early detection to improve melanoma patient survival.

CONFLICT OF INTEREST

Founder and major shareholder—Digital Derm Inc.—MoleMapCD (total body photography), Consulting and Grants—Electro-Optical Sciences Inc.—Melafind (melanoma detection device), and Consulting and Grants—Spectral Image Inc.—Dermatologic Diagnostics (no product yet).

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REFERENCES

A'Hern RP (2007) Sentinel-node biopsy in melanoma. *N Engl J Med* 356:418

Cochran AJ, Thompson JF (2008) Lymphatic mapping and sentinel node biopsy: the data unclouded by speculation. *Arch Dermatol* 144:687–8

Gervasoni JE Jr, Sbayi S, Cady B (2007) Role of lymphadenectomy in surgical treatment of solid tumors: an update on the clinical data. *Ann Surg Oncol* 14:2443–62

Gonzalez U (2007) Cloud over sentinel node biopsy. *Arch Dermatol* 143:775–6

Graham CH, Rivers J, Kerbel RS, Stankiewicz KS, White WL (1994) Extent of vascularization as a prognostic indicator in thin (<0.76 mm) malignant melanomas. *Am J Pathol* 145:510–4

Grichnik JM (2007) Sentinel-node biopsy in melanoma. *N Engl J Med* 356:418–9

Grichnik JM (2008) Melanoma, nevogenesis and stem cell biology. *J Invest Dermatol* 128: 2365–80

Guggenheim MM, Hug U, Jung FJ, Rousson V, Aust MC, Calcagni M *et al.* (2008) Morbidity and recurrence after completion lymph node dissection following sentinel lymph node biopsy in cutaneous malignant melanoma. *Ann Surg* 247:687–93

Kanzler MH, Levitt L, Lin A (2007) Sentinel-node biopsy in melanoma. *N Engl J Med* 356:419

Lipsker D (2007) Sentinel-node biopsy in melanoma. *N Engl J Med* 356:419

Massberg S, Schaerli P, Knezevic-Maramica I, Kollnberger M, Tubo N, Moseman EA *et al.* (2007) Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. *Cell* 131:994–1008

Minnebaev MM, Bakhtiozin VF, Musin MS (1981) [Lymph circulation dynamics and protein-cellular responses of central lymph during the early postresuscitation period]. *Biull Eksp Biol Med* 91:281–3

Morton DL, Elashoff R (2008) Sentinel node biopsy: facts to clear the alleged clouds. *Arch Dermatol* 144:685–6

Morton DL, Thompson JF, Cochran AJ, Mozzillo N, Elashoff R, Essner R *et al.* (2006) Sentinel-node biopsy or nodal observation in melanoma. *N Engl J Med* 355:1307–17

Murakami T, Cardones AR, Hwang ST (2004) Chemokine receptors and melanoma metastasis. *J Dermatol Sci* 36:71–8

Niakosari F, Kahn HJ, McCready D, Ghazarian D, Rotstein LE, Marks A *et al.* (2008) Lymphatic invasion identified by monoclonal antibody D2-40, younger age, and ulceration: predictors of sentinel lymph node involvement in primary cutaneous melanoma. *Arch Dermatol* 144:462–7

Ohtani O, Ohtani Y, Carati CJ, Gannon BJ (2003) Fluid and cellular pathways of rat lymph nodes in relation to lymphatic labyrinths and Aquaporin-1 expression. *Arch Histol Cytol* 66:261–72

Retsas S (2007) Sentinel-node biopsy in melanoma. *N Engl J Med* 356:419

Takeuchi H, Fujimoto A, Tanaka M, Yamano T, Hsueh E, Hoon DS (2004) CCL21 chemokine regulates chemokine receptor CCR7 bearing malignant melanoma cells. *Clin Cancer Res* 10:2351–8

Thomas JM (2007) Sentinel-node biopsy in melanoma. *N Engl J Med* 356:418

Warren BA, Shubik P, Feldman R (1978) Metastasis via the blood stream: the method of intravasation of tumor cells in a transplantable melanoma of the hamster. *Cancer Lett* 4:245–51

