Active and secondhand smoke exposure throughout life and DNA methylation in breast tumors

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Abstract

Purpose: Tobacco smoke exposure has been associated with altered DNA methylation. However, there is a paucity of information regarding tobacco smoke exposure and DNA methylation of breast tumors.

Methods: We conducted a case-only analysis using breast tumor tissue from 493 postmenopausal and 225 premenopausal cases in the Western New York Exposures and Breast Cancer (WEB) study. Methylation of nine genes (*SFN, SCGB3A1, RARB, GSTP1, CDKN2A, CCND2, BRCA1, FHIT,* and *SYK*) was measured with pyrosequencing. Participants reported their secondhand smoke (SHS) and active smoking exposure for seven time periods. Unconditional logistic regression was used to estimate odds ratios (OR) of having methylation higher than the median.

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Results: SHS exposure was associated with tumor DNA methylation among postmenopausal but not premenopausal women. Active smoking at certain ages was associated with increased methylation of *GSTP1, FHIT,* and *CDKN2A* and decreased methylation of *SCGB3A1* and *BRCA1* among both pre- and postmenopausal women.

Conclusion: Exposure to tobacco smoke may contribute to breast carcinogenesis via alterations in DNA methylation. Further studies in a larger panel of genes are warranted.

Keywords

Breast cancer; DNA methylation; tobacco; secondhand smoke; epigenetics

Introduction

There is inconsistent evidence that active smoking and secondhand smoke (SHS) exposure are associated with an increased risk of breast cancer. In a meta-analysis, active and secondhand smoking were weakly associated with elevated risk of breast cancer [summary relative risk (SRR) ever active smoking = 1.10 (95% CI: 1.09; 1.12); SRR passive smoke exposure =1.07 (95%CI: 1.02–1.13)] (1). Menopausal status has been reported to modify the association between active or passive smoke exposure and risk of breast cancer. For instance, active smoking was associated with increased risk of premenopausal, but not postmenopausal breast cancer, in a large prospective cohort study of African American women (2). The measure of association between active smoking or SHS exposure and risk of breast cancer also varies with the timing and intensity of exposure (3–5). These differences in risk estimates may reflect these physiologic changes throughout life that change the vulnerability of breast tissue to carcinogens. Tobacco smoke contains a myriad of carcinogens, several of which have been demonstrated to accumulate in breast tissue (6), including polycyclic aromatic hydrocarbons (PAHs) (7).

Epigenetic mechanisms are one possible way for exogenous exposures to contribute to breast carcinogenesis. Aberrant methylation of CpG sites, which can result in genomic instability, silencing of tumor suppressor genes, or activation of oncogenes, is frequently reported in cancer cells (reviewed by: (8)). Patterns of DNA methylation are tissue-specific (9). Active cigarette smoking has been associated with altered DNA methylation in peripheral blood among healthy adults (10). Tobacco smoke exposure has also been associated with altered methylation in lung (11), colorectal (12, 13) and prostate (14) tumors. SHS exposure has been associated with DNA methylation patterns of peripheral blood in healthy adults (15) and lung tumor tissue from never smokers (16). Although DNA methylation patterns are somewhat alterable, smoking-associated methylation events have been observed in peripheral blood of former smokers up to 22 years after smoking cessation (17). Furthermore, *in utero* exposure to tobacco has been associated with alterations in DNA methylation in umbilical cord blood(18) as well as in blood of children between ages three and five (19, 20) and up to age 17 (21).

Tobacco smoke exposure at the time of diagnosis has been previously associated with DNA methylation in breast tumor tissue (22, 23). However, to our knowledge, the association between lifetime exposure to tobacco smoke and DNA methylation in breast tumor tissue

has not been assessed. Alterations in DNA methylation are posited to be an early event in breast carcinogenesis (24, 25). Thus, considering exposures throughout life may elucidate an etiologically relevant window of exposure and reduce the potential for exposure misclassification. For instance, we have previously reported that the early life exposures, birth weight, being breastfed, and maternal age at delivery, were associated with methylation of tumor suppressor genes in breast tumors (26). Given the relatively stable nature of DNA methylation and the relevance of early life exposures in breast cancer etiology, we hypothesized that active smoking and SHS exposure throughout life would be associated with DNA methylation of nine tumor suppressor genes or oncogenes reported to be methylated in breast tumors.

Methods

The Western New York Exposures and Breast Cancer (WEB) study is a large, population-based case-control study of breast cancer conducted from 1996–2001, the details of which have been described previously (27–29). Cases were 1170 women with primary, histologically confirmed, incident breast cancer that were between ages 35–79 years at diagnosis. Women with breast cancer were interviewed within one year of diagnosis, most within six months (64%). All participants provided informed consent, and the study protocol was approved by the Institutional Review Boards of the University at Buffalo and of all participating institutions. The analyses reported herein are restricted to women with breast cancer who had DNA methylation measured from tumor samples. Information regarding tobacco smoke exposure and methylation data was available for 493 postmenopausal cases and 225 premenopausal cases. We have previously reported that these women tended to have larger tumors and were more likely to have metastatic disease at the time of diagnosis when compared with WEB study cases not included in methylation analyses (29).

Exposure assessment

The tobacco exposure assessment methods in the WEB study participants have been described previously (27). Medical history, diet, lifetime alcohol consumption, residential history, occupational history, and smoking history were compiled through extensive in-person interviews and self-administered questionnaires. During the interview participants were queried about secondhand smoke exposure from household co-inhabitants and coworkers for seven age periods: (1) <21 years, (2) 21–30, (3) 31–40, (4) 41–50, (5) 51–60, (6) 61–70, (7) >70. Participants were asked the number of people they lived with who smoked cigarettes, cigars, or pipes. They were also asked to report the number of years that they resided with these smokers for each specified time period. Participants also were asked to report their own smoking behaviors during this time period.

