

Is There a Link Between Genome-Wide Hypomethylation in Blood and Cancer Risk?

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Abstract

Cancer cells display widespread genetic and epigenetic abnormalities, but the contribution to disease risk, particularly in normal tissue before disease, is not yet established. Genome-wide hypomethylation occurs frequently in tumors and may facilitate chromosome instability, aberrant transcription and transposable elements reactivation. Several epidemiologic case-control studies have reported genomic hypomethylation in peripheral blood of cancer patients, suggesting a systemic effect of hypomethylation on disease predisposition, which may be exploited for biomarker development. However, more recent studies have failed to reproduce this. Here, we report a meta-analysis, indicating a consistent inverse association between genomic 5-methylcytosine levels and cancer risk [95% confidence interval (CI), 1.2–6.1], but no overall risk association for studies using surrogates for genomic methylation, including methylation at the LINE-1 repetitive element (95% CI, 0.8–1.7). However, studies have been highly heterogeneous in terms of experimental design, assay type, and analytical methods. We discuss the limitations of the current approaches, including the low interindividual variability of surrogate assays such as LINE1 and the importance of using prospective studies to investigate DNA methylation in disease risk. Insights into genomic location of hypomethylation, from recent whole genome, high-resolution methylome maps, will help address this interesting and clinically important question. *Cancer Prev Res*; 5(12); 1345–57. ©2012 AACR.

Introduction

Epigenetics is the mitotically heritable control of gene expression and chromatin structure through covalent modification by DNA methylation, posttranslational modifications of histone proteins, and control of gene expression by noncoding RNAs (1, 2). DNA methylation is the addition of methyl groups to cytosines within CpG dinucleotides, to form 5-methylcytosine (5meC) and is catalyzed by the DNA methyltransferases (DNMT; refs. 2, 3). DNA methylation is dependent on the 1-carbon metabolism pathway, which requires dietary supply of folate, homocysteine and choline, and other micronutrients (4, 5). The role of DNA methylation as a negative regulator of transcription initiation at CpG islands (CGI), regions of high CpG density found in approximately 60% of genes, is well established, and is required for

silencing of developmental and tissue specific genes (2, 6, 7). DNA Methylation facilitates transcriptional repression independently through preventing binding of DNA polymerases and transcription factors, or through interaction with methyl binding domain proteins and other epigenetic mechanisms such as repressive histone marks (8, 9). The functional roles of DNA methylation elsewhere in the genome is less well understood; however, DNA methylation is thought to confer genomic stability and integrity, and high methylation at repetitive elements is proposed to protect against expression of transposable elements (TE) and endogenous retroviruses (10, 11). Epigenetic regulation is often studied in the context of environmental and population health, as widespread DNA methylation patterns are known to be affected by environmental, lifestyle, and demographic factors that affect complex disease risk, such as diet, carcinogen exposure, reproductive factors, and age (12, 13). Furthermore, this "plasticity" or susceptibility to chemical alteration of the epigenome provides promise for therapeutic intervention where abnormal epigenetic patterns occur in disease or in relation to disease risk (14). A current research topic is the potential of DNA methylation variability in normal tissues such as peripheral blood, for development of cancer predisposition biomarkers (15, 16). This review and meta-analysis aims to synthesise all of the current data and to answer the question of whether there is a link between genome-wide DNA hypomethylation and cancer risk.

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DNA Methylation and Cancer

DNA methylation dysregulation occurs in almost all cancers, and is becoming established as a hallmark of cancer (1, 11, 17). Most research has been concerned with local hypermethylation of promoter CpG islands, as transcriptional repression because of promoter hypermethylation of tumor suppressor genes, such as MLH1 and BRCA1 is implicated in driving several cancers (7, 8). However, the first described cancer-associated epigenetic phenomenon was the lower net percentage of 5meC in tumor tissue compared with equivalent normal tissue, known as "genome-wide" or "global" hypomethylation, that occurs frequently in all cancer types (18, 19). Despite considerable research, it remains unclear whether promoter hypermethylation and genome-wide hypomethylation are mechanistically linked, or are independent processes. (20, 21). Whole-genome DNA methylation (methylome) analysis has so far revealed that an overall increase in methylation variability (hypervariability) may be more prevalent than discrete changes in DNA methylation levels in cancer, suggesting a widespread loss of epigenetic control (9, 17, 22). Recent research has shown that most cancer-associated DNA hypermethylation occurs at "CpG shores," regions flanking CpG islands, rather than within CpG islands (17, 23). Furthermore, genome-wide hypomethylation is largely confined to large genomic "hypomethylation blocks" (9, 17), that tend to occur in regions that display intermediate methylation levels in normal tissue, termed partially methylated domains (PMD; refs. 17, 22), and regions of low CpG density (23). That hypomethylation is specifically associated with repetitive elements has been called into question, with only a modest enrichment of repeats in hypomethylation blocks (17, 24). Such apparent compartmentalization of hypomethylation may reflect higher order changes in chromatin structure (9). Whereas genome-wide hypomethylation has long been implicated in loss of transcriptional repression, recent evidence suggests that hypomethylation at PMDs or gene bodies may be often associated with gene repression through formation of repressive chromatin (24). Therefore, understanding of cancer epigenetics is rapidly advancing with the advent of whole-methylome sequencing, revealing a greater complexity and organization to the epigenome than was previously appreciated.

Causes and Consequences of Genome-Wide Hypomethylation

The cause of genome-wide hypomethylation remains poorly understood, and several factors have been implicated (11, 21). Dietary insufficiency of folate and other 1-carbon metabolism-dependent micronutrients, or germline or somatic mutations in members of the pathway can induce hypomethylation in tumor, and normal tissues including blood (4, 14, 25, 26). Exogenous exposures, such as carcinogen exposure may influence methylation by inducing DNA damage or by affecting DNMT enzyme activity (4, 11, 27, 28). Furthermore, the efficiency of DNA methylation may be affected by age (12, 29). Finally,

neoplasia may induce "field-effect" hypomethylation in cancer and surrounding histologically normal tissues, through sequestration of DNA methylation enzymes and substrates by rapidly proliferating, dividing cancer cells, resulting in failure of DNA methylation maintenance in surrounding tissue (11, 30–35).

