Relation Between Animal-Type Melanoma and Reduced Nuclear Expression of Glutathione S-Transferase π

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Background: Animal-type melanoma (ATM) is a rare variant of the tumor showing diffuse, heavily pigmented neoplastic cells in the dermis. Despite the high mean thickness of the lesions, reports seem to indicate a less aggressive behavior and a better survival rate for ATM compared with conventional melanoma, but the underlying pathways related to this favorable outcome are still unknown.

Observations: Five women and 2 men aged 20 to 92 years presented with pigmented skin nodules (n=5) or plaques (n=2), varying in size from 1.0 to 4.5 cm. Findings from microscopic examination showed monotypic-appearing melanocytes with abundant intracytoplasmic melanin in a nodular or fascicular arrangement (mean Breslow thickness, 4.97 mm). Immunohistochemical analysis of ATM cells demonstrated the typical positive staining for S-100, vimentin, HMB-45, and melan-A. The investigation of the π isofom of glutathione S-transferase, a family of enzymes involved in tumor progression, revealed that nuclear expression is reduced in ATMs compared with control melanomas, whereas results from cytoplasmic staining did not vary. One patient died of cardiac failure without evidence of disease progression; the remaining patients are disease-free at 3 (n=4) and 5 years (n=3).

Conclusions: Our findings confirm that ATM is a variant of melanoma with distinctive clinical and histological features. Low nuclear expression of glutathione S-transferase π expression is a characteristic of ATM and could add new insight to better understand the unusual biological behavior of this rare neoplasm.


ANIMAL-TYPE MELANOMA (ATM) is a rare but distinctive histopathological variant of melanoma recently described in the literature.1-4 The presence of large hyperpigmented cells in the dermis, the lack of in situ component or junctional nests,1,2,4 the absence of marked cytological atypia, and the peculiar clinical appearance2,4 characterize this tumor. Dick5 was the first who defined as an equine melanotic disease a cutaneous melanocytic proliferation characterized by nodules of heavily melanized cells in gray horses.5-7 The similarity between equine and human neoplasms was later suggested by Darier,8 who introduced the term melanosarcoma. Although only a limited number of cases have been reported, ATM seems to have a less aggressive behavior and a better survival rate compared with conventional melanomas with similar mean thickness, even though metastases may occasionally develop.4,6,9,10

We report herein the clinical, pathological, and immunohistochemical findings of 7 ATM cases observed in the last 6 years at our institutions. To date, the mechanisms influencing the outcome in patients with ATM remain uncertain.4 Glutathione S-transferase (GST) is a complex family of enzymes with significant functions in cancer prevention.11-13 Among the different GST isoforms, the π class (GST-π) is predominant in the skin.13 Levels of GST-π isoform have been reported to be increased in human melanoma cells compared with normal melanocytes.14-15 To achieve a better knowledge of this rare variant of melanoma, data concerning the nuclear and cytoplasmic GST-π expression in our series of ATM cases are also reported.

METHODS

Melanoma tissue samples were obtained from diagnostic biopsy specimens from surgical pathology archives of Tor Vergata University and San Gallicano Institute, Rome, Italy, over the last 5 years, according to the guidelines of local ethics committees. According to the literature1,4,16 we considered the following as diagnostic criteria of ATM: low mitotic rate, absence of marked atypia, clear intraepidermal proliferation or junctional nests, necrosis, skin ulceration, signs of regression, and perineural and/or lymphatic invasion.

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In all cases, a wide surgical excision was performed and the sentinel lymph node was also biopsied.\(^\text{10}\) In the largest lesion, a 1-mm-thick section was also obtained for electron microscopy (see the “Ultrastructural Evaluation” subsection). Careful analysis of familial and personal medical histories was integrated with clinical reexamination to exclude specific syndromes, including Carney complex or lentiginosis syndrome.\(^\text{17}\)

