Search for germline alterations in CDKN2A/ARF and CDK4 of 42 Jewish melanoma families with or without neural system tumours

To gain insight into the molecular mechanisms involved in the inherited predisposition to melanoma and associated neural system tumours, 42 Jewish, mainly Ashkenazi, melanoma families with or without neural system tumours were genotyped for germline point mutations and genomic deletions at the CDKN2A/ARF and CDK4 loci. CDKN2A/ARF deletion detection was performed using D9S1748, an intragenic microsatellite marker. Allele dosage at the p14ARF locus was analysed by quantitative real-time PCR employing a TaqMan probe that anneals specifically to exon 1β of the p14ARF gene. For detecting point mutations, diHPLC and direct sequencing of the coding sequences of CDKN2A/ARF and CDK4 was used. No germline alterations in any of the tested genes were detected among the families under study. We conclude that in the majority of Ashkenazi Jewish families, the genes tested are unlikely to be implicated in the predisposition to melanoma and associated neural system tumours.

British Journal of Cancer (2005) 92, 2278 – 2285. doi:10.1038/sj.bjc.6602629 www.bjcancer.com

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Keywords: Ashkenazi Jewish melanoma families; neural system tumours; CDKN2A/ARF; CDK4; germline mutations; inherited predisposition

Familial cutaneous malignant melanoma is a genetically heterogeneous condition linked to chromosome 9p21 in many, but not all families (Hussussian et al, 1994; Greene, 1999). To date, germline mutations in two high penetrance genes have been identified in some of these families, CDKN2A/ARF and CDK4. The CDKN2A/ARF gene encodes two distinct proteins, p16INK4a and p14ARF, and the result of alternative splicing of exons 1a and 1b, respectively. The p16INK4a protein, which belongs to the INK4 family of cyclin-dependent kinase inhibitors, plays a key role in arresting cell cycle progression at the G1 phase by inhibiting cyclins CDK4 and CDK6 and subsequently blocking their ability to phosphorylate the retinoblastoma protein Rb (Chin et al, 1998). The p14ARF protein is also involved in cell cycle regulation by interacting with different substrates in the p53 pathway (Pomerantz et al, 1998), and by binding to MDM2, also in the Rb pathway with resultant cell cycle arrest in both G1 and G2 phases (Xiao et al, 1995; Weber et al, 1999; Momand et al, 2000). In all, 20% of melanoma families were found to harbour genetic alterations at the CDKN2A/ARF gene (Goldstein, 2004). The other gene involved in familial predisposition to melanoma, CDK4, is a proto-oncogene that promotes cell cycle progression by phosphorylating the Rb protein. Germline mutations in CDK4 were detected in three melanoma families (Zuo et al, 1996; Soufir et al, 1998).

The familial clustering of both melanoma and neural system tumours (NST) was first reported in 1993 by Kaufman et al (1993) in a single family with eight family members over three generations who were diagnosed with cutaneous melanoma, cerebral astrocytoma or both. Azizi et al (1995) surveyed 904 melanoma Jewish-Israeli patients for the occurrence of NST in their family pedigrees. Melanoma-affected members within families, as well as first and second-degree relatives, were found to be at an increased risk for developing NST. A total of 15 families with a clustering of melanoma and a variety of NST were identified, and 10 patients with two primary tumours, melanoma and NST, primarily meningioma were described (Azizi et al, 1995). Similar familial clustering of melanoma and NST was described in French (Bahuau et al, 1997) and Finnish families (Paunu et al, 2002). Recently, melanoma and NST association was confirmed by epidemiological and population-based studies in Scandinavia (O’Neill et al, 2002; Hemminki et al, 2003; Nielsen et al, 2004). The familial clustering of melanoma and NST has been recognised and designated as the Melanoma and Neural System Tumour syndrome (MM-NST) (OMIM # 155755), and in a small subset of melanoma-NST kindreds germline mutations, mainly deletions affecting the CDKN2A/ARF gene and cosegregating with both tumours, were described.

In the present study, 42 Jewish, mainly Ashkenazi, melanoma families with \( n = 24 \) or without \( n = 18 \) were genotyped for germline sequence alterations in the CDKN2A/ARF and CDK4.
Genes. Mutational screening of 24 families with co-occurrence of melanoma and NST is the largest analysis reported thus far.

