

# Co-regulated expression of matrix metalloproteinase-2 and transforming growth factor- $\beta$ in melanoma development and progression

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**Abstract.** Previous studies have found that matrix metalloproteinase-2 (MMP-2) and transforming growth factor- $\beta$  (TGF- $\beta$ ) can be considered as biomarkers and indices of disease progression in several human cancers. In this study, we investigated the plasma levels of MMP-2 and TGF- $\beta$  and their correlation in 49 primary cutaneous melanoma and 10 metastatic melanoma. Plasma MMP-2 and TGF- $\beta$  levels in patients with primary melanoma were significantly higher than those of healthy controls. These protein levels were significantly higher in patients with metastatic melanoma. A positive correlation between plasma levels of MMP-2 and TGF- $\beta$  in melanoma patients supports the hypothesis that TGF- $\beta$  triggers the release of MMP-2. The immunohistochemistry analysis shows that MMP-2 and TGF- $\beta$  were highly expressed in tumor tissues as well as in matched plasma samples. This finding suggests that these proteins are released from tumor cells. Overall, our data indicate that MMP-2 and TGF- $\beta$  may represent novel diagnostic markers and therapeutic targets in melanoma and the determination of their concentration could be a useful diagnostic and prognostic indicator. TGF- $\beta$ , leading the tissue invasion mediated by MMP-2, is a strong promoter of tumor progression. Therefore, reducing or blocking the activity of TGF- $\beta$  may represent a promising target in therapeutic strategies for limiting the growth of melanoma.

## Introduction

Melanoma is one of the most aggressive human malignancies and its incidence is steadily increasing throughout the world. In melanoma, prognostic markers are needed that would help to refine the risk of progression, assess the clinical outcome

and select optimal treatment strategies (1-3). In normal epithelial cells, transforming growth factor- $\beta$  (TGF- $\beta$ ) acts as a strong inhibitor of cell growth (4).

During tumorigenesis, transformed epithelial cells frequently escape from TGF- $\beta$ -induced growth control; and once this has occurred, TGF- $\beta$  can act as a promoter of tumor progression (5,6). TGF- $\beta$  may then affect the plasticity and adhesion of tumor cells and induce tumor cell migration (7,8).

In melanoma, high TGF- $\beta$  expression is correlated with a more aggressive phenotype and increased local infiltration, suggesting that TGF- $\beta$  may also stimulate the invasion of tumor cells to promote tumor progression (9-11).

TGF- $\beta$  up-regulates the matrix metalloproteinase-2 (MMP-2), acting as a strong autocrine mediator of tumor cell invasion and degrading proteinases (12-14). After the components of the extracellular matrix (ECM) are degraded, tumor cells invade the surrounding tissue and the vascular or lymphatic vessels to form metastatic colonies at distant sites. Increased expression of matrix metalloproteinases (MMPs) renders the tumor cells capable of digesting essential tissue barriers especially basement membranes lining the blood vessels, thereby promoting cell motility (15,16).

Expression of MMP-2 protein was found to be correlated with decreased architectural organization, increased atypia and haematogenous metastasis (17-19). High expression and activation levels of TGF- $\beta$  and MMP-2 can be considered as biomarkers and indices of disease progression in several types of human cancers (20-23). Although, it has been demonstrated that TGF- $\beta$  and MMP-2 are expressed in melanoma (10,11,24,25), no evidence has been shown on their correlation with clinic pathologic features in melanoma patients.

We analyzed the circulating plasma levels and immunohistochemistry (IHC) expression of TGF- $\beta$  and MMP-2 in human melanoma patients, with tumor characteristic compared with controls.

## Patients and methods

**Plasma and tissues.** Plasma obtained from 59 patients with cutaneous malignant melanoma at different stages of disease and healthy controls, matched in age and sex, were provided by

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Table I. Socio-demographic and clinical characteristics of patients with melanoma and control subjects.

