LINE-1 hypomethylation and mutational status in cutaneous melanomas

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ABSTRACT

Epigenetic dysregulation is an important emerging hallmark of cutaneous melanoma development. The global loss of DNA methylation in gene-poor regions and transposable DNA elements of cancer cells contributes to increased genomic instability. Long interspersed element-1 (LINE-1) sequences are the most abundant repetitive sequence of the genome and can be evaluated as a surrogate marker of the global level of DNA methylation. In this work, LINE-1 methylation levels were evaluated in cutaneous melanomas and normal melanocyte primary cell cultures to investigate their possible association with both distinct clinicopathological characteristics and tumor mutational profile. A set of driver mutations frequently identified in cutaneous melanoma was assessed by sequencing (actionable mutations in *BRAF, NRAS,* and *KIT* genes, and mutations affecting the *TERT* promoter) or multiplex ligation-dependent probe amplification (MLPA) (*CDKN2A* deletions). Pyrosequencing was performed to investigate the methylation level of LINE-1 and *CDKN2A* promoter sequences. The qualitative analysis showed a trend toward an association between LINE-1 hypomethylation and *CDKN2A* inactivation (p=0.05). In a quantitative approach, primary tumors, mainly the thicker ones (>4 mm), exhibited a trend toward LINE-1 hypomethylation when compared with control melanocytes. To date, this is the first study reporting in cutaneous melanomas a possible link between the dysregulation of LINE-1 methylation and the presence of driver mutations.

Significance of this study

What is already known about this subject?

- DNA methylation plays an important role in silencing mobile elements in DNA, such as long interspersed element-1 (LINE-1) sequences.
- Retrotransposon reactivation leads to genomic instability, and is associated with cancer development and progression.
- Driver mutations confer proliferation advantages on cancer cells.
- The link between specific driver mutations and DNA methylation dysregulation of LINE-1 elements is poorly explored.

What are the new findings?

- The methylation level of LINE-1 sequences was evaluated in cutaneous melanomas. Our findings provide evidence that *CDKN2A* inactivation is associated with LINE-1 hypomethylation in melanomas.
- Primary melanomas, mainly the thicker ones (>4 mm), exhibited a trend toward LINE-1 hypomethylation.
- This is the first study showing a link between LINE-1 hypomethylation and the landscape of driver mutations in cutaneous melanomas.

How might these results change the focus of research or clinical practice?

- Epigenetic dysregulation, mainly DNA methylation, has been implicated in melanoma progression, posing as a useful diagnostic tool.
- Additional studies can clarify if melanoma samples carrying methylation dysregulation of LINE-1 sequences are linked to a worst prognosis.

INTRODUCTION

The worldwide incidence of cutaneous melanoma (CM) has increased in the past decades, with resulting high mortality rates and socioeconomic burden.[1] While melanoma is frequently detected in developed countries, in Brazil the reported incidence rates are modest.[2] Some well-known etiological factors of CM are ultraviolet exposure, especially those related to childhood sunburns and intermittent exposure, and familial predisposition.[3] To better understand CM development, one must consider histopathological aspects of the disease (eg, tumor growth, mitotic rate, presence of ulceration) which are important prognostic factors,[4] as well as the underlying molecular alterations. CMs exhibit several altered biological pathways, such as mitogen activated protein kinases (MAPK), opening a way for new treatment options. The MAPK pathway is responsible for the transduction of proliferative signals, and CMs often carry gain-of-function and actionable mutations in *BRAF, NRAS*, and *KIT* genes, all of them being a target for therapies (eg, Vemurafenib is indicated for patients with metastatic melanomas harboring BRAFV600E mutation).[5]

Cancer is the outcome of genetic and epigenetic alterations, the latter affecting mechanisms that control gene expression without modifying the underlying DNA sequence.[6] The covalent addition of methyl radicals to cytosines at CpG dinucleotides is a well-characterized epigenetic mark, being related to repression of gene expression.[7] Dysregulation of epigenetic mechanisms, particularly DNA methylation, plays a relevant role in CMs, being implicated in disease progression, and posing as a useful diagnostic tool, as reviewed elsewhere.[8] The long interspersed element-1 (LINE-1) sequences are retrotransposon elements comprising ~17% of the human genome, some of them still retaining the capacity to retrotranspose themselves to new genomic locations.[9] Loss of DNA methylation has been associated with increased retrotransposon activity,[10] and a meta-analysis study has revealed that LINE-1 hypomethylation is significantly associated with a wide range of cancer types.[11] Recently, we have demonstrated an association of LINE-1 dysregulation in the serum of melanoma-prone patients with metastasis development.[12]

