LINE-1 hypermethylation in peripheral blood of cutaneous melanoma patients is associated with metastasis

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Aberrant DNA methylation pattern is a well-known epigenetic marker of cancer cells. Recently, aberrant methylation was also reported in the peripheral blood of cancer patients and it could potentially serve as a biomarker for cancer risk. We investigated the methylation pattern of LINE-1 and other repetitive DNA elements in peripheral blood of cutaneous melanoma patients in order to search for an association with clinical characteristics. The patient cohort was composed by 69 unrelated melanoma patients, 28 of whom were hereditary cases (with or without CDKN2A mutations) and 41 were isolated (sporadic) melanoma cases. Methylation of LINE-1 was evaluated by pyrosequencing, whereas additional repetitive DNA sequences were assessed using Illumina 450K methylation microarray. Melanoma patients exhibited a higher, albeit heterogeneous, LINE-1 methylation level compared with controls. Hereditary melanoma patients carrying CDKN2A mutations showed a hypermethylated pattern of both LINE-1 and repetitive DNA elements compared with other patients. In particular, the methylation level at one specific CpG of LINE-1 was found to be correlated with the occurrence of metastasis. Our data suggest that LINE-1 hypermethylation in peripheral blood of melanoma patients is a potential epigenetic biomarker for metastasis occurrence. Melanoma Res 25:173–177 Copyright © 2015 Wolters Kluvier Health, Inc. All rights reserved.

Methylation of cytosines in CpG dinucleotides is a covalent modification associated with transcriptional repression [1]. This epigenetic mark regulates several normal biological processes, such as cellular differentiation [2], and is also implicated in diseases, including cancer [3].

The transposable element LINE-1 has been used as a surrogate marker for global methylation in several cancer studies [4,5]. The pattern of LINE-1 methylation in peripheral blood has been associated with cancer risk and differs among different cancer types; whereas hypomethylation has been reported for hepatocellular carcinoma [6] and gastric [7] and bladder cancer [8], hypermethylation has been described for renal [9] and colorectal cancer [10]. Recently, a study was performed in leukocytes of melanoma patients, which did not detect differences in LINE-1 methylation between patients and controls [11].

Introduction

Keywords: biomarkers, DNA methylation, leukocytes, LINE-1 elements, melanoma, metastasis, repetitive sequences

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We analyzed the methylation patterns of LINE-1 and other repetitive DNA elements as potential biomarkers for cutaneous melanoma in a cohort of Brazilian patients classified according to clinical characteristics.

Patients and methods

Patients and control groups

This retrospective study was performed at the A. C. Camargo Cancer Center (São Paulo, Brazil) after obtaining approval from the Internal Ethics Committee Board. Melanoma patients were referred to the Skin Cancer Department of the A. C. Camargo Cancer Center, which surveyed sex, age at blood draw, number of melanomas, Breslow thickness of melanoma high stage, and metastasis occurrence. Family history of cancer was assessed in the Oncogenetics Department, and genetic counseling was provided to all participants.

The study was based on blood samples from 69 unrelated cutaneous melanoma patients who had undergone no previous chemotherapy, who were classified in three groups: familial melanoma patients who were carriers of pathogenic CDKN2A mutations (\(n = 8\)), familial melanoma patients with CDKN2A-wild type (\(n = 20\)), and sporadic melanoma patients (\(n = 41\)). The familial melanoma patients who were
negative for CDKN2A mutations fulfilled at least one of the following criteria: they had at least three primary melanomas \((n=8)\); they had at least two primary melanomas, one of which was before 35 years of age \((n=3)\); and they had a family history in three or more relatives of two consecutive generations, one of whom was under 50 years of age \((n=9)\). The control group was composed of 51 individuals without cancer history, who were matched for age (range for each 10 years) and sex with the melanoma patients.

DNA samples extracted from peripheral blood were retrieved from the A. C. Camargo Cancer Center Biobank.

**Pyrosequencing and HM450K genome-wide methylation analysis**

Bisulfite modification was performed on 500 ng of DNA using the EZ DNA methylation kit (Zymo Research, Irvine, California, USA). Quantitative bisulfite pyrosequencing for LINE-1 was performed in DNA blood samples from 69 melanoma patients and 51 controls using PyroMark Q96-CpG LINE-1 (Qiagen, Hilden, Germany), which analyzed four CpG sites (position 305 to 331 – GenBank accession X58075). The methylation level was obtained using PyroMark Q96-CpG Software (Qiagen), which provides the percentage of methylated cytosines relative to the sum of methylated and unmethylated cytosines. All experiments were performed twice and samples with a variation coefficient greater than 10% were excluded.