Pyrosequencing

As described previously (29), candidate genes were selected because they had previously been reported to be frequently methylated in breast tumors (30–38). The genes assessed in this analysis were as follows: secretoglobin, family 3A, member 1 (*SCGB3A1*); cyclin-dependent kinase inhibitor 2A (*CDKN2A)*; fragile histidine triad *(FHIT*); glutathione S-

transferase pi 1 *(GSTP1)*; stratifin (*SFN*); *breast* cancer gene 1 *(BRCA1)*; retinoic acid receptor, beta (*RARB)*; cyclin D2 (*CCND2*); and spleen tyrosine kinase *(SYK)*.

The Pyrosequencing system (Qiagen, Valencia, CA) was used to detect methylated CpG sites in sequencing reactions. Archived tumor samples were micro-dissected from fixed microscope slides. Five hundred nanograms of genomic DNA were treated with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research, Orange, CA). Bisulfite-treated DNA was amplified with specific primers for each gene of interest. The Pyro Mark Assay Design program and the Pyro Q-CpG software were used for primer designs and data analysis, respectively. Average methylation levels of individual CpG sites for each DNA sample were calculated. Primer sets, *SYK* (Cat. # PM00151816), *SCGB3A1* (Cat. # PM00022687), *GSTP1* (Cat. # PM00151809), *CDKN2A* (Cat. # 972012), *CCND2* (Cat. # PM00051674), were purchased from Qiagen. Sample size varied for each gene primarily because individual samples did not pass quality control for some of the assays.

Statistical analyses

We have previously observed differences in the association between several exposures and methylation by menopausal status (29, 39, 40), thus, all analyses were stratified by menopausal status. Analyses of associations of secondhand smoke exposure with methylation were restricted to participants who reported smoking less than 100 cigarettes in their entire life (never smokers). Unconditional logistic regression was employed to estimate odds ratios (OR) and 95% confidence intervals (CI) for odds of methylation above the median by exposure to SHS or active smoking. Participants that reported living with a smoker were defined as exposed to SHS for that period. Similarly, participants that reported actively smoking during each age period were considered active smokers for that period. We also assessed the total number of years of living with a smoker and the total number of pack-years. Total years were dichotomized at the median number of years among exposed women specific to menopausal status.

Methylation was defined as a value above the median value for each gene, which we have used to dichotomize methylation previously (29). Because we have previously reported that methylation is associated with tumor characteristics we considered adjusting for them in addition to other potential confounders(29). Covariates considered for adjustment in regression models were age; age at menarche; parity; body mass index; alcohol consumption (lifetime number of drinks per usual drinking day); race; and tumor characteristics including, histological grade, tumor size, estrogen receptor (ER) status, progesterone receptor status (PR), Human Epidermal Growth Factor Receptor 2 (HER2) status, and triple negative status. We included covariates that were hypothesized to be confounders in the association between tobacco exposure and methylation (age and ER status) or that changed the association between average methylation and SHS by at least 10%. Final models were adjusted for age and ER status. Because age of smoking initiation and pack-years are correlated, we further adjusted active smoking analyses for lifetime pack-years of smoking. Only estimates based on categories with 5 or more participants are presented herein.

Results

Site specific methylation percentages were available for 4–10 CpG sites for each gene. Methylation of individual CpG sites was averaged for each gene for each participant. The median Spearman's p value for the correlation between site-specific methylation values and the average methylation value for each gene was 0.75. Median methylation values for each gene by select characteristics are presented in Table 1.

The associations between active smoking throughout life and DNA methylation in breast tumor tissue among premenopausal women are presented in Table 2. Methylation of *SCGB3A1* was inversely associated with active smoking at before age 21 (OR = 0.25, 95% CI: 0.09; 0.75), between ages 21 and 30 (OR = 0.30, 95% CI: 0.10; 0.91) and between ages 41 and 50 (OR = 0.19, 95%CI: 0.04; 0.81). Smoking before age 21 was associated with increased odds of methylation of *GSTP1* (OR = 2.56, 95% CI: 1.00; 6.58). Smoking between age 31 and 40 was associated with lower odds of methylation of *BRCA1* (OR = 0.09, 95% CI: 0.02; 0.38).

Active smoking before age 41 was not associated with methylation of any gene in tumors from postmenopausal women (results not shown). Active smoking between age 41–50 was associated with increased odds of methylation of *FHIT* (OR= 4.64, 95%CI: 1.60; 13.51). Between age 51–60, active smoking was associated with increased odds of methylation of *GSTP1* (OR= 2.28, 95% CI: 1.00; 5.20) (Table 3). Results for the association between smoking status (never, former, current) and methylation above the median are presented in Table 4. We did not observe evidence of an association between smoking status and methylation among premenopausal women. Among postmenopausal women, current smokers had the highest odds of methylation of *CDKN2A* (OR current smokers vs. never smokers 2.12, 95% CI: 0.96; 4.69, *p* for trend = 0.02).

The exposure distribution in our study was relatively homogeneous, with a small number of women who were not exposed to SHS in early life. Period-specific analyses of SHS exposure resulted in many cells with sample size less than five and are not presented. We did not observe any statistically significant associations between cumulative years of SHS exposure or pack-years among premenopausal women (results not shown). Among postmenopausal women, cumulative SHS exposure was inversely associated with methylation of *BRCA1* and *SYK* and total number of pack-years was associated with higher methylation of *CDKN2A* in tumor tissue (OR = 1.95, 95% CI: 1.07; 3.54) (Table 5). Since ER status could potentially be on the causal pathway between tobacco smoke exposure and methylation we also considered select analyses not adjusted for ER status and note that the results were virtually identical (not shown).

Discussion

In our study, active smoking and SHS exposure throughout life were associated with altered methylation of the promoter region of several candidate genes in breast tumor tissue, both positively and negatively. While previous studies of tobacco smoke exposure and breast cancer risk have been inconsistent, our findings provide suggestive evidence that tobacco

smoke alters DNA methylation, potentially a mechanism contributing to breast carcinogenesis. We observed that exposures decades prior to diagnosis were associated with changes in methylation patterns, which supports the hypothesis that epigenetic alterations are an early event in breast carcinogenesis.

