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Blood DNA methylation, nevi number and the risk of melanoma.

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Abstract

Background: Germline mutations determining increased cutaneous malignant melanoma (CMM) risk have been identified in familial and sporadic CMM cases, but they account only for a small proportion of CMM cases. Recent evidence suggests that germline epimutations (e.g. DNA methylation alterations), which can be inherited similarly to genomic mutations and can be detected in normal body cells (including blood), might increase cancer susceptibility.

Objectives: To identify germline epimutations of genes which were found to be mutated in familial CMM (p16^{INK4a}, p14^{ARF}, CDK4, MC1R, hTERT), immune and inflammatory genes (ICAM-1, TNFalpha), DNA mismatch repair gene (MLH1) and repetitive elements (ALU, LINE-1, HERV-w).

Methods: We measured DNA methylation using Bisulfite-Pyrosequencing in peripheral blood mononuclear cells (PBMCs) from 167 CMM cases and 164 sex and age-matched control. We used multivariable logistic regression models to evaluate the association between methylation levels and CMM status or presence of dysplastic nevi.

Results: We found an association between CMM risk and PBMC methylation levels of TNFalpha (OR=1.11, 95% CI=1.03-1.18), CDK4 (OR=0.76, 95% CI=0.64-0.91) and MLH1 (OR=1.12, 95% CI=1.02-1.22). In control subjects, risk of developing dysplastic nevi was associated with methylation levels of TNFalpha (OR=0.81, 95% CI=0.69-0.95), hTERT (OR=0.90, 95% CI=0.82-0.99), and ALU (OR=1.56, 95% CI=1.02-2.39).

Conclusions: Epimutations in CMM susceptibility genes and in genes involved in response to oxidative damage are associated with risk of developing CMM or dysplastic nevi. Further studies

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measuring methylation levels of these genes in prospectively collected samples are warranted to further elucidate their role in CMM development and progression.

Keywords

Methylation; cutaneous malignant melanoma; nevi; pyrosequencing

Introduction

The overall worldwide incidence of cutaneous malignant melanoma (CMM) is increasing faster than that of any other cancer.[1] Current estimates indicate that CMM incidence will double over the next-20 years in most Caucasian populations.[2] Late stage CMM is highly fatal. Once diagnosed with metastatic CMM, most patients die of their disease within 2 years.[3] Approximately 10-13% of CMM cases are clustered in families, whereas the remaining are diagnosed as sporadic CMM.[4] Germline mutations have been identified in both familial and sporadic CMMs.[5] However, even mutations of p16^{INK4a}, the most common of the germline mutations in CMM, have been detected in approximately 20 – 40% of melanoma – prone families (reviewed in [6]), and in 0.2% to 3% of sporadic CMM cases of Northern European descent.[6-8] Other genetic determinants associated with CMM – e.g. germline mutations of p14^{ARF} and CDK4, and variants in melanocortin 1 receptor (MC1R, associated with fair pigmentation), Telomerase reverse transcriptase (hTERT), Microphthalmia-associated Transcription Factor (MITF) and (BAP1) – taken together account for an even lower proportion of CMM attributable risk.[9-14]

Epimutations due to increased DNA methylation are very common somatic events, and can also arise in the germline and cause soma-wide transcriptional silencing of a gene.[15] Somatic epimutations are restricted to the tumor tissue and precursor lesions. Recent evidences suggest that germline epimutations, which can be inherited similarly to what happens for genomic mutations and thus can be detected in normal body cells (including peripheral blood), might increase cancer susceptibility.[16-19]

We therefore hypothesize that alterations in blood DNA methylation of specific genes, which have been linked with heritable traits that are not related to differences in the DNA sequence,[20, 21] might contribute to determine the risk of CMM unaccounted for by genetic mutations.