### HISTOLOGICAL AND IMMUNOHISTOCHEMICAL EVALUATION

Serial 4-µm-thick paraffin sections were stained with hematoxylin-eosin or used for immunohistochemical analysis. For the latter, after deparaffinization, endogenous peroxidase activity was blocked with 0.2% hydrogen peroxide (20 minutes) and the sections were incubated with normal goat serum (30 minutes). When necessary, pigment was bleached according to the method used by Orchard and Calonje.\(^\text{18}\) Sections were exposed for 1 hour to the following monoclonal antibodies: anti-p65 (B N F-p65 subunit (Santa Cruz Biotechnology Inc, San Diego, California; 1:100), which gave similar results. Diaminobenzidine and red chromogen amino ethyl carbazole were used as final chromogens. As a control for immunohistochemical evaluation, a group (n=10) of consecutive patients with superficial spreading melanomas in vertical growth phase (mean [SD] tumour thickness, 2 [0.65] mm) was enrolled. The mean (SD) age of the control patients (52 [13] years) did not differ from that of the ATM group. Immunoreactivity was estimated at an original magnification of ×200 in at least 10 fields by 2 of the authors (S. Costantini and A.F.), with an intraobserver reproducibility greater than 95%. Immunostaining results were graded according to the percentage of positive cells as follows: 0, negative or less than 1% positive cells; 1, less than 25% positive cells; 2, between 25% and 50% positive cells; and 3, greater than 50% positive cells. For GST-\(\pi\), the same score was also used to calculate nuclear staining.

### ULTRASTRUCTURAL EVALUATION

For ultrastructural study, small tissue fragments were post-fixed in Karnowsky fixative and embedded in epoxy resin. Semithin sections were prepared, stained with toluidine blue, and evaluated by light microscopy. Then appropriate areas were selected, and thin sections were examined under a Philips Morgagni electron microscope (FEI, Brno, Czech Republic).

### RESULTS

#### CLINICAL FINDINGS

Among 316 registered melanomas diagnosed in our 2 departments during the last 6 years, we found 7 cases fitting the diagnosis of ATM. The patients included 5 women and 2 men, with a mean age of 54 years. The clinical and pathological features of these cases are summarized in the Table. Skin lesions presented as heavily pigmented nodules (n=3) or plaques (n=2), with the largest ranging from 1.0 to 4.5 cm. The clinical appearance of patient 4 is shown in Figure 1A. Sentinel lymph node biopsy specimens were obtained in all patients, and they did not disclose evidence of metastasis. Cutaneous satellite lesions were observed in patients 3 and 5. Staging procedures confirmed stage IA to IIB disease, according to American Joint Committee on Cancer (AJCC) criteria.\(^\text{10}\) The follow-up period was 3 (patients 1, 2, and 4) and 5 years (patients 5, 6, and 7). All patients received adjuvant immunotherapy with low-dose interferon alfa-2b. After 1 year, 1 patient (patient 3) died of cardiac failure without evidence of disease progression; the remaining patients are currently alive and well and clinically disease-free. The 2-year survival rate compared with that of the previous reported series is shown in Figure 2.

### HISTOLOGICAL AND IMMUNOHISTOCHEMICAL FINDINGS

In all the lesions, microscopic examination revealed a variable exophytic, heavily pigmented neoplasm in the dermis and epidermis (Figure 1B), with a thickness ranging from 0.7 to 10.0 mm (mean thickness, 5.0 mm; Table) and Clark level IV to V. The presence of melanocytes impinging on the epidermis and/or the dermoepidermal junction was rare and was focal and observed in only 14.3% and 28.6% of cases, respectively, whereas clear intraepidermal proliferation or junctional nests were absent. The ATM cells were arranged in a nodular or fascicular fashion, showing a monotypic appearance with round to oval nuclei, delicate chromatin, inconspicuous basophilic nucleoli, and heavy intracytoplasmic accumulation of melanin (Figure 3). Areas of conventional melanoma were not seen. Only a few mitotic figures could be detected after an extensive search (≤1-3/mm\(^2\)). In patient 3, a focal accumulation of neoplastic cells with large, pale,
and foamy cytoplasm, round and eccentrically placed nuclei, and prominent nucleoli was detected (Figure 3F). In these clear cells, nuclear pleomorphism was slight and mitotic figures were rare. Electron microscopy revealed large indented nuclei with abundant cytoplasm containing numerous single melanosomes varying in size; melanosomes were mainly in the early stage of maturation and sometimes had a pleomorphic appearance, which was in accordance with previously reported findings.1