Several tumorigenic events may result from genome-wide hypomethylation. These include chromosomal instability, which may facilitate gene-dosage alterations, mutations, genetic recombination, large deletions, or translocations (2, 11, 36). Hypomethylation may facilitate altered expression of oncogenes and/or tumor suppressors, as well as aberrant transcription of noncoding RNAs via transcriptional read-through subsequent to a loss of repression at repetitive DNA (37–39). Expression of repetitive elements is a feature of many cancers, with largely unknown consequences (37, 40). Whether genome-wide hypomethylation represents an early or causative tumorigenic event or a passive consequence of cancer remains unknown (reviewed elsewhere (21)). The detection of hypomethylation in cancer precursor lesions and normal adjacent tissue, and the induction of cancers in animal models with experimentally induced hypomethylation suggest a causative role (30, 41–43). However, the occurrence of many cancers without apparent hypomethylation, and the progression of hypomethylation with cancer stage suggest a passive role (20, 21).

Transposable Element Hypomethylation

Because of the conventional view that genome-wide hypomethylation is associated with repetitive elements, surrogate assays for genome-wide methylation have been developed specifically targeting consensus sequences within repetitive elements, including LINEs (Long Interspersed Nucleotide Element 1, LINE1), SINEs (Alu), and Satellite repeats (Sat2; refs. 44, 45). LINE1 (hereafter referred to as "L1") is the only autonomous [capable of independent retrotransposition (transposition via an RNA intermediate)], and most highly expressed TE in the human genome, comprising approximately 17% of the human genome, with more than 500,000 copies (11, 46, 47). A full length L1 element is approximately 6 kb long with a bidirectional, noncanonical promoter, and 2 open reading frames coding for an endonuclease and retrotransposition machinery proteins (11, 46, 47). L1 transcription is largely regulated by DNA methylation of the 5' promoter; however, most L1 elements are truncated and cannot be transcribed (37, 38, 40). Approximately 100 L1 elements are functionally capable of retrotransposition; however, only a few contribute to the vast majority of retrotransposition events (46). Alu elements, of which there are multiple families, are the most common TE in the human genome, with approximately 1.1 million copies, comprising roughly 11% of the genome (11, 47). Alu elements are nonautonomous and require the L1 transposition machinery for transposition (11). Satellite repeats (including Sat2) are short tandemly repeated noncoding DNA, frequently in centromeric and heterochromatic regions of chromosome 1 (48). Both L1

and Alu elements are heavily methylated in normal somatic tissue, however, hypomethylation of both, especially L1, is often detectable in tumors (20, 30, 49). Whereas transcription of TEs are required for their transposition, transposition-independent consequences of transcription may have functional consequences, as an estimated 7% of the human transcriptome is derived from transcription start sites within L1 elements (37) and hypomethylation induced transcription of L1 elements within host gene introns can effect host gene transcription through RNA interference (39) and through driving host gene ectopic expression from the L1 antisense promoter (50).

DNA Methylation Variability and Cancer Risk

We and others have hypothesized that the epigenetic variability may contribute to risk of cancer development (14, 15, 51–53). "Epigenetic epidemiology" refers to the investigation of epigenetic patterns associated with disease risk, and such research holds great potential for cancer prevention and cancer risk-biomarker development, especially where abnormal epigenetic patterns may be detectable in easily accessible tissues such as blood, which is suitable for population screening (15, 16).

Methylation variability may contribute to, or be predictive of, risk of tumorigenesis in other tissues for several reasons. Many innate cancer risk factors, such as age, anthropometric factors, and genetic factors affect epigenetic patterns in blood (54–56). For example, rare epimutations in MLH1 and MSH2, which consist of local DNA hypermethylation events detectable in all tissues and conferring high risk of colorectal cancer, have been shown to occur because of genetic polymorphisms (57, 58). Blood DNA methylation is also affected by exposure to environmental and lifestyle cancer risk factors, such as smoking, alcohol, and other carcinogens (59, 60), therefore, blood methylation may provide useful biomarkers for acquired or environmentally induced cancer risk (12, 13). Finally, inflammation predisposes to risk of many cancers (61, 62), and affects DNA methylation (63), suggesting that blood methylation may reflect immune effects on cancer risk.

Promising for this avenue of research is the identification of a variable methylated region within the ATM gene associated with breast cancer risk (64). Furthermore, hypermethylation of BRCA1 is reportedly associated with increased prevalence of BRCA1-methylated breast tumors (65, 66), suggesting a functional link between blood detectable DNA methylation and disease histology (67–69). High-throughput discovery studies have identified cancer-associated DNA methylation signatures in blood of patients with breast (69), ovarian (68), bladder (67), and head and neck cancers (70), providing potential cancer diagnostic biomarkers.

The most widely studied putative epigenetic risk marker for cancer is genome-wide or "global" DNA methylation in blood. The studies investigating this, their findings, and the research approaches used, will be discussed further.

