies for benign breast disease, first-degree history of breast cancer, and menopausal status] with the study outcome, promoter hypermethylation of *APC* and *RASSF1A* in nonmalignant breast tissue, and the study covariate, number of benign breast biopsies. We used logistic regression analysis to determine the association of the study predictor variable, physical activity (lifetime physical activity, physical activity over the previous 5 years, and physical activity over the previous year), with the study outcome and the study covariate, number of benign breast biopsies, adjusting for covariates when they were significantly associated with the study outcome at $P < 0.05$. We used Statistical Analysis Software version 9.0 for all of the analyses.

Results

Table 1 presents the study participant characteristics in terms of breast cancer risk and menopausal status. All of the study participants were White with a mean age of 43 years; their mean BMI did not exceed or fall below the healthy weight range (>25 kg/m^2; ref. 38); their alcohol intake was not considered to be excessive (<15 g/d; ref. 34); and their age at menarche and parity status were representative of the general female population (25, 34). However, a large proportion of the study participants had a higher frequency of two breast cancer risk factors (25), a history of benign breast biopsies (29%) and a higher family history of breast cancer in a first-degree relative (78%; ref. 39). In addition, the study participants had a high frequency for another breast cancer risk factor, a history of exogenous estrogen use (oral contraceptives at 91% and hormone replacement therapy at 62%, if postmenopausal; refs. 40, 41). In addition, 4 of 45 (9%) women had taken the antiestrogen tamoxifen sometime in the past.

[4] D. Euhus, D. Bu, R. Ashfaq, C. Lewis, unpublished data.

Table 1. Study participant characteristics (*N* = 45)

Age, y (mean ± SD)	43 ± 7
Race/ethnicity	
White	45 (100%)
Non-White	0 (0%)
Age at menarche, y (mean ± SD)	12 ± 2
Parity (mean ± SD)	1 ± 1.1
BMI, kg/m^2 (mean ± SD)	23 ± 5
Lifetime physical activity, MET-h/wk/y (mean ± SD)	89.4 ± 14.0
Benign breast biopsies	13 (29%)
Family history of breast cancer	
Yes	35 (78%)
No	10 (22%)
Alcohol (≥15 g/d)	
Yes	0 (0%)
No	45 (100%)
Oral contraceptive use	
Ever	41 (91%)
Never	4 (9%)
Antiestrogen	
Ever	4 (9%)
Never	41 (91%)
Postmenopausal	16 (36%)
Hormone replacement therapy	10 (62%)
Tumor suppressor gene promoter hypermethylation	
APC	10 (22%)
RASSF1A	12 (27%)

In the univariate analyses, only two of the selected study participant characteristics that have been found to be associated with breast cancer risk (34-36) were associated with promoter hypermethylation of *APC* and *RASSF1A* (Table 2). Increasing age at menarche ($P = 0.01$ and $P = 0.04$ for ages 12-13 and ≥14 years, respectively) and ≥2 benign biopsies ($P = 0.01$) were positively and significantly associated with promoter hypermethylation of *APC*. None of the study participant characteristics listed in Table 2 were found to be significantly associated with the number of benign breast biopsies.

The multivariate and univariate logistic regression analysis results were related to the association of the study predictor variable, physical activity (measured in MET-hours per week) for a lifetime, over the previous 5 years, and the previous year, with the study outcome and the covariate, number of benign breast biopsies, as follows. Although not significant, lifetime physical activity [odds ratio (OR), 0.57; 95% confidence interval (95% CI), 0.22-1.45; $P = 0.24$], physical activity in the previous 5 years (OR, 0.62; 95% CI, 0.34-1.12; $P = 0.11$), and physical activity in the previous year (OR, 0.72; 95% CI, 0.43-1.22; $P = 0.22$) were inversely related to promoter hypermethylation of *APC* in nonmalignant breast tissue (Table 3). In comparison, the association of physical activities for a lifetime (OR, 1.09; 95% CI, 0.60-1.98, $P = 0.77$), previous 5 years (OR, 0.93; 95% CI, 0.68-1.25; $P = 0.62$), and previous year (OR, 1.07; 95% CI, 0.79-1.46; $P = 0.66$) with promoter hypermethylation of *RASSF1A* in nonmalignant breast tissue was not only nonsignificant but also was weak and unclear on direction (Table 3). However, as noted in Table 4, the physical activity components assessed were inversely associated with the study covariate, number of benign breast biopsies, which was trending toward significance for lifetime physical activity (OR, 0.41; 95% CI, 0.16-1.01; $P = 0.05$) and was significant for physical activity in the last 5 years (OR, 0.57; 95% CI, 0.34-0.94; $P = 0.03$).

