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INVESTIGATIVE REPORT

Chronically Sun-damaged Melanomas Express Low Levels of Nuclear Glutathione-S-transferase-π: An Epidemiological and Clinicopathological Study in Italy

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The detoxifying enzyme glutathione–s–transferase pi (GST–π) is present in keratinocytes and melanocytes and exerts a protective role against tumour progression. Melanomas close to melanocytic naevus remnants occur less frequently on sun-exposed areas, whereas solar dermal elastosis, hallmark of chronic sun-damage, characterise melanomas on sun-exposed skin. We evaluated the expression of GST-π in 113 melanomas associated to melanocytic naevus remnants or to solar dermal elastosis, classified according to clinical characteristics, history of sun exposure, histological subtypes and AJCC staging. Chronically sun-damaged melanomas, identified by moderate–severe solar dermal elastosis, showed a lower nuclear GST-π expression and a higher thickness than those related to melanocytic naevus remnants ($p<0.03$). Multivariate logistic regression analysis demonstrated that male gender and chronic sun-exposure are independent risk factors significantly associated to melanomas localised on the trunk (OR=3.36, 95% CI: 1.31–8.65; OR=5.97, 95% CI: 1.71–20.87). If confirmed on a larger series, lower expression of nuclear GST-π in melanoma cells could represent a possible marker of chronically sun-damaged melanoma pathogenesis. *Key words: melanoma; solar dermal elastosis; melanocytic naevus remnant; glutathione-s-transferase.*

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Many efforts have been made to assess the role of exogenous or endogenous risk factors and the prognostic value of differentiation markers in melanoma development and progression (1, 2). Fair-skin, a family history of melanoma, predisposing genotypes, large number of melanocytic naevus count and chronic and/or intermittent sun exposure influence or characterise multiple melanoma pathways (3). Melanomas associated to melanocytic naevus remnants affect younger patients and occur less frequently on chronically sun-damaged skin, whereas melanomas with extensive to histological solar dermal elastosis, signs of chronic sun-damage, have been linked to increased survival rates (4–6). We studied glutathione-s-transferase (GST), a six-class complex family of enzymes, which act as biological agents of detoxification and deactivation of oxidative stress (7) in tumour progression (8). The pi class of GST (GST-π), which is the predominant isoenzyme in the skin (9), is variably expressed in normal keratinocytes and melanocytes (10), but is increased in human melanoma cells with no further distinction among more or less aggressive tumours (11). We reported that in animal-type melanoma, a rare variant with a relatively good prognosis despite thickness, nuclear GST-π is reduced compared to the different variants of melanoma with equivalent depth (12). In this study, we analysed the GST-π nuclear and cytoplasmic expression in 113 melanomas either associated to melanocytic naevus remnants or to solar dermal elastosis. Our series was classified according to clinical characteristics, history of sun exposure, histological subtypes and AJCC staging. Furthermore, body site, presence of melanoma-associated melanocytic naevus remnants (M/MN), degree of melanoma-associated solar dermal elastosis (M/SDE), and nuclear and cytoplasmic GST-π isoform expression in melanoma cells were statistically evaluated as possible independent risk factors in the development of melanoma.

MATERIAL AND METHODS

Patients and epidemiological assessment

The relationship among demographic, anamnestic and clinicopathological characteristics was investigated in 113 consecutively enrolled melanoma patients diagnosed at the Department of Dermatology, Tor Vergata University of Rome, Italy, during 2 years of observation. Patients with metastatic disease were excluded from the study. Local Ethics Committee approved the study and all participants gave their written consent. A structured questionnaire was proposed to the patients during their first follow-up visit by a trained physician. We collected

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detailed information about socio-demographic characteristics and age at first melanoma diagnosis. Furthermore, according to the described link between incidence of melanoma and sun exposure modalities (13), we recorded all data on acute/intermittent (< 15, 15–60, > 60 days/year) or chronic sun exposure – either for leisure or occupational causes – as well as histories of childhood sunburns, habit of tanning lamps and protective sunscreen usage for more or less than 10 years. For each patient, we evaluated body site of the primary lesion (head/neck, trunk, extremities), skin photo type (I–IV), eye colour (blue, green, brown, black), hair colour (red, blond, brown, black), signs of solar damage (freckling tendency, solar lentigo, actinic keratoses), melanocytic naevus count, presence of non-melanoma skin cancers, concurrent not neoplastic dermatoses.