Current Methods for Investigation of Genome-Wide Hypomethylation

A challenge in investigating genome-wide DNA methylation in population studies is the lack of cost-effective, high-throughput assays that have both wide genome coverage and high resolution (45). Early studies used methods that measure genomic 5meC content using 5meC-specific antibodies (71), methyl-acceptance assays (72) and high-performance liquid chromatography (HPLC) (73). Such methods, however, give no information about the spatial arrangement or genomic location of DNA methylation and require large amounts of input DNA, making them unsuitable for population studies using precious patient samples. Widespread DNA methylation patterns can be measured using methylation sensitive restriction enzymes with relatively high resolution; however, methylation analysis is biased toward regions of high CpG density, as the enzyme restriction sites occur at sequences such as CCGG, and CGCG for the *HpaII* and *HhaI* restriction enzymes, respectively (45, 74). Most DNA methylation assays are based on bisulphite conversion of DNA, where incubation of DNA with sodium bisulfite causes deamination of unmethylated, but not methylated, cytosines to uracil (45). Sequencing of bisulphite converted DNA represents the "gold standard" for DNA methylation analysis (16, 75); however, real-time PCR, restriction enzyme based [combined bisulphite restriction analysis (COBRA; ref. 76)], and microarray-based methods (75) are also used. Most popular and convenient for population studies is the measurement of methylation at the repetitive elements such as L1, Alu, and Sat2, which are distributed at high frequency throughout the human genome (11, 44). These are considered "surrogate" measures of genome-wide DNA methylation as their methylation is thought to reflect genome-wide methylation levels (77). However, the efficacy of these surrogate assays for genome-wide methylation has been questioned (refs. 14, 78; discussed below), and a reinterpretation of the results reported using these assays is warranted.

Comparison of Published Reports of Genome-Wide Methylation in Blood and Cancer Risk

Twenty-three publications have reported population based cancer case-control studies investigating blood genomic DNA methylation in relation to cancer risk (Table 1). It is important to note that the comparability of these studies is limited by several differences between each, including cancer type, assay, study design (prospective/retrospective), length of time between blood-draw and diagnosis, sample size, sex (male/female/mixed), ethnicity, cancer treatment exposure, and analytical/statistical methods. Furthermore, populations at different cancer risk are included, for instance, one report included elderly men at high cancer risk (79), whereas another included Asian women at lower risk of breast cancer (80). Some reports included more than 1 "study," because of use of different assays, different patient populations, or different study designs, so altogether there were 34 individual studies of genome-wide methylation

Table 1. Study details abstracted from all reports included in meta-analysis and all relevant studies excluded

Reference	Author	Publication date	Journal	Cancer type	Measurement	Assay	Categorical split	First author	Reported restricted ethnicity	Country/ continent	n (case/ctrl)	Prospective/retrospective	Newly diagnosed	Pre/postchemo-therapy	Cases (description)	Controls (description)
72	Pufulete	5/2/2003	GASTRO-ENTEROLOGY	Colorectal	5meC	Methyl acceptance	Tertiles	Mixed	No	UK	28/76	Retrospective	Yes	Pretreatment	Cancer screening	Unaffected on screening
72	Pufulete	5/2/2003	GASTRO-ENTEROLOGY	Colorectal adenoma	5meC	Methyl acceptance	Tertiles	Mixed	No	UK	35/76	Retrospective	Yes	Pretreatment	Cancer screening	Unaffected on screening
31	Lim	1/1/2008	GASTRO-ENTEROLOGY	Colorectal	5meC	HPLC and MS	Tertiles	Female Lim	No	US	115/115	Retrospective	Prospective	Pretreatment	Cancer screening	Unaffected on screening
106	Moore	4/9/2008	LANCET ONCOL	Bladder	5meC	HPCE and hpall*	Quartiles	Mixed Moore	No	Spain	775/397	Retrospective	Yes	Unreported	Hospital based	Hospital based
73	Choi	7/7/2009	CARCINO-GENESIS	Breast	5meC	Mass spectrometry	Tertiles	Female Choi	No	U.S.	176/173	Retrospective	Yes	Pretreatment	Hospital-based	Patients (unrelated conditions)
99	Hou	10/15/2010	INT J CANCER	Gastric	Alu	Pyrose-quencing	Tertiles	Mixed Hou	Caucasian	Poland	302/421	Retrospective	Yes	Mixed	Hospital based	Cancer registry
79	Zhu	3/22/2011	CANCER CAUSE CONTROL	All (combined)	Alu	Pyrose-quencing	Quartiles	Male Zhu	No	Boston	205/486	Retrospective	No	Posttreatment	Baseline screening of prospective population cohort	Unaffected at baseline and follow up
97	Gao	1/31/2012	BRIT J CANCER	Gastric	Alu	Pyrose-quencing	Quartiles	Female Gao	No	Shanghai	192/381	Prospective	Prospective	Pretreatment	Incident cases from prospective cohort	Unaffected on follow up
48	Wu	6/7/2012	CARCINO-GENESIS	Breast	Alu	MethylLight	Quartiles	Female Wu	No	US/ Australia	266/334	Retrospective	No	Posttreatment	High-risk families	Unaffected sisters
74	Xu	2/27/2012	FASEB J	Breast	LUMA	CCGG*	Quintiles	Female Xu	No	US	1055/1101	Retrospective	Yes	Posttreatment	Cancer registry, first primary breast cancer	Randomly selected (digit dialing)
101	Wilhelm	3/1/2010	CLIN CANCER RES	Bladder	LINE1	Pyrose-quencing	Deciles	Mixed Wilhelm	No	US	285/465	Retrospective	Yes	Unreported	Cancer registry (hospitals)	From previous study
88	Mirabello	3/17/2010	BMC MED GENET	Testicular	LINE1	Pyrose-quencing	Tertiles	Male Mirabello	No	US	114/151	Retrospective	No	Posttreatment	High-risk families	Unaffected relatives
99	Hou	10/15/2010	INT J CANCER	Gastric	LINE1	Pyrose-quencing	Tertiles	Mixed Hou	Caucasian	Poland	300/419	Retrospective	Yes	Mixed	Cancer registry (hospitals)	Randomly selected (registry)
79	Zhu	3/22/2011	CANCER CAUSE CONTROL	All (combined)	LINE1	Pyrose-quencing	Quartiles	Male Zhu	No	Boston	30/487	Prospective	Prospective	Pretreatment	Incident cases from prospective elderly male cohort	Unaffected at case selection
79	Zhu	3/22/2011	CANCER CAUSE CONTROL	All (combined)	LINE1	Pyrose-quencing	Quartiles	Male Zhu	No	Boston	204/478	Retrospective	No	Posttreatment	Baseline screening of prospective population cohort	Unaffected at case-selection
104	Rusiecki	4/3/2011	EPIGENOMICS	Gastric	LINE1	Pyrose-quencing	Tertiles	Mixed Rusiecki	No	Oman	110/104	Retrospective	Yes	Unreported	Hospital based	Donors/ government workers
85	Liao	6/11/2011	PLOS ONE	Renal cell	LINE1	Pyrose-quencing	Quartiles	Mixed Liao	Caucasian	Europe	328/654	Retrospective	Yes	Unreported	Hospital based	Patients (unrelated conditions)