Here, LINE-1 methylation level was assessed in CMs to investigate a possible association with clinicopathological features as well as recurrent driver mutations such as BRAFV600E, *NRAS* (exon 3), *KIT, TERT* promoter, and *CDKN2A*.[13 14]

MATERIAL AND METHODS

Samples selection and DNA extraction

Tumor samples were selected on the basis of their availability, and all specimens were reanalyzed by a pathologist of the AC Camargo Cancer Center (ACCCC—São Paulo, Brazil). We used samples presenting: (i) >80% of tumor cells, and (ii) absence of necrotic areas and/or inflammatory infiltrate. This study comprised 20 frozen specimens of primary CMs, and 7 paired metastatic tissues retrieved from the ACCCC Biobank. This retrospective project was approved by the local Ethics Committee of the Institution (CEP ACCCC 1765/13).

Data about histopathological characteristics of CMs (histological subtype, tumor thickness, ulcerative status, presence of inflammatory infiltrate, and mitotic rate) were collected on the clinical database of the ACCCC, and are summarized in figure 1.

Primary cultures of melanocytes in early passages (P1–P13) were used as control samples for DNA methylation analysis. These primary cultures of melanocytes were isolated from the foreskin samples of three young healthy donors obtained at the University Hospital of University of São Paulo (CEP HU/USP 943/09). The melanocyte cultures were established as previously described.[15 16]

DNA samples were extracted utilizing a standard phenol: chloroform protocol. The presence of melanin in DNA samples after the standard extraction protocol prompted us to use a purification column against polyphenolic compounds (OneStep PCR Inhibitor Removal Kit- Zymo Research). After this purification step, high-quality DNA samples were available for further genetic and epigenetic analysis.

Screening of driver mutations

Capillary sequencing on a 3130xl Genetic Analyzer (Applied Biosystems, Life Technologies, Carlsbad, California, USA) was conducted for screening of actionable mutations in the genes *BRAF* (V600E),[17] *NRAS* (exon 3),[18] *KIT* (exons 11 and 13),[19] and *TERT* promoter,[14] utilizing the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems), according to the manufacturer's protocol. The PCR conditions for the amplification of target regions were as follows: 0.2 μM of primers; 0.2 mM of dNTPs; 1 U of GoTaq Polymerase (Promega), and 50 ng of genomic DNA. The cycling conditions were an initial denaturation step of 95°C for 15 min, followed by 38 cycles of 95°C for 15 s, annealing temperature for 15 s, and 72°C for 45 s; and a final extension step (75°C) for 10 min. PCR products were visualized in a 1% agarose gel, and 1 μL of the PCR product was utilized for sequencing reactions. Primers for the amplification of the *BRAF* and *KIT* regions were generously given by the ACCCC molecular diagnostic sector, and are available on request. Primers for the amplification of *NRAS* exon 3 were: forward: 5′- GCATTGCATTCCC TGTGG-3′, reverse: 5′- CCCTAGATTCTCAATGTC AAAC-3′. Primers for *TERT* promoter amplification were obtained elsewhere.[14] Alignment using RefSeq annotation and variant calls were performed using the CLC Genomics Workbench software (CLC Bio).

Genomic deletions of the entire *CDKN2A* sequence were investigated utilizing the SALSA multiplex ligation-dependent probe amplification (MLPA) P419 CDKN2A/2B-CDK4 probemix (MRC Holland), according to the manufacturer's instructions; three DNA samples with the *CDKN2A* diploid copy number were used as controls. Data were analyzed with the Coffalyser software (MRC Holland), using the default parameters in the block analysis method. Normalized values in the range of 0.7–1.3 were considered as the diploid copy number threshold; tumors presenting median values for *CDKN2A* probes below 0.7 were considered to be deleted.

The methylation status of the *CDKN2A* promoter was obtained by pyrosequencing using the PyroMark Q96 CpG p16 kit (Qiagen Technologies, Hilden, Germany) on bisulfite converted DNA samples (EZ DNA Methylation-Gold Kit, Zymo, Irvine, California, USA); the amplified fragment contains seven CpG dinucleotides (present at positions +148 to +182 in exon 1 of the gene). Tumors exhibiting a median methylation level of all seven CpGs above 25% were considered hypermethylated when compared with control melanocytes. Both promoter hypermethylation and genomic deletions were considered as events resulting in *CDKN2A* inactivation.

