DNA methylation abnormalities at gene promoters are extensive and variable in the elderly and phenocopy cancer cells

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ABSTRACT Abnormal patterns of DNA methylation are one of the hallmarks of cancer cells. The process of aging has also been associated with similar, albeit less dramatic, changes in methylation patterns, leading to the hypothesis that age-related changes in DNA methylation may partially underlie the increased risk of cancer in the elderly. Here we studied 377 participants aged 85 yr from the Newcastle 85+ Study to investigate the extent of, and interindividual variation in, age-related changes in DNA methylation at specific CpG islands. Using highly quantitative pyrosequencing analysis, we found extensive and highly variable methylation of promoter-associated CpG islands with levels ranging from 4% to 35%, even at known tumor suppressor genes such as TWIST2. Furthermore, the interindividual differences in methylation seen across this elderly population phenocopies multiple features of the altered methylation patterns seen in cancer cells. Both aging- and cancer-related methylation can occur at similar sets of genes, both result in the formation of densely methylated, and likely transcriptionally repressed, alleles, and both exhibit coordinate methylation across multiple loci. In addition, high methylation levels were associated with subsequent diagnosis of leukemia or lymphoma during a 3-yr follow-up period (P=0.00008). These data suggest that the accumulation of age-related changes in promoter-associated CpG islands may contribute to the increased cancer risk seen during aging.—Gautrey, H. E., van Otterdijk, S. D., Cordell, H. J., Newcastle 85+ study core team, Mathers, J. C., Strathdee, G. DNA methylation abnormalities at gene promoters are extensive and variable in the elderly and phenocopy cancer cells. FASEB J. 28, 3261–3272 (2014). www.fasebj.org

Key Words: epigenetics • aging • CpG island • cell type specific

Cancer is one of the leading causes of death worldwide. The risk of almost all types of cancer increases with age, and population aging is expected to be one of the primary drivers of the predicted dramatic increases in cancer incidence in the next 20 yr (1). Thus, despite the improved success of treatment of most types of cancer, it is likely that the burden of cancer in the world’s population will continue to increase.

DNA methylation, which occurs almost exclusively at CpG dinucleotides, is an important epigenetic mechanism for controlling gene expression (2). In particular, hypermethylation of gene promoters that have high densities of CpG sites, known as CpG islands, is associated with chromatin compaction and consequent loss of gene expression (3). This is a critical mechanism for control of expression of imprinted genes, for X-chromosome inactivation, and for many germ cell-specific genes (2). However, although the majority of human genes possess a promoter-associated CpG island, these generally remain methylation free, even in tissues where the associated gene is not expressed (3). The development of cancer is associated with dramatic shifts in the pattern of DNA methylation (4). These include global reduction in genome-wide levels of DNA methylation, highly variable methylation at CpG island shores, and increased methylation of promoter-associated CpG islands. This increased CpG island methylation is often associated with gene silencing and the altered DNA methylation has been shown to be required for maintaining transcriptional inactivation of the linked gene (5). Many genes known to be important in cancer development, including the tumor sup-

Abbreviations: ALL, acute lymphoblastic leukemia; MNC, mononuclear cell; PBL, peripheral blood leukocyte; SNP, single nucleotide polymorphism; WBC, white blood cell
pressor genes MLH1 and BRCA1 (6), are inactivated by this mechanism.

However, these methylation changes are not unique to cancer, and similar, albeit less dramatic, changes are also seen in apparently healthy individuals during aging (7). Cross-sectional studies have reported that global levels of DNA methylation reduce with age (8), although cohort studies, in which methylation was measured in the same individual several years apart, have shown that the accumulation of age-related methylation changes can be highly individual (9). Furthermore, promoter-associated CpG islands can exhibit increasing levels of DNA methylation with increasing age (7).

Based on these observations, we hypothesized that the gradual acquisition of abnormalities in the pattern of DNA methylation during aging increases cancer risk and thus partially underlies the strong link between older age and greater risk of most types of cancer (10). In addition, it has been speculated that the altered methylation will likely result in age-related changes in gene expression, which may have subsequent impacts on the development of other age-related illnesses (11). Several age-related illnesses are associated with changes in DNA methylation at specific genes, suggesting a potentially significant role for altered DNA methylation in the development of these disorders (11).

If the acquisition of age-related methylation changes varies widely between individuals, this could play a key role in differential susceptibility to the development of cancer and other diseases associated with aging. However, the extent of, and interindividual variability in, CpG island methylation in apparently healthy people across the life course is not well understood. To begin to assess whether differences in the acquisition of altered methylation patterns during aging impacts on health, we have examined CpG island methylation in peripheral blood DNA from a large cohort of 85-yr-olds from the Newcastle 85+ Study (12). This strategy has the advantage that, because all participants are the same age, all variation detected represents interindividual variation with no confounding due to differences in chronological age. Furthermore, by studying a population of the oldest old, any interindividual differences in age-associated methylation changes are likely to be maximized.