The methylation profile of several repetitive DNA elements was performed in a subset of 39 melanoma patients (eight familial melanoma with CDKN2A mutations, 19 familial melanoma CDKN2A-wild type, and 12 sporadic cases) and 12 controls using Infinium HumanMethylation 450K BeadChips (Illumina, San Diego, California, USA), according to the manufacturer’s instructions. The Bioconductor IMA package was applied for quality control of array methylation data. We removed from further analyses probes that (a) lacked \(\beta\)-values, (b) contained single-nucleotide polymorphisms, (c) mapped at sexual chromosomes, or (d) had detection \(P\)-values greater than 1e-05. Normalization and probe type bias adjustment were achieved by beta mixture quantile dilation using the ChAMP package (http://www.bioconductor.org/packages/release/bioc/html/ChAMP.html). Information regarding HM450K probes mapped within repetitive sequences was obtained from the data set GSE42409 (available at the Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo), and only probes with total overlap (50 mers) were retrieved.

We evaluated the influence of both sex and age on the methylation levels of LINE-1 using Fisher’s test. DNA methylation differences between groups were tested for significance using either the Mann–Whitney test or the Kruskal–Wallis with Dunn’s multiple comparison post-test. After considering LINE-1 as a continuous variable, we also categorized the LINE-1 methylation level based on the median of the methylation values of the control group: hypomethylated when patients exhibited LINE-1 level lower than that of controls and hypermethylated when patients had LINE-1 level at least the same level as controls; differences were tested for significance using Fisher’s test. All statistical analyses were performed using the GraphPad PRISM (GraphPad Software Inc., La Jolla, California, USA) statistics software package.

**Results**

Quantitative bisulfite pyrosequencing for evaluation of the methylation pattern of four LINE-1 CpGs was performed for 69 patients and 51 control individuals. After exclusion of samples with variation coefficient above 10% between replicates, data from 48 melanoma patients and 42 controls were recovered. Considering all CpGs, neither controls nor melanoma patients exhibited significant differences in LINE-1 methylation level according to sex (controls \(P=0.06\); patients \(P=0.17\)) or age at blood draw (<40 years; controls \(P=0.99\); patients \(P=0.17\)). These two characteristics were not associated with LINE-1 methylation level in either controls or melanoma patients (sex \(P=1\); age at blood draw \(P=0.38\)). Therefore, statistical adjustments for these factors were not performed.

Table 1 presents the methylation data for the entire LINE-1 sequence (average of the four investigated CpGs) and each CpG position, comparing melanoma patients with controls, and groups of patients with different clinical characteristics with each other. The \(P\)-values in Table 1 refer to the statistical analysis of the means of the methylation levels of compared categories. Methylation levels were slightly heterogeneous between controls and melanoma patients across the four analyzed CpGs (Fig. 1a), and the CpG position 1 showed a statistically significantly higher methylation level compared with the other CpG positions in both groups (controls \(P<0.01\), patients \(P<0.01\)). However, melanoma patients exhibited a significantly higher average LINE-1 methylation level compared with controls, as well as at the CpG positions 2 and 4 (Fig. 1a; Table 1).

Methylation levels at CpG positions 2, 3, and 4 showed statistically significant differences among melanoma groups (Table 1): CDKN2A mutation carriers showed hypermethylation compared with both CDKN2A-wild-type melanoma patients (CpG positions 2 and 4) and sporadic melanoma patients (CpG position 4) (Fig. 1b). Subsequently, patients and controls were categorized as hypomethylated or hypermethylated on the basis of the median LINE-1 methylation levels of the control group (average LINE-1 methylation = 76.26%; CpG position 1 methylation = 81.50%; CpG position 2 methylation = 74.20%; CpG position 3 methylation = 73.49%; CpG position 4 methylation = 75.59%). Considering the
significant difference among different melanoma
and controls (Fig. 1c). However, there was a statistically
differences were observed between melanoma patients
sequences (\(22,436\) CpG sites mapped within repetitive
DNA elements in addition to
replicative LINE-1, SINE, and others) were
obtained from HM450K experiments of a subset of these
patients (\(n=39\)) and controls (\(n=12\)). No significant
differences were observed between melanoma patients
and controls (Fig. 1c). However, there was a statistically
significant difference among different melanoma
subgroups: patients carrying CDKN2A mutations showed
hypermethylation at repetitive DNA elements compared
with patients without CDKN2A mutations (Fig. 1d).