LINE-1 methylation analysis

To analyze the *LINE-1* methylation status, pyrosequencing was performed with the PyroMark Q96 CpG LINE-1 kit (Qiagen Technologies, Hilden, Germany). The sequence contained four CpG dinucleotides (position 305 to 331—GenBank accession X58075), for which the median methylation value was calculated for each sample. A standard methylation curve for correction of the detected methylation levels was made using a commercially available DNA set (EpiTect PCR control DNA set—Qiagen), which has samples with known genome-wide methylation levels (100% and 0%).

We compared the distribution of the LINE-1 methylation values of CMs grouped according to their

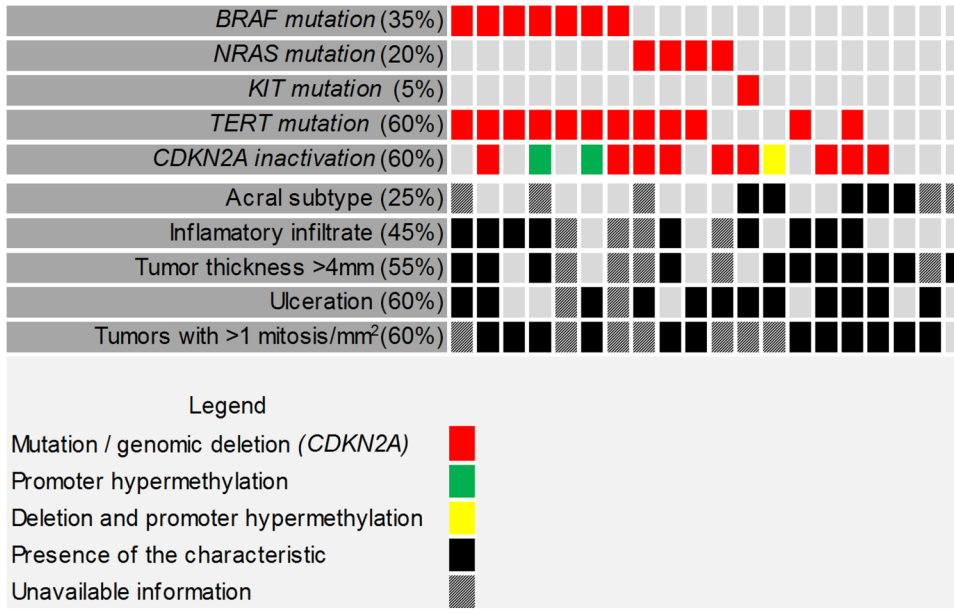

Figure 1 Panel of cutaneous melanoma driver mutations (top), and clinicopathological characteristics (bottom) of 20 primary melanoma samples. In this panel, each sample is represented in one column; the corresponding mutational and clinical data are presented in different rows. In this figure, we used the American Joint Committee on Cancer tumor-node-metastasis (TNM) staging system to group patients according to mitotic rate. Mutations in hot spots of *BRAF*, *NRAS*, and *KIT* genes, and in the *TERT* promoter, were tested by capillary sequencing, whereas *CDKN2A* deletions and promoter methylation were investigated by MLPA and pyrosequencing, respectively. The color scheme is indicated in the figure.

clinicopathological data (tumor thickness, presence of ulceration or inflammatory infiltrate, and mitotic rate) and mutational status (*BRAF*, *NRAS*, *KIT*, *TERT* promoter or *CDKN2A* inactivation) using the Mann-Whitney test ($p<0.05$). When grouping tumor thickness and mitotic rate in more than two categories (according to Thompson *et al*[20]), we used the Kruskal-Wallis test. For the statistical comparison of LINE-1 methylation levels between the group of seven primary tumors and their paired metastatic tissues, we also used the Mann-Whitney test ($p<0.05$). The LINE-1 methylation status of CMs was categorized in either hypomethylation (values ≤50% were considered hypomethylation) or hypermethylation. This association between driver mutations and LINE-1 methylation status was tested through Fisher's exact test ($p<0.05$). For all statistical analyses, we utilized the GraphPad Prism 5 software.