Welner RS, Kincade PW (2007) Stem cells on patrol. *Cell* 131:842–4

Absence of Germline Epimutation of the *CDKN2A* Gene in Familial Melanoma

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TO THE EDITOR

Melanoma occurs in approximately 10% of cases in families with multiple affected members (De Snoo *et al.*, 2003). In 20–57% of patients tumor susceptibility can be attributed to germline inactivating mutations in an allele of the *CDKN2A* gene (Goldstein *et al.*, 2007). A small minority of patients is predisposed to the development of melanoma through activating mutations in the *CDK4* gene (Goldstein *et al.*, 2006). However, in most cases of familial melanoma the genetic defect predisposing to melanoma development is unknown, which impedes genetic counseling. The *CDKN2A* gene encodes the p16 and $p14^{ARF}$ tumor suppressor proteins by means of different promoters and reading frames. Inactivation of p16 function through deletion, point mutation or promoter hypermethylation is a common and critical event in the pathogenesis of melanoma (Sharpless and Chin, 2003; Singh *et al.*, 2008). In addition, $p14^{ARF}$ may be genetically or epigenetically inactivated in sporadic melanoma and germline mutations in *CDKN2A* solely affecting $p14^{ARF}$ function have been identified in familial melanoma (Harland *et al.*, 2005; Furuta *et al.*, 2006). Patients with familial melanoma not related to germline mutation in *CDKN2A* or *CDK4* cannot be distinguished on the basis of clinical features. Germline mutations in different genes are assumed as a cause of melanoma predisposition in these patients (Bishop *et al.*, 2007).

Recently, an alternative mechanism of tumor predisposition, epimutation or germline promoter hypermethylation, has been found. In a subset of patients with hereditary nonpolyposis colorectal

cancer who did not harbor mutations in the *MLH1* or *MSH2* susceptibility genes, promoter hypermethylation of one allele of the *MLH1* gene was demonstrated in nearly all somatic cells, originating in the germline (Suter *et al.*, 2004; Martin *et al.*, 2005). Promoter hypermethylation is associated with transcriptional silencing and leads to functional inactivation of the affected allele. Although some uncertainty has remained concerning the exact mechanisms and patterns of inheritance of these epigenetic alterations, transmission of germline epimutation of *MLH1* to offspring has been demonstrated (Hitchins *et al.*, 2007). The purpose of this study has been to evaluate if melanoma predisposition might be caused by germline epimutation of the *CDKN2A* gene, in those cases not associated with germline mutations in *CDKN2A* or *CDK4*.

We selected 22 unrelated Dutch patients with familial melanoma, in whom melanoma had developed and who had at least one first-degree or two otherwise related family members diagnosed with melanoma (Table 1). Mutations in *CDKN2A* had been excluded by sequence analysis of its exons and introns. Also exon 2 of *CDK4*, harboring activating hotspot mutations, was analyzed in these patients. The promoters of p16 and $p14^{ARF}$ were analyzed for hypermethylation using two complementary methods: methylation-specific melting curve analysis (MS-MCA) and direct bisulfite sequence analysis (BSA; Paul and Clark, 1996; Worm *et al.*, 2001). Sensitivity of MS-MCA and BSA was validated by detection of p16 and $p14^{ARF}$ methylation in mixtures of methylated DNA (Chemicon, Hampshire, UK) and unmethylated semen DNA as well as in DNA from the colon cancer cell lines Colo205 and SW48, previously demonstrated to exhibit promoter hypermethylation of p16 and $p14^{ARF}$, respectively (Zheng *et al.*, 2000). DNA from patients with familial melanoma was isolated from peripheral blood mononuclear cells (PBMCs). DNA (1μg) was bisulfite-converted using the EZ DNA methylation kit (Zymo Research, Orange, CA). Primer sequences were designed to amplify CpG islands located in promoter regions of p16 and $p14^{ARF}$ and are available upon request (Figure 1a). Amplicons, both containing 10 CpG dinucleotides, were analyzed by MS-MCA and BSA. For MS-MCA, following PCR amplification DNA melting curves were acquired on a MyiQ real-time PCR detection system by measuring the fluorescence of SYBR Green during a temperature transition from 65 to 95 °C. BSA was performed as previously described (Van Doorn *et al.*, 2005). The studies were approved by the Leiden University Medical Center institutional review board and were conducted according to the Declaration of Helsinki Principles. Patient consent was obtained.

