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SHORT REPORT

Barbara Sarter · Tiffany I. Long · Wan H. Tsong
Woon-Puay Koh · Mimi C. Yu · Peter W. Laird

Sex differential in methylation patterns of selected genes in Singapore Chinese

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Abstract To date there have been few reports of a gender difference in methylation levels of genes. When examining the methylation levels of four autosomal genes (*ESR1*, *MTHFR*, *CALCA* and *MGMT*) in the white blood cells of a random sample of Singapore Chinese Health Study cohort participants ($n = 291$), we encountered an unexpected gender differential. Using MethyLight technology, we calculated a gene-specific percentage of methylated reference (PMR) value, which quantified the relative level of gene methylation for each study subject (134 males and 157 females). Two summary methylation indices were constructed by assigning gene-specific rank scores. We then used ANCOVA to compare logarithmically transformed individual PMR values and summary methylation indices by age and gender simultaneously. Adjustment was made for plasma homocysteine. For *ESR1*, for which a large proportion of subjects were negative for methylation, we also used polytomous regression to compare methylation across age and gender. Increasing age and the male gender independently predicted increasing PMR values for *CALCA* and MGMT. For the *MTHFR* gene, male gender was associated with higher PMR values ($P = 0.002$), while age was not ($P = 0.75$). Neither age nor gender had any statistically significant influence on the PMR values for *ESR1* ($P = 0.13$ and 0.96, respectively). Our data suggest that gender is at least as strong a predictor of methylation level in the four genes under study as age, with males showing higher PMRs.

B. Sarter · T. I. Long · W. H. Tsong · M. C. Yu · P. W. Laird (✉)
Kenneth Norris Comprehensive Cancer Center,
Keck School of Medicine,
University of Southern California,
Los Angeles, CA 90089, USA
E-mail: plaird@usc.edu
Tel.: +1-323-8650650
Fax: +1-323-8650102

W.-P. Koh
National University of Singapore, Singapore

Introduction

The Singapore Chinese Health Study is a prospective cohort investigation of diet and cancer risk among 63,000 Chinese men and women, aged 45–74 years, who were enrolled during 1993–1998 (Hankin et al. 2001; Esteller 2003). We examined the methylation levels of four autosomal genes (*ESR1*, *MTHFR*, *CALCA* and *MGMT*) in the white blood cells of a random sample of cohort participants ($n = 291$). Below, we report an unexpected finding of a sex differential in methylation levels of these genes.

Materials and methods

The DNA was extracted from leukocytes collected from 134 males and 157 females cohort subjects. Sodium bisulfite conversion of genomic DNA was conducted. Methylation levels of the promoter regions were determined using MethyLight technology as described elsewhere (Eads et al. 2000). Primers and probes included a methylated set for the gene of interest and two reference sets, comprising the β-actin (*ACTB*) and collagen 2A1 (*COL2A1*) genes, to normalize the input DNA. Using the reference DNA, we calculated a gene-specific percentage of methylated reference (PMR) value, which quantified the relative level of gene methylation, for each study subject. The number of subjects with non-informative PMR values were: *ESR1*, 3; *MTHFR*, 2; *CALCA*, 2 and *MGMT*, 1. There were 283 subjects with informative PMR values for all four genes under study.

The PMR is a relative measure, so we converted the PMR for each gene to two ranked indices for our data analysis. The summary methylation indices (S1 and S2) for the four studied genes were constructed as follows. For S1, we assigned four rank values (range, 1 to the maximum number of informative subjects for each gene) to each study subject according to their respective PMR values for each gene. Their gene-specific rank values

Table 1 Adjusted means (95% confidence interval) of S1 and S2, two indices summarizing the extent of methylation in promoter regions of *ESR1*, *MTHFR*, *CALCA* and *MGMT*, according to gender and age, Singapore Chinese Health Study

	S1	S2
Total men ($n=129$)	6.40 (5.97, 6.82)	4.26 (3.85, 4.68)
55–59 years ($n=42$)	5.60 (4.92, 6.28)	3.38 (2.72, 4.04)
60–64 years ($n=35$)	5.61 (4.86, 6.36)	3.58 (2.85, 4.32)
65–69 years ($n=34$)	7.43 (6.66, 8.19)	5.24 (4.49, 5.99)
70+ years ($n=18$)	6.95 (5.89, 8.02)	4.85 (3.80, 5.89)
Total women ($n=154$)	5.41 (5.03, 5.79)	3.33 (2.96, 3.70)
55–59 years ($n=64$)	5.26 (4.69, 5.83)	3.23 (2.67, 3.79)
60–64 years ($n=34$)	5.19 (4.44, 5.94)	3.00 (2.26, 3.74)
65–69 years ($n=25$)	5.35 (4.47, 6.23)	3.26 (2.39, 4.12)
70+ years ($n=31$)	5.84 (5.06, 6.64)	3.84 (3.06, 4.61)
***P* for trend (age)**	0.003	0.002
***P* value (gender)**	0.001	0.002

were then summed, and the summation was divided by 100 to form the S1 index. For S2, we first assigned three tertile values (0 = 1st tertile, 1 = 2nd tertile, 2 = 3rd tertile) to each study subject according to their respective PMR values for the three genes, *MTHFR*, *CALCA* and *MGMT*. For the *ESR1* gene, which had a high percentage of zero PMR values (which were assigned a score of zero), below median positive values were assigned a score of 1, and above median values were given a score of 2. These gene-specific ranked scores were then summed to form S2 (range, 0–8).

We used the analysis of covariance method (Snedecor and Cochran 1967) to compare logarithmically transformed individual PMR values and summary methylation indices (S1 and S2) by age and gender simultaneously, with adjustment for logarithmically transformed values of plasma homocysteine. Since a large proportion of study subjects (50%) were negative for *ESR1* methylation, we also used the polytomous regression method (Hosmer and Lemeshow 1989) on three levels of *ESR1* methylation (zero, below median positive values and above median positive values) to compare *ESR1* methylation across age and gender. Results of the two parallel analyses were similar. All *P* values quoted are two-sided. The *P* values less than 0.05 are considered statistically significant.

Results

Increasing age and the male gender independently predicted increasing PMR values for *CALCA* and *MGMT* (*P* for age = 0.02 and 0.0006, respectively; *P* for gender = 0.03 and 0.0003, respectively). For both genes, the increase in PMR values by age was more pronounced in men than women (*P* for age × gender interaction = 0.01 and 0.0001, respectively). For the *MTHFR* gene, the male gender was associated with higher PMR values ($P=0.002$), while age was not ($P=0.75$). Neither age nor gender had any statistically significant influence on the PMR values for *ESR1* ($P=0.13$ and 0.96, respectively).

Both summary methylation indices, S1 and S2, showed strong and independent associations with age and gender. Older age and the male gender were related to increased methylation scores (Table 1).

Discussion

To date there have been few reports of a gender difference in methylation levels of genes. Our data suggest that gender is at least as strong a predictor of methylation level in the genes under study as age, with males showing higher PMRs. The lack of a significant influence of gender and age on the PMR values of *ESR1* may be due to the large number of zero PMR values.

Aberrant methylation is an epigenetic change that can modulate expression of cancer-related genes (Esteller 2003; Jones 2002; Lee et al. 2003; Levine et al. 2003; Wajed et al. 2001); therefore, the finding of a gender differential in promoter hypermethylation of three of four studied genes has potentially important implications. More study is needed to better define possible gender differences in methylation patterns, how they alter gene expression especially in disease-related genes, and their clinical significance as predictive markers or targets for therapy.

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