To test this hypothesis we measured DNA methylation of the same genes which were found to be mutated in familial CMM (p16^{INK4a}, p14^{ARF}, CDK4, MC1R, hTERT) in 167 sporadic CMM cases and 164 frequency-matched controls, previously investigated for genetic mutations.[22] Moreover, we investigated: two genes known to impact immune and inflammatory response (ICAM-1 and TNFalpha)[23] since CMM is considered as a representative model for cross talk between immune responses and malignancy;[24] a DNA mismatch repair gene (hMLH1), representing the first example of germline epimutation in cancer;[16, 25] three repetitive elements (ALU, LINE-1, HERV-w), since reduced levels of repetitive elements methylation in blood have been associated with genomic instability and cancer risk.[26]

Methods

Study Subjects

This study has been previously described.[21, 22] In brief, 183 cases of CMM were recruited between 1994 and 1996 at the Dermatology Department of the Maurizio Bufalini Hospital of Cesena, Italy. The 179 sex and age-matched control subjects were living in the same geographic area and included 134 spouses or close friends of the CMM patients, 14 outpatients referred to the hospital because of minor accidental trauma, and 31 healthy volunteers from hospital personnel. All study participants underwent a full body skin examination performed by a single dermatologist to characterize phenotypes (pigmentation characteristics, freckling, type and total number of nevi, presence of skin lesions, and presence of other skin cancers), completed a standardized in-person questionnaire, assisted by trained interviewers and donated a blood sample collected in heparin tubes. DNA was available for 167 CMM cases and 164 controls. Diagnosis of dysplastic nevi, defined as number of common melanocytic nevi on the back of subjects were confirmed on standardized photographs by an expert oncologist blinded to CMM status. To be defined as dysplastic, a nevus had to be 5 mm or larger, had to be predominantly flat, and had to have at least two of the following criteria: variable pigmentation, indistinct borders, and irregular outline.[27]

DNA extraction and bisulfite treatment

Genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs), using the Nucleon Extraction and Purification kit (Amersham Life Science, UK), following the manufacturer's instructions.

500 ng DNA (concentration 50 ng/μl) was treated using EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol. Final elution was performed with 30 μl of M-Elution Buffer.

DNA methylation analysis

Analysis of DNA methylation was performed using previously published methods,[28, 29] with minor modifications. Briefly, a 50 μl PCR was carried out in 25 μl of GoTaq Green Master mix (Promega, Madison, WI, USA), 1 pmol of the forward primer, 1 pmol of the biotinylated reverse primer, 50 ng of bisulfite-treated genomic DNA and water. PCR cycling conditions and primer sequences are reported in Table 1. The biotin-labelled primers were used to purify the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden) and the Sepharose beads containing the immobilized PCR product were purified, washed, denatured using a 0.2 M NaOH solution, and washed again using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Inc., Westborough, MA), as recommended by the manufacturer. Then, 0.3 μM pyrosequencing primer was annealed to the purified single-stranded PCR product and pyrosequencing was performed using the PyroMark MD System (Pyrosequencing, Inc.). The degree of methylation was expressed as percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines (%5mC). Every sample was tested three

times for each marker to confirm reproducibility and increase precision of our results. The average of the three replicates was used in statistical analyses.

Statistical analysis

Phenotypic characteristics of cases and controls were compared by the χ^2 test or Fisher's exact test for categorical variables and for continuous variables by the Wilcoxon rank sum test.

We computed odds ratios (ORs) and 95% confidence intervals (CIs) for the association of methylation levels with case/ control status using multivariable logistic regression models. To reduce the number of adjustment variables, and to account for correlations between the pigmentation characteristics of an individual, we used factor analysis with varimax rotation among those individuals who did not have missing values on eye colour, hair colour, skin type, nevi number and presence of dysplastic nevi. Factor analysis identified two factors: factor1 ("pigmentation factor"), corresponding to pigmentation characteristics including eye colour, hair colour and skin type, and factor2 ("nevi factor") represented by number of nevi and presence of dysplastic nevi. The interaction between nevi number, presence of dysplastic nevi, pigmentation factor, nevi factor and DNA methylation was assessed in multivariable logistic models. All analyses were repeated stratified by the number of nevi (≤ 20 , > 20), adjusted for age, sex and pigmentation factor.