Immunohistochemical investigation revealed diffuse and strongly positive staining for S-100 protein, vimentin, HMB-45, and melan-A, similar to the control melanoma group (Figure 4 and Figure 5). Melan-A and HMB-45 immunoreactions in ATM spindle-shaped and polygonal areas were similar. Also, variable positive staining for melan-A in ATM clear cells was observed (Figure 4F). In all cases investigated, neoplastic cells were CD68 negative (not shown). Furthermore, we investigated the expression of GST-π (Figure 5). In melanocytes of adjacent skin, GST-π immunoreactions were mainly cytoplasmic, similar to the control melanomas (Figure 5A); however, unlike the control melanomas, GST-π expression in ATM cells was mainly cytoplasmic. Semiquantitative evaluation confirmed lower nuclear GST-π immunostaining in ATMs compared with control melanomas (Figure 6; P < .01); GST-π cytoplasmic staining was similar between the 2 melanoma groups. In all melanomas, NF-κB–p65 immunostaining was mainly in the cytoplasm, with no significant differences between the 2 groups, although in 20% of controls, a local nuclear NF-κB staining was also observed (not shown). Finally, the proliferative index, expressed as the mean (SD) percentage of Ki-67–positive cells, did not significantly differ when comparing ATMs (17% [1.9%]) and conventional melanomas (22% [4.2%]).

COMMENT

In the present work, we describe 7 cases of ATM. All lesions in our series featured the histological findings characteristic of ATM.1–4 Most of the authors of previously published series recognize ATM as a rare but distinct variant of human melanoma,1–4 although a small number of investigators do not agree on its existence.19 Microscopically, ATM must be differentiated from other heavily pigmented melanocytic lesions, such as cellular or malignant blue nevus, pigmented spindle cells nevus, melanophage-rich regressed superficial spreading melanoma, and epithelioid blue nevus. Cellular blue nevus is a benign neoplasm that may show a striking pigment synthesis.20 Malignant blue nevus is believed to be the consequence of malignant transformation in a preexisting cellular blue nevus,21 and cells are overly malignant with marked pleomorphism and mitoses. Prognostically, malignant blue nevus is an aggressive neoplasm with a high propensity for
The main distinguishing feature of the pigmented spindle cell nevus of Reed is the superficial plaquelike structure of the lesion, in contradistinction to the deeply invasive extent of ATM. Second, although both lesions show melanocytic monotypism, elongated and spindled pigmented spindle cells prevail in pigmented spindle cell nevus as opposed to the prevalent polygonal structure of ATM. In regressed superficial

**Figure 3.** Microscopic appearance of animal-type melanoma on hematoxylin-eosin–stained sections. A, A heavily pigmented neoplasm occupies the whole dermis (original magnification ×20). B, Focal impinging of dermoepidermal junction. C, Cytoplasmic accumulation of abundant coarse melanin in neoplastic cells arranged in nodules or large sheets (original magnification ×40). D, Polygonal and spindle-shaped neoplastic cells (original magnification ×100) with, E, rounded nuclei and occasional prominent nucleoli (original magnification ×200). F, In patient 3, clear cells with large foamy cytoplasm and mild nuclear pleomorphism is also present (original magnification ×200).
spreading melanoma, there are background foci of typical melanoma with conventional malignant cytomorphic features and a much lesser degree of neoplastic pigmentation compared with ATM. Regressed melanoma may contain dermal mononuclear and pigmented cells mimicking ATM. In this latter case, immunohistochemical analysis can help define the histiocytic origin of the cell infiltrate. Zembowicz et al recently described a new entity,

Figure 4. Immunohistochemical examination of nuclear and cytoplasmic glutathione S-transferase expression in animal-type melanoma (ATM) using red aminoethyl carbazole as chromogen. Positive HMB-45 (A) and S-100 (B) immunostaining of neoplastic cells arranged in nodular fashion (original magnification ×20). Large hyperpigmented epithelioid cells with positive HMB-45 immunostaining (original magnification ×100 [C] and ×200 [D]). Positive melan-A cells in a fascicular array (original magnification ×100) (E) and variable positive staining for melan-A in ATM clear cells in patient 3 (original magnification ×200) (F).
pigmented epithelioid melanocytoma, as a provisional histological entity encompassing both ATM and epithelioid blue nevus, based on the impossibility of distinguishing these 2 entities only by histological features. Although other authors criticized this view, those cases of pigmented epithelioid melanocytoma not associated with Carney complex are in essence ATM.