	Cases of melanoma		Controls		$\chi^2$
	n	(%)	n	(%)	
Sex					
Male	36	(61)	14	(40)	p=0.05
Female	23	(39)	21	(60)	
Age (years)					
$\leq 55$	25	(42.4)	22	(62.8)	p=0.05
$> 55$	34	(57.6)	13	(37.2)	
Type of cancer					
Primary cutaneous melanoma	49	(83)			
Metastatic melanoma	10	(17)			
Type of primary melanoma					
Superficial spreading	23	(46.9)			
Nodular	26	(53.1)			
Breslow thickness					
$\leq 2.0$	25	(51)			
$> 2.0$	24	(49)			
Clark's level					
I-II	30	(61.2)			
III-IV	19	(38.8)			
Ulceration of primary melanoma					
Absent	27	(55.1)			
Presente	22	(44.9)			
Not assessable	0				
Sentinel lymph node					
Negative	26	(53)			
Positive	23	(47)			

the Section of Plastic and Reconstructive Surgery, University of Catania, and collected at the Department of Biomedical Sciences, University of Catania. Healthy controls were recruited from among the institute personnel and all were in excellent health at the time of the study. Clinical staging of patients were performed on a pathological basis according to the new American Joint Committee on Cancer 2001 classification system (26).

Tumor biopsy specimens were analyzed from all 59 melanoma patients. Among these, 49 patients showed primary melanoma, at different stages (ranging from 0.1 to 15.9 mm in thickness, Clark's level II-V); 10 melanoma patients showed metastatic disease with predominantly lymph node involvement. As control groups, 35 healthy controls and 15 benign melanocytic nevi (BMN) tissue samples were used. Each patient gave informed consent for the use of biological material for research purposes.

The socio-demographic characteristics of cases and controls and the distribution of cases of melanoma, according to clinical features at diagnosis, are shown in Table I.

**Measurement of MMP-2 and TGF- $\beta$  proteins.** Circulating MMP-2 and TGF- $\beta$  levels were quantitatively analyzed by solid-phase enzyme linked immunosorbent assay (ELISA),

using specific commercial kits (R&D Systems Europe Ltd., Abingdon, UK). Were prepared 7 scale dilutions of the standard solution included in the kit. Each sample was tested in duplicate. Spectrophotometer reading at 450 nm was performed by EIA reader (Opsys, MR, ThermoLab System, Franklin, MA, USA) and, considering the optical density, it was possible to detect the concentration of the related proteins in question. The detection limit of MMP-2 and TGF- $\beta$  is 0.047 and 4.61 pg/ml, respectively.

**Immunohistochemical staining.** After fixation in 10% buffered formaldehyde, the processing of sample and immunohistochemical (IHC) procedures were done according to the instructions of the manufacturer. Expression of MMP-2 and TGF- $\beta$  was assessed using a standard streptavidin-biotinylated peroxidase (HRP) complex method on an automated platform. Mouse monoclonal anti-MMP-2 (R&D Systems) and mouse monoclonal anti-TGF- $\beta$  antibody (Santa Cruz Biotechnology, Inc.) were used for detected the immunohistochemical expression on serial sections of melanoma.

**Statistical analysis.** Statistical analysis was performed using SPSS software, version 12.0. The differences in plasma levels of MMP-2 and TGF- $\beta$  were analyzed using the Wilcoxon's