Among controls we used linear regression models to assess the association of subject characteristics with methylation levels. We also computed ORs and 95% CIs for the association of the presence of dysplastic nevi and DNA methylation levels using logistic regression models adjusting for age and sex. We further adjusted the OR for the "pigmentation factor". Statistical analysis was performed with the use of SAS software, version 9.2 (SAS Institute). A two sided P value of <0.05 was considered statistically significant.

Results

Study Population

As expected, pigmentation characteristics were significantly different between cases and controls (hair, eye and skin color, presence of freckles, tanning ability to prolonged sun exposure, presence of DN and number of nevi) (Supplementary Table 1).

Moreover, cases had lower methylation levels than controls in CDK4 gene (mean cases=2.24%: mean controls=2.72%; p-value=0.0076) and in MLH1 gene (mean cases=11.0%: mean controls=10.25%; p-value=0.01) (Table 2).

Melanoma characteristics of cases are presented in Supplementary Table 2.

DNA methylation and Dysplastic Nevi risk in controls

We explored associations between gene specific methylation levels and subjects characteristics and found several suggestive associations (Table 3).

In a logistic regression model adjusted for age and gender, there was a suggestive negative association between DN risk and hypermethylation of hTERT (OR=0.90, 95% CI = 0.82-0.99) and TNF-alpha (OR=0.81, 95% CI = 0.69-0.95) (Table 4). In contrast, hypermethylation of ALU was associated with an increased risk of DN (OR=1.56, 95% CI = 1.02-2.39).

This pattern persisted when pigmentation factor was added to the regression model, for hTERT (OR=0.91; 95% CI = 0.84-1.00), TNF-alpha (OR=0.82; 95% CI = 0.70-0.96) and ALU (OR=1.51, 95% CI = 1.00-2.28). We did not include nevi factor in the model, since it included the presence of DN and nevus count.

DNA methylation and CMM risk

In logistic regression analyses adjusted for age and gender, the risk of CMM was positively associated with methylation levels of the p16^{INK4a} gene (OR 1.28, 95% CI = 1.03-1.59) and MLH1 gene (OR 1.14, 95% CI = 1.01-1.27) (Table 5). When we adjusted additionally for the pigmentation and nevi factors, the OR for p16^{INK4a} methylation was no longer significant (OR=1.14, 95% CI = 0.97-1.34), while the risk of CMM was positively associated with methylation of TNF-alpha gene (OR=1.11, 95% CI = 1.03-1.18) and MLH1 (OR=1.12, 95% CI = 1.02-1.22) and negatively associated with CDK4 (OR=0.76, 95% CI = 0.64-0.91).

We observed a different pattern of methylation when we stratified analyses by nevi number (Table 6). Among individuals with <20 nevi, we found a positive association between methylation of ICAM-1 (OR 2.45, 95% CI = 1.01-5.94), TNF-alpha (OR 1.49, 95% CI = 1.17-1.90) and ALU (OR 2.37, 95% CI = 1.13-4.95) and risk of CMM (Table 6). In contrast, among subjects with more than 20 nevi, we found an association of CMM risk with methylation of CDK4 (OR=0.57, 95% CI = 0.42-0.77) and MLH1 (OR=1.29, 95% CI = 1.12-1.50) (Table 6). The interaction term between number of nevi and DNA methylation was significant in the logistic model for ICAM-1 (p-value=0.02), TNF-alpha (p-value<0.01), ALU (p-value<0.01), LINE-1 (p-value=0.02), CDK4 (p-value<0.01), and MLH1 (p-value=0.02) (table 6).

As a sensitivity analysis, we also stratified the number of nevi variable as above and below the median (number of nevi=34) obtaining similar results (data not shown).

Discussion

In a case-control study of CMM from Italy, we found an association between methylation levels in blood DNA of TNFalpha, CDK4 and MLH1 and risk of CMM. Moreover, methylation levels of TNFalpha, hTERT, and ALU were associated with risk of developing dysplastic nevi in control subjects. For the first time, we found that methylation of specific genes, detectable in surrogate tissues such as PBMCs, could be involved in CMM development.

