Aberrantly Methylated Gene Marker Levels in Stool: Effects of Demographic, Exposure, Body Mass, and Other Patient Characteristics

David A Ahlquist*1, William R Taylor1, Tracy C Yab1, Mary E Devens1, Douglas W Mahoney2, Lisa A Boardman1, Steven N Thibodeau3, Hongzhi Zou4, Michael J Domanico4, Barry M Berger4 and Graham P Lidgard4

1Division of Gastroenterology and Hepatology, Mayo Clinic, USA
2Department of Biostatistics, Mayo Clinic, USA
3Department of Laboratory Medicine and Pathology, Mayo Clinic, USA
4Exact Sciences Corporation, Madison, WI, USA

Abstract

Background: Selected aberrantly methylated genes represent sensitive candidate stool markers for colorectal cancer (CRC) screening. We assessed the impact of demographic, exposure, body mass, and other patient variables on stool levels of highly informative methylated gene markers — BMP3, NDRG4, vimentin, and TFPI2.

Methods: We studied freezer-archived stools from 500 patients with normal colonoscopy (median age 64 (range 44-85); 53% women). On supernatants from thawed aliquots, target gene sequences were purified by hybrid capture; bisulfite treated, and assayed using the analytically-sensitive QuARTS method (quantitative allele-specific real-time target and signal amplification). The reference human gene β-actin was assayed along with the 4 methylated genes.

Results: Only age significantly influenced all methylated marker levels in stool (p<0.0001 for each). The relative increase per standard deviation of age was greatest with TFPI2 at 49.4% and least with BMP3 at only 0.21%; levels of β-actin did not change across age. Other demographic variables (sex, race, and residence), exposures (smoking, alcohol, or analgesic use), family or personal history of colorectal neoplasia, body mass, and diabetes mellitus had no effect on methylated marker levels.

Conclusions: Although stool levels of candidate methylated markers increase with age to variable extents, most common clinical covariates have no effect.

Impact: These findings have important implications on CRC screening compliance, as patients using a stool test that incorporates these markers would not have to make life-style or medication adjustments. Furthermore, age effect can be mitigated by adjustment of cut-off levels based on age or by selection of markers least influenced by age.

Keywords: Stool DNA; Methylated gene markers; Cancer screening; Specificity

Introduction

Stool DNA testing has emerged as a patient-friendly, noninvasive, and easily distributable new approach to colorectal cancer (CRC) screening that achieves high detection rates of both CRC and clinically significant precancerous lesions [1,2]. Incorporating key advances, including next generation analytical technology and broadly informative marker panels, prototype stool DNA tests in recent case-control studies have yielded sensitivities for curable stage CRC of 87-98% and for adenomas >1 cm of 64% and higher with increasing size at specificity cut-off ≥ 90% [3-5]. An understanding of factors that contribute to non-specificity is critical to optimizing test configuration and clinical use of this screening approach. Yet, relatively little has been reported about the effects of common clinical variables on stool DNA marker levels. This is particularly true of aberrantly methylated gene markers which, to date, have proven to be the most informative panel elements in prototype stool DNA tests [3-5].

Aberrant methylation of the promoter region of numerous genes occurs early during colorectal carcinogenesis [6-10]. While there exists remarkable molecular heterogeneity across colorectal neoplasms, we [3,10,11] and others [7,9,12,13] have found that several aberrantly methylated gene markers alone or in combination almost perfectly discriminate CRC and adenomas from normal colorectal mucosa at the tissue level. However, many of these candidate markers fail on stool application due to non-specificity resulting from high marker background levels [3,10]. In a recent large study [3], we selected four methylated gene markers (BMP3, NDRG4, vimentin, and TFPI2) that maintained both high sensitivity and high specificity for detection of CRC and advanced adenomas on stool testing. The effects of common clinical covariates on these 4 candidate methylation markers have not been evaluated.

Based on observations across different tissues, rates of aberrant methylation of some genes may be affected by various non-neoplastic factors. Such factors may include demographic variation, particularly age [14], exposures such as smoking [15], alcohol intake [15,16], or analgesic use [17], and body mass [18] or diabetes mellitus [19]. Furthermore, aberrant methylation on some genes has been detected in histologically normal mucosa at points distant from colorectal neoplasms [20] and such molecular field defects could conceivably contribute to non-specificity on stool testing.
Ideal markers for CRC screening would have high specificity and be unaffected by common non-neoplastic factors. Use of such ideal markers would obviate the need to alter medications or daily routines—interventions which could threaten patient compliance.