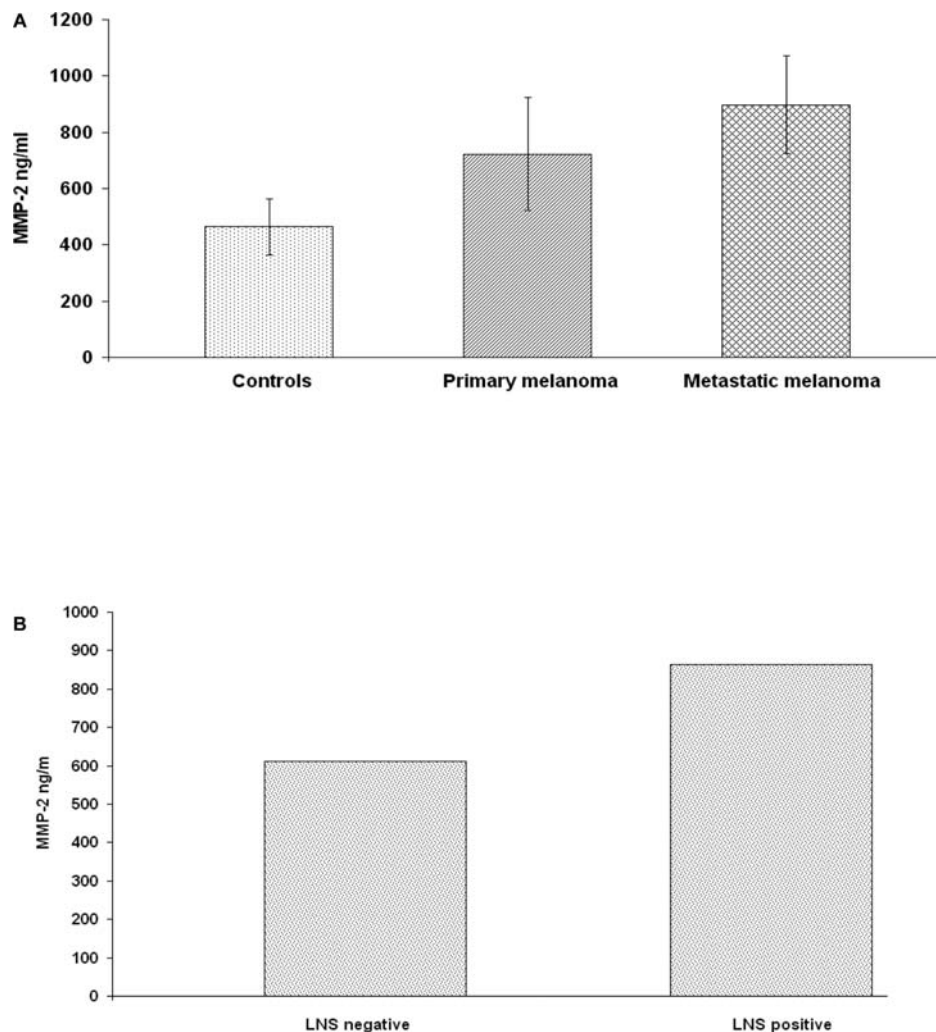


Figure 1. MMP-2 plasma levels in melanoma patients. (A) MMP-2 plasma levels in primary and metastatic melanoma patients; (B) MMP-2 plasma levels in melanoma patients with negative and positive sentinel lymph-nodes.

signed ranks test. Comparison of socio-demographic and clinical characteristics between healthy controls and melanoma patients was performed with the  $\chi^2$  test. Correlation between MMP-2 and TGF- $\beta$  was calculated with two-sided Spearman rank correlation test.  $P < 0.05$  was regarded as significant.

## Results

**Plasma levels of MMP-2.** The plasma levels of MMP-2 in primary and metastatic melanoma and in healthy controls are shown in the Fig. 1. Patients with metastases had a higher level of MMP-2 than patients without metastasis (mean, 898 vs. 722 ng/ml;  $p = 0.008$ ) and healthy controls (mean, 898 vs. 464 ng/ml;  $p < 0.0001$ ) (Fig. 1A). In primary melanoma these levels remain statistically higher than in healthy controls ( $p < 0.0001$ ). Moreover, among melanoma patients at different Clark levels, there was no statistically significant difference of MMP-2 plasma levels ( $p = 0.5$ ). Increased levels of MMP-2 were observed according to Breslow thickness, although no statistic significance was observed ( $p = 0.09$ ). In contrast, significant difference was found in patients with positive vs. negative sentinel lymph node melanoma ( $p = 0.009$ ) (Fig. 1B).

**Plasma levels of TGF- $\beta$ .** The plasma levels of TGF- $\beta$  in primary melanoma, metastatic melanoma and in TGF- $\beta$  healthy controls are shown in Fig. 2. A statistically significant difference was noted between the patients with primary melanoma and healthy controls. Higher plasma TGF- $\beta$  levels was found in melanoma patients than in controls (mean, 301 vs. 195.7 pg/ml;  $p < 0.0001$ ). Patients with metastatic melanoma showed a higher level of TGF- $\beta$ , statistically significant, compared with patients with primary melanoma (mean, 374 vs. 301 pg/ml;  $p = 0.03$ ) and healthy controls (mean, 374 vs. 195.7 pg/ml;  $p < 0.0001$ ) (Fig. 2A). In addition, median TGF- $\beta$  plasma determinations showed significant difference among patients with positive vs. negative sentinel lymph nodes melanoma ( $p < 0.0001$ ) (Fig. 2B), at different Clark stages ( $p < 0.005$ ) (Fig. 2C) and at high Breslow thickness ( $> 2$ ) vs. low Breslow thickness ( $< 2$ ) ( $p < 0.01$ ) (Fig. 2D).