Epimutations occurring in a germline cell are believed to be maintained throughout development, so all somatic cells originated from the germline would carry these

epimutations. Methylation alterations have been frequently observed in non-tumor surrogate tissue, such as leukocytes and buccal cells, of cancer patients, suggesting that DNA methylation changes in cancer may be systemic and detectable in peripheral blood (surrogate tissue) (reviewed in).

Therefore, what we observed in peripheral blood samples may reflect the environment in the melanocytes, and could have an impact on the development of melanocyte-related traits (like nevi or dysplastic nevi) and diseases (like CMM).

CMM tissue methylation have been recently evaluated using a genome-wide approach, which showed that melanoma is characterized by a diffuse epigenetic deregulation with many genes showing tumor-selective aberrant methylation.[30] In this study, highly recurrent methylation of the HOXA9, MAPK13, CDH11, PLEKHG6, PPP1R3C, and CLDN11 genes was established. This evidence follows a previous study, showing genomic loss of 5-hydroxymethylcytosine in CMM.[31] The changes observed in CMM tissue, however, might be the result of processes that occur later in the pathogenesis of cancer, given the complex modification occurring in the fully developed cancer phenotype.

DNA methylation in peripheral blood has been previously investigated in one single study, which evaluated LINE-1 methylation in CMM-prone families. This study, however, found no significant evidence that changes in global LINE-1 methylation in peripheral blood were associated with familial CMM risk.[32] This negative result was also confirmed in our study.

Alterations in DNA methylation, in particular in LINE-1 elements, have been also reported to be independent prognostic factors in melanoma patients. Methylation as prognostic marker might help in achieving a more appropriate clinical management of cutaneous melanoma.[33]

We surmise that DNA hypermethylation (i.e., epimutations) in the loci that harbor the known CMM-related genetic variations might determine CMM risk. Because these loci have functions that have been shown to be causally associated with CMM risk, they represent prime candidates for CMM epimutations.

Among the genes whose methylation levels were associated with CMM, the MLH1 may play an important role. MHL1 is a major mismatch repair gene involved in maintaining the stability of the genome.[16] Germline hypermethylation of MLH1 have been found in peripheral tissues (including PBMCs) of colon cancer families with no MLH1 genetic mutations,[16, 25] emphasizing the role of this gene in epimutation-related phenotypes. This mechanism might be generalized to different kind of cancer, including CMM. If confirmed, MLH1 epimutation analysis could provide a tool for CMM risk stratification.

Another gene found to be associated with CMM risk was CDK4, one of the two major high penetrance susceptibility genes associated with familial CMM.[12] CDK4, in conjunction with the D-type cyclins, mediates progression through the G1 phase when the cell prepares to initiate DNA synthesis. Activating mutations in this gene result in a predisposition of individuals to the development of CMM.[34, 35] Hypomethylation of this gene, resulting in

increased CDK4 expression, might induce uncontrolled proliferation and might have a functional effect similar to the one exerted from CDK4 mutation on CMM risk.

TNFalpha was also associated with CMM risk. TNF-alpha is a pro-inflammatory cytokine which has been shown to have an important role in tumorigenesis,[36] tumor progression,[37] and tumor invasiveness.[38] TNF-alpha may also favor the formation of a permissive microenvironment for tumor invasion[39]. A direct association between the development of cancer and inflammation has long-been appreciated. From one side, there is evidence to suggest the inflammatory and immune systems may inhibit the development of cancer, from the other side long-standing inflammation secondary to chronic infection or irritation predisposes to cancer.[40] We found that subjects carrying dysplastic nevi tended to have lower methylation levels of TNF-alpha, while CMM cases were associated with increased TNF-alpha promoter methylation. It is possible that the TNF-alpha-related pro-inflammatory environment contribute to the stability of the dysplastic nevi, which are strong risk factors for melanoma but rarely progress to CMM.[41] Conversely, increased methylation levels and related decreased expression of TNF-alpha may exercise a lower protection towards CMM development. This is just speculative at this point and needs to be further explored in a larger population.

