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Osteopontin/Matrixmetalloproteinase-9 Pathway Activation is Associated with Melanoma Progression

THESIS

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Introduction

The incidence of cutaneous melanoma is increasing (1). Although several progresses have been made in terms of immuno and chemotherapy, the mortality rate for melanoma remain very high as it is very aggressive (2-5).

Current molecular information indicates that melanoma should be viewed as a heterogeneous group disorders with molecularly distinct defects in important cellular and molecular processes that include cell cycle regulation, cell signalling as adhesion molecules, angiogenetic factors and genetic alterations (6). The heterogeneity of these molecular alterations may impact on melanoma diagnosis, prognosis, and treatment. On these bases there is a need to identify novel marker that can be used for individual target therapy.

The invasion of tumor cells is a complex multistage process. To facilitate the cell motility, invading cells need to change the cell–cell adhesion properties, rearrange the extracellular matrix (ECM) environment and reorganize their cytoskeletons. Matrix metalloproteinases (MMPs) play a major role in the regulation of cancer cell migration, extracellular matrix (ECM) invasion, and metastasis by degrading the ECM proteins and enabling tumor cells both to escape from a tumor and to invade adjacent tissues (7-8). MMPs, growth factors, cytokines have been shown to regulate tumor cell invasion through autocrine or paracrine pathways. Previous study demonstrated the expression of MMP-1, MMP-2 and MMP-9 in human cancers (9-14). Of them, MMP-9 has been found to play a role in the ECM degradation associated with metastasis of cutaneous melanoma (15-17). The activation of MMP-9 in human cancer may be associated with the release of osteopontin (OPN), a secreted multifunctional phosphoprotein that has been implicated as an important mediator of tumor metastasis (18-20).

In detail, OPN expression has been linked to tumorigenesis and metastasis in a wide range of cancers including melanoma (12, 21-27). In melanoma cancer, previous studies, suggested a role for increased OPN tissue expression in the malignant transformation of melanocytes and

as an important determinant of tumor progression (28-31). Aggressiveness of malignant melanoma has been associated with high OPN expression and its over expression correlates with advanced tumor stages (30-31). OPN is a substrate for several extracellular proteases and is cleaved *in vitro* and *in vivo* by thrombin coagulation factor and MMPs. OPN contributes to malignancies through both inhibition of apoptosis and activation of various matrix-degrading proteases such as activation of MMP-2 and MMP-9 (18, 32-34). OPN up-regulates MMP activities modulating multiple signaling pathways via focal adhesion kinase (FAK), extracellular signal regulated kinase (ERK) and NF-kB (35) that regulate cytoskeletal organization, cell motility, cell growth, and the ability of the cell to escape from apoptosis. Recent data, obtained using *in vitro* melanoma cell lines, suggested the involvement of OPN in the activation of MMP-9, which ultimately controls cell migration, ECM invasion and tumor growth (32, 36-38).

In melanoma, both the RAS-RAF-MEK-ERK (MAPK) and the PI3K-AKT (AKT) signalling pathways are constitutively activated through multiple mechanisms. Mutations of the B-Raf gene have been proposed to contribute to melanoma development (39-41). Increased activity of the MAPK pathway prevents apoptosis and induces cell cycle progression. Disregulation of Ras/Raf/MEK/ERK pathway plays a key role in pathogenesis of several human cancers (42); mutations at upstream membrane receptors, NRAS and B-Raf as well as genes in other pathways (e.g., PIK3CA, PTEN, AKT), which serve to regulate Raf activity, promote constitutive ERK signalling, stimulating proliferation and survival and providing essential tumour growth and maintenance functions (43). Effects of PTEN deletion on PI3K/Akt and Raf/MEK/ERK activation in melanoma cancer are schematized in Figure 1.

Therapies targeting the mutant activity of components of the MAP kinase cascade could stop progression of malignant tumors by slowing tumor growth and inducing tumor cell death (44-45). The MAPK pathway plays an important role in melanoma cell proliferation and survival, with ERK being constitutively activated in up to 90% of melanomas (39, 46-47). In this

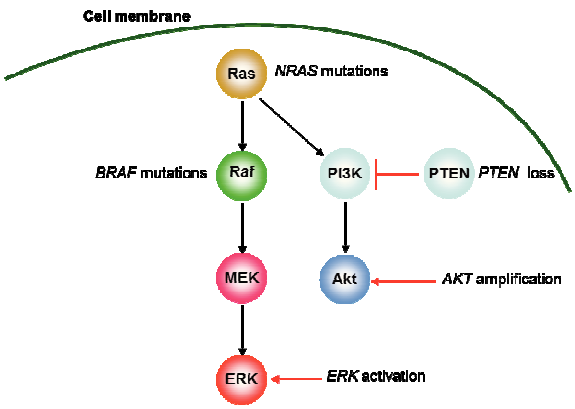


Figure 1. Raf-MEK-ERK and PI3K-Akt pathways

disease, ERK hyperphosphorylation is most commonly due to mutations of NRAS (15%-30%) and especially B-Raf (50%-70%) genes (41, 48-49). The aberration in the NRAS gene often is a substitution of leucine for glutamine at residue 61; this change impairs GTP hydrolysis and maintains the protein in constitutively-activated state (50). Mutations in other RAS isoforms are rare in melanoma, suggesting an activity context-dependent of specific RAS isoforms (51). The most frequent B-Raf mutation, which accounts for more than 90% of melanoma tumors with B-Raf activation, is a glutamic acid for valine substitution at codon 600 in exon 15, (Val600Glu; B-Raf^{V600E}) (42); this mutation introduces a conformational change in protein structure due to glutamic acid that acts as a phosphomimetic between the Thr598 and Ser601 phosphorylation sites, leading to constitutive activation of the protein with a large increase in the basal kinase activity (52); the resulting hyperactivity of the MAP kinase pathway promotes tumor development (42, 53-54). B-Raf^{V600E} mutant protein also promotes vascular development by stimulating autocrine vascular endothelial growth factor (VEGF) secretion (55). Interestingly, genetic alterations in NRAS and B-Raf rarely coexist in melanoma (42, 49, 56), suggesting that mutant B-Raf or NRAS proteins by themselves is sufficient to activate the MEK-ERK pathway.

