CDKN2A and MC1R analysis in amelanotic and pigmented melanoma

Paola Ghiorzo[a], Lorenza Pastorino[a], Maria A. Pizzichetta[c], Riccardo Bono[d], Paola Queirolo[b], Renato Talamini[c], Giorgio Annessi[d], William Bruno[a], Sabina Nasti[a], Sara Gargiulo[a], Linda Battistuzzi[a], Maria C. Sini[e], Giuseppe Palmieri[e] and Giovanna Bianchi Scarrà[a], on behalf of the Italian Melanoma Intergroup

Amelanotic melanoma (AM) is a rare subtype of melanoma with little or no clinically visible pigment; it is more difficult to diagnose than pigmented melanoma (PM), and has a worse prognosis. In the attempt to find a genetic explanation for the distinction between AM and PM, we conducted a case–case study, matching AM and PM patients, and testing them for germline mutations in high- (p16INK4A, p14ARF, CDK4) and low-penetrance (MC1R) melanoma susceptibility genes. Similar CDKN2A mutations were found in both sets of melanomas. A p14ARF splice germline mutation was detected for the first time in an Italian family with AM. This rare mutation, which has been described only once previously, may be involved in predisposition to the amelanotic phenotype in combination with germline MC1R variants and coordinate somatic expression of pigmentation genes and their regulators. ***Melanoma Res***

19:142–145 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Melanoma Research 2009, **19:**142–145

Keywords: amelanotic melanoma, CDKN2A, MC1R, mutation, p14ARF

[a]Dipartimento di Oncologia, Biologia e Genetica, Università di Genova, [b]Istituto Nazionale per la Ricerca sul Cancro, Genova, [c]Centro di Riferimento Oncologico, Aviano, [d]Istituto Dermopatico dell'Immacolata, Roma and [e]Istituto di Chimica Biomolecolare, CNR, Sassari, Italy

Correspondence to Dr Paola Ghiorzo, Dipartimento di Oncologia, Biologia e Genetica, Università di Genova, V.le Benedetto XV, 6, Genova 16132, Italy
Tel: +39 10 3538982; e-mail: paola.ghiorzo@unige.it

Paola Ghiorzo and Lorenza Pastorino equally contributed to this study

Received 15 April 2008 **Accepted** 28 January 2009

Amelanotic melanoma (AM) is a rare subtype of melanoma with little or no clinically visible pigment. Depending on whether the term is only used to define lesions entirely lacking pigment or also lesions in which less than 25% is pigmented, AMs account for 2–8% of all melanomas [1]. Their atypical appearance often results in delayed diagnosis and worse prognosis than with pigmented melanoma (PM).

Low cutaneous melanin content, such as that seen in AM, leads to inefficient repair of ultraviolet-radiation-induced DNA damage and is thus associated with melanoma risk [2]. Mutations in the high-penetrance melanoma susceptibility genes *p16INK4A* and *p14ARF* play a prominent role in DNA repair, in addition to being involved in cell-cycle control [3,4]. Certain MC1R alleles are also associated with melanoma susceptibility through their impact on DNA repair, independent of their role in melanin regulation [5,6].

We have conducted a case–case study, matching AM and PM patients, testing them for germline mutations in high (p16INK4A, p14ARF, CDK4) and low-penetrance (MC1R) melanoma susceptibility genes and evaluating the somatic expression of genes involved in the pigmentary pathway.

Forty cases of AM and 78 cases of PM were consecutively enrolled at the participating centers. Diagnosis of AM was confirmed by histology [7]. The two groups were matched by age (± 5 years), sex, and residence, and were administered a standardized questionnaire on phenotypic features and family history. All participants provided their informed consent, and the study was approved by a local ethics committee.

The two p16INK4A germline mutations, which are most frequently identified in Italian melanoma patients, the G101W and E27X founder mutations [8], were each detected in one PM case. Among the 40 AM cases, one harbored G101W and another was positive for the p14ARF g.193 + 1 G > A mutation. A variant of unknown functional significance (–25CT) was also identified in this series but not in 100 healthy individuals. Overall, the mutation rate was higher, if not significantly, in the AM group ($P = 0.06$). The G101W-positive AM patient had previously developed multiple PMs and belonged to a melanoma family. No tissue samples from this kindred were available.