RESULTS

A high frequency of alterations was detected in the majority of the tested genes: 60% of the CMs harbored mutations in the MAPK pathway genes (35% *BRAF*; 20% *NRAS*; and 5% *KIT* (figure 1). *CDKN2A* loss of function alterations, including genomic deletions and promoter hypermethylation, were identified in 60% of the CM samples: 50% of genomic deletions, and 15% of promoter hypermethylation; one of the CM samples presented a heterozygous *CDKN2A* deletion and promoter hypermethylation in the remaining allele. Additionally, mutations in the *TERT* promoter were detected in 60% of the tumors. Only three CMs did not present detectable mutations in the sequences investigated here. Mutations in MAPK were mutually exclusive, whereas *TERT* promoter mutations were frequently detected simultaneously with MAPK alterations. We also screened the seven metastatic tissues for the presence of driver mutations, and they presented a mutational profile similar to their matched primary tumors, with the exception of two cases (see online supplementary figure S1). The three primary cultures of melanocytes used as controls were wild-type for all tested alterations (data not shown).

LINE-1 methylation levels were compared between primary CMs and melanocyte controls, and between primary CMs grouped according to the following pathological characteristics: ulcerative status, presence of inflammatory infiltrate, tumor thickness, and mitotic rate. We observed that CMs presented a heterogeneous level of LINE-1 methylation when compared with controls (figure 2A), with loss of methylation, although not statistically significant. Similarly, CMs with >4 mm, and those presenting an inflammatory infiltrate, also exhibited lower levels of LINE-1 methylation when compared with thinner tumors (figure 2B), and CMs without an inflammatory infiltrate (data not shown), respectively. We did not observe any trend in LINE-1 methylation levels when comparing tumor thickness according to T staging, as also when separating tumors according to their mitotic index (see online supplementary figure S2). The LINE-1 methylation levels in metastatic tissues when compared with their matched primary tumors were heterogeneous (figure 2C; the methylation levels for all samples are listed in online supplementary table S1).

We also investigated a possible association in CMs between the investigated driver mutations and the LINE-1 methylation status. The threshold was established at 50% of methylation to categorize the measured LINE-1