On these bases, the aim of the present study was to investigate the clinical significance of OPN/MMP-9 pathways activation in melanoma, especially in the context of B-Raf^{V600E} mutation.

Materials and Methods

Patients and samples

A total of 148 melanoma patients with B-Raf^{V600E} and B-Raf^{WT} genes were included in the retrospective study. Blood from 119 patients with cutaneous melanoma at different stage of disease, obtained prior to surgery, were collected and stored at the Department of Bio-medical Sciences, University of Catania, Catania, Italy and was used for the analysis of protein concentrations of OPN and MMP-9. Of the remaining 29 melanoma samples, an additional set of peripheral blood mononuclear cells (PBMCs) was used for analyses of cell stimulation. PBMCs and plasma were obtained from a group of 53 healthy subjects (control patients) who served as a control group. B-Raf^{V600E} mutation status was assessed in our laboratory. The local scientific ethics committee approved all procedures. All participants provided written consent for blood collection. Blood was centrifuged (300 g for 10 min at 4^{o} C) and the separated plasma was placed in aliquots and stored at -80^{o} C until analysis.

Plasma assays of OPN and MMP-9. The OPN and MMP-9 plasma concentrations were measured using ELISA kits (R&D Systems, Europe). The MMP-9 assays recognized both pro and active forms. Plasma samples were diluted and the immunoassay was performed according to the instructions of the manufacturer. All assays were done in triplicate. The minimum detectable dose (MDD) of OPN and MMP-9 was less than 0.024 and 0,156 ng/mL, respectively. The optical density was measured at 450 nm using a microplate reader (Thermo-Lab Systems). Activities of MMP-9 were measured by specific Biotrak MMP-9 activity assay kits (Amersham Pharmacia Biotech UK Limited, Little Chalfont, UK) according to the manufacturer's instructions. Plasma were diluted 1:100 and 1:32 for the determination of MMP-9 activities, respectively. The appropriate standards were included in each assay. In order to measure the total content of the MMPs, activation of the pro form of the MMPs was performed using p-aminophenylmercuric acetate (APMA).

Immunohistochemistry and evaluation of immunostaining for OPN and MMP-9. OPN, and MMP-9 expression were detected by immunohistochemistry on serial sections obtained from melanoma specimens using specific antibody: mouse monoclonal anti-OPN antibody (Santa Cruz Biotechnology, Inc.) and rabbit polyclonal anti–MMP-9 antibody (Chemicon). The processing of samples and immunohistochemical procedures were done according to the instructions of the manufacturer. Immunolabelled and sampled tumor sections were examined using a Leica DMRB microscope (10× and 40× magnification) (Leica, Wetzlar, Germany), the images were photographed with a Canon G-9 camera (Canon, Japan) and analysed using Image J software. Four randomly chosen fields of view were assessed in the melanoma biopsies. A section was considered negative or positive according to the absence or presence of cytoplasmic staining. Three independent observers, blinded from the clinical data, analysed separately the immunostaining reactions for both OPN and MMP-9. Immunoreactivity was assessed by the amount of positive malignant cells. For both antibodies, the tumor was considered positive if more than 5% of malignant cells revealed a positive staining. We evaluated the immunohistochemical expression of the positive cells as follows: negative, no cells stained; low, 1–10% of cells stained; moderate, 15–30% of cells stained; and high, 40% or more cells stained.

Gelatin zymography. Since the proteolytic activity of metalloproteinases is crucial for their biologic effect in tumors we used gelatinolytic zymography to determine the activity of MMP-9 in plasma and in PBMCs from controls and melanoma patients. This technique distinguishes between active and latent forms of MMP-9. Gelatinolytic activity of MMP-9 in plasma was analyzed using gelatin zymography as described (57). Gel images were acquired with a Duoscan T1200 scanner (AGFA), and MMP-9 levels were quantified by densitometric analysis of the bands by the Image-Pro Plus 4.1 program and expressed as integrated absorbance.

Chemicals. DHMEQ, kindly provided by Prof. K. Umezawa (Keio University, Kanagawa, Japan), was synthesized as previously described (58). All chemicals were purchased from Sigma Aldrich Co (Milan, Italy). Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Chemicon; ELISA kits were R&D Systems Europe (Abingdon, Oxfordshire, UK) and Active Motif, (Rixensart, Belgium).

Isolation of peripheral blood mononuclear cell (PBMC) - NF-kB activity and MMP-9 production. PBMCs were obtained using a Ficoll gradient. After isolation, PBMC pellets were collected and stored immediately at −80°C until analysis. PBMCs (1 x 10^6 cells/mL), from melanoma patients and healthy controls, were cultured in RPMI 1640 medium supplemented with 1% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/mL penicillin and 100 µg/mL streptomycin (Gibco Life Technologies Inc.). MMP-9 and NF-kB was analysed in supernatants and nuclear extract, of PBMCs isolated from 29 melanoma patients and 53 control patients, respectively. PBMCs (following over- night incubation in serum-free medium) were stimulated with or without 100ng/ml of recombinant human OPN (R&D Systems, Minneapolis, MN) for 24 h (each condition was performed in triplicate), with or without pre-incubation (1h) with DHMEQ (10µg/ml). The concentration of MMP-9 was determined with human MMP-9 ELISA kit (R&D System) from short-term culture of isolated PBMCs.