Morphological and immunohistochemical investigations

Haematoxylin & eosin-stained tumour sections were retrospectively investigated. The following histological parameters were considered: the histologic subtype (superficial spreading melanoma: SSM; nodular melanoma: NM; lentigo maligna melanoma: LMM), the tumour thickness (Breslow's thickness, Clark's levels), the presence (M/MN$^+$) or absence (M/MN$^-$) of melanoma-associated melanocytic naevus remnant, the latter documenting benign melanocytes located within or in the immediate vicinity (less than 1 field at 400 × magnification) of the melanoma cells, the presence (M/SDE$^+$) or absence (M/SDE$^-$) of melanoma-associated solar dermal elastosis, graded as mild and moderate/severe (14). Presence or absence of melanocytic naevus remnant could not be investigated in 4 of the overall series, as the primary tumour sections were not available from other Institutions. Immunohistochemistry was performed as reported (15) on serial 4 mm-thick paraffin sections using monoclonal anti-HMB-45 (1:40, Ylem, Avezzano, Italy), anti-Melan-A (1:50, Neomarkers, Fremont, USA), anti-S-100 (1:200, Neomarkers), and anti-vimentin (1:40, Ylem). Monoclonal anti-GST-π immunostaining (1:100, Novocastra, Newcastle, UK) was performed as earlier reported (12) and repeated using a rabbit polyclonal antibody (Santa Cruz, USA), which gave similar results (not shown). Immunoreactivity using 3-amino-9-ethylcarbazole as chromogen was estimated at × 200 magnification in at least 10 fields and graded as follows: 0: negative or < 1% positive, 1: < 25%, 2: between 25–50% and 3: > 50% of cells (8). All measurements were performed blinded by 10 of the authors (ADS, AO), with an interobserver variability of < 5%.

Statistical analysis

Patients with melanoma were stratified according to clinico-pathologic features. Analyses of two-by-two contingency tables were performed to assess the association between site-specific and the presence or absence of an associated naevus and other possible risk factors. Pearson's Chi-square test was performed and crude odds ratio (OR)s with their confidence intervals (95% CI) were estimated using the first category as reference group. Significance of OR can be noticed from its CI. Multivariable logistic regression was used to evaluate independent risk factors for all considered outcomes. Models were fitted on the basis of the results of the univariate analysis and considering the effect of potential confounding variables. Continuous variables were categorised according to biological considerations or conventional cut-off points. Immunohistochemical results were analysed by means of Student's *t*-test and ANOVA. The differences were considered statistically significant for values of $p < 0.05$. Immunohistochemical nuclear expression of GST-π was then correlated to the clinico-pathological features via Spearman's rank correlation test. All statistical analyses were performed with STATA 11.0 (Stat Corp, College Station, Texas, USA).

RESULTS

The distributions of 113 melanomas, according to patients' socio-demographic, clinical and histological characteristics are reported in Table SI[1]. Approximately 50% of the melanomas were localised on the trunk, 45.1% on the extremities and 4.4% on the head/neck region. Of the tumours, 87.6% were SSMs, 6.2% NMs, and 6.2% LMMs. Almost 90% (89.4%) of all lesions had a Breslow's thickness < 0.90 mm and 10.6% > 0.91 mm.