MATERIALS AND METHODS

Patients

Jewish families with a history of melanoma and NST were recruited to the study. The inclusion criteria (based on OMIM’s definition) were a minimum of two cancers in the pedigree, one being melanoma and the other NST, or an individual harbouring both tumours.

Additional 18 Jewish melanoma families without NST, having at least two or more individuals with melanoma, or multiple melanomas in a single family member – as minimal inclusion criteria – were also included.

The families had been recruited between the years 1997 and 2003. The study had been approved by the Institutional Ethics Committee of the Sheba Medical Center, Israel. All participants signed a written informed consent prior to being enrolled in the study. Demographic details, including country of birth of the probands, their parents and grandparents, were collected using a self-response questionnaire. Classification to ethnic groups was done according to the country of birth of the grandparents on both the maternal and paternal sides, provided that one or both parents were either from the same origin, or Israeli-born. Families with both sets of grandparents from Eastern and Central European countries were classified as Ashkenazi. Families originating from Spain, North-Africa, Balkans, or Iraq, Iran, Yemen and Egypt were classified as Sephardic. Families containing individuals with multiple melanomas. Nota-

Genetic alterations detection

DNA preparation Genomic DNA was extracted from peripheral blood leukocytes using the Puregene™ Genomic DNA Isolation Kit (Gentra Systems, Minneapolis MN, USA), using the manufacturer’s recommended protocol.

Mutation analysis For detecting CDKN2A/ARF and CDK4 gene coding region sequence alterations, exons 1a, 1b and 2 of CDKN2A/ARF and exon 2 of CDK4 were screened by dhPLC (denaturing high performance liquid chromatography), by using PCR and dhPLC analysis conditions previously described (Laud et al, 2003). Briefly, PCR was carried out in a final volume of 20 μl containing 100 ng genomic DNA, 1× HotStar Taq DNA Polymerase buffer with 1.5 mM MgCl₂ (Qiagen), 4 pmol of each primer, 1 UI HotStar Taq DNA Polymerase (Qiagen) and 2.5 mM dNTPs. For PCR amplification of each exon, a touch down protocol was used as follows: initial denaturation and HotStar Taq Polymerase activation at 95°C for 15 min; six cycles of 30 s at 95°C, 30 s at 66°C (the annealing temperature decreasing by 2°C at every two cycles), 30 s at 72°C; followed by 40 cycles of 30 s at 95°C, 30 s at 60°C and 30 s at 72°C. Heteroduplex analyses were carried out on an automated dhPLC instrument (WAVE, Transgenicom, CA, USA). DNA samples with known germline mutations at CDKN2A/ARF locus were used as positive controls.

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Samples displaying abnormal profiles were subsequently bi-directionally sequenced using the BigDye™ Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions on an ABI Prism 377 instrument (Applied Biosystems, Foster City, CA, USA).

CDKN2A/ARF deletion detection Since CDKN2A/ARF deletions were previously identified in melanoma-NST families (Bahuau et al, 1998, Randerson-Moor et al, 2001), deletions were sought only in this subset of families (n = 24). Deletion genotyping was performed using the D9S1748 microsatellite marker located adjacent to CDKN2A exon 1β. The PCR amplifications were carried out in a final volume of 25 μl, the reaction mix containing: 1× HotStar Taq DNA polymerase buffer with 1.5 mM MgCl₂ (Qiagen, Chatsworth, CA, USA), 1 UI HotStar Taq DNA polymerase (Qiagen), 4 pmols of each primer and 0.2 mM dNTPs. Primer sequences are available through The Genome Database (http://www.gdb.org). The forward primer was fluorescently labeled with the 6-FAM at its 5’ extremity. The PCR products were loaded on a 6%/7 M urea denaturing polyacrylamide gel in an ABI Prism 377 (Applied Biosystems, Foster City, CA, USA) device along with the ROX 350 (Applied Biosystems, Foster City, CA, USA) internal marker standard. Genotypes were analysed using the GeneScan software (Applied Biosystems, Foster City, CA, USA). Since the homozygous status could possibly be due to the loss of an allele, homozygous samples were further analysed for allele dosage (Barrois et al, 2004) at the p14ARF locus by quantitative real-time PCR using an ABI Prism 7700 instrument (Applied Biosystems, Foster City, CA, USA). A TaqMan probe that anneals specifically to the exon 1β of the p14ARF gene, marked with a fluorescent reporter dye (FAM) and a quencher dye (TAMRA), was used. By calculating the ratio initial copy number of p14ARF/initial copy number of GAPDH, we obtained the normalized gene dose. The PCR was performed in triplicate for each sample in a final volume of 50 μl, the reaction mix containing for the GAPDH gene 1× TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA, USA), 15 pmols of each primer and probe and 25 ng DNA. For the exon 1β of CDKN2A, same quantities were used, with the exception of the TaqMan Universal Master Mix which was replaced by 1× TaqMan PCR Core Reagent Buffer (Applied Biosystems, Foster City, CA, USA), 2.5 mM dNTPs, 5% glycerol, 5 mM MgCl₂ and 1.25 UI AmpliTaqGold DNA polymerase (Applied Biosystems, Foster City, CA, USA). Amplification conditions were: 2 min at 50°C, 10 min at 95°C (20 min for p14ARF) followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. We used as positive control the haploid cell line HL60 kindly provided by Juliette Moor and Julia Newton Bishop from Genetic Epidemiology Division, Cancer Research UK, St James’s University Hospital, Leeds, UK (Randerson-Moor et al, 2001).