The p14ARF g.193 + 1 G > A-positive AM case was found to belong to an unreported melanoma family comprising

0960-8931 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CMR.0b013e32832a1e18

five melanoma cases overall. The proband had previously developed two PMs (no tissue samples were available from these melanomas) and his mother had developed an AM. We have no information about the pigmentation of the other three melanomas in the family. The proband's amelanotic lesion (superficial spreading melanoma, Clark III, Breslow 0.73 mm, vertical growth phase) was partially, weakly pigmented. His mother's AM (nodular melanoma, Clark IV, Breslow 2.2 mm) was seen to lack pigmentation at histology (Fig. 1a). They were both classified phototytpe III according to the Fitzpatrick classification.

The proband's mother, children, and two siblings were found to be p14ARF g.193 + 1 G > A-positive. One of the siblings was affected by a neural system tumor (NST).

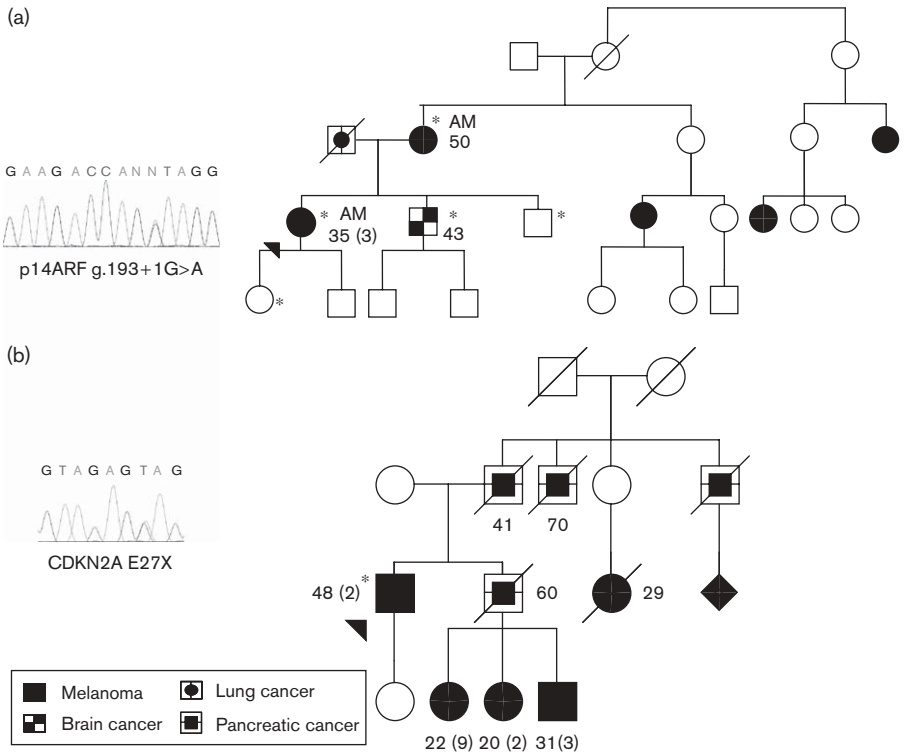
To our best knowledge, this rare germline mutation has been previously identified in a single kindred from the Netherlands displaying melanoma and one case of NST [9]. Our p14ARF-positive kindred also displayed melanoma and NST, confirming the association between p14ARF mutations and NST [10,11].

The finding that the two mutation-positive AM cases developed both pigmented and amelanotic lesions may suggest that inheritance of these mutations, in combination with somatic alterations in other modifying factors and/or genes acting as downstream effectors, played a role in determining the AM phenotype.

The E27X-positive PM case was found to belong to a previously undescribed family including three cases of multiple melanoma and four of pancreatic cancer (PC) among first-degree relatives (Fig. 1b). The proband had previously developed two other PMs, one of his nieces had developed nine and another niece had developed two. His father was diagnosed with PC at the age of 41 years. One brother and two uncles were also affected by PC. None of the relatives was available for molecular testing.