**MATERIALS AND METHODS**

**Study population**

DNA was extracted from peripheral blood leukocytes from 377 participants from the Newcastle 85+ study. The analysis was initially performed as a pilot study on 50 samples (consisting of 25 survivors, at 3 yr follow-up, and 25 nonsurvivors). After successful analysis of the pilot study samples the analysis was extended to all participants with a previous cancer history \((n=113)\), all participants with an incident cancer diagnosis \((at 3 \text{ yr follow-up}, n=72, 20 \text{ of whom were also in the previous cancer history group})\), and a control group without a history of cancer or an incident diagnosis \((n=212)\). The methodology for the Newcastle 85+ Study has been reported (12, 13). In brief, members of the 1921 birth cohort living in Newcastle or North Tyneside (Northeast England) were recruited at approximately age 85 through general practice patient lists. People living in institutions and those with cognitive impairment were included. The research complied with the requirements of the Declaration of Helsinki. Ethical approval was obtained from the Newcastle and North Tyneside 1 Research Ethics Committee (reference number 06/Q0905/2). Written informed consent was obtained from participants, and where people lacked capacity to consent, for example, because of dementia, a formal written opinion was sought from a relative or care giver. Information on previous cancer history and incident cancer diagnoses (at 3 yr) was obtained from the general practice medical records. In the UK, patients are registered with a single general practice, which acts as a gatekeeper to secondary care and receives details of all hospital admissions and outpatient attendances. The review of general practice records included hospital correspondence to ensure that all recorded disease diagnoses were extracted irrespective of where and when the diagnosis was made.

To allow analysis of methylation at different ages another 150 DNA samples were obtained. These consisted of 50 DNA samples extracted from cord blood from newborns and 50 extracted from peripheral blood of their mothers (median age 28 yr old) from the North Cumbria Community Genetics Project (14) and 50 peripheral blood DNA samples from the 50-yr-old participants from the Newcastle Thousand Families Study (15). Although the samples from newborn and mother groups were from related individuals, there were no significant correlations between mother-child pairs for any of the genes included in this study (for more details of relationships between maternal and child DNA methylation in this cohort, see refs. 16, 17).

**DNA modification and PCR**

We modified 200 ng of genomic DNA with sodium bisulfiten using the Methylenzyme One-Step DNA Modification Kit (Epigentek, Brooklyn, NY, USA) as per the manufacturer’s instructions. All samples were resuspended in 15 μl of TE, and 1 μl of this was used for subsequent PCR reactions. The samples were amplified in 25 μl volumes containing 1× manufacturer’s buffer, 1 U of FastStart Tag polymerase (Roche, Welwyn Garden City, UK), 1–4 mM MgCl₂, 10 mM dNTPs, and 75 ng of each primer. PCR was performed with 1 cycle of 95°C for 6 min, 40 cycles of 95°C for 30 s, 57–63°C for 30 s, and 72°C for 30 s, followed by 1 cycle of 72°C for 5 min. For each set of primers (listed in Table 1) 1 of the forward or reverse primers included a 5‘ biotin label to allow for subsequent analysis by pyrosequencing.

**Quantitative DNA methylation analysis using pyrosequencing**

Following PCR amplification sequencing was performed using a PSQ 96MA pyrosequencer (Qiagen, Hilden, Germany), as per the manufacturer’s protocol. For all loci, assays were performed in duplicate and values averaged between the duplicates. Only samples that were passed by the pyrosequencer were included, and to further ensure a high degree of accuracy, only runs in which single peak heights were in excess of 200 were included. Samples in which the repeats varied in excess of 2% methylation were repeated 1 additional time and the average taken across the 3 repeats.