This study exploits a large freezer archive of properly collected stool samples with an accompanying well-characterized clinical database to address the effects of common variables on stool levels of aberrantly methylated BMP3, NDRG4, vimentin, and TFPI2.

Materials and Methods

Study design and participants

This blinded cross-sectional study, designed and coordinated at the Mayo Clinic, was approved by the Mayo Institutional Review Board and endorsed by the industry collaborators. The 500 study participants were randomly selected from a cohort of roughly 2400 fully consented asymptomatic adults whose stools were collected and archived within 3 years of a screening or polyp surveillance colonoscopy that showed no colorectal pathology. Clinical data (demographic, medications, lifestyle exposures, family or personal history of colorectal neoplasia, body mass index, and presence of diabetes mellitus) were obtained prospectively at the time of recruitment and from Mayo Clinic records.

Stool processing and assays

Sample processing, marker selection, primer sequences, and all analytical methods used in this study have been described in detail [3,11]. Spontaneously passed stools were collected in a preservative buffer, homogenized and aliquoted upon laboratory receipt, and promptly stored at -80°C. Stools received later than 3 days from defecation were excluded from archival storage.

Four aberrantly methylated gene markers (BMP3, NDRG4, vimentin, and TFPI2) were selected for evaluation, as we had found these markers to be most discriminant for colorectal neoplasia on earlier marker triage studies on tissue and stool [3,11]. Next generation analytical methods used included direct hybrid capture of target genes from thawed fecal supernatant, an optimized rapid bisulfite treatment process, and the quantitative allele-specific real-time target and signal amplification (QuARTS) assay [3,11]. Stool levels of methylated gene markers were normalized as % of total human DNA (estimated by β-actin content).

Statistical methods

The association of clinical characteristics with individual methylated gene marker levels in stools from patients with normal colonoscopy was tested with the non-parametric Kruskal-Wallis test. Box-plots summarize the distribution of methylated marker levels across these clinical characteristics. To estimate the continuous age related changes on marker levels, a robust linear regression model was fit to down weight the influence of outlying observations. For this model, marker levels, a robust linear regression model was fit to down weight the influence of outlying observations. For this model, marker levels were analyzed on the natural log scale to dampen data skewness with advancing age (p<0.0001 for each). In contrast to the methylated gene markers, β-actin levels (plotted as dotted gray line) were unaffected by age (p=0.32). Distributions by age of observed stool levels of methylated TFPI2, the marker most affected by age. C. Similar stool level distributions by age of β-actin, which served as the un-methylated human control gene.

Figure 1: Relationship of age to stool marker levels. A. Relative changes per standard deviation of age are plotted for each marker. All four methylated gene markers (plotted as solid colored lines per inserted key) increased progressively with advancing age (p<0.0001 for each). In contrast to the methylated gene markers, β-actin levels (plotted as dotted gray line) were unaffected by age (p=0.32). B. Distributions by age of observed stool levels of methylated TFPI2, the marker most affected by age. C. Similar stool level distributions by age of β-actin, which served as the un-methylated human control gene.

B-actin

Normalized levels (log)

Age

C

40-49 (25) 50-59 (152) 60-69 (177) 70-79 (109) 80+ (37)

10 8 6 4

P=0.32

Tissue and stool premalignant lesions are most likely to be unaffected by common non-neoplastic factors. Use of such ideal markers would obviate the need to alter medications or daily routines—interventions which could threaten patient compliance.

Their median age was 64 years (range 44-85), 52.6% were women, and 89.9% were Caucasian. Distributions on demographic, exposure, and other clinical covariates are summarized in figures.

Participants

The 500 participants were asymptomatic adults with normal colorectal findings on screening or polyp surveillance colonoscopy. The 500 participants were asymptomatic adults with normal colorectal findings on screening or polyp surveillance colonoscopy.
Demographic variables

Age: Of all the common clinical factors evaluated in this study, only age substantively and significantly affected stool levels of the methylated gene tested (age-related gene methylation). All four methylated gene increased progressively with age, p<0.0001 for each marker (Figure 1A). The relative increase per standard deviation of age was greatest with TFPI2 at 49.4% and least with BMP3 at only 0.21%; the age related methylation of TFPI2 was significantly greater than that of the others (p<0.001 for comparison against vimentin, NDRG4, or BMP3). The significant serial rise in observed stool levels of methylated TFPI2 with advancing age is illustrated in figure 1B. In contrast, stool levels of the unmethylated human control gene β-actin did not change with age (p=0.32), as illustrated by similar distributions of observed levels at all age intervals (Figure 1C).

