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Brief Communication

Biomarkers measured in buccal and blood leukocyte DNA as proxies for colon tissue global methylation

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Abstract: There is increasing interest in clarifying the role of global DNA methylation levels in colorectal cancer (CRC) etiology. Most commonly, in epidemiologic studies, methylation is measured in DNA derived from blood leukocytes as a proxy measure of methylation changes in colon tissue. However, little is known about the correlations between global methylation levels in DNA derived from colon tissue and more accessible tissues such as blood or buccal cells. This cross-sectional study utilized DNA samples from a screening colonoscopy population to determine to what extent LINE-1 methylation levels (as a proxy for genome-wide methylation) in non-target tissue (e.g., blood, buccal cells) reflected methylation patterns of colon mucosal tissue directly at risk of developing CRC. The strongest Pearson correlation was observed between LINE-1 methylation levels in buccal and blood leukocyte DNA (r = 0.50; N = 67), with weaker correlations for comparisons between blood and colon tissue (r = 0.36; N = 280), and buccal and colon tissue (r = 0.27; N = 72). These findings of weak/moderate correlations have important implications for interpreting and planning future investigations of epigenetic markers and CRC risk.

Keywords: LINE-1 DNA methylation, global DNA methylation, colorectal adenoma, colorectal cancer, correlation

Introduction

There is increasing interest in clarifying the role of aberrant genome-wide DNA methylation as a useful biomarker of cancer risk in population-based epidemiologic studies. Global methylation status of easily accessible tissues (mainly blood cells) is commonly used as a surrogate measure of methylation changes at less accessible cancer target-sites despite a lack of supporting research to suggest that these measures are correlated.

Global DNA hypomethylation, an early fundamental event in the development of colorectal cancer (CRC) [1-3], is characterized by a genome-wide decrease in the total number of methylated cytosines within CpG (cytosine-phosphate-guanine) dinucleotide pairs (CpG sites), the majority (80%) of which are located in repetitive sequences such as LINE-1 (long interspersed nuclear element-1) repeats, that occur across the genome [4]. The role of global hypomethylation as a biomarker of CRC risk has been investigated in epidemiologic studies by comparing global DNA methylation levels in normal appearing (healthy) colon mucosal tissue between patients with CR adenoma (precursor to the vast majority of CRCs) or CRC, and subjects without colorectal tumors. Lower global methylation levels in normal colon tissue biopsies from patients with CR adenoma [5, 6] or CRC [5] as compared to those without CR pathology support the premise that global hypomethylation in normal background colon tissue represents a useful biomarker that may be indicative of an underlying predisposition to developing CR tumors.

However, obtaining colon tissue biopsies for a large population-based study may not be feasible. Consequently, global methylation as a pre-

dictor of CRC risk has also been investigated using peripheral blood leukocytes as a substitute for colon tissue biopsies with results suggestive of a potentially important trend towards increasing risk of CR adenoma [6, 7] but not CRC [6, 8] associated with lower global methylation levels in these surrogate samples. In addition, a recent prospective study of 863 participants enrolled in the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial reported no association between global methylation and subsequent CRC diagnosis using DNA derived from blood leukocytes collected between 0 and 10 years (median of 3 years, SD: 2.7) prior to selection for the PLCO trial [9].

From an ethical and a practical perspective, it is advantageous for population-based epidemiologic studies of methylation markers of colorectal cancer risk to use accessible sources of DNA, such as blood leukocytes and buccal cells, as proxy measures of methylation changes in colon tissue. However, to date, no published studies have reported correlations between global DNA methylation levels at these different sites. Our objective was to determine to what extent LINE-1 methylation levels (as a proxy for genome-wide methylation) in readily accessible non-target tissue (e.g., blood, buccal cells) reflected methylation patterns of less accessible target tissue directly at risk of developing CRC (e.g., colon mucosal tissue). It is essential to understand these relationships in order to interpret and plan future investigations.