For each locus between 3 and 6 consecutive CpG sites were measured, and the methylation value for each loci was taken...
| Gene  | Forward primer | Reverse primer | Sequencing primer | CpG site | Distance from TSS | Genomic region covered |
|-------|---------------|----------------|-------------------|---------|-------------------|----------------------|
| APOE  | GGAGTTTTATAATTGGATAAGT | AAAATCCCAACTCTTTTCT | GGGATTTTTGAGTTTTTATT | 3       | -24 → +123 | chr1: 44905758–44905904 |
| EPHA10 | ATTATTTATGGGAATTTATTTAG | CCCACCAATTAATATTC | TATTTATGGGAATTTATTTAGTA | 3       | +28 → +143 | chr1: 37765181–37765295 |
| ESR1  | GGAGATATATTTTAAAGTGGAGGT | CCTAAAAAGAAAAACACACACC | TGGGATTGTATTTGTTTT | 5       | +92 → +294 | chr6: 151807643–151807843 |
| HAND2 | CCAAATTTTTAATTTATTATA | GGATTTTTTATAAGATTTATTTT | ATTTTATGCCAAACCCTTC | 5       | -29 → +185 | chr4: 173530198–173530412 |
| HLB9  | TAGTGYGGTTTGTTTAGGA | ATCACTACACATCTAAAG | GAAGTYGTGTTGATTTTTT | 4       | -161 → +61 | chr7: 157010837–157010616 |
| HOXA4 | TACACTTCAACATTTAAATAGTTTTG | GATTTTTTATTTTTTATTAG | CTTTTTATGCCAAACCCTTC | 4       | +31 → +219 | chr7: 27130749–27130562 |
| HOXB9 | TCTACCTCAGCTACAACTACTATCAC | ATTTTATGCCAAACCCTTC | AATTTTATGCCAAACCCTTC | 4       | +321 → +397 | chr7: 27143608–27143342 |
| HOXA5 | CGTGGTTTTAGTTTTAGTTT | ATTTTATGCCAAACCCTTC | AATTTTATGCCAAACCCTTC | 3       | +31 → +219 | chr7: 27130749–27130562 |
| HOXD4 | GAAATTTATTTTTTAGGGT | CTTTTTATGCCAAACCCTTC | AATTTTATGCCAAACCCTTC | 3       | +321 → +397 | chr7: 27143608–27143342 |
| IGF2  | GAGTTTATTTTTTAGGGT | ATTTTATGCCAAACCCTTC | AATTTTATGCCAAACCCTTC | 3       | +321 → +397 | chr7: 27143608–27143342 |
| MLH1  | ATTTTTATTTTTTAGGGT | ATTTTATGCCAAACCCTTC | AATTTTATGCCAAACCCTTC | 3       | +321 → +397 | chr7: 27143608–27143342 |
| p15   | GAGTTTATTTTTTAGGGT | ATTTTATGCCAAACCCTTC | AATTTTATGCCAAACCCTTC | 3       | +321 → +397 | chr7: 27143608–27143342 |
| RARB2 | TATGTAGATTATATAAGTTGTTTGT | AATTTTATGCCAAACCCTTC | GAGTTTATTTTTTAGGGT | 4       | -207 → +41 | chr9: 22009107–22009354 |
| RASSF1A | GAGTTTATTTTTTAGGGT | ATTTTATGCCAAACCCTTC | GAGTTTATTTTTTAGGGT | 5       | +136 → +454 | chr3: 30340801–30340503 |
| TUSC3 | GAATATTAGTTTTTTAGTTT | TAATTTTTTTTTTTTTTTTTT | CCAATTACCAACCCTTAC | 6       | +25 → +115 | chr8: 15540246–15540336 |
| TWIST2 | AAGAGATTATTTTACTCCTAACCCTT | GAGTTTATTTTTTAGGGT | CCAATTACCAACCCTTAC | 4       | +26 → +208 | chr2: 238848006–238848239 |

*Based on GRCh38 primary assembly.*
as the mean of all CpG sites measured at that loci. Methylation values at consecutive CpG sites were strongly correlated, with correlation coefficients between consecutive CpG sites ranging 0.93–0.98 (TWIST2), 0.87–0.93 (TUSC3), 0.63–0.87 (HAND2), 0.80–0.86 (HOXD4), 0.42–0.64 (EPHA10), and 0.96–0.98 (HOXA5).

 Primer design was performed using the manufacturer’s provided PyroMark software. For initial assessment of all assays pyrosequencing was carried out on samples of known methylation status (5%, 10%, 15%, 20%, 50%, 70%, and 100% methylated), which were produced by diluting peripheral blood derived DNA (from a young adult volunteer) into in vitro methylated DNA (Millipore, Watford, UK). Only assays that produced correlations with $r^2$ of $≥0.90$ were used for further analysis.

## Derivation of overall methylation level

To produce a more accurate estimate of the comparative levels of age-related CpG island methylation in the samples, the values for each of the 5 age-related loci examined in the full sample set (EPHA10, HOXD4, HAND2, TUSC3, TWIST2) were averaged. The values at the 5 loci were weighted prior to averaging to ensure an equal contribution from each locus to the final average.

The percentage of lymphocytes in each peripheral blood sample was highly variable (8–56%) and was found to correlate strongly with overall methylation levels. To correct for this effect a correlation analysis was performed between overall methylation and lymphocyte percentage. The slope of the regression line produced was calculated to determine the average increase in methylation per 1% increase in lymphocyte percentage (slope=0.1811). This was then used to correct all values back to the average percentage of lymphocytes in the population (29.0%).