RESULTS

Clinical features of the study participants

The study population included (a) 25 probands and 11 unaffected relatives from 24 families with pedigrees displaying cutaneous melanoma and NST and (b) 20 probands from 18 melanoma families without NST, among them 13 families with pedigrees containing two or more melanoma-affected individuals and five families containing individuals with multiple melanomas. Nota-

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British Journal of Cancer (2005) 92(12), 2278 – 2285

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this category, there were one melanoma and one NST in each family (Table 1). The male:female (M:F) ratio among the affected cases was 1. The melanoma and NST were diagnosed at the age range of 22–74 years and 10–86 years, respectively. Of the 24 families in this subgroup, 22 were of Ashkenazi origin, one out of 24 was Sephardic (#107), and one out of 24 heterogeneous (#121). Major phenotypic features of the patients, available in 13 out of 25 probands, were variable with no specific pattern. Additional cancers that were reported in this series included colon cancers in four families; breast cancer in three families; lung cancer in two families; liver cancer in two families; and renal, gastric, laryngeal, pleural and nonmelanoma skin cancer each in one family (Table 2). Examples of pedigrees showing melanoma families with NST are presented in Figure 1.

Table 1  The distribution of melanoma-NST pedigrees according to number of tumours

| No. of tumours | Family # | Total n (%) |
|----------------|----------|-------------|
| NST x 2 and MM x 2 | 120 | 1 (0.5) |
| MM x 2 and NST x 1 | 105, 109, 110 | 6 (25.0) |
| MM x 1 and NST x 1 | 112, 117, 119 | 14 (58.0) |
| NST x 2 and MM x 1 | 116, 122, 113 | 3 (12.5) |
| Total | 24 (100.0) |

*MN = melanoma, NST = neural system tumours.

Among the 18 melanoma families without NST (Table 3), the M:F ratio among the melanoma patients was 1:2, and the age at diagnosis was in the range of 25–88 years. Of 18 families in this series, 17 were of Ashkenazi origin. Family #321 was of heterogeneous Romanian (Ashkenazi)/Turkish-Greek (Sephardic) origin. Major phenotypic features of the melanoma patients, not available for two out of 20 probands included dermato-heliosis and solar keratosis (15 out of 18), freckles (15 out of 18) and AMS X

Table 2  Distribution of melanoma-NST pedigrees by tumour type and family affiliation