Melanoma characteristics according to body site

As shown in Table I, melanomas in the male group were mostly located on the trunk, whilst the female group showed a greater presence of melanomas on the extremities (OR 3.39; 95% CI: 1.43–8.12). Melanoma on the extremities were almost exclusively SSMs (96%), whereas on the trunk other variants were not infrequent (NMs (7.0%) and LMMs (8.8%)). In our series, we did

Table I. *Demographic and clinico-pathological findings according to body sites. Crude odds ratio (OR) of developing melanoma on the trunk and extremity, respectively (*n = *108)*

	Extremity (n = 51) n (%)	Trunk (n = 57) n (%)	OR (95% CI)
Age at diagnosis (mean ± SD)	47.5 ± 13.8	50.9 ± 16.2	
Gender			
Female	33 (64.7)	20 (35.1)	–
Male	18 (35.3)	37 (64.9)	3.39[a] (1.43–8.12)
Histologic type			
SSM	49 (96.0)	48 (84.2)	
NM	2 (4.0)	4 (7.0)	
LMM	0 (–)	5 (8.8)	
Acute/intermittent sun exposure			
< 15 days/year	21 (41.2)	15 (26.8)	–
15–60 days/year	26 (51.0)	29 (51.8)	1.56 (0.62–3.99)
> 60 days/year	4 (7.8)	12 (21.4)	4.20 (0.98–19.4)
Chronic solar exposure			
No	46 (90.2)	34 (60.7)	–
Yes	5 (9.8)	22 (39.3)	5.95[a] (1.87–20.11)
Solar lentigo and actinic keratoses[b]			
No	17 (34.7)	12 (21.8)	–
Yes	32 (65.3)	43 (78.2)	1.90 (0.73–4.98)
Melanoma-associated melanocytic naevus[c]			
No	35 (71.4)	34 (61.8)	–
Yes	14 (28.6)	21 (38.2)	1.54 (0.63–3.83)
Melanoma-associated solar dermal elastosis[c]			
Mild	23 (46.0)	20 (37.1)	–
Moderate/Severe	27 (54.0)	34 (62.9)	1.45 (0.66–3.17)

[a]These effects remained significant in the final logistic regression model (Male gender: adjusted OR: 3.36 (95% confidence interval (CI): 1.31–8.65; Chronic solar exposure: adjusted OR: 5.97 (95% CI: 1.71–20.87).
[b]For these variables population number was different [b](n = 107) [c](n = 104).
SSM: superficial spreading melanoma; NM: nodular melanoma; LMM: lentigo maligna melanoma.

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not have a significant number of melanomas diagnosed on the head/neck region, typical site for sun-damaged melanomas. Melanomas with associated histological features of moderate/severe M/SDE$^+$ or M/MN$^+$ did not differ according to the body site of location. Considering the acute/intermittent sun exposure, the risk was 4-fold higher for patients exposed for more than 60 days/year (OR 4.20; 95% CI: 0.98–19.4) compared to those with less than 15 days/year exposure. Patients with chronic sun exposure displayed about a 6-fold higher risk of developing melanomas on the trunk (OR 5.95; 95% CI: 1.87–20.11). Patients with tumours on the trunk were more likely to have solar lentigo and actinic keratoses (78.2%), than those with melanoma on the extremities (65.3%), although the difference did not reach a significant level. After adjustment for potential confounders, the variables significantly associated to melanoma of the trunk were male gender (OR 3.36; 95% CI: 1.31–8.65) and chronic sun exposure (OR 5.97; 95% CI: 1.71–20.87). Associations with other variables have been reported in Table SII[1].

Melanoma characteristics according to the presence of associated melanocytic naevus remnant

As shown in Table II, 74 (67.98%), were M/MN$^-$, whereas 35 (32.1%) were M/MN$^+$. The prevalence of M/MN$^+$ did not vary according to gender or sun exposure habits, while the presence of an associated melanocytic naevus remnant was inversely associated to moderate/severe M/SDE$^+$ (OR 0.36; 95% CI:0.15–0.85). Multivariate analysis showed that only moderate/severe M/SDE$^+$ was significantly negatively associated to M/MN$^+$ (OR 0.36; 95% CI: 0.13–0.92). Patients with > 50 melanocytic naevi accounted for 34.3% of the M/MN$^+$ compared to 24.3% of the M/MN$^-$ group. Furthermore, Breslow's thickness ≥ 0.91 mm, as well as Clark's level ≥ 3, were more frequent in M/MN$^-$ than in M/MN$^+$ (13.5 vs 5.7% and 50 vs 37.1%, respectively) (data not shown).