## Bisulfite sequencing

Sodium bisulphite modification and PCR was carried out as above. PCR products were then cloned using the TA Cloning Kit (Invitrogen, Paisley, UK), as per the manufacturer’s protocol. Next, 2 μl of the ligation product was transformed into One Shot TOP10 Chemically Competent E. coli cells (Invitrogen) and single colonies obtained by plating on LB agar plates containing kanamycin (50 μg/ml). Vector DNA was isolated and purified by lysis of bacterial cells and adsorption of DNA using QIAprep Spin Miniprep kit (Qiagen, Crawley, UK) according to the manufacturer’s protocol and eluted in 50 μl of elution buffer. The presence of the PCR amplified region of interest was confirmed by EcoRI (Fermentas, York, UK) restriction enzyme digestion, which cuts the vector on either side of the incorporated PCR product. Sequencing of the inserts was then carried out using the Sp6 or T7 sequencing primers (Source Bioscience, Nottingham, UK).

## Isolation of specific hematopoietic cell types

CD19+ B lymphocytes and CD14+ monocytes were isolated using the DynaBeads system (Invitrogen), according to manufacturer’s protocol. These cell populations were isolated from mononuclear cell populations, which were obtained after density gradient centrifugation of whole blood samples using Ficoll-Hypaque (BiochromAG, Berlin, Germany). We collected 15 ml of whole blood in an EDTA tube and resuspended in 15 ml PBS. The blood-PBS mixture was carefully layered on top of 15 ml of Ficoll-Hypaque and centrifuged for 20 min at 800 g. This resulted in several layers of cell types, including a layer of peripheral blood mononuclear cells. This mononuclear cell layer was carefully removed and put into a fresh tube. Mononuclear cells were then split into 3 and used directly for DNA isolation or for isolation of CD19+ or CD14+ cells.

The presence of the DynaBeads still attached to the isolated cells prevented assessment of purity by FACS analysis of cell surface markers, and so purity of the isolated cell populations was validated using RT-PCR for the specific cell surface markers used to isolate the populations.

The levels of lymphocyte subpopulations in peripheral blood samples taken from Newcastle 85+ participants were determined using flow cytometry (Becton Dickinson FACScan Flow Cytometer, Oxford, UK) for B cells (CD19+) and T cells (CD3+, CD56+), as described previously (18).

## Statistical analysis

Correlations between methylation levels and fractions of specific cell types and correlations in methylation levels between the individual genes assessed were investigated by calculating the Pearson correlation coefficient. The Pearson correlation coefficient was also used to investigate relatedness in methylation at neighboring CpG sites at individual loci, as determined by pyrosequencing analysis. The Student’s t test was used to assess differences in methylation at specific loci or average methylation (of TWIST2, TUSC3, HOXD4, HAND2, and Epha10) between cohorts of different ages, with different histories of cancer diagnosis and between sorted cell types. Coefficients of variation in the initial 50 samples assessed were calculated by dividing the sd by the mean methylation at each individual locus. The Mann-Whitney U test and Fisher’s exact test were also used where indicated.

## RESULTS

### Genes methylated in hematological malignancies show highly variable DNA methylation in peripheral blood from 85-yr-olds

DNA methylation levels in peripheral blood samples were assessed using pyrosequencing analysis at 15 candidate genes (see Table 2), in an initial subset of 50 participants from the Newcastle 85+ Study. Pyrosequencing provides robust quantification of methylation levels so that even low levels of interindividual variation can be quantified. The assays interrogated CpG sites in CpG islands surrounding the transcriptional start sites of the respective genes (except for IGF2 where it targeted the imprinted DMR0 region), since these regions would be most likely to influence expression of the corresponding gene. The candidate genes were selected to include: genes known to be methylated in hematological malignancies (EPHA10, HOXD4, HAND2, HLBX9, TUSC3, TWIST2, p15 (19–23), and the authors’ unpublished data); genes known to exhibit age or malignancy-associated methylation in other tissues (ESRI, RARB2, RASSF1A, MLH1) (24–27); and genes implicated in other age-related diseases (APOE) (28). The imprinted gene IGF2 and HOXA4, and HOXA5, which exhibit high levels of methylation throughout life (29, 30), were included as positive controls.
Methylation levels (averaged across all CpG sites assayed) were relatively high for several candidate genes including EPHA10, HOXD4, HAND2, TUSC3, and TWIST2, which had maximal methylation levels of 18–35%. However, interindividual variation in methylation levels was also substantial (see Fig. 1A–C for examples). Furthermore, such higher levels of methylation were not seen for all genes examined, and methylation levels for some genes (such as MLH1, RASSF1A, and RARB2) exhibited lower or near undetectable levels (Fig. 1D–F). As shown in Table 2, higher levels of CpG island methylation were more common in