| Family # DNA# | Proband | Affected relatives | Unaffected relatives |
|---------------|---------|--------------------|----------------------|
| (n = 24) (n = 25) Gender | MM (°) | NST (°) | MM | NST (°) | MM | NST (°) | MM | NST (°) | DNA# Gender Age |
| 116 115 F | 68 64 (c) BCC (66) — — | — | Mother Lung (uncle) | 139 F 35 |
| 120 218 M | 51 51 (c) BCC | — — | — | Pleura (father) |
| 105 88 F | 58 68 (c) — | — — | — | — |
| 113 165 M | 57 19 (x) BCC | — | Lung (mother) | 194 M 65 |
| 117 153 F | 55 56 (c) — — | — | — | Breast (mother) |
| 109 27 M | 67 — | — Brother (a) | — | 67 M 45 |
| 99 66 F | 40 — | — Daughter | — | — |
| 118 73 F | 22 — | — Mother (48) | — | 41 M 14 |
| 101 15 H | 52 BCC (37) | — — | — | — |
| 119 — — — Aunt (d) Sister | Breast (sister, mother) | 12 M 53 |
| 102 109 M | 67 — | — Grandson (24) (e) | — | 133 F 45 |
| 103 6 M | 40 — | — Mother (71) (a) Colon (mother) | BCC/SCC Larynx (father) Liver (grand-mother) | — — |
| 105 13 H | 67 — | — — | | — — |
| 106 160 M | 74 — | — Father (68) (x) | — | 166 M 75 |
| 108 131 M | 67 — | — Brother (57) (x) | — | — — |
| 111 113 F | 66 — | — Mother (x) | — | — — |
| 115 137 F | 24 — | — Grand-mother (86) (x) | — | — — |
| 123 222 F | 56 — | — Father (84) (x) | Colon (mother) | — — |
| 124 223 M | 36 — | — Cousin (30) (x) | Renal (father) Gastric (aunt) | — — |
| 107 83 F | 43 — | — Daughter (10) (x) | — | — — |
| 121 220 F | 37 — | — Grand-father (x) Breast (mother) | — | — — |

*MN = melanoma, NST = neural system tumours, (a) = glioblastoma multiforme, (b) = oligodendroglioma, (c) = meningioma, (d) = glioma, (e) = neurilemmoma, (f) = malignant peripheral schwannoma, (g) = brain germinoma, (h) = medulloblastoma, (x) = NST, pathologic type unspecified.
Mutational analyses of the CDKN2/ARF and the CDK4 genes for point mutations

Overall, nine samples displayed different chromatographic profiles. Sequence analyses revealed a G to A transition at position 442 leading to a missense mutation at codon 148 (Ala148Thr) in all nine patients: patients #5, #83 and #115, all unrelated, among the melanoma-NST families (Table 4); and patients #15, #111, #114, #116, #124, #134, all unrelated among the melanoma families without NST (Table 5).
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Detection of CDKN2A/ARF gene deletions

A total of 30 individuals among the melanoma-NST families were genotyped using the D9S1748 microsatellite marker, located adjacent to exon 1 of CDKN2A/ARF gene on chromosome 9p21. A total of 12 samples displayed a heterozygous status, that is, two alleles without genomic deletion (Table 4); And 18 samples displaying homozygous profiles for this locus were selected for further analysis, since the homozygous status could indicate the loss of an allele by a large deletion encompassing exon 1β. Gene dosage for these samples, as well as six samples not analysed for the D9S1748 microsatellite marker, showed no deletions, therefore, all individuals presented two alleles (Table 4).

DISCUSSION

In the present study, no bona fide pathogenic germline alterations were identified in the CDKN2A/ARF and CDK4 loci among 42 Jewish, primarily Ashkenazi Israeli families, with a seemingly inherited predisposition to cutaneous melanoma, and in some, clustering of melanoma with NST, for deletions and point mutations in the CDKN2A/ARF and CDK4 loci. The only sequence variation identified in nine DNA samples was G to A transition at position 442 leading to a missense mutation at codon 148 (Ala148Thr). The Ala148Thr missense mutation is considered as a polymorphism based on several observations: it has been previously reported in individuals from the general, average risk, and heterogeneous populations. It is unlikely that the familial clustering of melanoma and NST is due to chance, as both tumours are relatively rare cancers in Israel, with age standardised incidence rates of 7.7 and 8.4 per 10^5, respectively, in female subjects, and 7.6 and 10.3 per 10^5, respectively, in male subjects (Aizizi et al., 1995). All the melanoma families with NST in the present study withstand the OMIM criteria for the melanoma-NST syndrome. Yet, without identifying a mutation, cosegregating within the current series of families with both melanoma and NST, we cannot unequivocally determine the proportion of families that truly represent melanoma-NST syndrome. However, 10 families (42% of the series), having at least two melanoma or two NST probands, are strongly suggestive of an inherited predisposition for developing melanoma and NST.