For measurement of NF-kB activation, nuclear fractions were prepared from PBMCs (5–7x10^6 cells per extraction) during batch processing using a Nuclear Extract kit (Active Motif, Rixensart, Belgium). Binding of NF-kB to its consensus DNA sequence was measured by ELISA using Trans-AM™ NF-kB p65 Transcription Factor Assay Kit (Active Motif), according to the instructions of the manufacturer. Briefly, nuclear extract protein (5 µg/well) was incubated in 96-well plates coated with immobilized oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3') containing a consensus (5'-GGGACTTTCC-3') binding site for the p65subunit of NF-kB. NF-kB binding to the target oligonucleotide was

detected by incubation with primary Ab specific for the activated form of p65 (Active Motif), visualized by anti-IgG horseradish peroxidase –conjugated secondary antibody. At the end of incubation, the developing and stop solution were added, and an optical density of 450 nm (OD 450) was read on a Wallac Victor 1420 multilabel counter (Perkin Elmer Life Sciences, Shelton, CT).

Statistical analysis. The values of plasma OPN, MMP-9 and MMP9 activity were described as median, minimum and maximum of values. Differences in distribution of OPN, MMP-9, and MMP-9 activity between healthy controls and melanoma cases were tested through by non-parametric Wilcoxon Test or non-parametric $\chi 2$ tests (59). Correlation between markers were evaluated by means of Pearson's correlation scores (59). Odds ratios (OR) and the corresponding 95% confidence intervals (CI) were calculated using the multiple logistic regression models adjusted for sex and age (60). Two-tailed - *P*-values < 0.05 were considered to be significant.

Melanoma Cell Lines. A375 and A2058 melanoma cell lines were obtained recently from the ATTC (Rockville, Maryland, USA) and maintained in RPMI medium (Lonza Group Ltd, Basel, Switzerland), supplemented with 2 mmol/l L-glutamine, 100 IU penicillin and 100 µg/ml streptomycin and 10% heat-inactivated fetal calf serum (FBS) (Lonza). All cells were cultured at 37°C in 5% CO2 atmosphere at constant humidity. Each cell line ($1x10^6$ cells) were plated in ten Dishes (100 mm) (JETBIOFIL, Guangzhou Jet Bio-Filtration Products, Co., Ltd) containing 10 ml of RPMI with 1% of FBS. After 24h the supernatant from each dish was replaced with 10% FBS RPMI and 8 µM and 20 µM of PLX4032 (Selleck Chemicals, Catania, Italy) diluted in DMSO (VWR-Prolabo) for A375 and A2058, respectively. While control experiments were performed using DMSO alone. Cells were harvested for RNA after incubations for different time intervals and subjected to RT-PCR.

RT-PCR. Total cellular RNA was extracted from cultured cell lines with Pure link RNA mini Kit (Ambion, Life Technologies, USA) according to the manufacturer's instructions. Reverse

transcription was carried out using M-MLV reverse transcriptase (Invitrogen, California, USA determine source), 5X First Strand Buffer (Invitrogen) and random primers (Invitrogen). Semiquantitive PCR was carried out by applying standard conditions. Glucose-6-phosphate dehydrogenase (G6PD) mRNA expression was used for normalization. Semiquantitive PCR was carried out using DreamTaq DNA Polymerase (Thermo Fisher Scientific Inc., USA), 10X DreamTaq buffer (Thermo Scientific) and dNTP Mix (Thermo Scientific). The following primers were used: MMP-9 FW: GAACCAATCTCACCGACAGG; MMP9 RW: CCACAACTCGTCATCGTCG; G6PD FW: ACGTGATGCAGAACCACCTACTG; G6PD RW: ACGACGGCTGCAAAAGTGGCG.

RESULTS

Levels of OPN and MMP-9. The socio-demographic characteristics of cases and controls and then distribution melanoma cases according to clinical characteristics at diagnosis, is presented in Table 1. The majority of cases were nodular melanoma (52.9%), without ulceration (55.5%), and with negative sentinel lymphnode (65.6%). Breslow thickness was >4mm for 27 cases (22.7%) and there had been invasion of reticular dermis or subcutaneo us fat in 17.6% of cases.

Table 1. Distribution of 119 melanoma cases 53 control subjects according to sociodemografic

	Melanoma cases		**Controls**		χ^2
	n = 119	**(%)**	**n = 53**	**(%)**	
Sex					
Men	73	(61.3)	25	(47.2)	
Women	46	(38.7)	28	(52.8)	p=0.08
Age (years), median range	58 (29-76)		49 (25-63)		
<50	26	(21.9)	27	(50.9)	
50-59	42	(35.3)	18	(34.0)	
≥60	51	(42.9)	8	(15.1)	p<0.01
Hystological subtype					
Suderficially spreading	56	(47.1)			
Nodular	63	(52.9)			
Breslow thickness (mm)					
≤2.0	58	(48.7)			
2.1-4.0	34	(28.6)			
>4.0	27	(22.7)			
Clark's level					
2.Invasion to basal layer epidermis	51	(42.9)			
3.Invasion to papillary dermis	47	(39.5)			
4.Invasion to reticular dermis	11	(9.2)			
5.Invasion to subcutaneous fat	10	(8.4)			
Ulcerate lesion					
No	66	(55.5)			
Yes	49	(41.2)			
Not evaluated	4	(3.4)			
Sentinel lymph node					
Negative	78	(65.6)			
Positive	41	(34.5)			

The levels of OPN, MMP-9 and MMP-9 activity are shown in Figure 2. For all considered markers, the levels were considerably higher among melanoma cases than healthy controls, in particular for those who had a positive sentinel lymphnode and nodular histotype (Figure 3).