Materials and methods

The subjects for this analysis included a subset of participants recruited for a clinic-based cross-sectional study investigating the relationship between several biomarkers of DNA methylation and the risk of colorectal adenomas. Briefly, 330 men and women, aged 40-65 undergoing a screening colonoscopy in Kingston, Ontario, were eligible to be included in the study population. Indications for colonoscopy included a positive family history of colorectal cancer or adenoma, a positive fecal occult blood test (FOBT) result or average risk screening. Patients with a previous diagnosis of inflammatory bowel disease (ulcerative colitis or Crohn's disease), known genetic disorders that predispose to CRC (hereditary nonpolyposis CRC, familial adenomatous polyposis) or any gastrointestinal abnormality detected at a previous colonoscopy (adenoma, hyperplastic polyp or cancer) were not recruited. In addition, patients diagnosed at the current colonoscopy with inflammatory bowel disease, serrated or sessile serrated adenomas, hyperplastic polyp(s) only or colorectal cancer were excluded from the eligible study population.

After obtaining informed consent, all eligible participants completed a questionnaire and provided a fasting venous blood sample. Blood was centrifuged within 45 minutes of collection and the buffy layer (blood leukocytes) was removed and stored at -20°C until DNA extraction. Also, during the colonoscopy, two pinch biopsies of healthy, normal appearing colon mucosa were obtained from the descending colon 10 cm apart and at least 10 cm from any lesion, polyp or mucosal abnormality. Tissue specimens were immediately placed in cell lysis solution (5-Prime DNA Isolation kit, Inter Medico, Markham, ON, Canada) and stored at -20°C. In addition, on the colonoscopy day, for 72 of the 330 eligible subjects, two separate buccal samples were collected by gently rubbing the inside of each cheek for 30 seconds using a disposable sterile cytological brush (5-Prime ArchivePure DNA buccal cell kit). Brushes, after air drying for 15 minutes, were placed in cell lysis solution and stored at -20°C until DNA extraction.

DNA was extracted from buccal brush samples, blood leukocytes and colon mucosal biopsy tissue using the 5-PRIME DNA isolation kit (Inter Medico, Markham, ON, Canada) according to instructions provided by the manufacturer and purified DNA was stored at -20°C until methylation analysis. High-resolution melting (HRM) profile analysis - a quantitative methylation-sensitive real-time florescence-based PCR method - was used to quantify global methylation in extracted DNA and is described in detail elsewhere [10]. Briefly, prior to HRM, DNA was bisulfite-converted using established protocols (Qiagen EpiTect Bisulphite Modification Kit); this chemical modification of cytosine bases at CpG sites permits differentiation between unmethylated and methylated cytosines. Due to low DNA yields from each buccal brush, the two buccal cell DNA samples were pooled prior

Table 1. Mean LINE-1 methylation levels and Pearson's correlation coefficients (and *P*-values) for buccal cell, blood leukocyte and colon tissue comparisons

Comparison	N	Mean (SD): % LINE-1 methylation	*P*-value*	Pearson's r (*p*-value)
Buccal cell	72	85.28 (4.33)	< 0.01	0.27 (0.02)
Colon tissue		90.17 (3.64)		
Blood leukocyte	280	87.20 (3.90)	< 0.01	0.36 (< 0.01)
Colon tissue		88.34 (3.58)		
Blood leukocyte	67	86.35 (3.68)	0.07	0.50 (< 0.01)
Buccal cell		85.41 (4.47)		

*P-value from paired t-test.

to bisulfite conversion. The HRM assay was designed to specifically target eight CpG sites within the LINE-1 repetitive sequence promoter region as a reliable surrogate measure of global methylation levels [11, 12].

Triplicate measures of LINE-1 methylation were obtained for each of the two colon tissue biopsies, blood leukocytes and the pooled buccal cell DNA samples. Individual triplicate measures were excluded where PCR values were not satisfactory due to a high PCR threshold crossing point (Cp value > 27). Individual triplicate outliers (defined as > 10% difference from each of the remaining triplicate values) were also excluded from the analysis. A single average percent methylation value was calculated for colon tissue, blood and buccal DNA for each subject by averaging all remaining triplicate measures after applying these criteria. The intra-assay coefficients of variation, which assess agreement between triplicate measures of LINE-1 DNA methylation for buccal, blood and colon tissue samples, were 2.17%, 1.81% and 1.84% respectively.