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Table 1. Study details abstracted from all reports included in meta-analysis and all relevant studies excluded (Cont'd)

Reference	Author	Publication date	Journal	Cancer type	Measurement	Assay	Categorical split	First author	Reported restricted ethnicity	Country/continent	n (case/ctrl)	Prospective/retrospective	Newly diagnosed	Pre/postchemo-therapy	Cases (description)	Controls (description)
87	Di	10/12/2011	J ZHEJIANG UNIV-SC B	Hepatocellular	LINE1	Pyrose-quencing	Quartiles	Di	No	China	315/956	Retrospective	Yes	Pretreatment	Hospital based	Unaffected at case selection
97	Gao	1/31/2012	BRIT J CANCER	Gastric	LINE1	Pyrose-quencing	Quartiles	Gao	No	Shanghai	192/382	Prospective		Pretreatment	Incident cases from prospective female cohort	Unaffected on follow up
74	Xu	2/27/2012	FASEB J	Breast	LINE1	Pyrose-quencing	Quintiles	Xu	No	US	1064/1100	Retrospective	Yes	Posttreatment	Cancer registry, first primary breast cancer	Randomly selected (digit dialing)
80	Cash	3/1/2012	INT J CANCER	Bladder	LINE1	Pyrose-quencing	Tertiles	Cash	Han Chinese	China	510/528	Retrospective	No	Posttreatment	Cancer registry	Recruited from registry
15	Brennan	5/1/2012	CANCER RES	Breast	LINE1	Pyrose-quencing	Quartiles	Brennan	White	UK	252/255	Prospective	Prospective	Pretreatment	Incident cases from prospective female cohort	Unaffected on follow up
15	Brennan	5/1/2012	CANCER RES	Breast	LINE1	Pyrose-quencing	Quartiles	Brennan	No	Europe	263/257	Prospective	Prospective	Pretreatment	Incident cases from prospective female cohort	Unaffected on follow up
89	Wu	5/12/2012	CARCINO-GENESIS	Hepatocellular	LINE1	Pyrose-quencing	Quartiles	Wu	No	Taiwan	305/1254	Prospective	Prospective	Pretreatment	Incident cases from prospective female cohort	Unaffected on follow up
47	Wu	6/7/2012	CARCINO-GENESIS	Breast	LINE1	Pyrose-quencing	Quartiles	Wu	No	US/Australia	279/340	Retrospective	No	Posttreatment	High-risk families	Unaffected sisters
76	Hsiung	1/16/2007	CANCER EPIDEM BIOMAR	Head and neck	LRE1	COBRA*	Tertiles	Hsiung	No	Boston	278/526	Retrospective	Yes	Posttreatment	Hospital based	Recruited from registry
89	Wu	5/12/2012	CARCINO-GENESIS	Hepatocellular	Sat2	MethylLight	Quartiles	Wu	No	Taiwan	305/1254	Prospective	Prospective	Pretreatment	Incident cases from prospective cohort	Recruited from registry
47	Wu	6/7/2012	CARCINO-GENESIS	Breast	Sat2	MethylLight	Quartiles	Wu	No	US/Australia	266/333	Retrospective	No	Posttreatment	High-risk families	Unaffected sisters
26	Cho	12/30/2010	ANTICANCER RES	Breast	Alu	MethylLight	NA	Cho	No	Turkey	40/40	Retrospective	Yes	Pretreatment	Hospital based	Healthy volunteers
69	Widschwendter	7/16/2008	PLOS ONE	Breast	Alu	MethylLight	NA	Widschwendter	No	Germany	169/180	Retrospective	Prospective	Pretreatment	Hospital based	Unaffected on routine health check
73	Choi	7/7/2009	CARCINO-GENESIS	Breast	LINE1	Pyrose-quencing	NA	Choi	No	US	19/18	Retrospective	Yes	Pretreatment	Hospital based	Unaffected relatives/visitors
26	Cho	12/30/2010	ANTICANCER RES	Breast	LINE1	MethylLight	NA	Cho	No	Turkey	40/40	Retrospective	Yes	Pretreatment	Hospital based	Healthy volunteers
98	Piyathilake	5/27/2011	NUTRITION	CIN*	LINE1	Pyrose-quencing	NA	Piyathilake	No	US	103/273	Prospective	Prospective	Pretreatment	Incident CIN cases from prospective cohort	Women with 1 or less CIN
83	Patchsung	5/15/2012	PLOS ONE	Bladder	LINE1	COBRA	NA	Patchsung	No	Thailand	61/45	Retrospective	Yes	Unreported	Hospital based	Healthy elderly volunteers