Other demographic variables: Those examined had no substantive effect on methylated gene marker levels in stool, including sex, race, and location of residence within the United States (Figures 2A-2C. Data are shown for NDRG4 and BMP3 only in this and subsequent figures, as these two markers have been selected for use in the optimized clinical assay [3,5] currently the subject of a pivotal clinical CRC screening study (ref: http://www.clinicaltrials.gov/ct2/show/NCT01397747?term=Deep-C&rank=1). However, the slightly higher marker levels in whites compared to non-whites did reach statistical significance.

Exposure variables

Smoking: Stool levels of candidate methylated gene markers were not influenced by smoking status (Figure 3A), as distributions were similar in current, previous, and never smokers.

Figure 2: Effects of other demographic variables on methylated marker levels in stool. Distributions are shown for both NDRG4 and BMP3 genes according to (A) sex, (B) race, and (C) residence within regions of the United States.
Alcohol consumption: Neither the type (beer, wine, distilled liquor [data not shown]) nor the frequency of alcohol consumption affected stool levels of any of the four methylated gene markers studied (Figure 3B).

Non-steroidal anti-inflammatory drugs (NSAIDS): Stool levels of methylated gene markers were unaffected by NSAID use, based on similar distributions in participants reporting no use, daily cardiovascular prophylactic doses, occasional therapeutic doses, or regular therapeutic doses (Figure 3C).

Personal or family history of colorectal neoplasia

Neither a personal history of prior colorectal polyps (Figure 4A) nor a first degree relative with colorectal CRC or polyps (Figure 4B) was associated with changes in distribution of methylated gene marker levels in stool.

Metabolic factors

Stool marker levels were comparable between participant subsets that were classified as normal weight, overweight, or obese based on body mass index criteria (Figure 5). The presence or absence of diabetes mellitus did not significantly influence stool levels of the methylated markers BMP3 (p=0.91), NDRG4 (p=0.37), TFF12 (p=0.36), or vimentin (p=0.23) or of the unmethylated marker β-actin (p=0.94); (data not shown).

Discussion

This investigation examined the influence of common clinical
variables on stool levels of methylated gene markers which, based on demonstrated high discrimination for colorectal neoplasia in both tissue [10,11] and stool [3] studies, had been selected as candidates for application in a CRC screening application. Age proved to be the only factor that affected stool marker levels. All four aberrantly methylated gene marker levels increased progressively with advancing age, but to considerably variable extents. Importantly, none of the other common clinical variables substantively impacted stool marker levels, including other demographic factors (sex, race/ethnicity, and geographic residence), exposures (smoking, alcohol, and NSAID use), a personal history of colorectal polyp or relatives with colorectal neoplasia, or metabolic factors (body mass or diabetes mellitus). These novel observations should help guide clinical use of emerging next generation stool DNA tests.

The age-effect must be considered in the selection and optimal use of methylated genes for CRC screening by stool DNA tests. It is well established that aberrant methylation of many genes occurs with
progressive frequency as individual's age and that methylation rates vary widely across ages and different tissues [14,21]. While causes of this age-related process are multifactorial and not fully understood, strong evidence supports that aberrant methylation of promoter regions on tumor suppressor genes predisposes to carcinogenesis [14]. Aberrant gene methylation may occur in normal-appearing colorectal mucosa [20], a field effect which could increase with age and potentially contribute to elevated background methylated gene marker levels in stool. In addition, high stool marker levels in some patients with reportedly normal colonoscopy may reflect the well-established increase in prevalence of colorectal neoplasms with age [22] and variable false negative rates of colonoscopy [23,24]. From a practical standpoint with respect to CRC screening, age-related false positive stool tests can be minimized by selecting methylated gene markers like BMP3 which are negligibly affected by age or by adjusting positivity cut-off levels according to age.

While methylation levels of some genes in certain tissues may vary with smoking [15], alcohol [15,16], or NSAID [17] use, such exposures did not affect stool levels of the candidate colorectal neoplasia markers studied. These findings suggest that it is not necessary for patients to alter these common lifestyle or medication exposures as preparation for testing by stool DNA assays incorporating these candidate markers. Obviating the need for patients to change habits or daily routines could help remove barriers to screening and increase patient adherence to clinical screen recommendations.