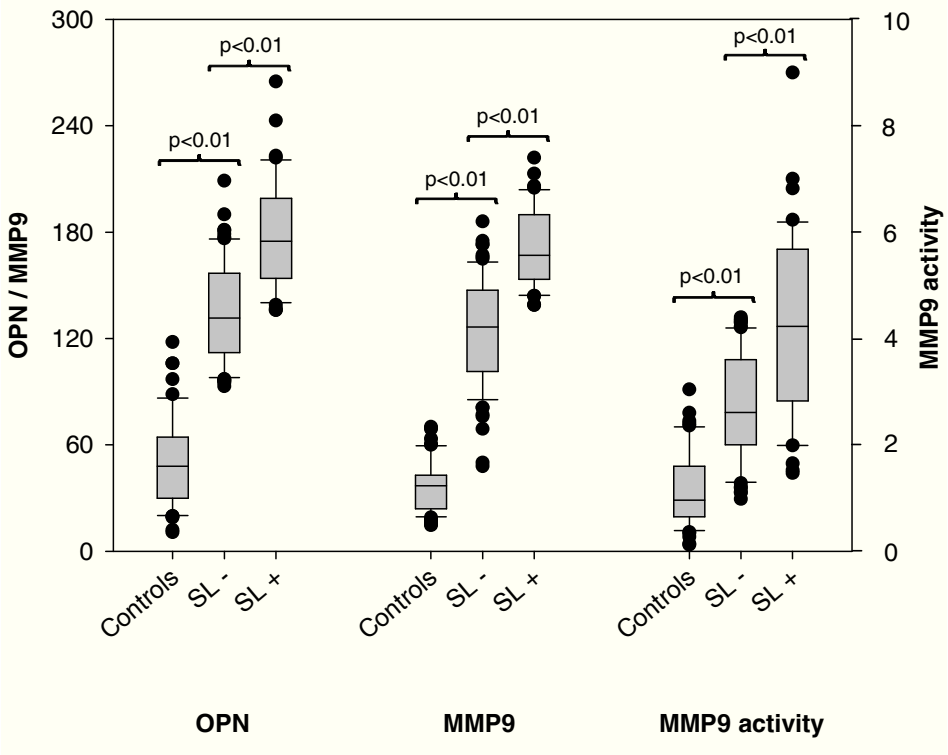


Figure 2. Distribution of OPN, MMP9, and MMP9 activity markers among 53 controls and 119 melanoma cases, according to sentinel lymph node (SL) status. P-values were calculated by means of Wilcoxon test.

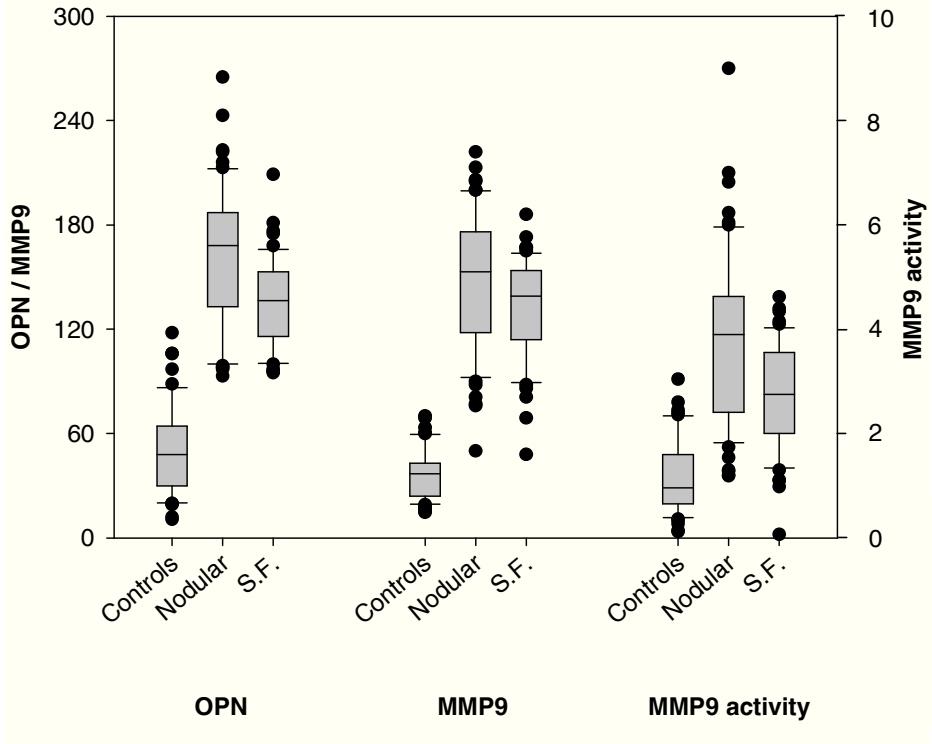


Figure 3. Distribution of OPN, MMP-9, and MMP-9 activity markers among 53 controls and 119 melanoma cases, according to histological type (nodular vs. superficial spread melanoma). S.F. = Superficially spreading melanoma.

Linear correlations between OPN, MMP-9 and MMP-9 activity were high (r>0.50) among both melanoma cases and healthy subjects (Figure 4).