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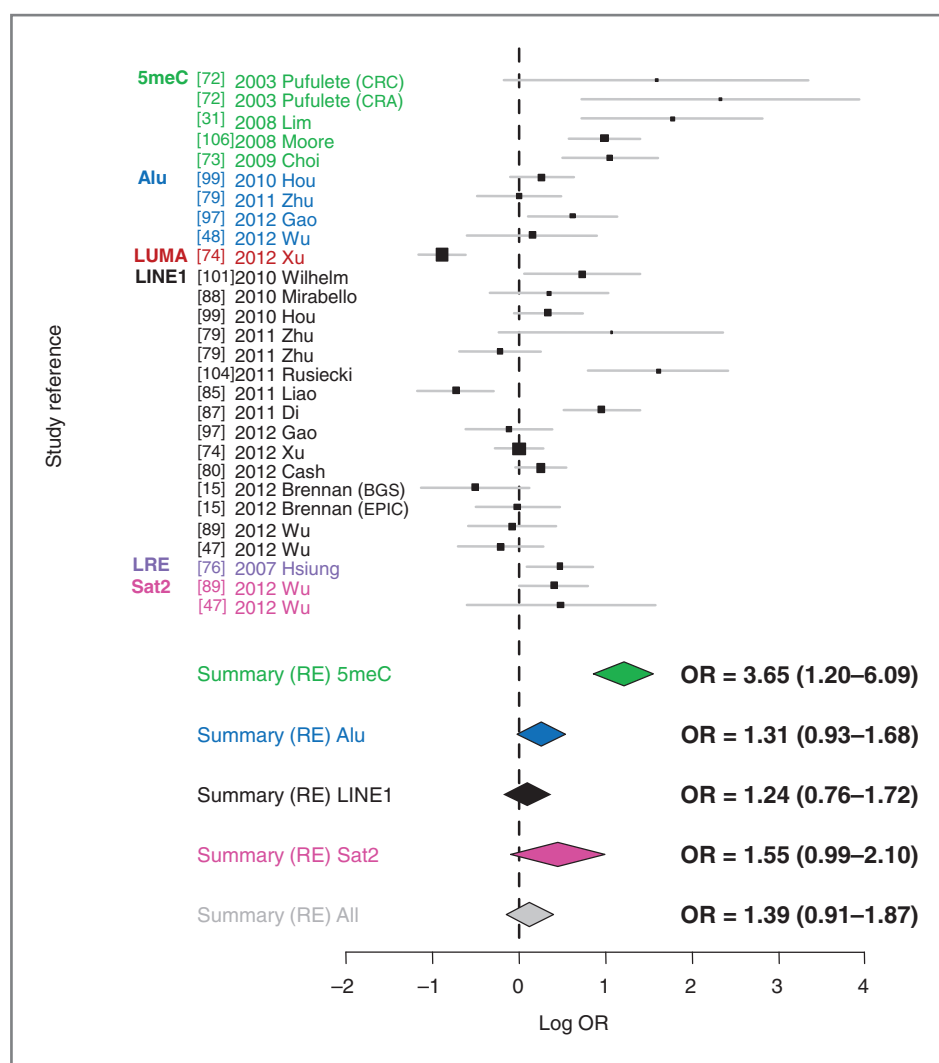
Table 1. Study details abstracted from all reports included in meta-analysis and all relevant studies excluded (Cont'd)

Reference	Author	Publication date	Journal	Cancer type	Measurement	Assay	Categorical split	Sex	First author	Reported restricted ethnicity	Country/continent	n (case/ctrl)	Prospective/retrospective	Newly diagnosed	Pre/postchemo-therapy	Cases (description)	Controls (description)
82	Pobsook	11/12/2010	CLIN CHIM ACTA	Oral	LINE1	COBRA	NA	Mixed	Pobsook	No	Thailand	34/43	Retrospective	Yes	Pretreatment	Hospital based	Volunteers
26	Cho	12/30/2010	ANTICANCER RES	Breast	Sat2	MethylLight	NA	Female	Cho	No	Turkey	40/40	Retrospective	Yes	Pretreatment	Hospital based	Healthy volunteers

NOTE: Categorical split represents the level of categorization of DNA methylation used in the study. Country/continent refers to the place of sample collection. Prospective/retrospective and pre/postchemotherapy indicate whether blood samples were collected specifies whether blood samples were collected before or after diagnosis and neo-adjuvant chemotherapy, respectively. "Newly diagnosed" indicates whether retrospectively collected samples were collected from patients reported as "newly" or "recently" diagnosed. Cases and controls give a brief indication of the way in which case and control subjects were recruited and defined. "Reported restricted ethnicity" indicates whether publications for each study reported exclusion of study participants based on race. "No" indicates that no race exclusion was reported. Other values indicate the race to which the sample group was restricted.

and cancer incidence/prevalence (Table 1). The greatest limitation to comparison between studies was reporting of data analyses. Twenty-six studies reported an odds ratio (OR) for cancer associated with DNA methylation, generated by categorical analysis. Categorical analysis compares the OR for cancer for individuals displaying methylation within the lowest category (tertile, quartile, or decile) of methylation, compared with individuals within the highest (reference) category. The choice of categorical "split" for methylation affects the OR, with fewer categories providing more conservative analysis, and this varied between studies (Table 1). OR was the most consistently reported, comparable, and representative (of overall results) factor between studies, and was, therefore, used for meta-analysis. Therefore, 8 studies reporting risk analysis at the mean/median level only (none of which showed significant case-control differences) could not be included, resulting in an overestimate of any positive effects (refs. 69, 73, 79, 81-83; Table 1). We have recently reported methylation analysis of blood L1 methylation in 2 population-based prospective case-control studies for breast cancer risk, but did not report categorical analysis (78). However, for the purpose of comparison with other studies, we have included categorical analyses of these in this review. In addition, as 4 studies (3 reports; refs. 31, 84, 85) used the lowest, rather than the highest methylation category as the reference category, inverse ORs were calculated for these studies. Importantly, use of different methylation "split" (tertile, quartile, quintile, or decile) for categorical analysis between reports is a potential confounding factor; however, this cannot be easily corrected without obtaining the raw data for each study. A meta-analysis of many of the relevant studies was recently reported by Woo and colleagues (86), which did not include 5 relevant and recent reports (64, 84, 87-89). Therefore, we report a revised meta-analysis including these studies (Fig. 1). A thorough literary search was carried out to identify all case-control studies relationship between genomic DNA methylation in blood, and either cancer incidence or prevalence (Table 1). Included were studies investigating any cancer type and using any quantitative measurement of genome-wide DNA methylation, including 5meC measures and surrogate assays. Excluded were studies measuring methylation at single-locus sites, or measuring methylation in tissues other than blood. Summary estimates were weighted by sample size, using random-effects models because of significant interstudy heterogeneity. (Full details of search strategy and meta-analysis are provided in supplementary methods.) The overall summary estimate OR for all using a random effects model was OR, 1.4 (0.9-1.9), suggesting no overall association with cancer risk. Summary ORs for studies using L1, Alu, and Sat2 repetitive elements were not significantly associated with cancer risk. It appears that total 5meC genomic content is the most consistent association with cancer prevalence, as all 5 studies identified significant associations between hypomethylation and cancer prevalence in categorical analysis, 4 of which also found significant association at the mean level. Though the effect sizes are