A comparison of the distribution of MC1R variants in AM and PM patients showed that high-risk 'R' variants – D84E, R142H, R151C, R160W, D294H, I155T – [12] conferred an increased risk of AM compared with PM (odds ratio: 2.31, 95% confidence interval: 1.06–5.03, $P = 0.035$). After adjusting for skin color, the difference was no longer significant, but the trend remained unvaried (odds ratio: 2.09, 95% confidence interval: 0.94–4.64, $P = 0.069$). It has been shown that activated

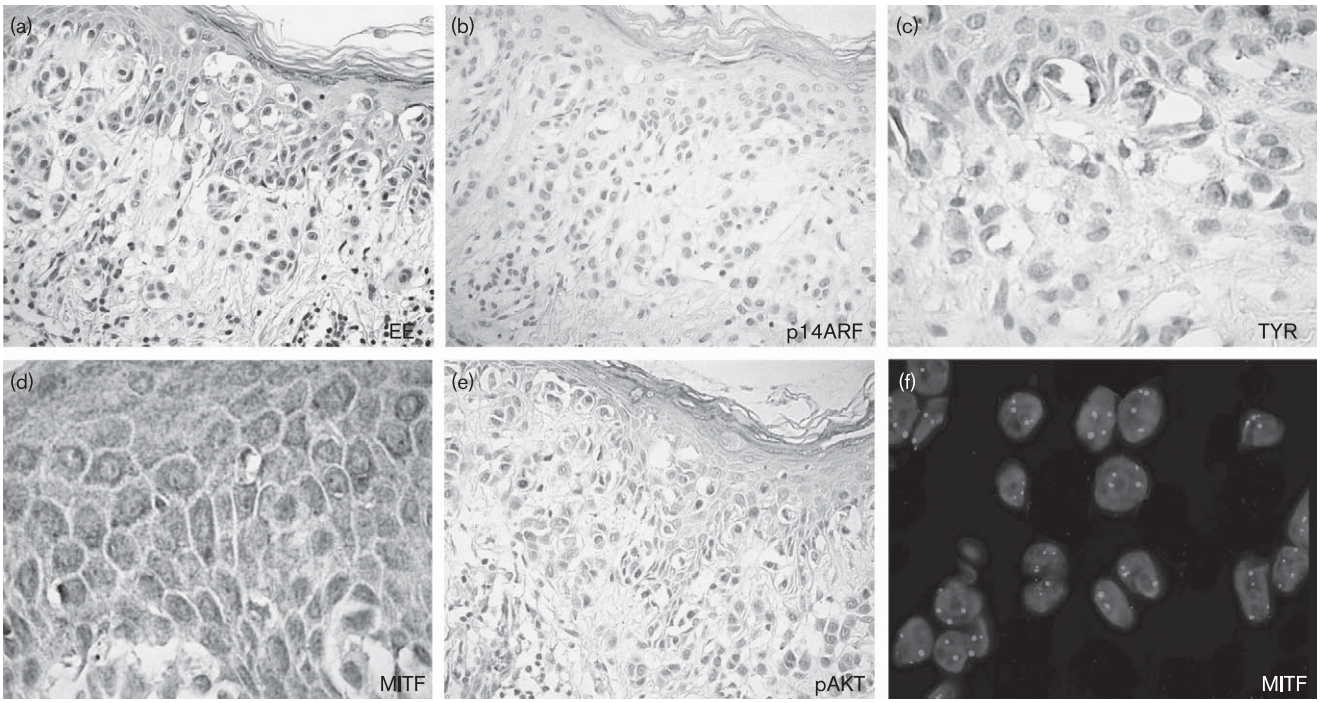
Fig. 1



(a) Pedigree of the amelanotic melanoma (AM) case harboring the p14ARF g.193 + 1 germline mutation. (b) Pedigree of the pigmented melanoma case harboring the CDKN2A E27X germline mutation. Mutation carriers are indicated by an asterisk, the arrow indicates the proband. Ages at diagnosis are indicated under each symbol and the number of primaries is indicated in brackets.