Table II. *Demographic and clinico-pathological findings according to the presence/absence of melanoma-associated melanocytic naevus (M/MN). Crude odds ratio (OR) of developing melanoma-associated melanocytic naevus (*n = *109)*

	M/MN$^-$ (*n* = 74) *n* (%)	M/MN$^+$ (*n* = 35) *n* (%)	OR (95% CI)
Age at diagnosis (mean ± SD)	51.27 ± 15.77	48 ± 14.7	
Gender			
Female	36 (48.6)	18 (51.4)	–
Male	38 (51.4)	17 (48.6)	0.89 (0.40–2.01)
Acute/intermittent sun exposure			
< 15 days/year	22 (29.7)	13 (37.1)	–
15–60 days/year	39 (52.7)	17 (48.6)	0.76 (0.31–1.86)
> 60 days/year	13 (17.6)	5 (14.3)	0.88 (0.27–2.87)
Chronic solar exposure			
No	54 (73.9)	25 (71.4	–
Yes	19 (26.0)	10 (28.6)	1.13 (0.46–2.81)
Melanoma-associated solar dermal elastosis			
Mild	24 (32.4)	20 (57.1)	–
Moderate/Severe	50 (67.6)	15 (42.9)	0.36[a] (0.15–0.85)
Total melanocytic naevus count			
< 10	37 (50.0)	11 (31.4)	–
10–50	19 (25.7)	12 (34.3)	2.12 (0.78–5.82)
> 50	18 (24.3)	12 (34.3)	2.24 (0.81–6.20)

[a]This effect remained significant in the final logistic regression model (Moderate/severe solar dermal elastosis: adjusted OR: 0.36 (95% confidence intervals (CI): 0.13–0.92).

Melanoma characteristics according to the presence of solar dermal elastosis

Patients with melanoma were also stratified according to M/SDE severity (Table SIII[1]). As expected, M/SDE$^+$ was less evident in younger patients. In particular, moderate/severe M/SDE$^+$ was associated to male gender (OR 1.91; 95% CI: 0.88–4.15) and to other concurrent not neoplastic dermatoses (OR 2.99; 95% CI: 1.02–8.78), namely psoriasis and seborrhoeic dermatitis. Moreover, moderate/severe M/SDE$^+$ compared to mild M/SDE$^+$ series tended to have Breslow's thickness ≥ 0.91 mm (15.4 vs 4.6%; OR 3.82), Clark's level ≥ 3 (53.9 vs 34.1%; OR 2.26), a history of chronic sun exposure (30.8 vs 20.5%; OR 1.72), and numerous solar lentigo and actinic keratoses (76.9 vs 68.1%; OR 1.56). The results of multivariate logistic regression model confirmed that males are more likely to develop moderate/severe M/SDE$^+$ (adjusted OR = 1.91; 95% CI: 0.88–4.15).

GST-π expression

As shown in Fig. 1, GST-π detection in melanoma cells was both cytoplasmic and nuclear, in agreement with the literature (8–10). Semiquantitative evaluation (Fig. 2) revealed differences in GST-π nuclear staining (ANOVA, $p < 0.0002$). In particular, GST-π nuclear staining of M/MN$^-$-M/SDE$^+$ was significantly lower than M/MN$^+$-M/SDE$^-$ ($p < 0.002$) and between M/MN$^+$-M/SDE$^+$ and M/MN$^-$/SDE$^+$ ($p < 0.0001$), whereas GST-π cytoplasmic staining did not differ. High statistical correlation between nuclear GST-π intensity and Breslow's thickness was documented via Spearman's rank test ($\rho = 0.60$, $p < 0.03$). Moreover, we documented an inverse correlation between nuclear GST-π intensity and moderate/severe solar dermal elastosis ($\rho = -0.61$, $p < 0.03$), but not with the presence or absence of melanocytic naevus remnant. No significant differences were observed comparing nuclear and cytoplasmic GST-π melanoma cells immunoreactivity in those groups stratified for body site and gender (data not shown). Immunohistochemical investigation also showed a diffuse and strong positive staining for S-100 protein, vimentin, HMB-45 and Melan-A in melanoma cells, with no significant differences among different stratified subgroups (data not shown).