We observed that SHS exposure and active smoking were associated with both increased and decreased methylation of breast cancer related genes. Active and secondhand tobacco smoke exposure has been associated with increased and decreased methylation in studies of peripheral blood leukocytes of healthy adults and children (10, 41–44). Furthermore, treatment of four breast cancer cell lines with benzo(a)pyrene induced both hypomethylation and hypermethylation of repeat elements as well as tumor suppressor genes (45). Tobacco smoke exposure has also been associated with altered methylation tumors from lung (11), colorectal (12, 13) or prostate (14) tissue.

There have only been two prior reports on the association between exposure to cigarette smoke and patterns of methylation in breast tumors (22, 23). Active smoking was associated with hypermethylation of *ERa* in a study of 137 Iranian women (23). While we did not measure methylation of ERa; we did find that active smoking at different points in the life course was associated with increased odds of methylation of *GSTP1, FHIT,* and *CDKN2A*.

Our results were largely consistent with the results of White et al. (22). Synthetic log use, another source of PAH exposure, was associated with lower methylation of *BRCA1* (OR = 0.44, 95%CI: 0.30; 0.60) (22), and we found that SHS exposure and active smoking at certain points in time were associated with decreased methylation of *BRCA1*. Conversely, smoking was associated with increased methylation of *BRCA1* in DNA from peripheral blood leukocytes in a study of women without breast cancer (46). Methylation is tissue-specific (9) and these discordant findings could also be attributed to differences in the methods used to measure methylation.

We found that active smoking was associated with lower odds of methylation of *SCGB3A1* among premenopausal women only. Conversely, White et al reported that synthetic log use was associated with increased methylation of *SCGB3A1* (OR = 2.14, 95%CI: 1.34; 3.42) among all breast cancer cases (22). This discrepancy could be attributed to differences in the method used to measure methylation between studies, we used pyrosequencing while the previous study used the MethyLight assay, and the targets of the primers may be different. Alternatively, the impact of PAH exposure on methylation of *SCGB3A1* may vary by the timing or intensity of exposure. Furthermore, although both SHS and synthetic log use are sources of exposure to PAHs, they also contain different constituents that may also differentially impact DNA methylation.

SHS exposure after age 61 and active smoking between ages 41 to 50 was associated with increased methylation of *FHIT*. Our analyses of SHS exposure after age 61 were based on a very small number of cases. Previous studies have indicated that inactivation of *FHIT* via methylation contributes to the development of smoking-related lung cancer. For instance, increased methylation of *FHIT* was detected in bronchial lavage samples of cancer-free men and women who reported smoking for more than 40 pack-years (47). Compared with those

who had smoked less than 20 pack-years, those who had smoked greater than 40 pack-years were at a 13.10-fold increased odds of having methylation of *FHIT* (95% CI: 4.94; 63.96) (47). Mice exposed to environmental tobacco smoke for 30 days experienced a loss of *FHIT* expression as detected by immunohistochemistry, while mice exposed to filtered air did not (48). Our results extend the association between tobacco exposure and increased methylation of *FHIT* to breast tumor tissue.

The results of our study should be considered in the context of several limitations and strengths. Exposure to both SHS and active smoke were obtained by self-report. We utilized a case-only analysis where cases were not aware of the methylation status of their tumors nor the hypothesis of our study. Thus, bias from differential recall is less of a concern in our study. Furthermore, exposures were queried through an extensive in-person interview with trained interviewers, which further reduced potential recall bias. However, there is likely non-differential exposure misclassification resulting from error in recall of exposures in the past. Another source of potential exposure misclassification is the metric we used to estimate SHS exposure. SHS exposure was based on the participants' report of living with at least one person who smoked. Thus, this metric assumes that these individuals smoked in the presence of the participant, that the number of individuals smoking in a home did not impact the association, and that most SHS exposure occurred in participants' homes. Although the overall sample size of our study was relatively large, the analyses for certain sub-groups, especially analyses of exposure in later ages were hindered by sparse data. We made many comparisons and some of our findings could be due to chance. We did not adjust for multiple comparisons because we selected genes that were biologically relevant and did not rely on statistical significance to interpret our results (22, 49).

There are several important strengths of our study as well. It was population-based with a relatively large number of cases providing tumor tissues. We also assessed exposures throughout the life-course as opposed to only assessing recent exposures. These measures, while imperfect, may better target the etiologically relevant window of exposure. However, we note that several non-significant associations for different points in time were observed in the same direction as the statistically significant results we observed.

In conclusion, we found that SHS and active smoking throughout the life-course were associated with altered methylation of several breast cancer-related genes in DNA from breast tumor tissue. Our results support the hypothesis that exposure to tobacco smokes affects breast cancer risk and that epigenetic alterations in response to these exposures are an early event in breast carcinogenesis. Further studies across larger panels of genes with more subjects are warranted.

Acknowledgments

References

1. Macacu A, Autier P, Boniol M, Boyle P. (2015) Active and passive smoking and risk of breast cancer: a meta-analysis. Breast Cancer Res Treat. 154: 213–24. [PubMed: 26546245]

2. Rosenberg L, Boggs DA, Bethea TN, Wise LA, Adams-Campbell LL, Palmer JR. (2013) A prospective study of smoking and breast cancer risk among African American women. Cancer causes & control : CCC. 24: 10.1007/s10552-013-0298-6.