Lack of germline mutations in the CDKN2A/ARF and CDK4 loci has been recently reported by Loo et al. (2005) among 22 Ashkenazi Jewish families with an apparent inherited predisposition to melanoma. Taken together with the data reported herein, it appears that in over 60 Ashkenazi Jewish melanoma families, no germline alteration in CDKN2A/ARF and CDK4 loci underlie the
### Table 4  Mutation detection analysis in p16, p14 and CDK4 genes of melanoma-NST pedigrees (n = 24)

| Family # (n = 24) | DNA # (n = 36) | P16 sequencing analysis | P14 deletion analysis by D9S1748 | Quantative TaqMan analysis of p14 sequencing | p14 sequencing | CDK4 sequencing |
|-------------------|----------------|-------------------------|----------------------------------|---------------------------------------------|----------------|----------------|
| 101               | 154            | WT                      | Hmz                              | 2n                                          | WT             | WT             |
| 102               | 185*           | WT                      | Htz                              | ND                                          | WT             | WT             |
| 103               | 109            | WT                      | Hmz                              | 2n                                          | WT             | WT             |
| 104               | 13             | WT                      | Htz                              | ND                                          | WT             | WT             |
| 105               | 88             | WT                      | Hmz                              | 2n                                          | WT             | WT             |
| 106               | 160            | WT                      | Htz                              | ND                                          | WT             | WT             |
| 107               | 166*           | WT                      | Htz                              | ND                                          | WT             | WT             |
| 108               | 83             | Ala148Thr               | Hmz                              | 2n                                          | WT             | WT             |
| 109               | 131            | WT                      | Htz                              | ND                                          | WT             | WT             |
| 110               | 27             | WT                      | Hmz                              | 2n                                          | WT             | WT             |
| 111               | 66             | WT                      | Hmz                              | 2n                                          | WT             | WT             |
| 112               | 67*            | WT                      | Hmz                              | 2n                                          | WT             | WT             |
| 113               | 113            | WT                      | Htz                              | ND                                          | WT             | WT             |
| 114               | 169            | WT                      | Hmz                              | 2n                                          | WT             | WT             |
| 115               | 194*           | WT                      | Hmz                              | 2n                                          | WT             | WT             |
| 116               | 73             | WT                      | Hmz                              | 2n                                          | WT             | WT             |
| 117               | 116            | WT                      | Hmz                              | 2n                                          | WT             | WT             |
| 118               | 79             | WT                      | Hmz                              | 2n                                          | WT             | WT             |
| 119               | 137            | WT                      | Htz                              | ND                                          | WT             | WT             |
| 120               | 139*           | WT                      | Hmz                              | 2n                                          | WT             | WT             |
| 121               | 131            | WT                      | Htz                              | ND                                          | WT             | WT             |
| 122               | 100            | WT                      | Hmz                              | 2n                                          | WT             | WT             |
| 123               | 105            | WT                      | Htz                              | ND                                          | WT             | WT             |
| 124               | 111            | WT                      | HАЗ                             | ND                                          | WT             | WT             |
| 125               | 78             | WT                      | ND                               | ND                                          | WT             | WT             |
| 126               | 79             | WT                      | ND                               | ND                                          | WT             | WT             |
| 127               | 112            | WT                      | ND                               | ND                                          | WT             | WT             |
| 128               | 113            | WT                      | ND                               | ND                                          | WT             | WT             |
| 129               | 114            | WT                      | ND                               | ND                                          | WT             | WT             |
| 130               | 115            | WT                      | ND                               | ND                                          | WT             | WT             |
| 131               | 116            | WT                      | ND                               | ND                                          | WT             | WT             |
| 132               | 117            | WT                      | ND                               | ND                                          | WT             | WT             |
| 133               | 118            | WT                      | ND                               | ND                                          | WT             | WT             |
| 134               | 119            | WT                      | ND                               | ND                                          | WT             | WT             |
| 135               | 120            | WT                      | ND                               | ND                                          | WT             | WT             |
| 136               | 121            | WT                      | ND                               | ND                                          | WT             | WT             |
| 137               | 122            | WT                      | ND                               | ND                                          | WT             | WT             |
| 138               | 123            | WT                      | ND                               | ND                                          | WT             | WT             |
| 139               | 124            | WT                      | ND                               | ND                                          | WT             | WT             |
| 140               | 125            | WT                      | ND                               | ND                                          | WT             | WT             |
| 141               | 126            | WT                      | ND                               | ND                                          | WT             | WT             |
| 142               | 127            | WT                      | ND                               | ND                                          | WT             | WT             |
| 143               | 128            | WT                      | ND                               | ND                                          | WT             | WT             |
| 144               | 129            | WT                      | ND                               | ND                                          | WT             | WT             |
| 145               | 130            | WT                      | ND                               | ND                                          | WT             | WT             |
| 146               | 131            | WT                      | ND                               | ND                                          | WT             | WT             |
| 147               | 132            | WT                      | ND                               | ND                                          | WT             | WT             |
| 148               | 133            | WT                      | ND                               | ND                                          | WT             | WT             |
| 149               | 134            | WT                      | ND                               | ND                                          | WT             | WT             |
| 150               | 135            | WT                      | ND                               | ND                                          | WT             | WT             |
| 151               | 136            | WT                      | ND                               | ND                                          | WT             | WT             |
| 152               | 137            | WT                      | ND                               | ND                                          | WT             | WT             |
| 153               | 138            | WT                      | ND                               | ND                                          | WT             | WT             |