Table 1. Characteristics of included patients with familial melanoma

Patient number	Gender	Age at diagnosis	Affected family members
1	Female	49	Mother, two daughters
2	Female	39	Two sisters, one aunt, one female cousin
3	Male	25	Grandfather, two uncles
4	Female	27	Brother, one female cousin
5	Female	32	Mother, third-degree relative
6	Female	40	Son, brother
7	Female	54	Two sisters, one female cousin
8	Male	28	Father, mother, sister
9	Male	54	Daughter, one female cousin
10	Male	48	Two sisters, brother
11	Male	63	Sister, female cousin
12	Male	27	Sister, aunt, uncle
13	Female	58	Son
14	Female	40	Brother
15	Male	40	Sister
16	Male	30	Father
17	Male	60	Daughter
18	Male	63	Daughter
19	Male	42	Two female cousins, male cousin
20	Female	72	Daughter
21	Male	52	Sister, female cousin
22	Female	45	Twin brother

MS-MCA of the unmethylated p16 promoter sequence results in a peak with a melting temperature (T_m) of 79.6 °C, whereas the methylated sequence results in a T_m of 82.8 °C. For the $p14^{ARF}$ promoter, the T_m is 81.2 °C for the unmethylated sequence and 84.6 °C in case of methylation. Depicted in Figure 1b are melting curve profiles for the p16 and $p14^{ARF}$ promoters of a mixture containing 50% methylated DNA and 50% unmethylated DNA showing two T_m peaks in the upper graphs and for DNA from the cancer cell lines in the middle graphs. Melting curve profiles of DNA samples isolated from PBMCs of 22 patients with familial melanoma are presented in the lower graphs. All patient samples showed melting curve profiles with a T_m of 79.6 °C for p16 and 81.2 °C for $p14^{ARF}$, indicating that the CpG islands in the promoters of p16 and $p14^{ARF}$ contained no or scarcely any methylated CpG dinucleotides. To confirm these findings, PCR products of the 22 bisulfite-converted DNA samples were subjected to sequence analysis. BSA showed that indeed CpG dinucleotides in the p16 or $p14^{ARF}$ promoter were not methylated in any of the patient samples (Figure 1c).

Taken together our results indicate that at least in the Dutch population familial melanoma is not caused by