3. Catsburg C, Miller AB, Rohan TE. (2015) Active cigarette smoking and risk of breast cancer. Int J Cancer. 136: 2204–9. [PubMed: 25307527]

4. Dossus L, Boutron-Ruault MC, Kaaks R, et al. (2014) Active and passive cigarette smoking and breast cancer risk: results from the EPIC cohort. Int J Cancer. 134: 1871–88. [PubMed: 24590452]

5. Gram IT, Park SY, Kolonel LN, et al. (2015) Smoking and Risk of Breast Cancer in a Racially/Ethnically Diverse Population of Mainly Women Who Do Not Drink Alcohol: The MEC Study. Am J Epidemiol. 182: 917–25. [PubMed: 26493265]

6. Hecht SS. (2002) Tobacco smoke carcinogens and breast cancer. Environmental and molecular mutagenesis. 39: 119–26. [PubMed: 11921179]

7. MacNicoll A, Easty G, Neville A, Grover P, Sims P. (1980) Metabolism and activation of carcinogenic polycyclic hydrocarbons by human mammary cells. Biochemical and biophysical research communications. 95: 1599–606. [PubMed: 6774726]

8. McCabe MT, Brandes JC, Vertino PM. (2009) Cancer DNA methylation: molecular mechanisms and clinical implications. Clinical cancer research : an official journal of the American Association for Cancer Research. 15: 3927–37. [PubMed: 19509173]

9. Johnson KC, Koestler DC, Cheng C, Christensen BC. (2014) Age-related DNA methylation in normal breast tissue and its relationship with invasive breast tumor methylation. Epigenetics. 9: 268–75. [PubMed: 24196486]

10. Zhu X, Li J, Deng S, et al. (2016) Genome-Wide Analysis of DNA Methylation and Cigarette Smoking in a Chinese Population. Environmental health perspectives. 124: 966–73. [PubMed: 26756918]

11. Freeman JR, Chu S, Hsu T, Huang YT. (2016) Epigenome-wide association study of smoking and DNA methylation in non-small cell lung neoplasms. Oncotarget.

12. Weisenberger DJ, Levine AJ, Long TI, et al. (2015) Association of the colorectal CpG island methylator phenotype with molecular features, risk factors, and family history. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 24: 512–9.

13. Limsui D, Vierkant RA, Tillmans LS, et al. (2010) Cigarette smoking and colorectal cancer risk by molecularly defined subtypes. Journal of the National Cancer Institute. 102: 1012–22. [PubMed: 20587792]

14. Shui IM, Wong CJ, Zhao S, et al. (2016) Prostate tumor DNA methylation is associated with cigarette smoking and adverse prostate cancer outcomes. Cancer. 122: 2168–77. [PubMed: 27142338]

15. Reynolds LM, Magid HS, Chi GC, et al. (2016) Secondhand Tobacco Smoke Exposure Associations with DNA Methylation of the Aryl Hydrocarbon Receptor Repressor. Nicotine & tobacco research : official journal of the Society for Research on Nicotine and Tobacco.

16. Scesnaite A, Jarmalaite S, Mutanen P, et al. (2012) Similar DNA methylation pattern in lung tumours from smokers and never-smokers with second-hand tobacco smoke exposure. Mutagenesis. 27: 423–9. [PubMed: 22217548]

17. Ambatipudi S, Cuenin C, Hernandez-Vargas H, et al. (2016) Tobacco smoking-associated genome-wide DNA methylation changes in the EPIC study. Epigenomics. 8: 599–618. [PubMed: 26864933]

18. Ivorra C, Fraga MF, Bayon GF, et al. (2015) DNA methylation patterns in newborns exposed to tobacco in utero. Journal of translational medicine. 13: 25. [PubMed: 25623364]

19. Rzehak P, Saffery R, Reischl E, et al. (2016) Maternal Smoking during Pregnancy and DNA-Methylation in Children at Age 5.5 Years: Epigenome-Wide-Analysis in the European Childhood Obesity Project (CHOP)-Study. PLoS One. 11: e0155554.

20. Ladd-Acosta C, Shu C, Lee BK, et al. (2016) Presence of an epigenetic signature of prenatal cigarette smoke exposure in childhood. Environ Res. 144: 139–48. [PubMed: 26610292]

21. Lee KW, Richmond R, Hu P, et al. (2015) Prenatal exposure to maternal cigarette smoking and DNA methylation: epigenome-wide association in a discovery sample of adolescents and

replication in an independent cohort at birth through 17 years of age. Environmental health perspectives. 123: 193–9. [PubMed: 25325234]

22. White AJ, Chen J, Teitelbaum SL, et al. (2016) Sources of polycyclic aromatic hydrocarbons are associated with gene-specific promoter methylation in women with breast cancer. Environmental Research. 145: 93–100. [PubMed: 26671626]

23. Pirouzpanah S, Taleban FA, Atri M, Abadi A- R, Mehdipour P. (2010) The effect of modifiable potentials on hypermethylation status of retinoic acid receptor-beta2 and estrogen receptor-alpha genes in primary breast cancer. Cancer Causes & Control. 21: 2101–11. [PubMed: 20711807]

24. Johnson KC, Koestler DC, Fleischer T, et al. (2015) DNA methylation in ductal carcinoma in situ related with future development of invasive breast cancer. Clin Epigenetics. 7: 015–0094.

25. Muggerud AA, Ronneberg JA, Warnberg F, et al. (2010) Frequent aberrant DNA methylation of ABCB1, FOXC1, PPP2R2B and PTEN in ductal carcinoma in situ and early invasive breast cancer. Breast Cancer Res. 12: 7.

26. Tao MH, Marian C, Shields PG, et al. (2013) Exposures in early life: associations with DNA promoter methylation in breast tumors. Journal of developmental origins of health and disease. 4: 182–90. [PubMed: 25054684]

27. Bonner MR, Nie J, Han D, et al. (2005) Secondhand smoke exposure in early life and the risk of breast cancer among never smokers (United States). Cancer Causes & Control. 16: 683–9. [PubMed: 16049807]

28. Tao MH, Shields PG, Nie J, et al. (2009) DNA hypermethylation and clinicopathological features in breast cancer: the Western New York Exposures and Breast Cancer (WEB) Study. Breast cancer research and treatment. 114: 559–68. [PubMed: 18463976]

29. Callahan CL, Wang Y, Marian C, et al. (2016) DNA methylation and breast tumor clinicopathological features: The Western New York Exposures and Breast Cancer (WEB) study. Epigenetics. 1–10.

30. Xu X, Gammon MD, Jefferson E, et al. (2011) The influence of one-carbon metabolism on gene promoter methylation in a population-based breast cancer study. Epigenetics. 6: 1276–83. [PubMed: 22048254]

31. Feng W, Orlandi R, Zhao N, et al. (2010) Tumor suppressor genes are frequently methylated in lymph node metastases of breast cancers. BMC Cancer. 10: 1471–2407.