We found a protective role against DN for hTERT (human telomerase catalytic subunit) hypermethylation. The major role of telomerase is to catalyze the de novo addition of telomeric repeat sequences onto chromosome ends and thereby counterbalance telomere-dependent replicative aging. Two prospective studies have reported that shorter telomeres measured in peripheral blood, were associated with a decreased risk of CMM.[10, 42, 43] Moreover, two independent studies identified, with high frequency, mutations within the core promoter of hTERT.[44, 45] Although cancer genome is characterized by many recurrent mutations in the protein-coding regions of genes, regulatory regions are rarely mutated. In CMM, somatic mutations in regulatory regions of the genome may then represent an important tumorigenic mechanism. Similarly, hypermethylation of the hTERT promoter might play a critical role in the negative regulation of telomerase activity in normal human cells, thus limiting the DN growth.

The identification of alterations in peripheral blood DNA methylation possibly linked to an increased risk of developing CMM might be suggestive of a new mechanism, able to explain CMM onset in those patients which don't carry known genetic alterations.

We also observed an interaction between number of nevi and methylation levels of specific genes in the association with CMM risk. In particular, genes involved in inflammatory pathways, like ICAM-1[46] or TNF-alpha, were associated with CMM risk in subjects with low number of nevi, while methylation levels in susceptibility genes for CMM, like CDKN2A[6] or CDK4, were more strongly associated with CMM risk in subjects carrying many nevi. Although based on a small number of subjects, these findings are suggestive of at least two distinct pathways for CMM development. One, for subjects with "many" nevi, which may involve CMM susceptibility genes such as p16^{INK4a} and CDK4, either through genetic or epigenetic regulation, and the other for subjects with fair pigmentation and

consequent oxidative stress, which may involve genes in inflammation or DNA repair pathways.

The results we presented in this paper suggest that methylation level of specific loci may have an impact on CMM risk. However, because methylation levels in peripheral blood has been shown to be altered by several factors, including diet, environmental exposure and lifestyle habits [47], we cannot exclude that the observed changes might be due to other confounding factors.

An important limitation of this study is that, due to the case-control design, we were unable to exclude that aberrant DNA methylation we detected in blood is a consequence, rather than a cause, of the fully developed CMM. Given the early stage of the disease in our cases, it is however unlikely that the tumors could have strongly altered methylation levels in blood. Moreover, this limitation does not apply to our findings in control subjects related to the development of dysplastic nevi.

In conclusions, we found that epimutations in CMM susceptibility genes and in genes involved in response to oxidative damage are associated with risk of developing CMM or dysplastic nevi. Further study measuring methylation levels of these genes in large prospective studies is warranted to further elucidate their role in CMM development and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1.

Genomic location of the assayed CpGs and PCR primer sequences.

Gene	Chromosome	Promoter		CpGs	Primers: Forward (F), Reverse (R) and Sequencing (S)	
		Start	End		Reverse (R) and Sequencing (S)	
CDK4	12	58145399	58146355	58145667; 58145686; 58145695	F:TTTGGTAGTTGGTTATATGGTGA R:Biotin-CATAACCCACAAAAACCCCACTTC S:GGGGGTTTTTTTAGTTTG	
					F:AAGTATAGTTGAAGGAAGA R:Biotin-CAATCCCCCATCTCAACAA S: AGTTTAAGATTAAAGATTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
hTERT	5	1294804	1295862	1294894; 1294904; 1294912; 1294906	F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
ICAM-1	19	10242017	10242937	10242236; 10242218; 10242225	F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
MCIR	16	89984770	89985767	89985638	F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
MLH1	3	37009483	37010128	37009960; 37009968	F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
p14 ^{ARF}	9	21994219	21995123	21994500; 21994506; 21994509; 21994513; 21994511	F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
p16 ^{INK4a}	9	21974711	21975632	21975453; 21975458; 21975460; 21975477; 21975468; 21975471; 21975464	F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
TNF α	6	31650822	31651599	31651172; 31651155; 31651157	F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	
					F:GGGTTTGTGTTAAGGAGTTTAAAGT R:Biotin-AAACCCCAAACTACCTCCA S:AGTGTGTAGGGGAGGTAT	

Table 2.