Discussion
DNA hypomethylation in peripheral blood has been already
generated as a biomarker for cancer risk [12]. Genome-wide hypomethylation might induce activation of endogenous parasitic sequences, such as transposable elements, subsequent genomic instability, and oncogene expression [13,14]. Measurement of the methylation level of LINE-1, which represents 17% of the human genome, is an accurate method for evaluating the global DNA methylation pattern [15]. It is worth mentioning that two independent meta-analyses have confirmed that global hypomethylation in leukocytes could be used as a biomarker for cancer risk, although LINE-1 in particular could not reflect this global methylation status [4,5]. The LINE-1 methylation status associated with cancer risk appeared to vary between different cancer types, and both hypomethylation and hypermethylation were already reported [6–10]. Despite LINE-1 being, or not being, a suitable indicator of global DNA methylation status, it may still be an independent biomarker for cancer.

We evaluated the possible correlation between changes in the pattern of DNA methylation in leukocytes of repetitive sequences, mainly LINE-1, and the presence of cutaneous melanoma. Our data suggest that LINE-1 hypermethylation is associated with melanoma occurrence. In addition, a higher methylation level at one single LINE-1 CpG was significantly associated with

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\text{average LINE-1 methylation level, there was a significant association of methylation status with melanoma occurrence (} P = 0.03\text{), with a higher number of hypermethylated individuals in the melanoma group (72.9%); individual CpG positions did not show significant differences in this analysis.}
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To investigate the relevance of LINE-1 methylation in peripheral blood as a potential biomarker for melanoma, we tested for significant differences in LINE-1 methylation according to clinical characteristics (Table 1). In melanoma patients, there was a statistically significant association of CpG1 methylation level and metastasis occurrence. The status of CDKN2A (mutated or wild type) was not significantly associated with any of the analyzed clinical characteristics (number of melanomas \(P = 0.57\); Breslow thickness \(P = 1.00\); metastasis \(P = 0.56\)). However, the studied group was very small: among CDKN2A mutation carriers, only one patient showed Breslow thickness greater than 2 mm, two of them developed more than two melanomas, and metastases were not detected in this melanoma group.

Next, we investigated whether the methylation level of other repetitive DNA elements in addition to LINE-1 could be associated with melanoma. The methylation levels of 22,436 CpG sites mapped within repetitive sequences (LINE-1, SINE, LTR, and others) were recovered from HM450K experiments of a subset of these patients \((n=39)\) and controls \((n=12)\). No significant differences were observed between melanoma patients and controls (Fig. 1c). However, there was a statistically significant difference among different melanoma