Figure 1. Meta-analysis of ORs reported in studies investigating Genome-wide DNA methylation in peripheral blood DNA for cancer risk. Test for heterogeneity showed highly significant heterogeneity across all studies ($P < 0.001$), and specifically in the analysis of 5meC ($P < 0.001$) and LINE1 ($P < 0.001$), but not significant for Alu ($P = 0.121$) or Sat2 ($P = 0.827$). Squares represent the size of the study and are centered on the OR with whiskers representing the 95% CIs. Random effects (RE) model was used for all summary analyses and the width of the diamond represents the confidence intervals. CRC, colorectal cancer; CRA, colorectal adenoma; BGS, breakthrough generations study; EPIC, European Prospective Investigation into Cancer and Nutrition.



variable, with smaller studies showing the largest ORs, it appears evident that genomic 5meC content is reduced in blood of cancer patients, suggesting potential for biomarker development. Interestingly, these studies represent the earliest published reports investigating blood genomic methylation in relation to cancer risk, and no similar study has attempted to replicate these findings since. Given that most studies using surrogate assays are null for cancer risk, it may be worthwhile returning to 5meC measures in an effort to reproduce this association. Interestingly, the only study (84) investigating widespread genomic methylation, using the restriction enzyme and bisulphite-pyrosequencing-based lumino-metric methylation assay (LUMA), identified a strong protective effect of genomic DNA hypomethylation on breast cancer risk. This inconsistent finding was likely influenced by the use of restriction enzymes, which measure methylation mainly at CGIs, which tend to be unmethylated, meaning that the only detectable change would be an increase in methylation (84).

There is no overall association between methylation at L1 and cancer risk according to this analysis. Whereas early studies suggested an association of blood L1 hypomethylation with cancer, later studies have failed to reproduce this. Furthermore, the direction of effect on cancer prevalence of L1 hypomethylation was inconsistent between significant studies (85), suggesting that this association may have occurred because of chance. One consideration is that heterogeneity between cancer types may affect the overall findings from this meta-analysis. However, in 3 breast cancer studies there is no association of L1 methylation with either cancer incidence (64) or prevalence (73, 84). Of 5 prospective studies, only 1 (79) identified an association between low L1 methylation and cancer incidence, however, small sample size, combining of multiple cancer types, and selection of a very high-risk population (elderly males) may be confounding factors for this study. Four more recent, larger studies have failed to identify any such association in gastric (90), breast (64), or hepatocellular

(89) cancer, suggesting that L1 methylation is not a cancer risk factor.

Factors Affecting Epigenetic Epidemiologic Studies

Study design

Many retrospective studies investigating blood DNA methylation in cancer patients inappropriately use the term "risk" to describe associations between methylation and disease. Association with disease risk of blood DNA methylation variability can only be determined using prospective studies, with blood samples collected several years before disease development, because of the possibility of "reverse causality," that is, the alteration of blood DNA methylation by presence of active disease (15, 68, 89). Whereas the effects of active cancer on blood DNA methylation are unknown, proliferation of lymphocytes, or depletion of required substrates or enzymes may affect blood DNA methylation (91). In addition, blood methylation may be affected by the presence of circulating tumor cells (92), though the contribution to overall blood methylation of this small fraction of tumor cells may not be detectable. An important factor for prospective studies is latency, that is, the duration between sample collection and cancer diagnosis (15), as methylation may be affected by as yet undetected cancer in samples collected shortly before diagnosis. Furthermore, the degree to which latency affects methylation-risk association may help determine whether methylation variability confers long-term or transient risk, or whether methylation is likely to be an early tumorigenic event. Retrospective studies are useful for investigating the relationship between DNA methylation variability and cancer prevalence, and may be used for development of diagnostic cancer biomarkers (16, 68). A potential confounding factor in some retrospective studies is the possible effect of cancer treatment, as several commonly used neoadjuvant chemotherapeutic drugs are known to affect activity of the folate-mediated 1-carbon metabolism pathway (93, 94). Whereas this treatment-induced inhibition of DNA methylation could potentially explain the occurrence of genome-wide hypomethylation in posttreatment blood samples, 4 studies identifying significant associations between genomic hypomethylation and cancer prevalence, including 1 L1 study (87), and 3 5mC studies (31, 72, 73) used pretreatment sample only. Nonetheless, the exposure of cases, but not controls to cancer treatment may represent a confounding factor. Sample size is an important factor for case-control studies, and a confounding factor when comparing different population-based case control studies. Studies of small sample size ($n < 40$ cases; refs. 72, 79), may have little power to detect subtle methylation variability, perhaps leading to a bias toward nonsignificance, or to chance detection (16). Most of the studies relevant to this report were of small sample size. Conversely, larger sample sizes may lead to detection of statistically significant results with a very small overall effect size (95), which can be misleading. The largest study to date included 1,000 cases/control pairs, at $\alpha = 0.05$ this study would have 80%

power to detect a difference of 0.12 standard deviations. For the L1 assay, with a standard deviation of 1.8% across the population, this would equal a difference in means of 0.06%, which is well below the technical variability in this assay [0.5%–5% (78, 88, 89, 96–99)] and may be biologically meaningless.