In the literature, the prognosis of ATM is controversial. In a recent review, Milette and Ackerman do not consider ATM as a distinct variant of melanoma and describe its prognosis as unpredictable or unclear because of the limited evaluation of cases previously reported and the occurrence of metastasis. Antony et al reported 14 additional ATM cases with an excellent survival rate, even

Figure 5. Immunohistochemical investigation of nuclear and cytoplasmic glutathione S-transferase (GST–π) expression in animal-type melanoma (ATM) using red amino ethyl carbazole (A, B, D, and F) and diaminobenzidine (C and E) as chromogens. A, GST–π reactivity of normal skin is mainly cytoplasmic and stronger in the stratum basalis and less strong in the superficial layers (original magnification ×100). B, In the radial phase growth adjacent to a tumorigenic nodule of superficial spreading melanoma, malignant cells show strong cytoplasmic and nuclear GST–π staining (original magnification ×100). C and D, GST–π cytoplasmic expression in ATM cells is present, whereas nuclear staining is low and focal (original magnification ×200 [C] and ×400 [D]). E and F, In a tumorigenic nodule of superficial spreading melanoma, GST–π diffusely stains both the nucleus and cytoplasm (original magnification ×200 [E] and ×400 [F]).
in the presence of metastasis. A review of the 31 cases of ATM with available clinical information, including the cases in our present study, indicate a 2-year survival rate of 90%. The 5-year survival rate in 18 cases was similar (94%).

In conventional melanomas with similar thickness and no ulceration, 2- and 5-year survival rates are approximately 84% and 70%, respectively. Despite the high mean thickness, the only death in our ATM series was due to cardiac failure and not to disease progression. Although these percentages are preliminary and need to be confirmed by larger series, they suggest that, although metastasis can occur in patients with ATM, the overall survival and biological behavior seem longer and less aggressive, respectively, compared with conventional melanomas.

In addition to morphological features, we tried to further define the characteristics of ATM. To this end, we investigated our cases using immunohistochimical analysis. Routine investigation of ATM cells revealed the typical phenotype pattern of melanoma. In addition, in all ATMs investigated, we documented a reduced nuclear GST-π expression compared with control melanomas. Glutathione S-transferase is a 6-class complex family of enzymes that acts as biological agents of detoxification and is involved in tumor progression. In human epidermis, π isofrom is the predominant GST isoenzyme. Glutathione S-transferase immunoreactivity is stronger in the cytoplasm of basal keratinocytes and less evident in the upper layers of the epidermis. Melanocytes also show a diffuse cytoplasmic and a focal nuclear GST-π staining, whereas cytoplasm and nuclei of non-ATM melanoma cells is strongly stained. However, besides melanoma, expression of GST-π in other neoplasms can vary.

Glutathione S-transferases operate in synergy with specific proteins to confer a multidrug resistance to the melanoma and nonmelanoma tumor cells. The GST-π isoform is likely to make adducts with anticancer drugs and facilitate their transport out of the cells. GST-π nuclear amount does not depend on the cytoplasmic level. Low nuclear GST-π expression seems to confirm indirectly that ATM is a morphologically distinct entity that can be distinguished from conventional melanomas. Similarly, Zembowicz et al recently reported a further distinctive characteristic: the lower or absent levels of protein kinase A regulatory subunit 1α in ATM enclosed in a large group of pigmented epithelioid melanocytomas compared with conventional melanomas.

It is likely that the reduced GST-π nuclear level may influence the biological behavior of ATM. Nuclear GST-π has a greater protective role against anticancer drugs than the cytoplasmic GST-π isoform, in ovarian cancer, the 5-year survival rate of nuclear GST-π-positive patients is lower than that of cytoplasmic GST-π-positive patients. The distinction between cytoplasmic and nuclear localization can help to explain the lack of correlation between the overall GST-π level and melanoma progression. Another hypothesis is that the nuclear GST-π expression could indirectly influence the resistance of melanoma cells to the host immune system. In light of this view, it is of interest to notice that GST-π-deficient mice show higher levels of bone marrow–derived circulating white blood cells compared with controls, and nuclear GST-π expression may negatively influence the immune transductional response pathway.

In conclusion, our results are in accordance with the opinion that ATM is a distinct clinical and histopathological variant of melanoma, and, as an original finding, we observed a reduced expression of nuclear GST-π. If this enzymatic profile is confirmed in larger series of ATM cases, our observation could add new insights to the recognition of this rare variant of melanoma and to a better understanding of its apparently different biological behavior.

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