DNA methylation in Cases and Controls

Methylation (%5mC)	Cases (n=167)		Controls (n=164)		Wilcoxon p-value
	Mean (SD)	Median (q1-q3)	Mean (SD)	Median (q1-q3)	
HERV-w	93.95 (1.38)	94.12 (93.48-94.68)	93.9 (1.17)	94.11 (93.49-94.58)	0.620
hTERT	46.96 (6.44)	47.52 (42.95-50.86)	46.17 (6.46)	46.45 (42.61-50.46)	0.241
ICAM1	2.7 (0.52)	2.61 (2.33-2.99)	2.7 (0.66)	2.54 (2.29-2.96)	0.405
MC1R	17.92 (3.05)	18.09 (15.97-19.94)	17.81 (2.72)	18.08 (16.24-19.53)	0.721
p16	3.58 (1.5)	3.09 (2.45-4.49)	3.29 (1.36)	2.93 (2.22-3.99)	0.075
p14	1.48 (0.67)	1.34 (1.10-1.74)	1.41 (0.52)	1.36 (1.10-1.64)	0.751
TNF-alpha	13.03 (4.51)	12.40 (9.70-15.99)	12.33 (3.74)	12.05 (9.47-14.71)	0.312
ALU	23.02 (0.93)	23.05 (22.33-23.65)	22.95 (1.00)	23.15 (22.12-23.72)	0.853
LINE-1	75.83 (1.78)	75.85 (74.60-77.06)	76.01 (1.84)	76.30 (74.77-77.30)	0.309
CDK4	2.24 (1.38)	2.25 (1.33-3.10)	2.72 (1.35)	2.60 (1.98-3.24)	0.008
MLH1	11.00 (2.63)	11.12 (8.82-13.08)	10.25 (2.51)	10.32 (8.24-12.25)	0.012

Table 3:

Slope coefficients from linear regression models evaluating the association between DNA methylation and control subjects' characteristics.