| Gene     | Methylation range (%) | Mean (%) | Median (%) | sd (%) | CoV% | Associated with leukemia/lymphoma |
|----------|-----------------------|----------|------------|--------|------|----------------------------------|
| HOXD4    | 7–35                  | 14.8     | 13.3       | 6      | 0.399| Yes                               |
| EPHA10   | 7–31                  | 12       | 11.4       | 3.9    | 0.318| Yes                               |
| TUSC3    | 4–22                  | 8.9      | 8.7        | 3.4    | 0.38 | Yes                               |
| TWIST2   | 4–20                  | 8.4      | 7.8        | 3.3    | 0.395| Yes                               |
| HAND2    | 2–18                  | 7.3      | 6.6        | 2.8    | 0.382| Yes                               |
| HLXB9    | 1–15                  | 3.3      | 3.0        | 2.1    | 0.371| Yes                               |
| APOE     | 6–13                  | 8.4      | 7.9        | 1.9    | 0.22 | No                                |
| p15      | 2–12                  | 4.9      | 4.4        | 1.9    | 0.381| Yes                               |
| ESR1     | 4–11                  | 5.5      | 5.1        | 1.6    | 0.292| No                                |
| RARB2    | 1–5                   | 2.5      | 2.3        | 1      | 0.397| No                                |
| RASSF1A  | 1–4                   | 2.5      | 2.4        | 0.7    | 0.257| No                                |
| MLH1     | 1–2                   | 1.3      | 1.2        | 0.3    | 0.256| No                                |

**Control gene**

| HOXA5    | 28–92                 | 61.5     | 63.3       | 15.1   |      |                                    |
| HOX4     | 13–64                 | 41.5     | 42.4       | 11.8   |      |                                    |
| IGF2     | 30–50                 | 40.2     | 40.0       | 4.4    |      |                                    |

*CoV = Coefficient of variance.

Figure 1. Examples of variation in methylation in peripheral blood samples. Examples of genes with high levels (A–C) or low levels (D–F) of age-related methylation in Newcastle 85+ study samples. Methylation levels were assessed by pyrosequencing in each of the indicated genes in an initial sample set from 50 85+ study participants (labeled 1–50 along the x axis). The y-axis scale is the same for all genes (0–25%), except for HOXD4 (0–40%), because this gene exhibited the highest levels of methylation. Error bars indicate se.
genes known to be methylated in hematological malignancies. In contrast, genes known to exhibit age- or malignancy-associated methylation in other tissues or those implicated in nonmalignant disease generally had lower levels of methylation \((P=0.009, \text{Mann-Whitney } U \text{ test})\). As expected, high levels of methylation were found in the 5 control genes assessed (HOXA5, HOXA4, and IGF2) (Table 2). The patterns of genes hypermethylated in different types of cancer are known to be cell type specific (31), and the results presented here are consistent with the hypothesis that the patterns of genes that accumulate methylation during aging are similarly cell type specific and may target similar sets of genes to those that are highly methylated in cancers derived from the same cell type.

Methylation levels in this initial subset of 50 participants were correlated significantly with the percentage of lymphocytes in the WBC population \((r^2=0.148, P=0.006)\). Consequently, for subsequent studies (where indicated), methylation levels were corrected for lymphocyte percentage as described in Materials and Methods.

Genes that exhibit variable methylation in 85-yr-olds also exhibit age-related acquisition of methylation across the life course

Methylation levels at the 5 most highly methylated loci, the control loci and a largely unmethylated locus \((MLH1)\) were also assessed in DNA from newborns, young adults and 50-yr-olds and compared with the data obtained above from the initial subset of 50 participants from the Newcastle 85+ Study. While methylation of the 5 highly methylated loci was detectable at all ages, this increased significantly with increasing age \((i.e., \text{newborns}<\text{young adults}<50-\text{yr-olds}<85-\text{yr-olds})\), confirming that the acquisition of methylation at these loci was age related (Fig. 2, Supplemental Table 1). In contrast, the levels of methylation at the control loci, although consistently high, did not show age-related increases across the life course (Supplemental Fig. S1, Supplemental Table 1) and methylation of \(MLH1\) remained low/undetectable methylation in all age groups (Fig. 2G).

Age-related methylation exhibits multiple features of malignancy-associated methylation

As described above, there was significant overlap between the genes exhibiting higher methylation in DNA from peripheral blood in the subset of 50 participants from the Newcastle 85+ Study and those genes known to exhibit cancer-related methylation. To provide a more rigorous examination of this apparent concordance between aging and tumorigenesis, we examined DNA from an expanded set of samples derived from an additional 327 participants in the Newcastle 85+ Study. For this analysis we quantified methylation of the 5 genes that were most highly methylated during aging \((EPHA10, HOXD4, HAND2, TUSC3, \text{and } TWIST2)\) together with the highly methylated, but not age-related, gene \(HOXA5\). This expanded panel of samples confirmed the extensive and highly variable methylation at all 5 loci (Supplemental Fig. S2). One of the notable features of methylation in cancer is that methylation across multiple CpG islands is strongly correlated (32).