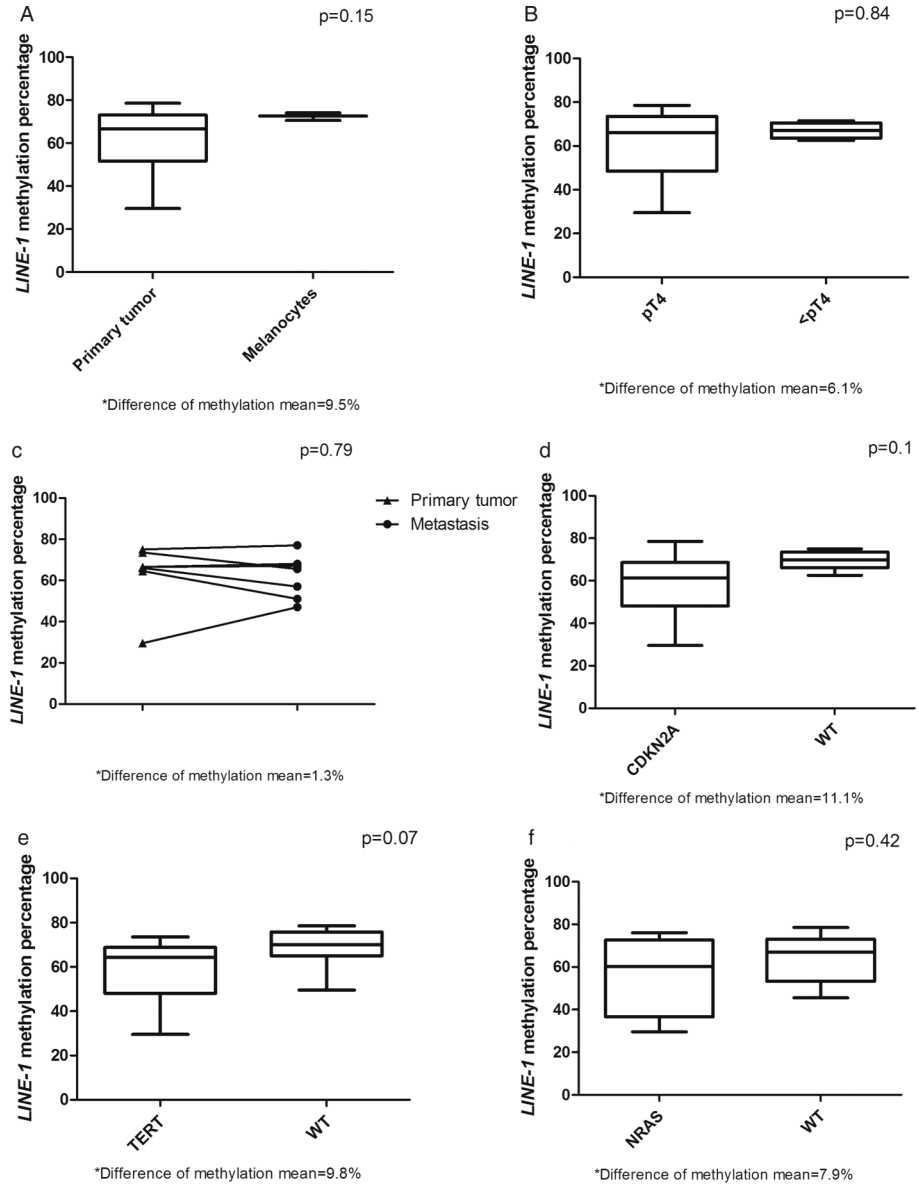

Figure 2 Analysis of the long interspersed element-1 (*LINE-1)* methylation levels obtained for primary cutaneous melanoma samples, metastatic melanomas, and primary cultures of melanocytes. The statistical analysis was performed comparing (A) primary tumors and melanocyte cultures; (B) tumors >4mm and <4 mm; (C) seven primary tumors and their paired metastatic tissues; and primary tumors with and without mutations affecting (D) *CDKN2A* inactivation, (E) *TERT* promoter mutation, and (F) *NRAS* mutation (from left to right).

methylation level of each sample in either hypomethylation or hypermethylation (methylation values ≤50% were considered as hypomethylation). A significant association (p=0.0547) was detected between LINE-1 hypomethylation and *CDKN2A* inactivation (genomic deletions and promoter hypermethylation considered as a group). The remaining driver mutations were not associated with categorized LINE-1 methylation status.

The set of driver mutations was also used to investigate quantitative differences in the *LINE-1* methylation level. BRAFV600E mutated tumors presented LINE-1 methylation levels very similar to the *BRAF* wild-type group, with a methylation difference of only 4.3%. Tumors harboring *CDKN2A* alterations, *NRAS* mutations, and *TERT* promoter mutations presented a relative loss of methylation in LINE-1 sequences when compared with the respective wild-type groups (figure 2D), with methylation differences of 11.1%, 7.9%, and 9.8%, respectively; however, none of these differences reached statistical significance.

DISCUSSION

In the present work, we reported a trend toward LINE-1 hypomethylation in primary CMs with thickness >4 mm when compared with thinner tumors. Tumor thickness is one of the most important prognostic factors in CM,[4] and our results point to a link between LINE-1 hypomethylation and a worse prognosis. This result finds support in the work of Tellez *et al*,[21] who detected LINE-1 hypomethylation in cell lines derived from primary and metastatic

melanomas, a finding later confirmed by Hoshimoto *et al*[22] in paraffin-embedded tissues. Furthermore, a correlation between advanced stages of this disease and loss of LINE-1 methylation was also observed by Hoshimoto *et al*, which is in agreement with our data of LINE-1 hypomethylation in pT4 tumors.

To date, few works have demonstrated a clear association between driver mutations, mainly BRAFV600E, and a distinct landscape of DNA methylation in CMs,[23 24] and they were mainly restricted to analysis of gene-rich regions. Here, we sought to explore the impact of CM driver mutations in the methylation level of LINE-1 sequences. We also included the study of the *CDKN2A* promoter methylation level because this gene plays a relevant tumor suppressor role by regulating p53 and RB1 pathways, and it is epigenetically repressed in a considerable number of tumors.[25 26] Interestingly, we detected a possible link between *CDKN2A* inactivation and LINE-1 hypomethylation. LINE-1 hypomethylation could have a key functional role in CMs since loss of methylation in the promoter regions of retrotransposons has been associated with increased retrotransposon activity, augmenting the genomic instability.[27 28] Therefore, a deficiency in control of cell cycle caused by *CDKN2A* inactivation, combined with LINE-1 demethylation, could be related to worse prognosis in patients with cancer. We are aware that any conclusion should be taken with caution owing to the small size of this cohort, as well as to the inherent limitations when using cell cultures as controls.[29] However, primary melanocyte cultures can be considered a proper DNA methylation control for melanomas; melanocytes are rare in the skin composition when compared to keratinocytes and fibroblasts,[30] and the utilization of normal skin as control would probably reflect the epigenetic background of these most common cell types.[31] Taking into account the limitations of this work, we propose a possible association of *CDKN2A* inactivating mutations with LINE-1 hypomethylation.