**Correlation between plasma MMP-2 and TGF- $\beta$  levels and the clinicopathological characteristics of patients with melanoma.** Table II shows a positive correlation between plasma MMP-2 and TGF- $\beta$  levels in melanoma patients. As expected, this correlation is very high in the group of patients with primary

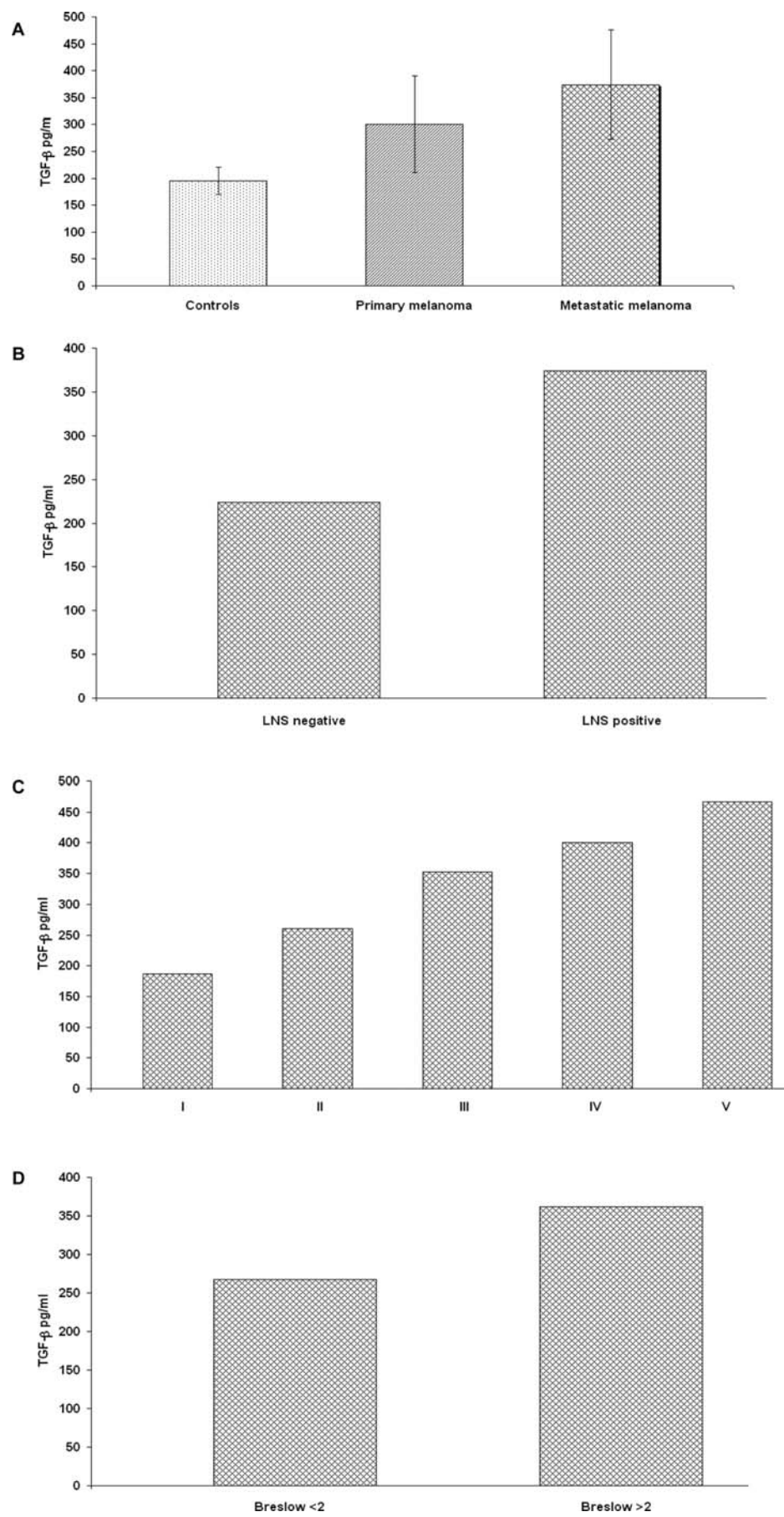


Figure 2. TGF- $\beta$  plasma levels in melanoma patients. (A) TGF- $\beta$  plasma levels in primary and metastatic melanoma patients; (B) TGF- $\beta$  plasma levels in melanoma patients with negative and positive sentinel lymph nodes; (C) TGF- $\beta$  plasma levels in melanoma patients with different Clark's levels; (D) TGF- $\beta$  plasma levels in melanoma patients with different Breslow thickness.