	HERV-W		hTERT		ICAM-1		MCIR		p16		p14	
	Beta	95% CI	Beta	95% CI	Beta	95% CI	Beta	95% CI	Beta	95% CI	Beta	95% CI
Age (for 1 year)	-0.02	(-0.03; 0.00)	0.07	(-0.01; 0.14)	0.00	(-0.01; 0.01)	0.07	(0.05; 0.10)	0.02	(0.01; 0.04)	0.01	(0.00; 0.01)
Gender (Males vs Females)	-0.21	(-0.57; 0.15)	3.38	(1.43; 5.32)	-0.09	(-0.30; 0.12)	0.97	(0.14; 1.80)	-0.17	(-0.61; 0.27)	-0.02	(-0.19; 0.14)
Nevus count (20 vs >20)	-0.26	(-0.64; 0.12)	0.51	(-1.62; 2.64)	-0.18	(-0.40; 0.04)	0.51	(-0.37; 1.40)	0.60	(0.14; 1.05)	-0.18	(-0.35; -0.00)
Displastic nevi (Yes vs No)	-0.49	(-0.96; -0.02)	-1.46	(-3.72; 0.81)	0.05	(-0.23; 0.32)	0.80	(-0.27; 1.87)	0.54	(0.01; 1.07)	-0.14	(-0.36; 0.08)
Chronic Sun Exposure (Yes vs No)	0.13	(-0.24; 0.49)	-0.38	(-2.41; 1.64)	0.08	(-0.14; 0.29)	-0.01	(0.86; 0.84)	-0.11	(-0.56; 0.33)	-0.09	(-0.26; 0.07)
Intermittent Sun Exposure												
Very little vs High	-0.39	(-0.88; 0.09)	1.70	(-1.02; 4.42)	0.20	(-0.09; 0.48)	0.82	(-0.31; 1.95)	0.05	(-0.55; 0.66)	-0.07	(-0.29; 0.15)
Some vs High	-0.27	(-0.70; 0.16)	1.68	(-0.72; 4.08)	0.11	(-0.14; 0.36)	0.05	(-0.95; 1.05)	0.03	(-0.50; 0.55)	-0.06	(-0.26; 0.13)
	TNF-alpha		ALU		LINE1		CDK4		MLH1			
	Beta	95% CI	Beta	95% CI	Beta	95% CI	Beta	95% CI	Beta	95% CI		
Age (for 1 year)	-0.17	(-0.20; -0.13)	-0.01	(-0.02; 0.00)	-0.02	(-0.04; 0.00)	0.00	(-0.02; 0.01)	0.03	(0.00; 0.05)		
Gender (Males vs Females)	0.97	(-0.20; 2.14)	-0.02	(-0.33; 0.29)	-0.51	(-1.08; 0.05)	0.10	(-0.33; 0.52)	-0.88	(-1.65; -0.11)		
Nevus count (20 vs >20)	0.13	(-1.11; 1.37)	-0.07	(-0.39; 0.26)	-0.05	(-0.65; 0.55)	0.24	(-0.20; 0.67)	-0.19	(-1.01; 0.63)		
Displastic nevi (Yes vs No)	-2.08	(-3.59; -0.57)	0.42	(-0.01; 0.84)	0.13	(-0.66; 0.91)	0.40	(-0.16; 0.96)	-0.51	(-1.54; 0.52)		
Chronic Sun Exposure (Yes vs No)	0.76	(-0.43; 1.94)	-0.09	(-0.41; 0.22)	-0.37	(-0.94; 0.20)	-0.27	(-0.70; 0.16)	-0.23	(-1.02; 0.55)		
Intermittent Sun Exposure:												
Very little vs High	0.07	(-1.46; 1.60)	-0.11	(-0.53; 0.30)	0.00	(-0.77; 0.76)	0.13	(-0.44; 0.71)	0.08	(-0.96; 1.12)		
Some vs High	2.06	(0.70; 3.42)	0.12	(-0.25; 0.49)	0.46	(-0.22; 1.13)	0.28	(-0.23; 0.79)	-0.66	(-1.59; 0.27)		

Table 4:

Logistic regression models showing OR and 95% CI of dysplastic nevi incidence for 1% increase in gene methylation (on control subjects).

Gene	Adjustment 1				Adjustment 2			
	OR	95%CI	p-value		OR	95%CI	p-value	
HERV-W	0.69	0.41	1.15	0.15	0.61	0.37	1.03	0.06
hTERT	0.90	0.82	0.99	0.03	0.91	0.84	1.00	0.04
ICAM-1	1.11	0.55	2.24	0.78	1.13	0.56	2.27	0.74
Mc1R	1.08	0.9	1.31	0.41	1.07	0.89	1.28	0.50
p16	1.43	0.93	2.21	0.10	1.47	0.96	2.24	0.07
p14	0.49	0.21	1.14	0.10	0.52	0.24	1.12	0.10
TNFAI1	0.81	0.69	0.95	0.01	0.82	0.70	0.96	0.01
ALU	1.56	1.02	2.39	0.04	1.51	1.00	2.28	0.05
LINE-1	1.12	0.89	1.41	0.35	1.08	0.86	1.35	0.50
CDK4	1.24	0.84	1.83	0.29	1.31	0.90	1.91	0.16
MLH1	0.91	0.75	1.11	0.36	0.91	0.76	1.09	0.30

1: Adjusted for age, sex

2: Adjusted for age, sex, pigmentation factor 1

Table 5:

Logistic regression models showing OR and 95% CI of melanoma incidence for 1% increase in gene methylation.