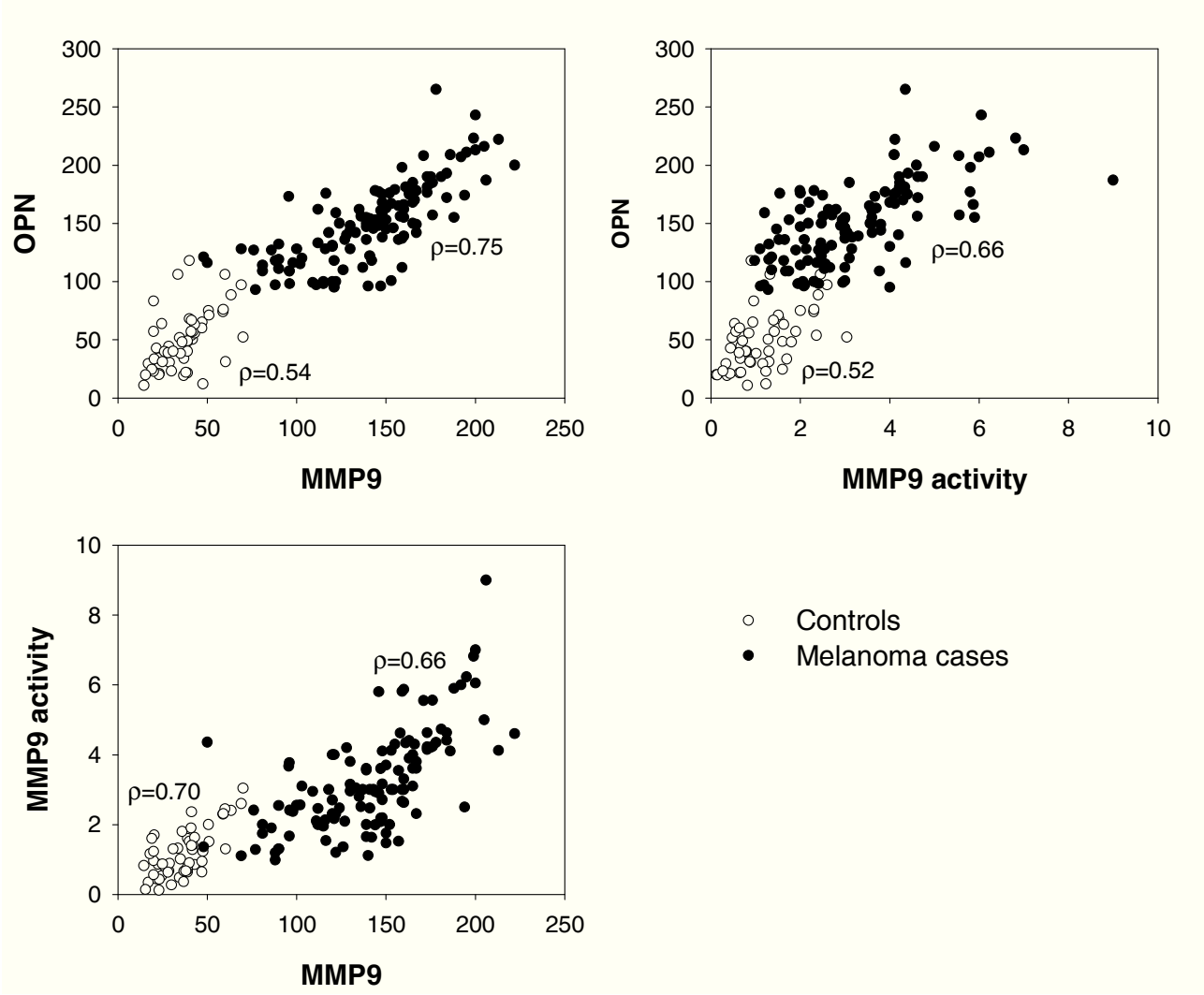


Figure 4. Correlations of expression of OPN, MMP9, and MMP9 activity among melanoma cases and controls. Linear correlations was evaluated through Pearson's correlation score (ρ).

The associations between high levels of OPN, MMP9, and MMP9 activity and clinical characteristics of the melanoma patients are reported in Table 2. OPN ≥150 ng/mL was directly associated with ulceration (OR=25.82; 95% CI: 6.54-102), tumor thickness ≥2mm (OR=21.78; 95% CI: 6.33-74.95), and, to a lesser extent, nodular type (OR=5.83; 95% CI: 2.05-16.58). MMP9 was strongly associated with positive sentinel lymphnode (OR=11.68; 95% CI: 3.49-39.08) and tumour thickness ≥ 2mm (OR=10.53; 95% CI: 2.95-37.57). MMP9 activity was not statistically associated with any of the considered clinical characteristics.

Table 2. Odds ratios (OR)[*] and corresponding 95% confidence intervals (CI) for nodular type, Breslow thickness >2mm, ulceration, and positive sentinel lymph node according to expression of OPN, MMP9, and MMP9 activity in 119 cases of melanoma.

	Hystological type[$]		**Breslow thickness (mm)**		**Ulceration**		**Sentinel lymphnode**	
	No/SS	OR (95% CI)	>2/≤2	OR (95% CI)	Yes/No	OR (95% CI)	Pos/Neg	OR (95% CI)
OPN								
<150	20/39	Reference	8/51	Reference	8/51	Reference	8/51	Reference
≥150	43/17	5.83 (2.05-16.58)	53/7	21.78 (6.33-74.95)	41/15	25.82 (6.54-102)	33/27	2.18 (0.70-6.81)
MMP-9								
<147	27/34	Reference	11/50	Reference	18/43	Reference	5/56	Reference
≥147	36/22	0.64 (0.22-1.88)	50/8	10.53 (2.95-37.57)	31/23	0.33 (0.08-1.40)	36/22	11.68 (3.49-39.08)
MMP-9 activity								
<3.05	28/36	Reference	18/46	Reference	17/46	Reference	13/51	Reference
≥3.05	35/20	1.25 (0.49-3.18)	43/12	2.27 (0.62-8.28)	32/20	2.21 (0.75-6.55)	28/27	0.55 (0.19-1.54)

[*]Estimates from multiple logistic regression models including terms for OPN, MMP9, and MMP9 activity. [$]No=Nodular type; SS=Superficially spreading type.