Pearson correlation coefficients were used to assess the relationships between overall average LINE-1 methylation values for buccal, blood leukocyte and colon tissue DNA, given that methylation levels were normally distributed for all cell types. Of the 330 eligible study participants, 72 subjects had methylation values for both buccal and colon tissue, 280 subjects had methylation results for blood and colon tissue, and 67 subjects were included in the blood/buccal correlation.

Results

Table 1 shows the distribution of mean LINE-1 methylation values and Pearson correlations for the three comparisons of interest. For the buccal/colon tissue and the blood/colon tissue comparisons, average LINE-1 methylation values were significantly higher in colon tissue. LINE-1 methylation levels were significantly correlated for buccal and colon tissue, and for blood and colon tissue (r = 0.27, P = 0.02 and r = 0.36, P < 0.01 respectively). A moderate correlation was observed for the blood/buccal comparison (r = 0.50, P < 0.01).

Discussion

This cross-sectional study utilized a screening colonoscopy patient population to assess correlations between LINE-1 methylation levels (as a surrogate measure of global DNA methylation) in DNA extracted from buccal cells, blood leukocytes and normal appearing colon mucosal tissue biopsies. The strongest correlation was observed between LINE-1 levels in buccal and blood leukocyte DNA. A moderate correlation was also found between blood and colon tissue LINE-1 methylation levels and a slightly weaker correlation was observed between buccal and colon tissue methylation. In addition, average LINE-1 methylation levels were highest in colon tissue and lowest in buccal cells.

To the best of our knowledge, this is the first study to compare LINE-1 methylation levels of healthy colon mucosal tissue with more accessible biologic samples (blood leukocytes and buccal cells). There is substantial evidence to suggest that LINE-1 methylation patterns are tissue and cell-type specific [2, 13-16], which may explain our weak/moderate correlations between LINE-1 methylation levels in buccal, blood leukocyte and colon tissue DNA. In addition, it is feasible that variation in methylation patterns between colon tissue, and blood-derived or buccal DNA may be explained by tissue-specific differences in susceptibility to the effects of lifestyle and environmental influences on methylation levels due to variation in exposure levels and responses to the same risk factors [13, 16].

The limitations of this study need to be considered. Sources of measurement error related to methylation measurement would affect buccal,

blood leukocyte and colon tissue LINE-1 methylation levels to the same extent as protocols were followed in the same manner for DNA extraction, storage and methylation measurement for all samples. Correlations will have been attenuated due to non-differential measurement error for each DNA source.

Our results are specific to DNA derived from buccal cells, blood leukocytes and colon tissue samples, as well as methylation levels of the sub-set of eight LINE-1 CpG sites targeted by the experimental method (HRM) used to quantify methylation in this study. Therefore, given evidence demonstrating variation in LINE-1 methylation profiles between different blood cell types and at different LINE-1 CpG sites, our findings may not be generalizable to other blood cell types, LINE-1 CpG sites or genome-wide methylation measures [13-15].

Participants in this cross-sectional study may be grouped based on findings at colonoscopy with subjects with no abnormality detected at colonoscopy assigned to the 'normal colonoscopy' (non-case) group and patients with one or more pathologically-confirmed tubular, tubulovillous or villous adenoma(s) comprising the adenoma (case) group. As case status based on colonoscopy results may have been a source of differential measurement error due to potential biologic effects of the disease process on methylation measures, all analyses were repeated stratified by colonoscopy results. However, correlations were not appreciably different between the combined and stratified analysis (data not shown).

In conclusion, though it can be argued that methylation measures in tissues at risk of developing cancer are superior markers of cancer risk, due to practical and ethical considerations, it is more feasible for epidemiologic studies to use accessible sources of DNA, such as blood leukocytes and buccal cells, as proxy measures of epigenetic changes in less accessible target-site tissues. LINE-1 (global) methylation status of accessible tissues (mainly blood) has been commonly used as a surrogate measure of target-site methylation changes in population-based studies of cancer risk (including colorectal cancer) [17-20]. However, few studies have examined the correlations between global DNA methylation levels at these different sites. Our findings of weak/moderate correlations between LINE-1 methylation levels of accessible cell types (blood leukocytes and buccal cells) and less accessible target tissue (colon tissue) have important implications for interpreting and planning future investigations of epigenetic markers and CRC risk.

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Disclosure of conflict of interest

None to declare.

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