![Figure 2](image-url)
To assess if a similar pattern is seen for age-related methylation, methylation levels (corrected for lymphocyte percentage) at all 5 loci were investigated for evidence of comethylation. As shown in Table 3, we found highly significant correlations between methylation of each of the 5 genes with methylation levels of any of the other genes (specific examples of participants with high and low methylation are shown in Supplemental Table 2). In contrast, no significant correlation was found between methylation levels for any of these 5 loci and that for the highly methylated, but not age-related, gene HOXA5.

A further feature of cancer cells is the presence of densely methylated alleles, in which most or all CpG sites in a promoter-associated CpG island are methylated. Such alleles have invariably been associated with lack of gene expression (33). To begin to investigate if the identified age-related methylation can lead to the appearance of densely methylated alleles, similar to those observed in cancer cells, we used bisulfite sequencing to investigate methylation at the allelic level in one of the participants with higher methylation at 2 loci (TWIST2 and HOXD4). As shown in Fig. 3A, methylation was unequally distributed, with some alleles exhibiting dense methylation at all or most CpG sites assessed, while other alleles remained largely methylation free. This demonstrates that age-related methylation can lead to the development of cancer-like densely methylated regions that would likely be transcriptionally inactive.

High methylation levels may be detectable prior to the diagnosis of lymphoid malignancy

The similarities between the processes of age- and cancer-associated DNA methylation raises the possibility that altered methylation in apparently healthy cells may precede the development of clinically detectable disease and that age-associated methylation may predispose individuals to the development of cancer (34). To provide an overall estimate of the extent of age-related CpG island methylation in each sample, the methylation values for all 5 age-related genes (corrected for lymphocyte percentage) were combined (as described in Materials and Methods) to produce an overall methylation level. Interestingly, when the distribution of these combined methylation levels was examined, 4 of the participants (who exhibited the highest methylation levels) were found to lie out with the normal distribution (Fig. 4). Of these, 2 participants were subsequently diagnosed with leukemia or lymphoma compared with none out of 373 participants without unusually high methylation levels ($P=0.00008$, Fisher exact test). As the diagnosis of malignant disease in these 2 patients was up to 2.5 yr after the baseline blood sample was taken, this illustrates that increased methylation can be detectable for a significant period of time before the clinical diagnosis of disease.

To determine if the patterns of age-associated methylation detected in this population were more generally associated with cancer development, methylation levels (corrected for lymphocyte percentage) were compared between participants who had had a diagnosis of any type of cancer prior to the study (excluding nonmelanoma skin tumors, $n=113$) and participants with no history of cancer diagnosis ($n=212$). Individuals with a previous diagnosis of cancer had significantly higher methylation levels, however the difference between individuals with and without a history of cancer was relatively small (10.40% vs. 10.06%, $P=0.04$). During a 3-yr follow-up period, 72 individuals were diagnosed with cancer, and methylation levels in this group of individuals were compared with those in the 212 cancer-free participants. This confirmed the link between increased methylation and cancer diagnosis, because individuals with an incident cancer diagnosis also exhibited significantly higher methylation levels at study baseline (10.59% vs. 10.06%, respectively, $P=0.008$). However, as for participants with a previous cancer diagnosis, the absolute increase was small. Overall, the results suggest that, at least for the genes examined in this study, dynamic increases in methylation levels may be detectable prior to cancer diagnosis only if examined in the corresponding tissue or cell type (e.g., peripheral blood for prediction of hematological malignancies). Small differences in the methylation levels of HOXA5 were also seen (mean methylation levels 62.2% for no cancer controls, and 60.4% and 64.19%, for previous cancer and incident cancer groups, respectively). However, these differences were not statistically significant and the direction of change was not consistent in the previous cancer and incident cancer groups.

**Table 3. Comethylation of genes in 85+ samples**

|     | TWIST2 | TUSC3 | HOXD4 | HAND2 | EPHA10 |
|-----|--------|-------|-------|-------|--------|
| TUSC3 | $r^\alpha = 0.60; P < 1 \times 10^{-7}$ |       |       |       |        |
| HOXD4 | $r = 0.55; P < 1 \times 10^{-7}$ | $r = 0.55; P < 1 \times 10^{-7}$ |       |       |        |
| HAND2 | $r = 0.29; P < 1 \times 10^{-7}$ | $r = 0.31; P < 1 \times 10^{-7}$ | $r = 0.38; P < 1 \times 10^{-7}$ |       |        |
| EPHA10 | $r = 0.32; P < 1 \times 10^{-7}$ | $r = 0.33; P < 1 \times 10^{-7}$ | $r = 0.20; P < 0.0001$ | $r = 0.32; P < 1 \times 10^{-7}$ |        |
| HOXA5 | $r = 0.04; P = 0.62$ | $r = -0.03; P = 0.72$ | $r = -0.002; P = 0.98$ | $r = -0.02; P = 0.77$ | $r = 0.003; P = 0.97$ |