32. Jeong YJ, Jeong HY, Lee SM, Bong JG, Park SH, Oh HK. (2013) Promoter methylation status of the FHIT gene and Fhit expression: association with HER2/neu status in breast cancer patients. Oncol Rep. 30: 2270–8. [PubMed: 23969757]

33. Marzese DM, Hoon DS, Chong KK, et al. (2012) DNA methylation index and methylation profile of invasive ductal breast tumors. J Mol Diagn. 14: 613–22. [PubMed: 22925694]

34. Hsu NC, Huang YF, Yokoyama KK, Chu PY, Chen FM, Hou MF. (2013) Methylation of BRCA1 promoter region is associated with unfavorable prognosis in women with early-stage breast cancer. PLoS One. 8: 6.

35. Klajic J, Fleischer T, Dejeux E, et al. (2013) Quantitative DNA methylation analyses reveal stage dependent DNA methylation and association to clinico-pathological factors in breast tumors. BMC Cancer. 13: 1471–2407.

36. Arai T, Miyoshi Y, Kim SJ, Taguchi T, Tamaki Y, Noguchi S. (2006) Association of GSTP1 CpG islands hypermethylation with poor prognosis in human breast cancers. Breast Cancer Res Treat. 100: 169–76. [PubMed: 16791478]

37. Zhang X, Shrikhande U, Alicie BM, Zhou Q, Geahlen RL. (2009) Role of the protein tyrosine kinase Syk in regulating cell-cell adhesion and motility in breast cancer cells. Mol Cancer Res. 7: 634–44. [PubMed: 19435818]

38. Cheol Kim D, Thorat MA, Lee MR, et al. (2012) Quantitative DNA methylation and recurrence of breast cancer: a study of 30 candidate genes. Cancer Biomark. 11: 75–88. [PubMed: 23011154]

39. Tao MH, Marian C, Nie J, et al. (2011) Body mass and DNA promoter methylation in breast tumors in the Western New York Exposures and Breast Cancer Study. The American journal of clinical nutrition. 94: 831–8. [PubMed: 21775555]

40. Tao MH, Marian C, Shields PG, et al. (2011) Alcohol consumption in relation to aberrant DNA methylation in breast tumors. Alcohol (Fayetteville, N.Y.). 45: 689–99.

41. Zeilinger S, Kühnel B, Klopp N, et al. (2013) Tobacco Smoking Leads to Extensive Genome-Wide Changes in DNA Methylation. PLoS ONE. 8: e63812.

42. Dogan MV, Shields B, Cutrona C, et al. (2014) The effect of smoking on DNA methylation of peripheral blood mononuclear cells from African American women. BMC Genomics. 15: 151. [PubMed: 24559495]

43. Gao X, Jia M, Zhang Y, Breitling LP, Brenner H. (2015) DNA methylation changes of whole blood cells in response to active smoking exposure in adults: a systematic review of DNA methylation studies. Clinical Epigenetics. 7: 113. [PubMed: 26478754]

44. Joubert BR, Haberg SE, Nilsen RM, et al. (2012) 450K epigenome-wide scan identifies differential DNA methylation in newborns related to maternal smoking during pregnancy. Environmental health perspectives. 120: 1425–31. [PubMed: 22851337]

45. Sadikovic B, Rodenhiser DI. (2006) Benzopyrene exposure disrupts DNA methylation and growth dynamics in breast cancer cells. Toxicology and applied pharmacology. 216: 458–68. [PubMed: 16926039]

46. Peplonska B, Bukowska A, Wieczorek E, Przybek M, Zienolddiny S, Reszka E. (2017) Rotating night work, lifestyle factors, obesity and promoter methylation in BRCA1 and BRCA2 genes among nurses and midwives. PloS one. 12: e0178792.

47. Kim H, Kwon YM, Kim JS, et al. (2004) Tumor-specific methylation in bronchial lavage for the early detection of non-small-cell lung cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 22: 2363–70. [PubMed: 15197197]

48. D'Agostini F, Izzotti A, Balansky R, Zanesi N, Croce CM, De Flora S. (2006) Early loss of Fhit in the respiratory tract of rodents exposed to environmental cigarette smoke. Cancer Res. 66: 3936–41. [PubMed: 16585223]

49. Savitz DA, Olshan AF. (1995) Multiple comparisons and related issues in the interpretation of epidemiologic data. Am J Epidemiol. 142: 904–8. [PubMed: 7572970]

Table 1.