Sample selection

Various patient characteristics are potential modifiers of both cancer risk and DNA methylation. Such factors may represent intermediate factors in the link between DNA methylation and disease risk, but may also represent potential confounding factors within the study. For example, where DNA methylation is affected by smoking, an association between DNA methylation and cancer risk may be apparent because of uneven numbers of smokers in the case and control groups. To reduce the effect of such potential confounders, case-control pairs should be matched on potential confounding patient characteristics, as well as technical factors such as experimental batch (16). Furthermore, statistical adjustment for these factors should be applied during analysis. Age is a strong modifier of DNA methylation for some genes (12, 100), and is the biggest risk factor for most cancers, therefore, adjustment for age is essential (12, 100). L1 methylation is significantly lower in females than males (80, 101, 102), probably because of differences in the X and Y chromosomes (103), therefore, population studies investigating genome-wide DNA methylation must be stratified by gender (79, 80, 101, 104). Whereas gender may represent a confounding factor for some of the included studies (72, 85), statistical adjustment may have helped to reduce this bias. Both L1 methylation and cancer risk are modified by ethnicity and environmental carcinogen exposures (55, 80, 99, 105). Hospital-based studies (106) may include controls confounded by conditions unrelated to the disease under investigation. Ideally, controls samples should represent healthy individuals, matched on all potential confounding factors, both technical and biological. Case-control studies nested within prospective cohorts remain the gold standard for this type of analysis (16, 107).

Assay measurement

Repetitive element surrogate assays provide a practical and cost-effective indicator of genomic hypomethylation in tumor tissues and cell lines, where L1 methylation is highly variable (108). However, in blood, L1 methylation displays little variability (109), and it remains unclear how sensitive such assays are to detect subtle DNA methylation variability. Methylation of TEs, particularly L1, is often reported as genome-wide or "global" DNA methylation. However, L1 pyrosequencing measures methylation at only 3 to 4 CpG sites within a pool of L1 elements based on a consensus sequence (44), and is, therefore, not representative of genome-wide methylation (14). The detection of genome-wide hypomethylation by repetitive-element assays in tumor DNA is likely due the

occurrence of a proportion of repetitive elements within hypomethylated domains, rather than to a specific enrichment at repetitive elements (17, 24). Key to the utility of repetitive element methylation as a surrogate for genome-wide methylation is the reported correlation between 5meC measured by HPLC, and methylation of L1 and Alu measured by MethyLight, a methylation-sensitive PCR-based system, in a panel of cell lines and tissues. However, these correlations were moderate ($r = 0.66$ and 0.70 for L1 and Alu, respectively), and the authors recommended using a composite measure of 2 repetitive elements, ALU-M2 and Sat2-M1 ($r = 0.85$), rather than individual elements, as a surrogate for genome-wide methylation. The only study to measure the correlation between L1 pyrosequencing and 5meC in blood, or indeed any tissue, failed to find any correlation ($r = -0.204$), though the sample size was small ($n = 27$; ref. 73). Whereas L1 methylation measured by pyrosequencing is reduced in cell lines exposed to DNA methylation inhibitors (44), and in many tumors, it is unclear whether L1 methylation reflects subtle genomic methylation variability in blood. This discrepancy may explain the stronger association with cancer risk of blood 5meC than blood L1 methylation.

Two prospective cancer risk studies detected blood hypomethylation at Alu (97) and Sat2 (89), but not at L1 in the same samples, and methylation of L1 and Alu are not correlated (99). This is inconsistent with these assays detecting "genome-wide methylation," but suggests that hypomethylation may be restricted to specific genomic sequences. Widely apparent is the greater variability of repetitive element methylation between different studies than between cases and controls within individual studies (14). L1 methylation varies with ethnicity (55, 105), likely because of L1 genetic variability (14, 90). L1 sequence heterogeneity may also cause cellular and allelic heterogeneity in L1 methylation (110), and may lead to underestimation of methylation levels (77). L1 pyrosequencing, however, displays little technical variation (108), and it will be important to determine whether measures of 5meC are as technically reproducible. L1 elements are differentially methylated at different genomic loci (50, 103, 111), and at different CpG loci within the L1 consensus sequence (98), therefore, assays preferentially amplifying different L1 elements or measuring methylation at different CpG sites may produce inconsistent results.

Finally, methylation variability at individual "functional" repetitive elements may provide greater biomarker potential than methylation of "global" repetitive elements, as hypomethylation of an aberrantly transcribed L1 within the *MET* oncogene, but not global L1 methylation, was detected in normal bladder in patients with bladder cancer (50).

Statistical analysis

Categorical analysis of DNA methylation using OR is the most frequently used measure of disease risk for case control

epigenetic epidemiology studies (16). Furthermore, categorical analysis is useful for investigating potentially non-linear relationships between methylation and disease risk and retains more power to detect significant differences compared with linear regression. However, different approaches may introduce bias, for instance, a prominent inconsistency between studies included in this report is the use of different levels of methylation categories, including tertile, quartile, quintiles, and deciles, when a prespecified analysis plan and statistical power should have been described and used for the study. The narrow ranges of DNA methylation reported, especially at L1, would mean that the difference in percentage methylation between categories would be far below the detection sensitivity of the assay, for instance the technical variation for pyrosequencing, one of the most quantitative assays currently available, is around 2% and 3% (64). In our study, we observed an intraclass correlation coefficient for LINE1 in blinded duplicate samples of 0 (95% CI, 0–0.61) that suggests higher within individual variability than between individual variability. A major problem with developing small methylation differences as biomarkers is the overlap between methylation of cases and controls or lack of specificity (14).