The levels of OPN, MMP9, and MMP9 activity was monitored in 18 melanoma cases who were followed-up actively (Table 3).

Table 3. Mean values and standard deviation (SD) of OPN, MMP-9, and MMP-9 activity in 53 controls and 18 melanoma cases at diagnosis and 3 months after surgery.

	n	Time of markes evaluation – Mean (SD)	
		Enrolment	3 months after surgery
OPN			
Controls	53	42.3 (25.3)	
Melanoma, SL Neg	4	189.0 (14.6)	34.3 (14.5)
Melanoma, SL Pos	14	208.1 (25.5)	45.6 (15.8)
MMP-9			
Controls	53	36.6 (14.2)	
Melanoma, SL Neg	4	162.6 (31.3)	30.0 (9.0)
Melanoma, SL Pos	14	186.6 (17.7)	31.4 (10.9)
MMP-9 activity			
Controls	53	1.18 (0.69)	
Melanoma, SL Neg	4	3.52 (1.32)	1.14 (0.48)
Melanoma, SL Pos	14	5.65 (1.33)	1.40 (0.57)

SL= Sentinel lymph node

At cancer diagnosis, the three markers were 3-to-6-fold higher than in healthy controls. However, three months after surgery, the levels of OPN, MMP9, and MMP9 activity was greatly reduced, falling down to the levels of healthy controls. Interestingly, melanoma cases with a positive sentinel lymph-node seemed to maintain an higher expression of OPN the negative ones (45.6 and 34.3, respectively). In addition, in three patients with melanoma in which metastasis were identified, the mean values of all markers increased (OPN from 235.6 to 361 ng/mL; MMP-9 from 192.3 to 275.3 ng/mL; MMP-9 activity from 5.72 to 12.05) ng/mL. The patients with metastasis were, at cancer diagnosis, all with positive sentinel lymph node melanoma (data not shown). However, the small sample since did not allow to draw final conclusions. However, the small sample since did not allow to draw final conclusions.

MMP-9 activity in melanoma patients. Zymography and densitometric quantitation of band intensity for MMP-9 revealed and confirmed what was shown by ELISA. Specifically, a significantly higher activity in patients with melanoma SL+ than in melanoma SL- and healthy subjects ($P < 0.0001$) was observed (Figure 5).

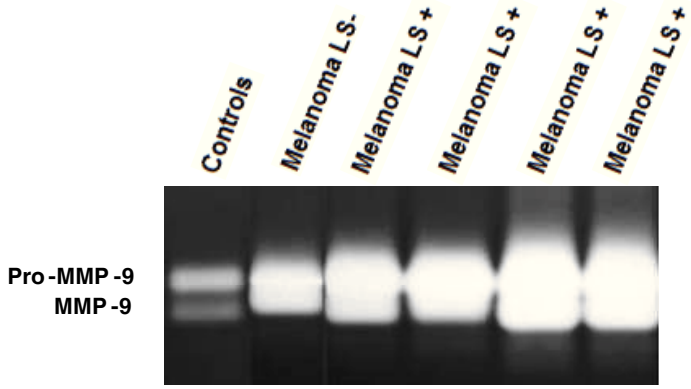
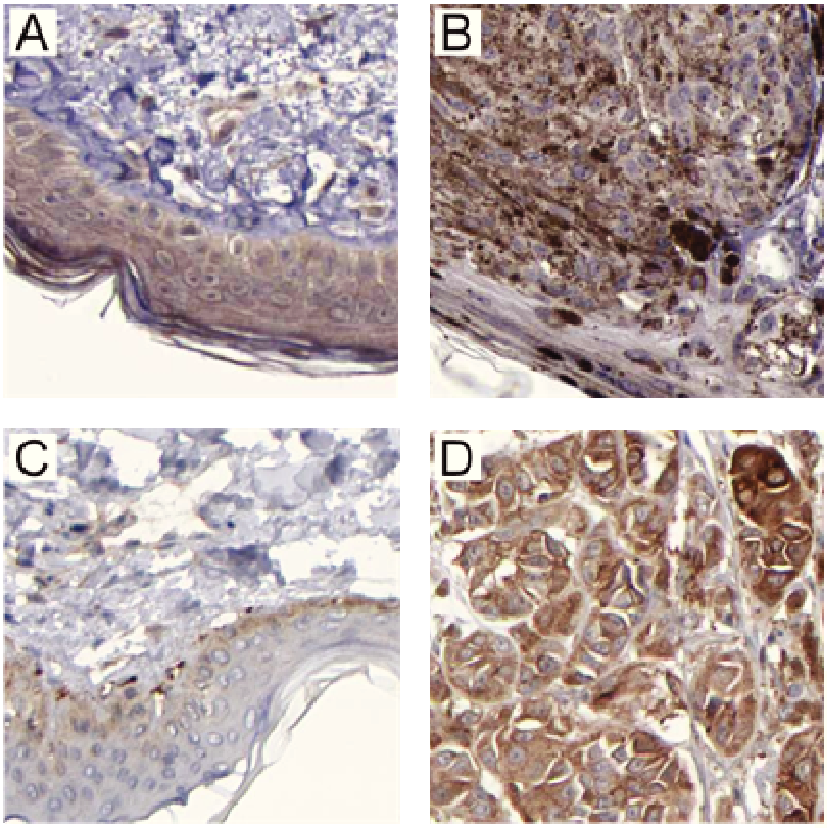


Figure 5. MMP-9 activity in plasma samples from melanoma patients. Profile of plasma gelatinases in zymography: control (lane 1), melanoma LS- (lane 2), melanoma LS + (lanes 3-6).