$\alpha$Pearson correlation coefficient.
DNA methylation is cell type specific and largely restricted to cells of lymphoid origin

As described above DNA methylation levels correlated with the proportion of lymphocytes in the WBC population ($r^2=0.32$, $n=377$, $P<1\times10^{-40}$ for the whole cohort) (Supplemental Fig. S3). DNA from individual cell types from participants within the 85+ Study was not available. However we obtained whole blood samples from young adults. Multiple different cell populations were then purified from these samples and methylation was assessed in DNA extracted from the different cell populations. This analysis showed that for the 5 loci exhibiting age-related methylation, DNA from total lymphocytes exhibited higher methylation than total peripheral blood leukocytes and that methylation levels in purified myeloid cells (monocytes) were significantly lower (examples in Fig. 3B). Even within the lymphoid compartment, methylation levels differed between cell types with B lymphocytes exhibiting significantly higher levels of CpG island methylation (Fig. 3B, Supplemental Table S3). While slight differences were seen in the nonage-related genes HOXA4 and HOXA5, these were far less pronounced (Supplemental Table 3). These results are again consistent with a link between age- and malignancy-related methylation, because all 5 loci are highly methylated specifically in lymphoid and not myeloid malignancies (22, 35, unpublished data).

The above analysis is consistent with the correlation that we observed between total methylation levels and proportion of lymphocytes in the WBC population in the Newcastle 85+ study samples. However, because B lymphocytes exhibit relatively high levels of methylation, it further suggests that this correlation may be primarily driven by the fraction of B lymphocytes. Thus we examined the correlations between methylation levels and the proportion of B lymphocytes and T lymphocytes in the WBC population in the Newcastle
DISCUSSION

Both tumor development and aging are associated with similar alterations in the patterns of genomic DNA methylation. Both processes result in loss of genome-wide methylation alongside gains in promoter-associated CpG island methylation. This has raised the possibility that accumulation of age-related methylation changes may increase the likelihood of malignant transformation and thus partly explain the strong link between older age and incidence of most cancer types (37). Here we sought to quantify the extent of, and interindividual variability in, CpG island methylation occurring during aging. This analysis demonstrated that even in cancer-free individuals, methylation levels at promoter-associated CpG islands can reach high levels, even at presumptive tumor suppressor genes such as TWIST2 and TUSC3. For some genes, we observed extensive variation in methylation not only between different age groups but also between individuals of the same age, raising the possibility that differential levels of accumulation of age-related methylation could play an important role in determining susceptibility to the development of age-associated disorders, particularly cancer.

This analysis found a clear similarity between the patterns of CpG island methylation resulting from aging and those previously attributed to the development of cancer. This included the altered methylation of a common set of genes, coordinate methylation of multiple loci at multiple sites throughout the genome, and the presence of densely methylated regions, which would likely lead to transcriptional inactivation. This raises 2 interesting possibilities. One potential explanation for this would be that the mechanisms responsible for cancer- and aging-related alterations in DNA methylation may be largely the same. Alternatively, it may be that a significant portion of the abnormal methylation found in cancer cells is not acquired during the disease process, but instead is produced by clonal expansion of cells that already contain the abnormal methylation patterns. If true, this would suggest that cells with altered methylation states may be at much greater risk for malignant transformation, as suggested by the epigenetic progenitor origin of cancer hypothesis (38).

Several studies have previously demonstrated an overlap between age- and cancer-related methylation. These include studies showing that both processes preferentially involve genes regulated by the polycomb group complexes (39), genes that exhibit bivalent chromatin domains (40), and genes associated with interindividual epialleles (41). Our results agreed with this link between age- and cancer-related methylation, because methylation levels were significantly higher in genes with a known link to leukemia compared with those chosen due to age- and cancer-related methylation in other tissues. However, by using highly quantitative methylation analysis our results also emphasize that the development of age-related methylation is not inevitable.