Median methylation values for selected genes, all cases, WEB study 1996–2001

Premenopausal	SFN	SCGB3A1	RARB	GSTP1	CDKN2A	CCND2	BRCA1	FHIT	SYK
				Median % methylation (IQR)					
Age									
30–39	62.5 (39.1)	67.8 (16.2)	3.3 (4.6)	5.6 (8.9)	3.0 (1.3)	9.2 (16.4)	3.8 (10.5)	6.8 (8.2)	2.5 (4.1)
40–49	53.6 (40.8)	66.8 (13.3)	5.4 (10.1)	7.2 (12.3)	2.6 (3.0)	6.7 (10.6)	4.2 (5.7)	6.4 (14.2)	2.8 (3.9)
50–59	51.9 (42.2)	61.6 (14.7)	4.3 (9.9)	5.8 (28.5)	3.6 (3.5)	13.5 (14.1)	4.1 (5.3)	1.7 (4.1)	2.0 (3.7)
ER status									
negative	59.4 (42.0)	63.0 (15.3)	5.2 (10.0)	6.4 (8.0)	2.9 (2.3)	5.4 (10.6)	3.9 (7.0)	2.3 (7.7)	2.6 (5.1)
positive	51.1 (41.3)	68.9 (12.2)	4.9 (10.1)	9.0 (15.9)	2.7 (3.4)	9.2 (12.7)	3.6 (4.6)	5.4 (13.4)	2.7 (3.6)
Smoking status									
never	53.0 (43.0)	67.2 (15.2)	4.7 (11.3)	6.1 (12.1)	2.7 (2.5)	8.5 (17.8)	4.3 (6.2)	3.5 (10.6)	2.1 (4.5)
former	56.5 (40.0)	64.4 (10.2)	4.8 (10.6)	8.2 (13.1)	2.8 (2.0)	6.9 (9.9)	3.8 (7.0)	4.7 (11.6)	2.8 (3.8)
current	55.4 (34.6)	67.0 (10.1)	5.4 (7.0)	7.2 (22.4)	3.5 (3.9)	6.8 (12.5)	4.4 (6.3)	5.9 (15.6)	3.8 (3.8)
Postmenopausal									
Age									
40–49	68.0 (37.4)	58.3 (14.3)	2.1 (4.6)	4.1 (28.4)	2.3 (2.2)	4.3 (7.0)	2.3 (7.9)	4.5 (3.5)	1.9 (2.0)
50–59	59.2 (35.3)	62.7 (11.8)	3.7 (6.9)	6.7 (10.4)	2.6 (1.9)	7.5 (11.9)	4.0 (5.1)	4.5 (15.5)	2.5 (4.0)
60–69	50.3 (39.7)	65.0 (14.1)	5.4 (12.2)	6.7 (11.9)	2.6 (2.4)	12.3 (23.0)	4.5 (6.6)	12.8 (21.8)	1.9 (3.8)
70	50.7 (46.4)	68.2 (19.5)	2.7 (5.1)	7.3 (14.6)	2.7 (3.7)	13.9 (19.2)	4.7 (5.1)	7.3 (20.8)	3.0 (5.3)
ER status									
negative	60.4 (29.0)	57.1 (19.1)	2.3 (10.8)	6.0 (8.2)	2.7 (2.3)	8.3 (18.4)	5.1 (8.4)	12.2 (22.3)	2.6 (3.3)
positive	51.6 (42.7)	65.4 (13.4)	3.5 (6.7)	6.7 (12.8)	2.6 (2.7)	9.7 (21.2)	4.5 (5.2)	7.6 (19.5)	2.2 (4.2)
Smoking status									
never	54.6 (39.0)	64.8 (14.7)	3.6 (8.9)	6.0 (13.0)	2.5 (2.0)	9.7 (22.0)	4.0 (5.6)	9.2 (21.7)	2.5 (4.1)
former	54.5 (39.8)	62.8 (14.4)	4.0 (9.2)	6.4 (12.7)	2.8 (3.0)	8.9 (21.7)	4.7 (6.3)	9.3 (17.1)	2.4 (4.3)
current	52.8 (40.8)	66.6 (13.6)	3.2 (8.1)	8.4 (10.0)	2.9 (3.2)	11.3 (11.4)	3.4 (6.4)	4.9 (15.0)	2.0 (3.4)

Abbreviations: IQR, inter-quartile range; ER, estrogen receptor; SFN, stratifin; SCGB3A1, secretoglobin, family 3A, member 1; RARB, retinoic acid receptor, beta; GSTP1, glutathione S-transferase pi 1; CDKN2A, cyclin-dependent kinase inhibitor 2A; CCND2, cyclin D2; BRCA1, breast cancer gene 1; FHIT, fragile histidine triad; SYK, spleen tyrosine kinase.

Table 2.

Association between active smoking with breast tumor DNA methylation, premenopausal cases

	Active smoking age <21				Active smoking age 21–30				Active smoking age 31–40				Active smoking age 41–50			
SMK	N >M	N <M	OR[a]	(95% CI)	N >M	N <M	OR[a]	(95% CI)	N >M	N <M	OR[a]	(95% CI)	N >M	N <M	OR[a]	(95% CI)
SFN																
No	33	39	1.00	(referent)	33	38	1.00	(referent)	45	47	1.00	(referent)	43	50	1.00	(referent)
Yes	30	30	1.22	(0.49; 3.00)	30	31	1.07	(0.40; 2.81)	19	22	0.49	(0.13; 1.89)	10	12	0.96	(0.26; 3.47)
SCGB3A1																
No	43	20	1.00	(referent)	40	19	1.00	(referent)	50	29	1.00	(referent)	51	30	1.00	(referent)
Yes	32	26	**0.25**	**(0.09; 0.75)**	35	27	**0.30**	**(0.10; 0.91)**	25	18	0.55	(0.16; 1.96)	9	12	**0.19**	**(0.04; 0.81)**
RARB																
No	38	31	1.00	(referent)	37	29	1.00	(referent)	50	39	1.00	(referent)	50	39	1.00	(referent)
Yes	37	24	1.29	(0.51; 3.27)	38	26	1.10	(0.42; 2.89)	25	17	0.96	(0.31; 3.02)	15	7	1.67	(0.46; 5.98)
GSTP1																
No	30	39	1.00	(referent)	31	37	1.00	(referent)	45	42	1.00	(referent)	44	46	1.00	(referent)
Yes	41	23	**2.56**	**(1.00; 6.58)**	40	25	1.98	(0.73; 5.41)	27	20	0.78	(0.25; 2.44)	18	8	1.78	(0.55; 5.75)
CDKN2A																
No	29	30	1.00	(referent)	26	28	1.00	(referent)	36	38	1.00	(referent)	36	42	1.00	(referent)
Yes	34	25	1.49	(0.54; 4.14)	37	27	1.66	(0.59; 4.65)	27	18	1.94	(0.59; 6.46)	14	11	1.51	(0.50; 4.62)
CCND2																
No	21	19	1.00	(referent)	18	18	1.00	(referent)	24	24	1.00	(referent)	24	27	1.00	(referent)
Yes	16	23	0.69	(0.18; 2.62)	19	24	1.09	(0.29; 4.01)	13	19	0.90	(0.20; 4.13)	5	9	0.66	(0.14; 3.03)
BRCA1																
No	40	43	1.00	(referent)	38	43	1.00	(referent)	55	50	1.00	(referent)	49	56	1.00	(referent)
Yes	29	39	0.66	(0.28; 1.53)	31	39	0.77	(0.32; 1.88)	15	32	**0.09**	**(0.02; 0.38)**	9	14	0.60	(0.19; 1.87)
FHIT																
No	14	22	1.00	(referent)	16	20	1.00	(referent)	20	26	1.00	(referent)	20	30	1.00	(referent)
Yes	15	17	1.50	(0.43; 5.27)	13	19	0.71	(0.17; 2.97)	9	13	0.80	(0.13; 5.08)	6	6	2.17	(0.38; 12.41)
SYK																
No	41	42	1.00	(referent)	41	42	1.00	(referent)	53	54	1.00	(referent)	53	52	1.00	(referent)