MC1R is required for human melanocyte survival and contrasts ultraviolet-radiation-induced damage; these effects were evident in tyrosinase-negative albino melanocytes, whereas they were absent in human melanocytes expressing loss-of-function MC1R [6,13]. The two related individuals who developed AM and carried the p14ARF mutation were positive for the R142H 'R' variant, which has been described as having reduced functional response, probably owing to defective G-protein coupling, or/and ligand affinity [12,14]. Gene expression profiling studies recently identified novel downstream effectors of p14ARF, whose regulation may contribute to melanoma development when p14ARF function is lost [15]. We conducted gene expression studies in the AM tissue of the patient harboring the p14ARF germline mutation. p14ARF expression was completely absent. Expression of both tyrosinase (the key enzyme in pigmentation) and MITF (the master regulator of pigmentation, which downregulates tyrosinase) was low. Interestingly, MITF expression was cytoplasmic and not nuclear, indicating that MITF was not active in these cells. Accordingly, MITF was not genomically amplified, as demonstrated by fluorescence in-situ hybridization analysis of the same lesion. Expression levels of pAKT, which inhibits MITF, were high (Fig. 2). This pattern of coordinate expression of pigmentation genes and their regulators is likely to have contributed to the determination of the AM phenotype in this lesion, and is consistent with results reported by previous murine and in-vitro studies [16–18]. Furthermore, our findings confirm that MC1R and p14ARF are also critical to DNA repair, and suggest that their germline variants, potentially along with other as yet unidentified downstream targets, may

Fig. 2



Immunohistochemical staining of the amelanotic melanoma lesion of the proband who harbors the p14ARF g.193+1 G>A mutation (b–e). Four-micron sections from paraffin-embedded tissue were deparaffinized according to standard methods and cemented with anti-MITF (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA), anti-pAKT (Ser473, Cell Signaling Technology, Danvers, Massachusetts, USA), anti-Tyrosinase (Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK), and anti-p14ARF (Novus Biologicals Inc., Littleton, Colorado, USA) antibodies after microwave antigen retrieval. Antigen localization was achieved using the streptavidin-biotin-peroxidase method, following the manufacturer's instructions (Vector Laboratories, Burlingame, California, USA). Staining was visualized with a diaminobenzidene chromogen kit (Ylem, Rome, Italy), which yields brown positive staining. Benign nevi and pigmented melanomas were used as positive control to confirm the efficacy of the staining procedure (data not shown). (a) Hematoxylin and eosin staining ($\times$ 20); (b) negative p14ARF staining ($\times$ 20); (c) weak tyrosinase expression ($\times$ 100); (d) cytoplasmic MITF staining ($\times$ 100); (e) positive pAKT expression ($\times$ 20). (f) Absence of MITF amplification after hybridization with probes specific for the MITF locus (red signals) and control centromere (green signals) on nuclei from same paraffin-embedded tissues. Double-colour fluorescence in-situ hybridization analysis was performed using a BAC clone, RP11 215K24 (supplied by the Sanger Institute of Molecular Cytogenetics, UK), as probe-specific for the MITF locus on chromosome 3. The specific probe was labeled by nick translation with Cy3 (GE Healthcare, Bucks, UK); the pericentromeric control probe was labeled with Spectrum Green (Vysis, Downer's Grove, Illinois, USA). Tissue slides were treated and hybridized as previously described [19]. MITF amplification was defined as the presence of three or more gene copies compared with two copies of the centromere control probe in at least 30% of 200 nonoverlapping nuclei.

also have predisposed our proband to the amelanotic phenotype.

This case–case study confirms that the G101W and E27X CDKN2A founder mutations are common in Italian melanoma families, and led to the identification of two previously unrecognized melanoma pedigrees: in the first, the E27X mutation is associated with PC and several cases of PM; in the second, the p14ARF g.193 + 1 G > A mutation (reported here for the second time in the literature) segregates in two cases displaying AM and is associated with NST. Studies on larger collections of AMs and matched PMs, correlated with somatic expression analyses, will help define the role of germline variants in known or as yet unidentified high/low-risk melanoma susceptibility genes in determining the amelanotic phenotype.

Acknowledgements

The authors acknowledge the support of the EU FP6 GenoMEL Network of Excellence (2005–2010). This study was partially funded by the Genoa Athaeneum Grant 2006 to GBS and ACC funding 2007 to PQ.

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