Expression of OPN and MMP-9 proteins in melanoma specimens. Immunohistochemical analysis was carried out to evaluate the expression and distribution of OPN and MMP-9 in the tumor specimens. Two examples of reactivity are presented in Figure 6. Very intense OPN immunostaining was observed in all tumor cells for all cases. MMP-9 immunostaining was observed in all tumor cells and in parts of the extracellular matrix for all cases. These results



clearly indicate that OPN and MMP-9 proteins, which are present at high plasma levels in patients with melanoma, may arise from cancer cells.

Figure 6. IHC of MMP-9 and OPN in normal skin and melanoma.

A: MMP-9 staining in normal skin; B: MMP-9 staining in melanoma; C: OPN staining in normal skin; D: OPN staining in melanoma.

Production of OPN, MMP-9 and NFkB activity from stimulated PBMCs. PBMCs from 53 healthy donors and 29 melanoma patients were cultured for 24 hours with and without OPN.

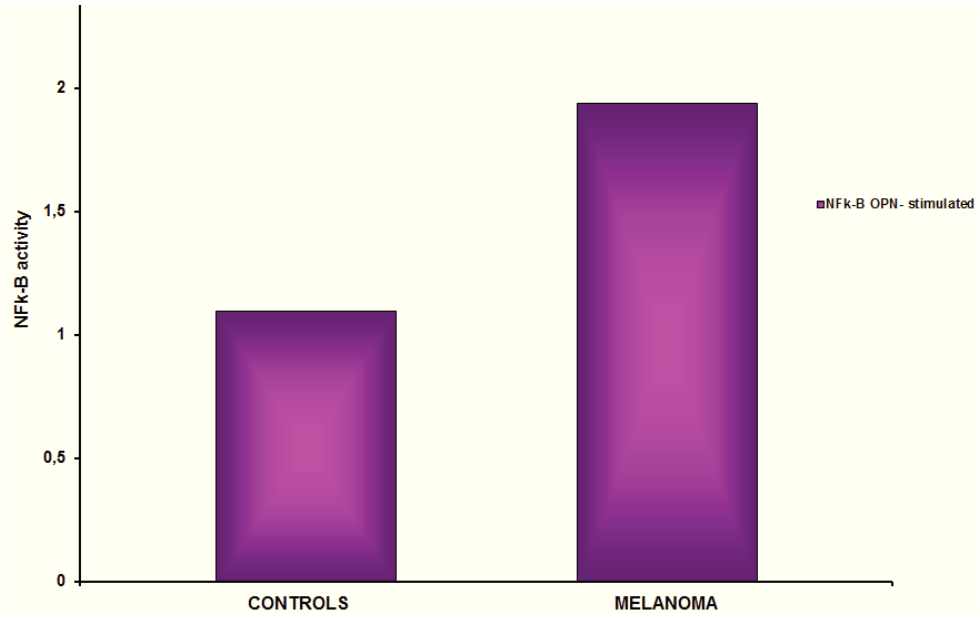


Figure 7. NFkB activity after treatment with OPN

According to the results obtained of OPN and MMP-9 in plasma from melanoma patients and healthy donors, similar data were obtained also in supernatants of

PBMCs (Data not shown). Similar trends were observed for the activity of NFkB after treatment with OPN (Figure 7). Figure 8 shows, in PBMCs from the two groups, the further production of MMP-9 after stimulation with OPN and its decrease after treatment with DHMQ, a NFk-B inhibitor (Figure 8).

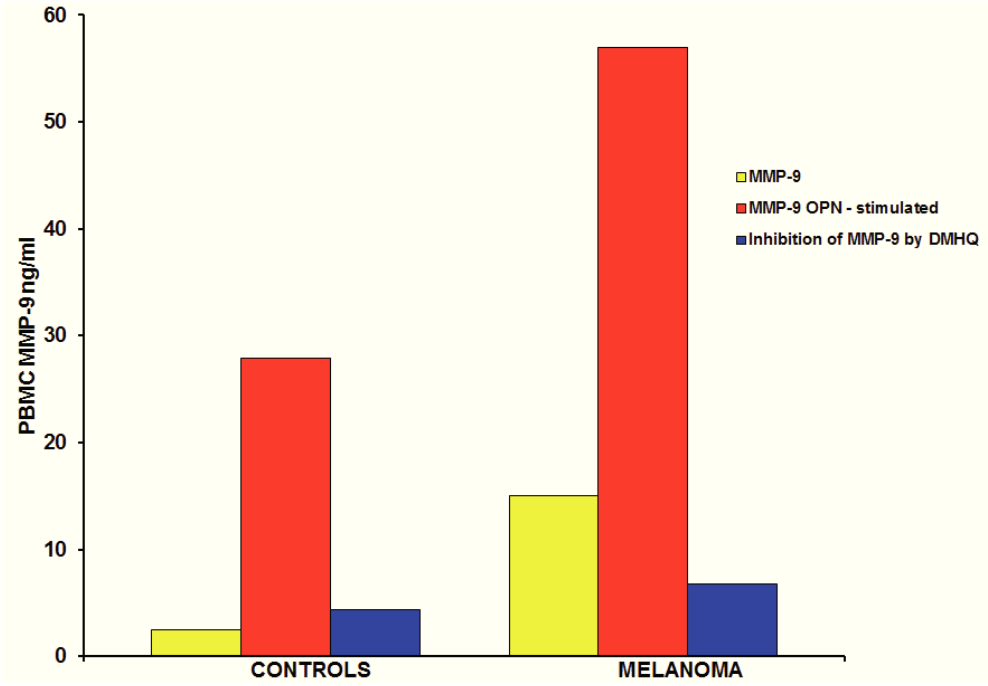


Figure 8. Production of MMP-9 after stimulation with OPN and its decrease after treatment with DHMQ

Expression of MMP-9 mRNA in melanoma cell lines treated with PLX4032. On the light that MMP-9 is expressed also in melanoma patients with the B-Raf^{V600E} mutation, we investigated whether MMP-9 may be an useful marker to monitor the response to treatment in patients that could benefit from B-Raf inhibition. Thus,