Discussion

The study indicates that lifetime physical activity and physical activity in the previous 5 years and the previous year were inversely associated with promoter hypermethylation of *APC* in nonmalignant breast tissue, with the trend toward significance being stronger when assessed in the 5-year period before the assessment of promoter hypermethylation of *APC* in nonmalignant breast tissue. Furthermore, similar results were noted when the relationship between these physical activity measurements and the study covariate, number of benign breast biopsies, was determined, with nearly significant and significant results for the inverse relationship of lifetime physical activity ($P = 0.05$) and physical activity in the previous 5 years ($P = 0.03$), respectively. Promoter hypermethylation of *APC* in nonmalignant breast tissue, as a measure of breast cancer risk, was also positively and significantly associated with the number of benign breast biopsies, as was noted previously by Lewis et al. (24). Thus, the significant and positive relationship between the number of benign breast biopsies and promoter hypermethylation of *APC* and the significant inverse relationship between lifetime physical activity and number of breast biopsies imply that there is an inverse relationship between promoter hypermethylation of *APC* and physical activity. In addition, promoter hypermethylation of *APC* in nonmalignant breast tissue was positively and significantly associated with later age at menarche, which for unknown reasons has previously been reported to increase breast cancer risk among women with benign breast disease, as opposed to later age at menarche decreasing the risk for breast cancer among women without benign breast disease (42).

Study limitations were likely due to a small sample size, possible physical activity exposure misclassification due to inaccurate study participant recall, and not accounting for all of the confounders. However, we did account for the most important known confounders in the analysis based on a literature review (34-36). One possible confounder that we did not account for in the data analyses was a history of antiestrogen use (tamoxifen) because its effect on the methylation status of tumor suppressor genes involved in breast carcinogenesis is unknown. Thus, if tamoxifen was a confounder, we expect that it would have reduced or reversed the promoter hypermethylation of *APC* and *RASSF1A*, further limiting our chances for identifying a significant inverse association between physical activity and promoter hypermethylation of these

Table 2. Selected study participant (*N* = 45) factors associated with promoter hypermethylation of *APC* and *RASSF1A*

Factors	Promoter hypermethylation			
	APC		*RASSF1A*	
	n (%)	*P*	*n* (%)	*P*
Age, y				
<50	8 (27)		9 (30)	
≥50	3 (20)	0.73	5 (33)	1.00
Age at menarche, y				
<12	0 (0)		4 (29)	
12-13	8 (35)	0.01	6 (26)	1.00
≥14	3 (38)	0.04	4 (50)	0.39
Parity				
0	3 (30)		2 (20)	
1-2	7 (29)	1.00	8 (33)	0.68
>2	1 (9)	0.31	4 (36)	0.64
Postmenopausal				
Yes	5 (17)		8 (28)	
No	6 (38)	0.16	6 (38)	0.52
BMI, kg/m^2				
<25	7 (28)		9 (36)	
≥25	4 (20)	0.73	5 (25)	0.43
No. benign breast biopsies				
0	5 (16)		8 (26)	
1-2	1 (14)	1.00	2 (29)	1.00
>2	5 (71)	0.01	4 (57)	0.18
Family history of breast cancer				
Yes	2 (20)		2 (20)	
No	9 (26)	1.00	12 (34)	0.47

Table 3. Association of physical activity with promoter hypermethylation of *APC* and *RASSF1A* (*N* = 45)

Physical activity (MET-h/wk)	Promoter hypermethylation	
	*APC**	*RASSF1A*
	OR (95% CI), *P*	OR (95% CI), *P*
Lifetime	0.57 (0.22-1.45), 0.24	1.09 (0.60-1.98), 0.77
Previous 5 y	0.62 (0.34-1.12), 0.11	0.93 (0.68-1.25), 0.62
Previous year	0.72 (0.43-1.22), 0.22	1.07 (0.79-1.46), 0.66

*Adjusted for age at menarche and number of benign breast biopsies.

tumor suppressor genes. However, the major limitation for the study was that the study design was not ideal for identifying a dose-response relationship between higher levels of physical activity and the outcomes assessed, primarily because of the homogeneity of the study population in terms of a low physical activity variance, as noted in Table 1 (mean lifetime physical activity, 89.4 ± 14.0 MET-h/wk), compared with a previously published result for women without breast cancer in a population-based study that used the same physical activity measure (127.8 ± 45.5 MET-h/wk; ref. 43). In addition, the study sample size was not large enough to investigate the effect of different physical activity intensities on promoter hypermethylation of *APC* and *RASSF1A*. A large cohort study (*N* = 90,509) among women at high risk for breast cancer and between the ages of 40 and 65 years showed that higher levels of intensity for physical activity were associated with a greater risk reduction in breast cancer (44). In addition, other genes, besides *APC* and *RASSF1A*, that undergo promoter hypermethylation during breast carcinogenesis may also be important markers of breast cancer risk; however, because their status as markers of breast cancer risk is unknown, they were not included in the study.