Table II. Correlation between plasma MMP-2 and TGF- $\beta$  level in melanoma patients.

	Primary melanoma		Metastatic melanoma	
	MMP-2	TGF- $\beta$	MMP-2	TGF- $\beta$
	r	r	r	r
MMP-2	-	0.54 (p<0.001)	-	0.61 (p<0.001)

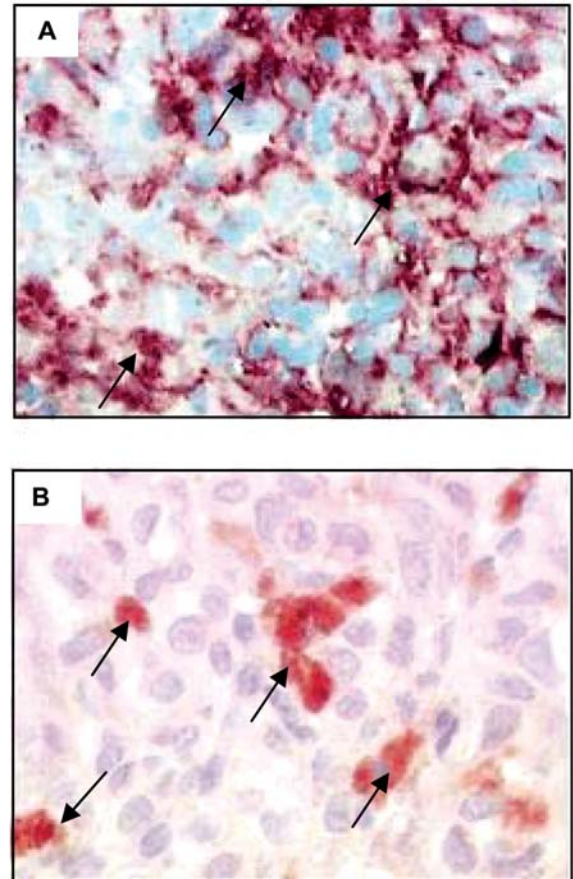
A.

Clinical characteristics	MMP-2 <700	MMP-2 ≥700	P-value
Sex			
Male	16	15	0.48
Female	8	10	
Age (years)			
≤57	10	16	0.11
>57	4	9	
Primary cutaneous melanoma			
Positive sentinel lymph node	6	17	0.003
Negative sentinel lymph node	18	8	
Clark's level			
I-II	17	13	0.1
III-IV	7	12	
Breslow thickness (mm)			
≤2.00	16	17	0.6
>2.00	9	7	
Ulceration of primary melanoma			
Absent	11	13	0.9
Present	11	14	
Not assessable	0	0	

B.

Clinical characteristics	TGF- $\beta$ <286	TGF- $\beta$ ≥286	P-value
Sex			
Male	15	16	0.9
Female	9	9	
Age (years)			
≤57	13	13	0.87
>57	11	12	
Primary cutaneous melanoma			
Positive sentinel lymph node	4	19	0.0001
Negative sentinel lymph node	20	6	
Clark's level			
I-II	21	9	0.001
III-IV	4	15	
Breslow thickness (mm)			
≤2.00	15	10	0.06
>2.00	8	16	
Ulceration of primary melanoma			
Absent	13	10	0.1
Present	9	17	
Not assessable	0	0	

MMP-2, matrix metalloproteinase-2; TGF- $\beta$ , transforming growth factor. Spearman rank correlation test. P<0.05 was regarded as significant.

Figure 3. Immunohistochemistry evaluation of MMP-2 and TGF- $\beta$  in a representative cutaneous melanoma tissue sample.

melanoma (r=0.53, p<0.001) and tends to be much higher in the metastatic group (r=0.61, p<0.0001). No correlation was found in the control group (data not shown).