In a previous work,[12] we analyzed the methylation pattern of LINE-1 in the blood of patients with CM, disclosing an association between LINE-1 hypermethylation and both melanoma and metastasis occurrence. Other studies have already reported aberrant methylation of repetitive sequences in blood of patients with cancer. Barry *et al*[32] reported Alu hypermethylation in blood of patients with prostate cancer. Additionally, both hypomethylation and hypermethylation of LINE-1 CpGs were detected in the blood of patients with pancreatic, colon, and gastric cancer.[33] Taken together, these recent reports demonstrate that the pattern of LINE-1 methylation in blood is quite variable, in a clear contrast to tumor samples, which in general exhibit a genome-wide hypomethylation. We have previously reported an apparent contrast between the LINE-1 methylation pattern detected in the blood of patients with melanoma (hypermethylation) and in primary melanomas (hypomethylation).[34] The biological explanation for this discrepancy, and even for methylation differences detected in blood, remains elusive. One possible source of methylation alterations in blood of patients with cancer could be the presence of a particular population of circulating tumor cells (CTCs), an event previously described in patients with melanoma, for *RASSF1A* and *RARB* genes.[35] Therefore, additional studies are required to clarify which cells are carrying the observed methylation changes in blood, and the possible association with melanoma risk that we have reported. It will be important to address if the contrasting LINE-1 methylation patterns identified in blood and melanoma samples indeed reflect the diverse methylation landscapes of different cell populations derived from the tumors themselves.

To date, this is the first study showing a link between LINE-1 hypomethylation and the landscape of driver mutations in melanomas, mainly *CDKN2A* inactivation. We hope these preliminary data could drive the attention to detect similar events in larger data sets.

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Competing interests None declared.

Patient consent Obtained.

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REFERENCES

1 Lens MB, Dawes M. Global perspectives of contemporary epidemiological trends of cutaneous malignant melanoma. *Br J Dermatol* 2004;150:179–85.
2 Instituto Nacional do Câncer José Alencar Gomes da Silva (INCA). Estimate/2014—Cancer Incidence in Brazil. 2014. http://www.inca.gov.br/estimativa/2014/index.asp?ID=1
3 Tucker MA. Melanoma epidemiology. *Hematol Oncol Clin North Am* 2009;23:383–95.
4 Balch CM, Soong SJ, Atkins MB, *et al*. An evidence-based staging system for cutaneous melanoma. *CA Cancer J Clin* 2004;54:131–49.
5 Tsao H, Chin L, Garraway LA, *et al*. Melanoma: from mutations to medicine. *Genes Dev* 2012;26:1131–55.
6 Eccleston A, DeWitt N, Gunter C, *et al*. Introduction epigenetics. *Nature* 2007;447:395.
7 Doerfler W. DNA methylation and gene activity. *Ann Rev Biochem* 1983;52:93–124.
8 Greenberg ES, Chong KK, Huynh KT, *et al*. Epigenetic biomarkers in skin cancer. *Cancer Lett* 2014;342:170–7.
9 Beck CR, Collier P, Macfarlane C, *et al*. LINE-1 retrotransposition activity in human genomes. *Cell* 2010;141:1159–70.
10 Tubio JMC, Li Y, Ju YS, *et al*. Mobile DNA in cancer. Extensive transduction of nonrepetitive DNA mediated by LINE-1 retrotransposition in cancer genomes. *Science* 2014;345:1251343.
11 Barchitta M, Quattrocchi A, Maugeri A, *et al*. LINE-1 hypomethylation in blood and tissue samples as an epigenetic marker for cancer risk: a systematic review and meta-analysis. *PLoS ONE* 2104;9:e109478.
12 De Araújo ES, Kashiwabara AY, Achatz MI, *et al*. LINE-1 hypermethylation in peripheral blood of cutaneous melanoma patients is associated with metastasis. *Melanoma Res* 2015;25:173–7.
13 Hodis E, Watson IR, Kryukov GV, *et al*. A landscape of driver mutations in melanoma. *Cell* 2012;150:251–63.
14 Huang FW, Hodis E, Xu MJ, *et al*. Highly recurrent TERT promoter mutations in human melanoma. *Science* 2013;339:957–9.
15 Brohem CA, Cardeal LB, Tiago M, *et al*. Artificial skin in perspective: concepts and applications. *Pigment Cell Melanoma Res* 2011;24:35–50.
16 Pennacchi PC, de Almeida ME, Gomes OL, *et al*. Glycated reconstructed human skin as a platform to study the pathogenesis of skin aging. *Tissue Eng Part A* 2015;21:2417–25.
17 Davies H, Bignell GR, Cox C, *et al*. Mutations of the BRAF gene in human cancer. *Nature* 2002;417:949–54.