Publication factors

According to the STROBE-ME (Strengthening the reporting of observational studies in epidemiology-molecular epidemiology) guidelines, all basic statistics of a biomarker measure distribution (mean, median, range, and variance) and details of all other analyses should be reported in studies investigating molecular biomarkers for disease risk (112); however, many reports do not include these, making direct comparison between studies difficult. Publication bias frequently affects meta-analyses of observational studies (113), and there is some evidence that it affects the current analysis.

All of the included reports showing significant association between L1 methylation and cancer included investigations of L1 methylation only, however all reports showing negative results for L1 also included studies showing significant associations between another methylation marker and cancer incidence/prevalence, consistent with a bias toward publication of significant associations. There appears to be a trend toward lower effect size with later publication date, as OR is significantly correlated with publication year among L1 methylation studies (spearman $r = 0.49$, $P = 0.05$). This appears to be independent of sample size, as the correlations between OR and sample size ($r = -0.19$), and sample size and year of publication ($r = 0.2$), are not significant. A funnel plot including L1 studies only (Supplementary Fig. S1) does not appear to show asymmetry or publication bias; however, the ability of this plot to indicate publication bias may be limited by the small number of studies ($n = 14$), and variable direction of effect in studies showing significant associations. A reporting bias exists whereby categorical analysis of methylation-risk associations tends only to be reported if it reveals statistically significant

results, and if the authors deem categorical analysis appropriate. The inclusion of categorical analysis of our previously reported L1 studies represents an attempt to address this bias; however, this could only be estimated for other studies that reported no association with cancer risk (Supplementary Fig. S2). As expected, inclusion of our studies, and other recent null studies, attenuated the significant association between L1 methylation and cancer risk reported by a recent meta-analysis (86). Inclusion of all relevant reports is critical to the accuracy of meta-analyses (95), therefore, publication and reporting biases may affect our ability to identify the true effect size.

Other Considerations for Epigenetic Risk Studies

A concern for all studies investigating blood DNA methylation in relation to cancer in other tissues is the tissue specificity of DNA methylation (91, 100). Several studies have indicated that methylation of repetitive elements is tissue specific, most variable in tumor tissue, and not correlated between tumor and blood (81, 98, 114). Consistently, evidence suggests that genomic hypomethylation in tumor and normal adjacent tissue of bladder and colon cancer was not detectable in blood (30, 50), suggesting that hypomethylation is restricted to the disease-affected tissue. Furthermore, approximately 36% of L1 elements display tissue-specific expression (37). Methylation variability between blood cell types is an important consideration for retrospective studies, as differential methylation may reflect cellular proliferation. A recent microarray-based study found that approximately 39% of CpG sites analyzed were differentially methylated between leukocyte subgroups (91). However, a recent report did not find any association between L1 methylation and blood cell count (115). Another potential caveat for all cancer biomarker studies is tumor subtype heterogeneity, where the biomarker under investigation is a feature of only 1 disease subtype. For example, in tumor tissue, L1 hypomethylation frequently occurs in cancers displaying chromosome instability, but rarely in cancers with microsatellite instability (20, 36, 116). Consistently, L1 hypomethylation is detectable in some colon tumor samples, but not others (20). Therefore, if blood L1 hypomethylation is associated with colorectal cancer risk, it may predict risk of some, but not all cancers.

Conclusions and Future Studies

Epigenetic epidemiology holds great promise for identifying biomarkers of cancer risk and understanding cancer etiology. It is important to investigate methylation variability at individual's loci in the context of widespread methylation changes associated with cancer; therefore, understanding the contribution of genome-wide DNA methylation to cancer susceptibility and development is needed. The challenges of investigating a genome-wide epigenetic phenomenon in population studies are formidable, because of the requirements of high genome coverage, highly quantitative DNA methylation measurement, and large sample size (16, 45). We conclude from

our meta-analysis that genome-wide DNA methylation, as measured by the surrogate L1 methylation assay, is not associated with cancer risk, shown most appropriately by several prospective cohort studies. However, there appears evidence for an association of genomic hypomethylation, measured using more representative assays, such as HPLC or methyl-acceptance assays, with cancer prevalence, though this remains to be validated in prospective studies.

A thorough investigation of the relationship between genome-wide DNA methylation and cancer risk would require whole-genome bisulphite sequencing of large cohorts of prospectively collected blood samples, with careful sample selection, case-control matching, and deep sequencing to achieve the sensitivity required to detect subtle DNA methylation variability (16, 45). Such an approach would provide information about genome-scale methylation changes as well as local methylation variability, thus providing candidate regions for risk biomarker development. An equivalent study using retrospectively collected blood samples may provide candidate diagnostic biomarkers. Whole-genome bisulphite sequencing is extremely costly, however, technological improvement will make this feasible in the coming years (45, 75). Whereas current research using whole-genome sequencing of small numbers of samples cannot provide information about methylation variability across populations, further classification of the regions that consistently undergo methylation changes in cancer may provide candidate regions for a sequence capture approach in population studies. New primer technology will enable the investigation of epigenetic regulation at specific "functional" repetitive elements, such as those L1 elements capable of retrotransposition (47, 50), or driving ectopic expression of neighboring genes (37, 50). Furthermore, availability of the first microarrays specifically covering repetitive elements will provide high-throughput methods of investigating repetitive element methylation on a genome scale (117). The most popular tool for current investigation of DNA methylation variability in population studies is the Illumina 450K methylation beadchip, which measures methylation at individual CpG sites across the entire human genome. These arrays are highly quantitative, high-throughput, and largely unbiased in terms of genomic coverage, making them suitable for biomarker discovery and epigenome-wide association studies (16). Use of these assays will likely provide interesting evidence for the implications of DNA methylation variability in disease susceptibility and development in coming years.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K. Brennan, J.M. Flanagan

Development of methodology: K. Brennan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Brennan, J.M. Flanagan

Writing, review, and/or revision of the manuscript: K. Brennan, J.M. Flanagan

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Brennan
Study supervision: J.M. Flanagan

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