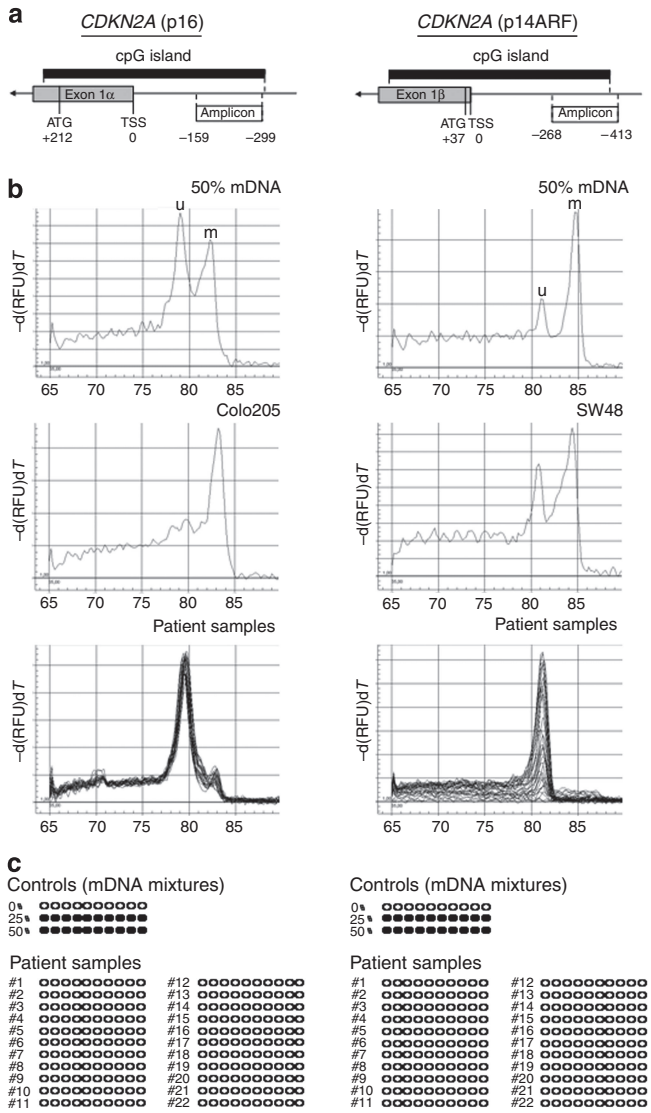

Figure 1. Methylation status of the p16 and p14^{ARF} promoters of the *CDKN2A* gene. (**a**) Schematic representation of the promoter regions of p16 and p14^{ARF} indicating the sequence amplified for MS-MCA and BSA, location of the CpG island and transcription start site (TSS). (**b**) MS-MCA melting curve profiles for mixtures of 50% completely methylated and 50% unmethylated bisulfite-converted DNA showing two peaks for the methylated (m) and unmethylated (u) DNA fraction, used as control (upper graphs). Melting curve profiles for the Colo205 and SW48 colon carcinoma cell showing biallelic hypermethylation of the p16 and monoallelic methylation of the p14^{ARF} promoter, respectively (middle graphs). MS-MCA results of the 22 patient DNA samples plotted jointly in one graph, showing a single peak at the T_m for unmethylated DNA (lower graphs). (**c**) Methylation density maps generated using BSA results of the p16 and p14^{ARF} CpG island amplicons. BSA of mixtures of completely methylated and unmethylated DNA: methylation could be detected when mixtures contained as little as 25% methylated DNA. In the patient samples none of the 10 interrogated CpG dinucleotides showed methylation (patient samples). Unmethylated cytosine bases are indicated as (○), methylated cytosines as (●).

germline epimutation of *CDKN2A*, in patients who lack mutations in this tumor suppressor gene. Although we have found no evidence of direct germline hypermethylation of *CDKN2A* gene promoters as a mechanism of tumor predisposition, other potentially heritable epigenetic events affecting the *CDKN2A* gene cannot be entirely excluded. The hypothesis has been advanced that alleles of certain genes, such as *MSH2* in a subset of patients with hereditary nonpolyposis colorectal cancer, may be prone to somatic hypermethylation because of their genetic or local chromatin structure, which would be associated with epigenetic silencing confined to specific tissues (Chan *et al.*, 2006; Suter and Martin, 2007). In this study, we exclude heritable epigenetic inactivation of *CDKN2A* as a mechanism causing susceptibility to the development of melanoma. The observed absence of *CDKN2A* epimutation implies that in patients with familial melanoma not related to mutations in this gene or in *CDK4*, heritable defects in other genes are implicated. The identification of additional melanoma susceptibility genes is currently actively pursued through genome-wide association studies on large groups of patients.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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REFERENCES

Bishop JN, Harland M, Randerson-Moor J, Bishop DT (2007) Management of familial melanoma. *Lancet Oncol* 8:46–54

Chan TL, Yuen ST, Kong CK, Chan YW, Chan AS, Ng WF *et al.* (2006) Heritable germline epimutation of *MSH2* in a family with hereditary nonpolyposis colorectal cancer. *Nat Genet* 38:1178–83

de Snoo FA, Bergman W, Gruis NA (2003) Familial melanoma: a complex disorder leading to controversy on DNA testing. *Fam Cancer* 2:109–16

Furuta J, Nobeyama Y, Umebayashi Y, Otsuka F, Kikuchi K, Ushijima T (2006) Silencing of peroxiredoxin 2 and aberrant methylation of 33 CpG islands in putative promoter regions in human malignant melanomas. *Cancer Res* 66:6080–6

Goldstein AM, Chan M, Harland M, Gillanders EM, Hayward NK, Avril MF *et al.* (2006) High-risk melanoma susceptibility genes and pancreatic cancer, neural system tumors, and uveal melanoma across GenoMEL. *Cancer Res* 66:9818–28

Goldstein AM, Chan M, Harland M, Hayward NK, Demenais F, Bishop DT *et al.* (2007) Features associated with germline CDKN2A mutations: a GenoMEL study of melanoma-prone families from three continents. *J Med Genet* 44:99–106

Harland M, Taylor CF, Chambers PA, Kukalizch K, Randerson-Moor JA, Gruis NA *et al.* (2005) A mutation hotspot at the p14ARF splice site. *Oncogene* 24:4604–8

Hitchins MP, Wong JJ, Suthers G, Suter CM, Martin DI, Hawkins NJ *et al.* (2007) Inheritance of a cancer-associated *MLH1* germ-line epimutation. *N Engl J Med* 356: 697–705

Martin DI, Ward R, Suter CM (2005) Germline epimutation: a basis for epigenetic disease in humans. *Ann N Y Acad Sci* 1054:68–77

Paul CL, Clark SJ (1996) Cytosine methylation: quantitation by automated genomic sequencing and GENESCAN analysis. *Biotechniques* 21:126–33

Sharpless E, Chin L (2003) The INK4a/ARF locus and melanoma. *Oncogene* 22:3092–8

Singh M, Lin J, Hocker TL, Tsao H (2008) Genetics of melanoma tumorigenesis. *Br J Dermatol* 158:15–21

Suter CM, Martin DI (2007) Inherited epimutation or a haplotypic basis for the propensity to silence? *Nat Genet* 39:573