18 Brose MS, Volpe P, Feldman M, *et al*. BRAF and RAS mutations in human lung cancer and melanoma. *Cancer Res* 2002;62:6997–7000.
19 Curtin JA, Busam K, Pinkel D, *et al*. Somatic activation of KIT in distinct subtypes of melanoma. *J Clin Oncol* 2006;24:4340–6.
20 Thompson JF, Soong SJ, Balch CM, *et al*. Prognostic significance of mitotic rate in localized primary cutaneous melanoma: an analysis of patients in the multi-institutional American Joint Committee on Cancer melanoma staging database. *J Clin Oncol* 2011;29:2199–205.
21 Tellez CS, Shen L, Estécio MRH, *et al*. CpG island methylation profiling in human melanoma cell lines. *Melanoma Res* 2009;19:146–55.
22 Hoshimoto S, Kuo CT, Chong KK, *et al*. AIM1 and LINE-1 epigenetic aberrations in tumor and serum relate to melanoma progression and disease outcome. *J Invest Dermatol* 2012;132:1689–97.
23 Hou P, Liu D, Dong J, *et al*. The BRAF(V600E) causes widespread alterations in gene methylation in the genome of melanoma cells. *Cell Cycle* 2012;11:286–95.
24 Thomas NE, Slater NA, Edmiston SN, *et al*. DNA-methylation profiles in primary cutaneous melanomas are associated with clinically significant pathologic features. *Pigment Cell Melanoma Res* 2014;27:1097–105.
25 Schinke C, Mo Y, Yu Y, *et al*. Aberrant DNA methylation in malignant melanoma. *Melanoma Res* 2011;20:253–65.
26 Stott FJ, Bates S, James MC, *et al*. The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *EMBO J* 1998;17:5001–14.
27 Hancks DC, Kazazian HH. Active human retrotransposons: variation and disease. *Curr Opin Genet Dev* 2012;22:191–203.
28 Taby R, Issa JP. Cancer epigenetics. *CA Cancer J Clin* 2010;60:376–92.
29 Nestor CE, Ottaviano R, Reinhardt D, *et al*. Rapid reprogramming of epigenetic and transcriptional profiles in mammalian culture systems. *Genome Biol* 2015;16:11.
30 Cichorek M, Wachulska M, Stasiewicz A, *et al*. Skin melanocytes: biology and development. *Postepy Dermatol Alergol* 2013;30:30–41.
31 Lee JS, Kim DH, Choi DK, *et al*. Comparison of gene expression profiles between keratinocytes, melanocytes and fibroblasts. *Ann Dermatol* 2013;25:36–45.
32 Barry KH, Moore LE, Liao LM, *et al*. Prospective study of DNA methylation at LINE-1 and Alu in peripheral blood and the risk of prostate cancer. *Prostate* 2015;75:1718–25.
33 Nüsgen N, Goering W, Dauksa A, *et al*. Inter-locus as well as intra-locus heterogeneity in LINE-1 promoter methylation in common human cancers suggests selective demethylation pressure at specific CpGs. *Clin Epigenetics* 2015;7:17.
34 de Araújo ÉS, Pramio DT, Kashiwabara AY, *et al*. DNA methylation levels of melanoma risk genes are associated with clinical characteristics of melanoma patients. *Biomed Res Int* 2015;2015:376423.
35 Koyanagi K, Mori T, O'Day SJ, *et al*. Association of circulating tumor cells with serum tumor-related methylated DNA in peripheral blood of melanoma patients. *Cancer Res* 2006;66:6111–17.