Our findings that neither a family history of colorectal neoplasia nor a personal past history of colorectal polyps had an effect on stool methylated marker levels have implications on the use of stool DNA testing. First, these data suggest that stool marker levels return to normal following polypectomy. Second, as stool marker levels were not elevated in these two groups at higher-than-normal risk for CRC but with normal colonoscopy, the door opens for rational consideration of stool DNA testing in the surveillance setting.

Given the alarming growth rates in the global prevalence of obesity [25,26] and diabetes mellitus [27], as well as the relationship of each to aberrant methylation of some genes in tissue studies [18,19], appraisal of their impact on stool levels of methylated gene markers is highly relevant. Furthermore, both obesity [28,29] and diabetes [30,31] may contribute to CRC risk. We found that none of the methylated gene markers evaluated in stool was quantitatively affected by these metabolic covariates, which simplifies the potential use in these markers for CRC screening.

Our study has both strengths and limitations. Specimens were obtained from a well-characterized archive with a prospectively collected clinical database and optimally collected and stored stools, and all assays were performed in blinded fashion using state-of-the-art technology. While the sample size of 500 patients provided excellent statistical power for most covariate analyses, non-white patient subsets were under-represented and further study is needed to assess marker level differences based on race or ethnicity. Effects of diet, various common medications, and other exposure variables on stool marker levels were not evaluated in our study, and such additional variables can be further explored in the future.

Based on results from this study suggesting that common clinical covariates have little effect on their specificity, and from additional sensitivity data demonstrating their synergy in detection of colorectal neoplasia [5,11], methylated BMP3 and NDRG4 have been chosen for inclusion in the marker panel of a new multi-marker stool test optimized for CRC screening [5]. The optimized stool test has been automated and is currently being validated in a pivotal multicenter screening study (Deep-C Study) comprising 10,000 average-risk adults. Study data will support a submission to the United States Food and Drug Administration for pre-market review and approval [ref:http://www.clinicaltrials.gov/ct2/show/NCT01397747?term=Deep-C&rank=1]. The primary outcomes of Deep-C will be sensitivity and specificity for the screen detection of CRC and clinically relevant precancers, and it represents an opportunity to further assess the relationship of common clinical covariates to test performance.

Acknowledgement

Funding for this study was provided by Exact Sciences Corporation, Edmond & Dana Gong, and Mayo Clinic. The authors thank Julie Simonson and Jacalyn McCormick for assistance with patient recruitment and clerical work. Preliminary data from this study were presented at the Annual Meeting of the American Association for Cancer Research; April 3, 2012; Chicago IL.

Disclosures

Mayo Clinic has licensed intellectual property to and is a minor equity investor in Exact Sciences; Dr. Ahlquist and Mr. Taylor were inventors of licensed technology. Dr. Ahlquist is a scientific advisor to Exact Sciences. Drs. Zou, Domanico, Berger, and Lidgard are employees of Exact Sciences.

References

1. Ahlquist DA (2010) Molecular detection of colorectal neoplasia. Gastroenterology 138: 2127-2139.
2. Kiesiel JB, Ahlquist DA (2011) Stool DNA screening for colorectal cancer: opportunities to improve value with next generation tests. J Clin Gastroenterol 45: 301-308.
3. Ahlquist DA, Zou H, Domanico M, Mahoney DW, Yab TC, et al. (2012) Next-generation stool DNA test accurately detects colorectal cancer and large adenomas. Gastroenterology 142: 248-256.
4. Ahlquist DA, Taylor WR, Mahoney DW, Zou H, Domanico M, et al. (2012) The stool DNA test is more accurate than the plasma septin 9 test in detecting colorectal neoplasia. Clin Gastroenterol Hepatol 10: 272-277.
5. Lidgard GP, Domanico MJ, Brunsma JJ, Gagrath ZD, Oldham-Haltom RL, et al. (2012) An Optimized Multi-marker Stool Test for Colorectal Cancer Screening: Initial Clinical Appraisal. Gastroenterology 142: S1.
6. Robertson KD, Jones PA (2000) DNA methylation: past, present and future directions. Carcinogenesis 21: 401-407.
7. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, et al. (2009) The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. Nat Genet 41: 178-186.
8. Das PM, Singal R (2004) DNA methylation and cancer. J Clin Oncol 22: 4632-4642.
9. Shen L, Toyota M, Kondo Y, Lin E, Zhang L, et al. (2007) Integrated genetic and epigenetic analysis identifies three different subclasses of colon cancer. Proc Natl Acad Sci U S A 104: 18654-18659.
10. Zou H, Harrington JJ, Shire AM, Rego RL, Wang L, et al. (2007) Highly methylated genes in colorectal neoplasia: implications for screening. Cancer Epidemiol Biomarkers Prev 16: 2686-2696.
11. Zou H, Allawi H, Cao X, Domanico M, Harrington J, et al. (2012) Quantification of methylated markers with a multiplex methylation-specific technology. Clin Chem 58: 379-383.
12. Hinoue T, Weisenberger DJ, Lange CP, Shen H, Byun HM, et al. (2012) Genome-scale analysis of aberrant DNA methylation in colorectal cancer. Genome Res 22: 271-282.
13. Lind GE, Danielsen SA, Ahlquist T, Merok MA, Andresen K, et al. (2011) Identification of an epigenetic biomarker panel with high sensitivity and specificity for colorectal cancer and adenomas. Mol Cancer 10: 85.
14. Issa JP (2002) Epigenetic variation and human disease. J Nutr 132: 2388S-2392S.
15. Vaissiere T, Hung RJ, Zaridze D, Moukeria A, Cuenin C, et al. (2009) Quantitative analysis of DNA methylation profiles in lung cancer identifies aberrant DNA methylation of specific genes and its association with gender and cancer risk factors. Cancer Res 69: 243-252.