Co-ordinate methylation across multiple promoter-associated CpG islands has been found in many types of cancer. For example, it has been suggested that there is a CpG island methylator phenotype in colorectal cancer in which a subset of tumors accumulate much more widespread methylation of CpG islands (42). Although many other studies have described a CpG island methylator phenotypes in other cancers, in the main these have been limited to identifying comethylation of genes, i.e., tumors in which high methylation of 1 gene is associated with increased likelihood of higher methylation at the other genes assessed in the study (43). Indeed, in a genome-wide study of childhood acute lymphoblastic leukemia (ALL), we have found that >1000 loci (including all 5 age-related genes investigated in the present study) exhibited coordinated methylation levels (i.e., their levels of methylation were significantly correlated, unpublished data). Thus the coordinate methylation of genes seen in the present study (as detailed in Table 3) appears to be analogous to the coordinate methylation previously reported in multiple types of cancer. Furthermore, as pointed out by Teschendorff and colleagues in a recent review, a key question regarding age-related methylation changes is whether they occur predominantly at individual CpG sites or are spatially related as in cancer (44). Here we show that not only do neighboring CpG sites show strong correlations in methylation levels, but that these correlations extend to multiple loci located on separate chromosomes. Thus the potential impact of age-related methylation on biological function has to be viewed not just in the context of changes at a single locus, but also coordinate changes in methylation across many loci.
The variability in methylation even among healthy individuals which we report here may be driven, at least in part, by underlying genetic variation. Twin and population studies have demonstrated that epigenetic variation can be associated with genetic variation (45, 46). However, these studies have predominantly found SNPs to be associated in cis with methylation at closely linked loci (46). In this study we found that variability at the age-related loci was correlated and the loci exhibit co-ordinate patterns of methylation. Thus any genetic variation underlying this variability would have to influence methylation levels at multiple loci, unlike the site-specific correlations observed to date.

The detection of higher levels of methylation in individuals prior to clinical diagnosis of leukemia/lymphoma raises the possibility that this could be used for early detection or even identification of high-risk individuals. This would be consistent with the observation that the 2 cases of leukemia/lymphoma that were subsequently diagnosed in the study population occurred in the participants with the highest baseline levels of methylation. It is also notable that while methylation of the genes examined increased with age, aberrant methylation of promoter-associated CpG islands of the same genes was detectable in PBL samples at all ages (even at birth), suggesting that cells with preexisting methylation abnormalities could underlie the development of cancer at all stages throughout the life course.

In addition, CpG island methylation levels were higher in participants with a cancer diagnosis (previous diagnosis or incident cancer). While the increases observed (vs. participants with no cancer history) were statistically significant, the absolute differences were very small and thus assessing methylation levels at the genes in this study would not provide useful information regarding overall cancer risk. However, analysis of an expanded set of loci or genome-wide methylation studies may identify loci whose methylation status is more predictive of risk of nonhematological cancers. Indeed, Brennan et al. have recently demonstrated that intragenic methylation at the ATM locus in peripheral blood samples is associated with risk of breast cancer development (47).

A potential explanation for the similarity in genes showing age- and cancer-related methylation could be that nonexpressed or functionally unimportant genes may be the predominant targets for both processes. Indeed, a number of recent studies have found relatively low correlations between aging-associated changes in methylation and gene expression (48, 49), and studies in cancer have also found that hypermethylation of promoter-associated CpG islands is predominantly associated with genes that are not expressed in the corresponding normal tissue (50). However, this is clearly not always the case. The TWIST2 gene exhibits tumor suppressor like properties in B cell leukemia and is expressed in normal differentiated B lymphocytes (22, 51), and yet methylation of this gene was not only present in peripheral blood samples, but is predominantly seen in B lymphocytes; the same cell type in which it appears to play a role in preventing malignant transformation. Subsequent bisulfite sequencing showed that this methylation was characterized by the presence of densely methylated alleles. We have observed similar densely methylated alleles in ALL and shown that this methylation maintains transcriptional repression of the gene (22). Since age-related methylation, like cancer-related methylation, can affect a large number of loci across the genome (39, 40), only a fraction of these epigenetic changes would need to target functionally relevant genes to result in significant biological effects.

Methylation levels varied between the different cell types in sorted peripheral blood samples from younger individuals and were largely lymphoid specific. Furthermore, even within in lymphoid cells, methylation levels were higher in B lymphocytes. This raises 2 interesting aspects. First, although B cells are one of the less common cell types in the blood, malignancies derived from B cells make up over half of hematological cancers. The propensity of B lymphocytes for malignant transformation may be explained, in part, by the intrinsically high levels of methylation observed in this cell type, further strengthening the link between age- and cancer-related methylation. Second, the conceptually simplest explanation for the development of age-related methylation is that such changes accumulate in stem cells over time, leading to gradual and permanent increases in CpG island methylation in the tissue derived from these stem cells. However, the cell type specificity seen here in cells derived from the same pool of stem cells is not fully compatible with this hypothesis and suggests that alternative mechanisms are at least partly responsible for age-related methylation. These could involve preferential lymphoid differentiation of stem cells with a high CpG island methylation content, differential expansion of short “seeds” of methylation in the different cell types (with the “seeds” accumulating in stem cells), or gradual deterioration of the fidelity of replicating DNA methylation patterns with increasing age, specifically in B lymphocytes. Methylation levels could be further influenced by the life span of the different cell types, as lymphoid cells are generally more long-lived than myeloid cells.

Overall these results strengthen the link between cancer- and age-related methylation patterns and show that age-related methylation in specific sets of genes can phenocopy the main features of altered methylation patterns seen in cancer cells, implying that the 2 processes may be interlinked.

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