Suter CM, Martin DI, Ward RL (2004) Germline epimutation of MLH1 in individuals with multiple cancers. *Nat Genet* 36:497–501

van Doorn R, Zoutman WH, Dijkman R, de Menezes RX, Commandeur S, Mulder AA *et al.* (2005) Epigenetic profiling of cutaneous T-cell lymphoma: promoter hypermethylation of multiple tumor suppressor genes including *BCL7a*, *PTPRG*, and *p73*. *J Clin Oncol* 23: 3886–96

Worm J, Aggerholm A, Guldberg P (2001) In-tube DNA methylation profiling by fluorescence melting curve analysis. *Clin Chem* 47: 1183–9

Zheng S, Chen P, McMillan A, Lafuente A, Lafuente MJ, Ballesta A *et al.* (2000) Correlations of partial and extensive methylation at the p14(ARF) locus with reduced mRNA expression in colorectal cancer cell lines and clinicopathological features in primary tumors. *Carcinogenesis* 21:2057–64

An Epinephrine-Dependent Mechanism for the Control of UV-Induced Pigmentation

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TO THE EDITOR

Tanning results from a coordinated set of signals that induce skin hyperpigmentation in response to exposure to UV radiation (Park *et al.*, 2008). This is classically thought to occur through the actions of proopiomelanocortin-derived peptides and α-melanocyte stimulating hormone (Abe *et al.*, 1969a, b; Wakamatsu *et al.*, 1997; Thody and Graham, 1998; Slominski *et al.*, 2000, 2004; Tsatmali *et al.*, 2000; Rousseau *et al.*, 2007) on the melanocyte melanocortin-1 receptor by increasing intracellular cAMP (Im *et al.*, 1998). However, as studies in proopiomelanocortin-deficient mice have shown that these mice retain the capacity to produce eumelanin even in the absence of α-melanocyte stimulating hormone or proopiomelanocortin-related peptides (Smart and Low, 2003; Slominski *et al.*, 2005), and animals with a nonfunctional melanocortin-1 receptor are still able to produce melanin in response to forskolin (Friedmann *et al.*, 1990; D'Orazio *et al.*, 2006), it is likely that alternate cAMP-dependent pathways can induce melanogenesis.

One alternate cAMP-dependent pathway involves the adrenergic receptor. The adrenergic receptors are pharmacologically divided into two subgroups, α and β, and both receptor subfamilies have been implicated in the control of pigmentation in frog skin (McGuire, 1970; Taylor and Teague, 1976) and human uveal melanocytes (Hu, 2000; Hu *et al.*, 2000). Human epidermal melanocytes express the α1-adrenergic receptor and β2-adrenergic receptor (B2AR; Schallreuter *et al.*, 1996; Scarparo *et al.*, 2000), and activation of the B2AR was shown to increase melanin synthesis (Gillbro *et al.*, 2004) whereas activation of the α1-adrenergic receptor had no effect (Schallreuter *et al.*, 1996). Human melanocytes also increase their expression of the B2AR in response to UV irradiation (Yang *et al.*, 2006), further suggesting a role for the B2AR in UV-induced hyperpigmentation.

Catecholamines are endogenous ligands for the adrenergic receptor and epinephrine has the greatest affinity for the B2AR. Indeed, epinephrine has been shown to increase melanin synthesis in human uveal melanocytes (Hu *et al.*, 2000) and increase intracellular cAMP in human epidermal melanocytes (Gillbro *et al.*, 2004). Epidermal melanocytes can synthesize the catecholamine norepinephrine but are unable to produce epinephrine as they do not express the enzyme phenylethanolamine-*N*-methyltransferase, which is necessary for synthesis of epinephrine (Gillbro *et al.*, 2004). Norepinephrine, though synthesized by melanocytes, does not seem to alter melanogenesis (Schallreuter *et al.*, 1996). However, keratinocytes possess the capacity to synthesize epinephrine (Schallreuter *et al.*, 1992; Pullar *et al.*, 2006). Thus, we hypothesized the existence of a paracrine interaction whereby keratinocytes secrete epinephrine in response to UV irradiation, which could then stimulate neighboring β-adrenergic receptors (BARs) on melanocytes to increase melanin synthesis.

Primary human keratinocytes and melanocytes were isolated from human neonatal foreskin and cultured in keratinocyte serum-free media (Cascade Biologics, Portland, OR) and phorbol-free

Abbreviations: B2AR, β2-adrenergic receptor; BAR, β-adrenergic receptor; PBS, phosphate buffer solution