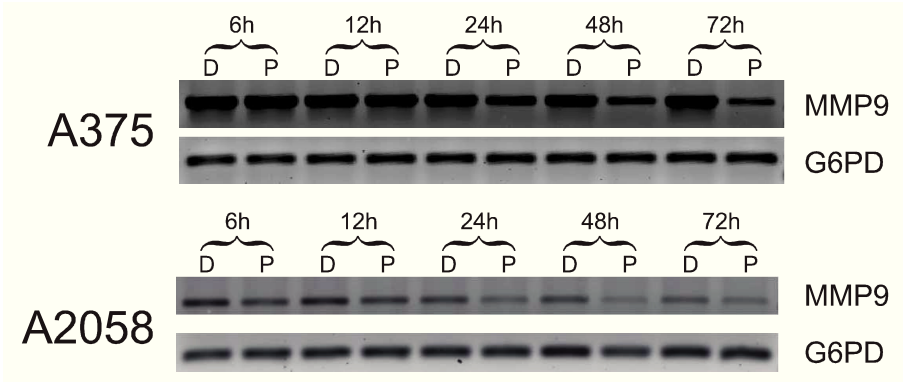


Figure 9. mRNA expression of MMP9 in melanoma cell lines treated with PLX4032
G6PD: glucose-6-phosphate dehydrogenase; **D:** DMSO; **P:** PLX4032;

we treated A375 and A2058 melanoma cell lines with PLX4032, a known B-Raf inhibitor. After 24 hours of treatment a decrease in MMP-9 mRNA transcripts levels were observed. This decrease was also observed at 48 and 72 hours (Figure 9). These findings suggest that MMP-9 may be a promising marker for the follow-up of melanoma patients treated with anti-B-Raf therapy. Clinical studies are needed to validate this hypothesis.

Discussion

Previous studies strongly support the crucial role of OPN in tumor progression through the regulation of highly integrated signaling events, including those critically dependent on MMP bioavailability (61). The results of the present study, including analysis of well-defined clinical material including more than 100 blood samples from melanoma patients, show a significant correlation between OPN and both levels and activity of MMP-9.

Several reports have implicated MMP-9 in the regulation of tumor cell migration and tumor-induced angiogenesis that result in the development and metastasis of several adenocarcinomas and the protein was detected immunohistochemically in tumor cells as well as in the stromal compartment (8, 62). Accordingly, our results indicate that MMP-9 is strongly associated with positive sentinel lymph-node and tumour thickness. Furthermore, a positive correlations between OPN and MMP9 was observed in both melanoma cases and healthy subjects.

Highest levels of OPN were directly associated with ulceration, tumour thickness and nodular type.

Intriguingly, in this study we the levels of OPN and MMP9 were monitored in 18 melanoma cases who were followed-up. These 18 cases, at the first diagnosis (time 0) expressed both markers 3-to-6 fold higher than healthy controls. Three months after surgery, those levels were significantly reduced. Furthermore, in three patients with melanoma in whom metastasis were identified after 3 months from the first surgery, the mean values of all markers increased. These data were also confirmed by functional experiments conducted on PBMCs from 53 healthy donors and 29 melanoma patients that were cultured for 24 hours with and without OPN. Stimulation with OPN in PBMCs increased the production of MMP-9, especially in melanoma patients. These data suggested that the OPN/MMP-9 pathway is associated with transformation and progression. Immunohistochemistry analysis was applied

to locate the origin/source of the OPN and MMP-9 expression in a fraction of melanoma samples. Our results are in agreement with previous data showing that OPN and MMP-9 are mostly confined with tumor cells. These data are also supported by our previous studies (12) raising the possibility that both markers are coregulated in tumor cells.

The mechanism by which OPN mediates MMP-9 activation in tumors and consequently increases cancer aggressiveness remains poorly understood; however, previous studies have indicated that OPN stimulates the secretion of urokinase-type plasminogen activator, which regulates pro–MMP-9 activation, cell motility, invasion, and tumor growth (37).

In this regard, the treatment with OPN in PBMCs from the small group of healthy donors and melanoma patients confirms the production of NFk-B activity in melanoma subjects. Additional experiments with PBMCs from the two groups revealed that MMP-9 levels decreased after treatment with OPN and then subsequently treated with DHMQ, a NFk-B inhibitor, suggesting a strong association between OPN and MMP-9 which is mediated by NFk-B.

Activation of OPN/MMP-9 pathway may vary between melanomas which are B-Raf^{V600E} and B-Raf^{WT}. If this is the case, such activation may be associated with clinical-pathologic features and/or with the response to therapy, but this have not been full investigated yet. In the present study, through the analysis of each patient's medical file, B-Raf^{V600E} mutation was detected in 38% melanoma patients and no differences of in both median OPN and MMP-9 plasma levels were observed between patients harbouring this mutations and the wild type group suggesting that B-Raf^{V600E} mutation does not induce any modification of the OPN/MMP-9 pathway. Therefore, on the light that MMP-9 is expressed also in B-Raf^{V600E} mutated melanoma patients, we investigated whether MMP-9 may be an useful marker to monitor the response to treatment in patients that can benefit from B-Raf inhibition by treating melanoma cell lines with PLX4032, a known B-Raf inhibitor.

After 24 hours of treatment, we observed a decrement of MMP-9 transcript levels, suggesting that MMP-9 may be a promising marker for the follow-up of melanoma patients treated with targeted B-Raf therapy.

Overall, the correlation between OPN and MMP-9 expression suggests the promise of the analysis of activation of the OPN/MMP-9 pathway as a marker for melanoma progression. Further clinical studies are needed to validate this hypothesis.

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