16. Tao MH, Marian C, Shields PG, Nie J, McCann SE, et al. (2011) Alcohol consumption in relation to aberrant DNA methylation in breast tumors. Alcohol 45: 689-699.

17. Shen R, Tao L, Xu Y, Chang S, Van Brocklyn J, et al. (2009) Reversibility of aberrant global DNA and estrogen receptor-alpha gene methylation distinguishes colorectal precancer from cancer. Int J Clin Exp Pathol 2: 21-33.

18. Lehmann U, Langer F, Feist H, Glockner S, Hasenreiber B, et al. (2002) Quantitative assessment of promoter hypermethylation during breast cancer development. Am J Pathol 160: 605-612.

19. Ling C, Groop L (2009) Epigenetics: a molecular link between environmental factors and type 2 diabetes. Diabetes 58: 2718-2725.

20. Shen L, Kondo Y, Rosner GL, Xiao L, Hernandez NS, et al. (2005) MGMT promoter methylation and field defect in sporadic colorectal cancer. J Natl Cancer Inst 97: 1330-1338.

21. Maegawa S, Hinkal G, Kim HS, Shen L, Zhang L, et al. (2010) Widespread and tissue-specific age-related DNA methylation changes in mice. Genome Res 20: 332-340.

22. DiSario JA, Foutch PG, Mai HD, Pardy K, Manne RK (1991) Prevalence and malignant potential of colorectal polyps in asymptomatic, average-risk men. Am J Gastroenterol 86: 941-945.

23. Baxter NN, Goldwasser MA, Paszat LF, Saskin R, Urbach DR, et al. (2009) Association of colonoscopy and death from colorectal cancer. Ann Intern Med 150: 1-8.

24. Brenner H, Hoffmeister M, Amrdt V, Stegmaier C, Altenhofen L, et al. (2010) Protection from right- and left-sided colorectal neoplasms after colonoscopy: population-based study. J Natl Cancer Inst 102: 89-95.

25. de Onis M, Blossner M, Borghi E (2010) Global prevalence and trends of overweight and obesity among preschool children. Am J Clin Nutr 92: 1257-1264.

26. Flegal KM, Carroll MD, Ogden CL, Curtin LR (2010) Prevalence and trends in obesity among US adults, 1999-2008. JAMA 303: 235-241.

27. Danaei G, Finucane MM, Lu Y, Singh GM, Cowan MJ, et al. (2011) National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. Lancet 378: 31-40.

28. Calle EE, Kaaks R (2004) Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nat Rev Cancer 4: 579-591.

29. Larsson SC, Wolk A (2007) Obesity and colon and rectal cancer risk: a meta-analysis of prospective studies. The Am J Clin Nutr 86: 556-565.

30. Inoue M, Iwasaki M, Otani T, Sasazuki S, Noda M, et al. (2006) Diabetes mellitus and the risk of cancer: results from a large-scale population-based cohort study in Japan. Arch Intern Med 166: 1871-1877.

31. Larsson SC, Orsini N, Wolk A (2005) Diabetes mellitus and risk of colorectal cancer: a meta-analysis. J Natl Cancer Inst 97: 1679-1687.