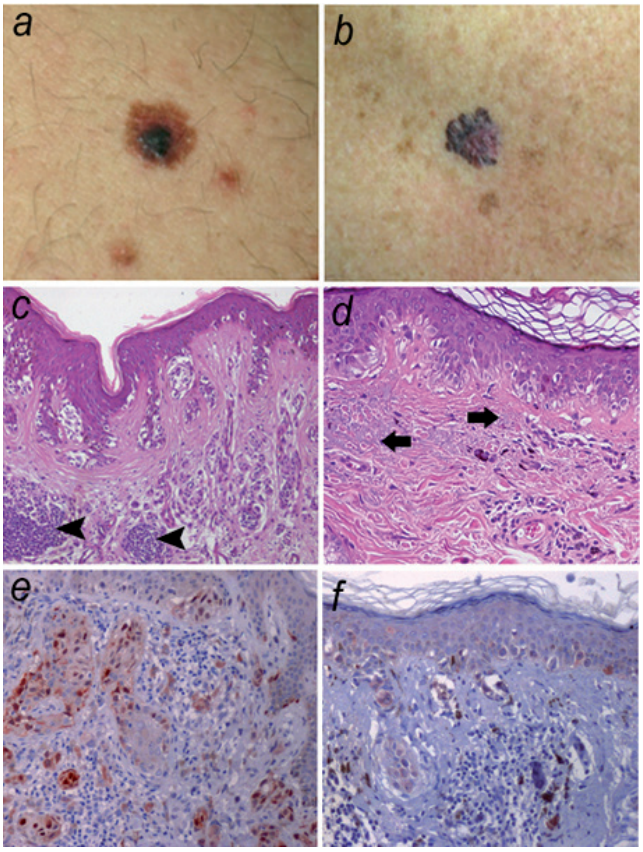

Fig. 1. Clinical images of (a) a melanocytic naevus-associated cutaneous melanoma (M/MN^+) and (b) cutaneous melanoma without naevus (M/MN^-) in chronically sun-damaged skin. Microscopic examination shows (c) a superficial spreading melanoma associated to intradermal naevus remnants (black arrowheads) with no solar dermal elastosis ($M/MN^+/SDE^-$) and (d) a superficial spreading melanoma with sun exposure-induced severe solar dermal elastosis (black arrows) ($M/MN^-/SDE^+$). Immunohistochemistry shows (e) diffuse cytoplasmic and nuclear GST-π expression in $M/MN^+/SDE^-$, whereas (f) in $M/MN^-/SDE^+$ nuclear GST-π staining is absent. [b, c: haematoxylin-eosin stain, d, e: 3-Amino-9-ethylcarbazole as chromogen, original magnification: ×200].

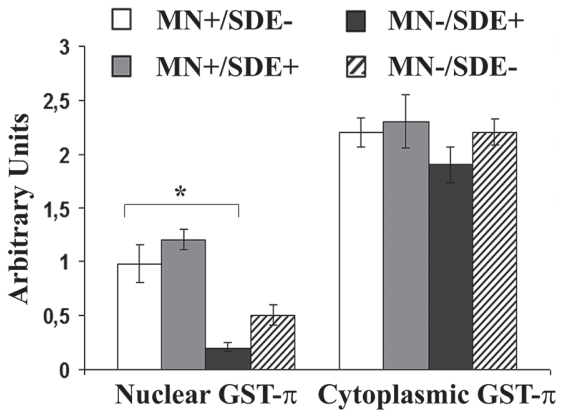

Fig. 2. Nuclear and cytoplasmic GST-π immunostaining in naevus–associated melanoma with no solar dermal elastosis ($M/MN^+/SDE^-$) (n=20), naevus–associated melanoma with moderate/severe solar dermal elastosis ($M/MN^+/SDE^+$) (n=15), melanoma without naevus remnants with moderate/severe solar dermal elastosis ($M/MN^-/SDE^+$) (n=50), melanoma without naevus remnants without moderate/severe solar dermal elastosis ($M/MN^-/SDE^-$) (n=24). Significant differences exist comparing nuclear GST-π expression of the 4 groups (ANOVA, $p<0.0002$), in particular between $M/MN^+/SDE^-$ and $M/MN^-/SDE^+$ (*$p<0.002$) and between $M/MN^+/SDE^+$ and $M/MN^-/SDE^+$ (**$p<0.0001$)) In contrast, GST-π cytoplasmic staining did not vary significantly.