| SMK | Active smoking age <21 | | | SMK | Active smoking age 21 –30 | | | SMK | Active smoking age 31–40 | | | SMK | Active smoking age 41–50 | | |
	N	OR[a]	(95% CI)		N	OR[a]	(95% CI)		N	OR[a]	(95% CI)		N	OR[a]	(95% CI)
	>M	<M			>M	<M			>M	<M			>M	<M	
Yes	40	31	1.42	(0.60; 3.37)	Yes	40	31	1.45	(0.57; 3.67)	Yes	29	19	2.78	(0.83; 9.31)	Yes	18	10	1.80	(0.58; 5.65)

[a] Odds ratios (OR) for methylation above the median value and 95% confidence intervals (CI) were estimated with unconditional logistic regression model adjusted for age in years, lifetime pack-years, and estrogen receptor status. Abbreviations: Smk, smoking; OR, odds ratio; *SFN*, stratifin; *SCGB3A1*, secretoglobin, family 3A, member 1; *RARB*, retinoic acid receptor, beta; *GSTP1*, glutathione S-transferase pi 1; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *CCND2*, cyclin D2; *BRCA1*, breast cancer gene 1; *FHIT*, fragile histidine triad; *SYK*, spleen tyrosine kinase

Table 3.

Association between active smoking and breast tumor DNA methylation, postmenopausal cases

	Active smoking age 41–50					Active smoking age 51–60			
	N		**OR**	**(95% CI)**			**N**		**OR**	**(95% CI)**
	>M	**<M**					**>M**	**<M**		
SFN										
No	83	93	1.00	(referent)		No	88	102	1.00	(referent)
Yes	48	46	1.00	(0.48; 2.08)		Yes	35	30	1.11	(0.49; 2.55)
SCGB3A1										
No	75	94	1.00	(referent)		No	79	100	1.00	(referent)
Yes	41	50	1.02	(0.47; 2.21)		Yes	31	32	1.76	(0.75; 4.15)
RARB										
No	84	107	1.00	(referent)		No	91	119	1.00	(referent)
Yes	46	65	0.89	(0.46; 1.74)		Yes	33	45	0.99	(0.47; 2.09)
GSTP1										
No	83	104	1.00	(referent)		No	95	108	1.00	(referent)
Yes	58	44	1.88	(0.94; 3.77)		Yes	41	27	**2.28**	**(1.00; 5.20)**
CDKN2A										
No	67	93	1.00	(referent)		No	79	97	1.00	(referent)
Yes	57	40	2.07	(0.93; 4.64)		Yes	40	27	1.64	(0.73; 3.70)
CCND2										
No	58	55	1.00	(referent)		No	60	60	1.00	(referent)
Yes	27	31	0.92	(0.34; 2.47)		Yes	21	20	1.47	(0.49; 4.43)
BRCA1										
No	111	102	1.00	(referent)		No	123	106	1.00	(referent)
Yes	66	58	1.25	(0.66; 2.34)		Yes	45	43	1.00	(0.50; 2.01)
FHIT										
No	51	51	1.00	(referent)		No	65	50	1.00	(referent)
Yes	37	23	**4.64**	**(1.60; 13.51)**		Yes	22	18	1.48	(0.51; 4.31)
SYK										
No	105	121	1.00	(referent)		No	116	126	1.00	(referent)
Yes	58	68	0.92	(0.49; 1.72)		Yes	40	50	0.80	(0.40; 1.59)

[a]Odds ratios (OR) for methylation above the median value and 95% confidence intervals (CI) were estimated with unconditional logistic regression model adjusted for age in years, estrogen receptor status, and lifetime pack-years. Abbreviations: M, median; OR, odds ratio; *SFN*, stratifin; *SCGB3A1*, secretoglobin, family 3A, member 1; *RARB*, retinoic acid receptor, beta; *GSTP1*, glutathione S-transferase pi 1; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *CCND2*, cyclin D2; *BRCA1*, breast cancer gene 1; *FHIT*, fragile histidine triad; *SYK*, spleen tyrosine kinase

Table 4.

Association between smoking status at interview and breast tumor DNA methylation, all cases, WEB study