Thus, although the study did not provide significant results about the association of physical activity with *APC* and *RASSF1A* promoter methylation status in nonmalignant breast tissue, it does provide indirect evidence to suggest that physical activity is inversely associated with promoter hypermethylation of *APC* in nonmalignant breast tissue. The basis for the inverse association of physical activity and promoter hypermethylation of tumor suppressor genes implicated in breast carcinogenesis, such as *APC*, is unknown; however, it is possible that physical activity prevents promoter hypermethylation of this tumor suppressor gene by decreasing circulating estrogen levels. Exercise interventions have been shown to decrease circulating estrogen levels in premenopausal and postmenopausal women (5, 6), and higher endogenous estrogen levels are linked with an increased risk for primary breast cancer (8, 9). There is also growing evidence that estrogens induce promoter hypermethylation of tumor suppressor genes implicated in breast carcinogenesis (12). Furthermore, given the overall study results, we believe that a randomized study would be the most effective and efficient approach to determine the effect of exercise on epigenetic markers for breast cancer risk, making provisions in the study to store tissue specimens that can be used in the future to study new markers of breast cancer risk. In addition, assessing the effect of exercise on circulating estrogen levels and their relationship with the promoter methylation status of *APC* and *RASSF1A* in nonmalignant breast tissue would provide data to further characterize the biological mechanisms involved in the development of breast cancer.

Additional support for conducting a randomized study in human subjects to test the effect of exercise on the promoter methylation status of *APC* and *RASSF1A* in nonmalignant breast tissue and potentially other genetic and molecular markers of breast cancer risk is that increasing intensity and duration of exercise have been shown to have a dose-dependent protective effect against carcinogen-induced mammary tumor progression in rats with *N*-methyl-*N*-nitrosourea (MNU) and 7,12-dimethylbenz(*a*)anthracene (DMBA; ref. 45). The study also showed that higher exercise intensity reduced mammary tumor occurrence induced by DMBA, but not by MNU, which may be due to an increase in DMBA metabolic deactivation because it requires metabolic activation by the mixed function oxidase system whereas MNU is a direct acting carcinogen (46). Thus, although the mechanism by which exercise decreases the progression of mammary carcinogens in this study is unknown, it is possible that exercise may promote the reversal or decrease in epigenetic changes induced by DMBA and MNU, such as tumor suppressor gene promoter hypermethylation.

Recent studies have shown that DMBA and MNU, as well as diethylstilbestrol and estradiol, induce promoter hypermethylation of tumor suppressor genes for which there is direct evidence that these epigenetic changes play a role in breast carcinogenesis (14). A recent study observed that there was reduced *FHIT* expression and *FHIT* promoter hypermethylation in rat mammary tumors induced by DMBA and MNU, and that these changes were reversed with the demethylating agent 5′-aza-2′-deoxycytidine (47). Thus, this study supports testing the hypothesis that physical activity is inversely associated with promoter hypermethylation of *APC* and *RASSF1A* in nonmalignant breast tissue. In addition, because diethylstilbestrol and estradiol have been shown to induce promoter hypermethylation of the tumor suppressor genes *p16* and *E-cadherin* in nontumor human breast cells (12), another hypothesis related to the study's hypothesis is that promoter hypermethylation of *APC* and *RASSF1A* in nonmalignant breast tissue is positively associated with circulating estrogen levels.

Conclusions

The study presents some of the first evidence to support testing the hypothesis that physical activity is inversely associated with promoter hypermethylation of tumor suppressor genes, such as *APC* and *RASSF1A*, in nonmalignant breast tissue. In addition, other genes that are known to undergo promoter hypermethylation during breast carcinogenesis and are likely to be associated with breast cancer risk need to be included, along with *APC* and *RASSF1A*, when testing this hypothesis in future studies. The study also indicates that a randomized exercise intervention study among women at high risk for breast cancer would provide the best opportunity to test this hypothesis, as well as to determine the relationship between promoter hypermethylation of these tumor suppressor genes and circulating estrogen levels. Furthermore, the results of the study emphasize the importance for further investigation on the association of physical activity with epigenetic markers for breast cancer, as well as other cancers for which there is evidence that physical activity reduces the risk for their development, because exercise interventions are safe and inexpensive and can easily be adopted by individuals in their own personal environments.

Table 4. Association of physical activity with number of benign breast biopsies (*N* = 45)

Physical activity (MET-h/wk)	No. benign breast biopsies	
	OR (95% CI)	*P*
Lifetime	0.41 (0.16-1.01)	0.05
Previous 5 y	0.57 (0.34-0.94)	0.03
Previous year	0.77 (0.52-1.15)	0.20

NOTE: Not adjusted for age, age at menarche, parity, menopausal status, BMI, and family history of breast cancer due to their insignificant association with number of benign breast biopsies.

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