DISCUSSION

GSTs play a role in the protection against the development of neoplasms (7). In humans, GST-π isoenzyme is widely distributed in normal and neoplastic tissues. In normal epidermis, GST-π immunoreactivity is stronger in the basal layer than in the superficial layers (8). Benign melanocytic naevi show a variable GST-π cytoplasmic staining, whereas malignant melanocytes express either cytoplasmic or nuclear GST-π (12). In order to define further phenotypic differences among M/MN^+, M/MN^- or moderate/severe M/SDE^+, we investigated the expression and the intracellular distribution of GST-π isoform. We documented that nuclear GST-π was very low or absent in M/MN^- whereas cytoplasmic GST-π did not differ. The observation that nuclear GST-π expression in M/MN^+ was similar regardless of the SDE status, could support the hypothesis that the naevus-dependent melanoma pathway might influence GST-π expression, exerting a protective role against its down-regulation as observed in sun exposure-associated melanoma cells. In fact, the level of nuclear GST-π expression correlated inversely with moderate/severe SDE and positively with Breslow's thickness, suggesting an adverse biological effect of nuclear GST-π expression in the progression of melanoma. A main limitation of our study is the small sample size, which limits the power to detect differences. Although further studies are needed, evidence of a different nuclear GST-π expression in M/MN^+ and M/MN^-, in accordance with GST-π prognostic significance in other neoplasms, could suggest a more aggressive behaviour in M/MN^+ (16). We previously reported that in animal-type melanoma, nuclear GST-π expression was markedly reduced compared to other variants of the same thickness (12). In invasive cervical carcinoma, a strong expression of GST-π is reported in the cytoplasm of malignant cells and, in a few cases, also in the nucleus (16). In ovarian carcinomas, low or absent GST-π expression is significantly associated to a patient's progression-free time and longer overall survival (17). Several studies have shown that GST can operate in synergy with the multidrug resistance proteins to confer resistance to several anticancer drugs (18, 19) – e.g. for melanoma cells with vincristine (20) – but also for head and neck tumours cells with cisplatin (21) and for glioblastoma cells with nitrosurea (22). GST-π expression potentiates cisplatin resistance by enhancing the rate of adducts between glutathione and platinum (23). The evidence of somatic genetic mutations in M/MN^+ also suggests a possible genetically determined aberrant intracellular distribution of GST-π.

It is likely that glutathione is crucial for antioxidant defence and that its rapid extrusion prevents c-Myc-induced apoptosis in melanoma cells. This assumption could lead to new therapeutic opportunities (24, 25). The nuclear localisation of GST-π may have a major functional impact by protecting the tumour cell from DNA damage and reducing apoptotic cell death induced by external stress and/or therapy. In a rat model of liver carcinogenesis, the disruption of tumour suppressor functions of Smad-dependent signalling is observed in nuclear GST-π positive proliferative lesions, thus suggesting a close link between aberration in the Smad-dependent signalling and GST-π (26).

In our study, the most prevalent melanoma subtype was thin SSM developed on the trunk of chronically sun-exposed males. The multivariate logistic regression analysis demonstrated that male gender and a chronic sun exposure were independent risk factors significantly associated to melanomas localised on the trunk. Conversely, tumours featuring as M/MN^+ were negatively associated to moderate-severe M/SDE^+. These findings are in accordance with the evidence that melanomas close to melanocytic naevi could have different risk factors compared to those with signs of solar damage (27, 28). However, Lee et al. (27) reported that melanomas on the head/neck region are linked to chronic patterns of sun exposure, whereas those of the trunk are associated to an intermittent pattern of sun exposure, supporting different pathogenic pathways and different prognosis (29).

Altogether, our experience made us hypothesise that a reduced nuclear GST-π expression, significantly lower in sun-damaged melanoma than in naevus-associated melanoma, could be a possible adjuvant marker of a chronically sun-damaged melanoma pathway.

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