Gene	Smoking status	Premenopausal				Postmenopausal			
		>M	<M	OR^a	(95% CI)	>M	<M	OR^a	(95% CI)
SFN	Never	30	37	1.00	(referent)	63	66	1.00	(referent)
	Former	25	21	1.45	(0.67; 3.13)	57	55	1.09	(0.66; 1.82)
	Current	9	11	1.00	(0.35; 2.92)	11	18	0.58	(0.25; 1.35)
	p for trend			0.72				0.45	
SCGB3A1	Never	39	19	1.00	(referent)	54	68	1.00	(referent)
	Former	22	20	0.35	(0.14; 0.89)	45	62	0.92	(0.54; 1.59)
	Current	14	8	0.46	(0.14; 1.50)	17	14	1.80	(0.79; 4.11)
	p for trend			0.09				0.35	
RARB	Never	36	29	1.00	(referent)	58	80	1.00	(referent)
	Former	27	18	1.19	(0.54; 2.62)	62	71	1.20	(0.74; 1.94)
	Current	12	9	1.04	(0.36; 2.98)	10	22	0.59	(0.26; 1.36)
	p for trend			0.84				0.59	
GSTP1	Never	28	36	1.00	(referent)	63	74	1.00	(referent)
	Former	29	17	2.18	(0.99; 4.79)	60	61	1.17	0.72; 1.92)
	Current	15	9	2.17	(0.78; 6.04)	18	14	1.62	(0.74; 3.54)
	p for trend			0.06				0.23	
CDKN2A	Never	26	27	1.00	(referent)	48	70	1.00	(referent)
	Former	24	21	1.15	(0.51; 2.59)	57	49	1.69	(1.00; 2.89)
	Current	13	8	1.59	(0.53; 4.72)	19	14	2.12	(0.96; 4.69)
	p for trend			0.43				**0.02**	
CCND2	Never	18	18	1.00	(referent)	41	36	1.00	(referent)
	Former	13	14	0.80	(0.27; 2.33)	35	41	0.73	(0.38; 1.40)
	Current	6	11	0.39	(0.11; 1.42)	9	9	1.02	(0.36; 2.92)
	p for trend			0.17				0.66	
BRCA1	Never	38	39	1.00	(referent)	76	78	1.00	(referent)
	Former	23	32	0.75	(0.37; 1.52)	84	58	1.50	(0.94; 2.37)
	Current	9	11	0.88	(0.31; 2.47)	17	24	0.75	(0.37; 1.53)
	p for trend			0.60				0.86	
FHIT	Never					41	36	1.00	(referent)
	Former					40	29	1.28	(0.66; 2.49)
	Current					7	10	0.69	(0.24; 2.05)
	p for trend							0.89	
SYK	Never	38	41	1.00	(referent)	79	85	1.00	(referent)
	Former	31	22	1.60	(0.78; 3.27)	70	77	0.99	(0.63; 1.54)
	Current	13	10	1.56	(0.59; 4.11)	14	28	0.56	(0.27; 1.16)
	p for trend			0.23				0.22	

^{a}Odds ratios (OR) for methylation above the median value and 95% confidence intervals (CI) were estimated with unconditional logistic regression model adjusted for age in years, estrogen receptor status, and lifetime pack-years. Abbreviations: M, median; OR, odds ratio; SFN, stratifin;

SCGB3A1, secretoglobin, family 3A, member 1; RARB, retinoic acid receptor, beta; GSTP1, glutathione S-transferase pi 1; CDKN2A, cyclin-dependent kinase inhibitor 2A; CCND2, cyclin D2; BRCA1, breast cancer gene 1; FHIT, fragile histidine triad; SYK, spleen tyrosine kinase

Table 5.

Association of cumulative secondhand smoke (SHS) exposure and lifetime pack-years active smoking (pkyrs) with breast tumor DNA methylation, postmenopausal cases, WEB study

	Secondhand smoke				Active smoking				
SHS	**N**		**OR**^{a}	**95% CI**	**Pkyrs**	**N**		**OR**^{a}	**95% CI**
	>M	**<M**				**>M**	**<M**		
SFN									
None	10	8	1.00	(referent)	None	63	66	1.00	(referent)
30	24	28	0.67	(0.23; 2.00)	18.9	30	38	0.82	(0.45; 1.49)
>30	29	30	0.78	(0.27; 2.30)	>18.9	38	35	1.12	(0.63; 1.99)
SCGB3A1									
None	10	6	1.00	(referent)	None	54	68	1.00	(referent)
30	20	33	0.33	(0.10; 1.08)	18.9	30	35	1.10	(0.59; 2.05)
>30	24	29	0.46	(0.14; 1.47)	>18.9	32	41	1.05	(0.57; 1.91)
RARB									
None	8	11	1.00	(referent)	None	59	80	1.00	(referent)
30	29	35	1.14	(0.40; 3.20)	18.9	34	44	1.04	(0.59; 1.82)
>30	21	34	0.86	(0.30; 2.48)	>18.9	37	49	1.01	(0.59; 1.75)
GSTP1									
None	9	7	1.00	(referent)	None	63	75	1.00	(referent)
30	34	27	1.03	(0.34; 3.14)	18.9	36	38	1.16	(0.65; 2.04)
>30	31	29	1.20	(0.39; 3.65)	>18.9	42	36	1.42	(0.81; 2.49)
CDKN2A									
None	8	8	1.00	(referent)	None	49	70	1.00	(referent)
30	27	23	0.85	(0.27; 2.62)	18.9	34	32	1.52	(0.83; 2.78)
>30	35	17	0.48	(0.15; 1.51)	>18.9	41	31	**1.95**	**(1.07; 3.54)**
CCND2									
					None	41	36	1.00	(referent)
					18.9	22	22	0.87	(0.41; 1.85)
					>18.9	22	28	0.71	(0.34; 1.46)
BRCA1									
None	14	5	1.00	(referent)	None	76	79	1.00	(referent)
30	32	34	0.33	(0.11; 1.03)	18.9	52	36	1.54	(0.91; 2.63)
>30	30	39	**0.27**	**(0.09; 0.83)**	>18.9	49	45	1.15	(0.68; 1.92)
FHIT									
					None	41	36	1.00	(referent)
					18.9	26	17	1.45	(0.67; 3.12)
					>18.9	21	22	0.89	(0.42; 1.91)
SYK									
None	14	5	1.00	(referent)	None	79	86	1.00	(referent)
30	34	38	**0.29**	**(0.09; 0.92)**	18.9	41	52	0.89	(0.53; 1.49)

	Secondhand smoke				Active smoking			
SHS	**N**		**OR**[a]	**95% CI**	**Pkyrs**	**N**		**OR**[a]	**95% CI**
	>M	**<M**				**>M**	**<M**		
>30	31	42	**0.23**	**(0.07; 0.73)**	>18.9	43	52	0.90	(0.54; 1.50)

[a]Odds ratios (OR) for methylation above the median value and 95% confidence intervals (CI) were estimated with unconditional logistic regression model adjusted for age in years and estrogen receptor status. Abbreviations: SHS, secondhand smoke; M, median; OR, odds ratio; SFN, stratifin; *SCGB3A1*, secretoglobin, family 3A, member 1; *RARB*, retinoic acid receptor, beta; *GSTP1*, glutathione S-transferase pi 1; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *CCND2*, cyclin D2; *BRCA1*, breast cancer gene 1; *FHIT*, fragile histidine triad; *SYK*, spleen tyrosine kinase