*Unaffected relatives.

### Table 5  Mutation detection analysis in p16, p14 and CDK4 genes of pedigrees of melanoma families without neural system tumours (n = 18)

| Family # (n = 18) | DNA # (n = 20) | P16 sequencing analysis | P14 deletion analysis by D9S1748 | Quantative TaqMan analysis of p14 sequencing | p14 sequencing | CDK4 sequencing |
|-------------------|----------------|-------------------------|----------------------------------|---------------------------------------------|----------------|----------------|
| 302               | 104            | WT                      | ND                               | ND                                          | WT             | WT             |
| 303               | 103            | WT                      | ND                               | ND                                          | WT             | WT             |
| 304               | 100            | WT                      | ND                               | ND                                          | WT             | WT             |
| 305               | 112            | WT                      | ND                               | ND                                          | WT             | WT             |
| 306               | 111            | WT                      | ND                               | ND                                          | WT             | WT             |
| 307               | 105            | WT                      | ND                               | ND                                          | WT             | WT             |
| 308               | 119            | WT                      | ND                               | ND                                          | WT             | WT             |
| 309               | 116            | Ala148Thr               | ND                               | ND                                          | WT             | WT             |
| 310               | 114            | Ala148Thr               | ND                               | ND                                          | WT             | WT             |
| 311               | 15             | Ala148Thr               | ND                               | ND                                          | WT             | WT             |
| 312               | 124            | Ala148Thr               | ND                               | ND                                          | WT             | WT             |
| 313               | 78             | WT                      | ND                               | ND                                          | WT             | WT             |
| 314               | 79             | WT                      | ND                               | ND                                          | WT             | WT             |
| 315               | 121            | WT                      | ND                               | ND                                          | WT             | WT             |
| 316               | 135            | WT                      | ND                               | ND                                          | WT             | WT             |
| 317               | 122            | WT                      | ND                               | ND                                          | WT             | WT             |
| 318               | 140            | WT                      | ND                               | ND                                          | WT             | WT             |
| 319               | 134            | Ala148Thr               | ND                               | ND                                          | WT             | WT             |
| 320               | 131            | Ala148Thr               | ND                               | ND                                          | WT             | WT             |
| 321               | 141            | WT                      | ND                               | ND                                          | WT             | WT             |
| 322               | 138            | WT                      | ND                               | ND                                          | WT             | WT             |

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ACKNOWLEDGEMENTS

Catalin Marian is a recipient of a ‘Marie Curie’ fellowship, EU ref. no. QLGA-GH-99-50406-15; his current address is Biochemistry Department, University of Medicine and Pharmacy of Timisoara, Romania. Karine Laud is a recipient of an IGR postdoctoral fellowship. This work was partly supported by PHRC regional Ile de France, Grant No. AOR 01 091. Alon Scope performed research as part of the requisite of the Scientific Council, Israel Medical Association, for Dermatology Specialty.
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