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\begin{array}{cccccccc}
\text{Overall LINE-1 methylation} & \text{CpG position 1} & \text{CpG position 2} & \text{CpG position 3} & \text{CpG position 4} \\
\hline
\text{N} (\%) & \text{Mean (SD)} & \text{N} (\%) & \text{Mean (SD)} & \text{N} (\%) & \text{Mean (SD)} & \text{N} (\%) & \text{Mean (SD)} \\
\hline
\text{Patients} & 48 (53.4) & 77 (2.1) & 47 (55.3) & 81 (4.8) & 47 (53.4) & 76 (2.3) & 47 (56.6) & 74 (2.3) & 46 (52.9) & 78 (2.9) \\
\text{Controls} & 42 (46.6) & 76 (1.8) & 38 (44.7) & 82 (1.8) & 41 (46.6) & 77 (1.8) & 36 (43.4) & 75 (3.1) & 41 (47.1) & 76 (2.4) \\
\hline
\text{P-value} & 0.03 & 0.77 & 0.01 & 0.11 & <0.01 \\
\hline
\text{Melanoma groups} & & & & & & & & & \\
\text{Sporadic} & 31 (64.6) & 74 (1.9) & 31 (65.9) & 82 (4.4) & 30 (63.8) & 76 (1.9) & 30 (63.8) & 75 (3.1) & 30 (65.2) & 77 (2.7) \\
\text{Hereditary CDKN2A-wild} & 13 (27.0) & 74 (2.4) & 12 (25.5) & 81 (5.2) & 13 (27.6) & 74 (1.9) & 13 (27.6) & 73 (2.8) & 12 (26.0) & 77 (2.5) \\
\text{Hereditary CDKN2A-mutated} & 4 (8.4) & 78 (1.0) & 4 (8.5) & 77 (4.8) & 4 (8.5) & 79 (2.9) & 4 (8.5) & 77 (2.6) & 4 (8.7) & 82 (1.3) \\
\hline
\text{Number of melanomas} & & & & & & & & & \\
\text{1} & 34 (70.8) & 77 (1.9) & 34 (72.3) & 82 (4.3) & 34 (72.3) & 76 (2.1) & 34 (72.3) & 75 (3.2) & 33 (71.7) & 78 (3.1) \\
\text{>1} & 14 (29.2) & 77 (2.4) & 13 (27.6) & 80 (5.9) & 13 (27.6) & 76 (3.0) & 13 (27.6) & 74 (2.8) & 13 (28.3) & 78 (2.2) \\
\hline
\text{Breslow thickness} & & & & & & & & & \\
\text{<2 mm} & 35 (77.7) & 77 (2.0) & 34 (77.3) & 81 (4.8) & 34 (77.3) & 76 (1.8) & 34 (77.3) & 75 (3.2) & 33 (76.7) & 78 (3.0) \\
\text{≥2 mm} & 10 (22.3) & 78 (2.1) & 10 (22.7) & 83 (4.9) & 10 (22.7) & 76 (3.7) & 10 (22.7) & 76 (2.1) & 10 (23.2) & 78 (2.7) \\
\hline
\text{Metastasis} & & & & & & & & & \\
\text{No} & 32 (72.7) & 77 (2.1) & 31 (72.1) & 80 (5.0) & 31 (72.1) & 76 (2.4) & 31 (72.1) & 75 (3.0) & 30 (71.4) & 78 (2.8) \\
\text{Yes} & 12 (27.3) & 78 (2.2) & 12 (27.9) & 84 (3.8) & 12 (27.9) & 75 (2.6) & 12 (27.9) & 74 (3.3) & 12 (28.6) & 78 (2.2) \\
\hline
\text{P-value} & 0.64 & 0.02 & 0.99 & 0.28 & 0.81 \\
\end{array}
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metastasis in melanoma patients. This could be partially explained by the relation between LINE-1 hypermethylation and DNA double-stranded breaks [10]. Hyland et al. [11] investigated the same target LINE-1 sequence in the only other methylation study in melanoma patients. In contrast to our findings, they did not find differences between melanoma patients and controls. This divergence can be because Hyland and colleagues studied a cohort composed of melanoma-prone families, whereas our melanoma group is heterogeneous with respect to the etiology of melanoma development. In addition, Jaffe and Irizarry [16] demonstrated the importance of cell heterogeneity in the DNA methylation analysis of peripheral blood. Peripheral blood contains a mixture of lymphocyte types, and detected differences in DNA methylation levels can arise from differences in the relative proportion of lymphocyte populations. Therefore, differences in the blood cell composition could influence the contrasting findings, reinforcing the relevance of accounting for cellular methylation levels of LINE-1 and repetitive DNA sequences in melanoma patients. (a, b) Methylation levels of four LINE-1 CpGs (positions 331 to 305, GenBank accession X58075) obtained using quantitative bisulfite pyrosequencing. (c, d) Methylation levels of 22,436 CpGs mapped in repetitive DNA elements measured using the HM450K platform (Illumina). (a) Melanoma patients exhibited significantly higher overall and specific LINE-1 methylations levels compared with controls (the Mann–Whitney test). (b) The methylation levels at LINE-1 CpGs positions 2 and 4 were significantly different among the groups of melanoma patients (the Kruskal–Wallis with Dunn’s multiple comparison post-test). (c) Repetitive DNA elements did not show significant difference in methylation levels between melanoma patients and controls (the Mann–Whitney test). (d) Familial melanoma patients carrying CDKN2A mutations showed a hypermethylated pattern of repetitive DNA elements compared with melanoma patients without mutations (Kruskal–Wallis with Dunn’s multiple comparison post-test). *P < 0.05.
heterogeneity in clinical practice. Although some previous studies have reported differences in LINE-1 methylation level according to sex [17] and age [18], they did not appear in our study.

Interestingly, melanoma patients carrying CDKN2A mutations exhibited higher methylation levels at LINE-1 and repetitive DNA elements compared with those without CDKN2A mutations. Hyland and colleagues also analyzed melanoma patients with and without CDKN2A mutations, but they did not find methylation differences. CDKN2A is the most relevant known melanoma susceptibility gene, and mutations in this gene are more commonly detected in patients with a familial melanoma history [19] or with multiple primary melanomas [20]. In a preceding study, we investigated the genome-wide DNA methylation profile of CDKN2A mutation carriers, revealing in these melanoma patients few differentially methylated CpGs, which were mainly related to underlying single-nucleotide polymorphisms [21]. Thus, the hypermethylated pattern found in this group of CDKN2A mutation carriers appears to be restricted to repetitive sequences. Further studies using prospective large cohorts of melanoma patients are needed to substantiate our findings and elucidate whether LINE-1 hypermethylation is a cause or a consequence of melanoma risk and metastasis occurrence.

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Conflicts of interest
There are no conflicts of interest.

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