Gene	Adjustment 1				Adjustment 2			
	OR	95%CI		p-value	OR	95%CI		p-value
HERV-W	0.89	0.73	1.09	0.27	1.04	0.87	1.24	0.68
hTERT	0.99	0.94	1.04	0.66	1.02	0.98	1.05	0.41
ICAM-1	1.18	0.73	1.91	0.49	0.99	0.68	1.45	0.97
Mc1R	0.97	0.87	1.08	0.53	0.99	0.91	1.07	0.81
p16	1.28	1.03	1.59	0.02	1.14	0.97	1.34	0.11
p14	0.87	0.53	1.43	0.58	1.16	0.81	1.68	0.42
TNFAI1	1.07	0.98	1.16	0.16	1.11	1.03	1.18	<0.01
ALU	1.23	0.90	1.68	0.20	1.11	0.88	1.40	0.38
LINE-1	0.98	0.84	1.15	0.84	0.96	0.85	1.08	0.51
CDK4	0.86	0.68	1.09	0.21	0.76	0.64	0.91	<0.01
MLH1	1.14	1.01	1.27	0.03	1.12	1.02	1.22	0.01

1: Adjusted for age, sex

2: Adjusted for age, sex, pigmentation factor 1, nevi factor 2

Table 6:

Logistic regression models for evaluating the association between DNA methylation and melanoma risk, stratified by nevus count.

Gene	NEVI 20 n=92				NEVI >20 n=208				p-value interaction	
	OR	95%CI		p-value	OR	95%CI		p-value		
<u>Adjusted for age and sex</u>										
HERV-W	0.85	0.65	1.10	0.22	1.17	0.89	1.53	0.26	0.11	
hTERT	0.99	0.90	1.08	0.77	1.02	0.98	1.07	0.36	0.22	
ICAM-1	2.28	0.99	5.25	0.05	0.86	0.55	1.37	0.53	0.04	
Mc1R	1.03	0.86	1.24	0.74	0.97	0.88	1.08	0.58	0.87	
p16	0.95	0.68	1.34	0.78	1.27	1.02	1.57	0.03	0.13	
p14	1.52	0.76	3.05	0.23	0.84	0.51	1.39	0.50	0.20	
TNF-alpha	1.34	1.11	1.62	<0.01	1.04	0.95	1.13	0.42	0.02	
ALU	2.39	1.24	4.60	<0.01	0.95	0.70	1.29	0.75	<0.01	
LINE-1	1.24	0.93	1.65	0.14	0.91	0.78	1.06	0.23	0.04	
CDK4	1.16	0.85	1.58	0.36	0.65	0.51	0.83	<0.01	<0.01	
MLH1	0.98	0.83	1.15	0.78	1.18	1.05	1.32	<0.01	0.08	
<u>Adjusted for age, sex and pigmentation factor 1</u>										
HERV-W	0.84	0.64	1.09	0.18	1.23	0.88	1.74	0.23	0.07	
hTERT	0.99	0.89	1.11	0.88	1.04	0.99	1.09	0.11	0.07	
ICAM-1	2.45	1.01	5.94	0.05	0.79	0.47	1.30	0.35	0.02	
Mc1R	1.08	0.89	1.32	0.43	0.96	0.86	1.08	0.50	0.99	
p16	1.02	0.69	1.50	0.93	1.24	0.97	1.59	0.08	0.30	
p14	1.18	0.53	2.63	0.69	0.66	0.36	1.20	0.18	0.43	
TNF-alpha	1.49	1.17	1.90	<0.01	1.00	0.91	1.09	0.94	<0.01	
ALU	2.37	1.13	4.95	0.02	0.86	0.60	1.22	0.39	<0.01	
LINE-1	1.26	0.89	1.77	0.19	0.86	0.72	1.02	0.09	0.02	
CDK4	1.09	0.79	1.52	0.60	0.57	0.42	0.77	<0.01	<0.01	
MLH1	0.97	0.81	1.17	0.76	1.29	1.12	1.50	<0.01	0.02	