We then compared plasma MMP-2 and TGF- $\beta$  levels among primary melanoma patients with different clinicopathological features. The potential association of plasma MMP-2 and TGF- $\beta$  levels with different clinicopathological features was examined in patients stratified according to MMP-2 (<700 and ≥700 ng/ml) and TGF- $\beta$  (<286 and ≥286 pg/ml) levels. Elevated MMP-2 (≥700 ng/ml; Table IIA) and TGF- $\beta$  (≥286 pg/ml; Table 2B) plasma concentrations were strongly associated with positive sentinel lymph nodes (p=0.003, p=0.0001; respectively). No association among sex, age, Breslow thickness, ulceration and MMP-2 and TGF- $\beta$  was found. However, only TGF- $\beta$  correlated with Clark's levels (p=0.001), in fact, we observed that TGF- $\beta$  concentration increased significantly with advanced Clark stages (stage II, p=0.015; stage III, p=0.008; stages IV-V, p=0.005; respectively). No association between MMP-2 and Clark's levels were found.

*Immunohistochemical staining of MMP-2 and TGF- $\beta$  in melanoma lesions.* To test whether the protein plasma levels of MMP-2 and TGF- $\beta$  derived from tumor cells, immunohistochemical staining was performed in cutaneous melanoma samples and in nevi. Overall, 38 of 49 primary tumors (77.5%) expressed MMP-2 and TGF- $\beta$  (Fig. 3). IHC analysis shows that both proteins were highly expressed in tumor tissues as

well as in matched plasma samples. The intensity of staining in metastatic melanoma cells was stronger than that in primary lesions (data not shown). As expected, MMP-2 and TGF- $\beta$  immunohistochemical staining was negative in nevi samples.

## Discussion

Recent studies strongly support the crucial role of TGF- $\beta$  in tumor progression through the regulation of MMP bioavailability. It was demonstrated that the release of TGF- $\beta$  in tumor microenvironment regulates pro-MMPs, triggering the secretion of these proteins, which intrinsically governs the behavior of various cells, leading to metastasis (12-14,21,27-30).

Many researchers have shown that TGF- $\beta$  induces the expression of MMPs (like MMP-2, MMP-9, MMP-10) in several human cancer cell lines, such as prostate (31), bladder (13), breast (32), endometrial (33), oral (29) and ovarian cancer (12). However, no previous study has investigated a possible correlation between serum levels of MMP-2 and TGF- $\beta$  in melanoma.

The results of the present study revealed that the plasma concentrations of both MMP-2 and TGF- $\beta$  were significantly higher in patients with melanoma than the concentration detected in healthy controls. In addition, patients with metastatic melanoma showed a higher level of MMP-2 and TGF- $\beta$ , compared with patients with primary melanoma and healthy controls, suggesting its potential usefulness as a marker of melanoma progression. These findings are in agreement with several studies, documenting an increased expression and secretion of the different TGF- $\beta$  isoforms in melanoma cell lines when compared with normal melanocytes (34,35). All these studies were inclined toward an increase in TGF- $\beta$  expression levels that correlates with tumor progression (9,11). The disruption of some components of the TGF- $\beta$  signaling pathway is associated with the evolution of melanoma (36).

The results of the present study are also in agreement with several reports that have implicated MMP-2 in the regulation of tumor cell migration and adhesion (19,20,37). Increased expression of MMP-2 was shown to correlate with an invasive phenotype (38,39), in addition, *in situ* studies of human melanocytic lesions demonstrated that MMP-2 expression is increased with architectural disorder, atypia and progression to melanoma (17,20,40).

The novelty of the present study is the documented concomitant increased plasma concentrations of TGF- $\beta$  and MMP-2 in melanoma patients. The combination of these proteins may be used as biomarkers to evaluate the aggressiveness of this pathological condition and may be helpful to monitor the efficacy of medical treatments.

Immunohistochemistry analysis was applied to discover the origin/source of both MMP-2 and TGF- $\beta$  melanoma samples. Our results show that these proteins are mostly released from tumor cells. These results are in agreement with previous data (9,41-43) and further support the possibility that both markers are co-regulated in melanoma cells. The release and the expression of these proteins suggests that these circulating factors arise from tumor and probably accumulate

as a result of an increased tumor burden in advanced stages of disease.

Based on the above, we believe that further studies focused on the reduction of MMP-2 expression by blocking the activity of TGF- $\beta$  may represent promising therapeutic strategy for an otherwise devastating disease.

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