Received: 17 August 2023 | Revised: 16 November 2023 | Accepted: 21 November 2023

DOI: 10.1111/cts.13694

ARTICLE

Expression pattern of osteopontin isoforms in malignant melanoma cell lines

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Abstract

Osteopontin (OPN) is a secreted integrin-binding protein that plays a role in inflammation, cellular viability, cell adhesion and migration, cancer development, and diabetes through different mechanisms. The splice variants of *OPN* can play essential roles in cancer development, progression, and metastasis formation; however, limited data are available about the role of *OPN* isoforms in human malignant melanoma. Our goal was to define the gene expression patterns of five *OPN* variants (*OPN4*, *OPN5*, *OPNa*, *OPNb*, and *OPNc*), integrin, and *CD44* receptor genes in primary and metastatic melanoma-originated cell lines ($n = 19$), and to explore the association of the expression patterns with clinicopathological parameters. We evaluated the invasive property of the cell lines and investigated the potential association between the invasion and gene expression of *OPN* isoforms. We found a significant rise in the expression of *OPNc* in the invasive cell lines compared to the noninvasive cells and detected significantly higher expression of the *OPN* splice variants in melanoma cell lines originating from more advanced stages tumors than cell lines originating from early-stage melanomas. The correlation analysis revealed that all five *OPN* variants positively correlated with *ITGB3* and *ITGA9*, whereas *OPN5* positively correlated with *ITGB1*, *ITGAV*, *ITGA6*, and *CD44*. OPN can activate extracellular signal-regulated kinase signaling through binding to α9β1 integrin, promoting melanoma tumor cell migration. It is possible that such associations between *OPN* splice variants and integrin receptors may play a role in melanoma progression. In conclusion, our findings suggest that high expression of *OPNc* correlates with the invasive behavior of melanoma cells.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

The expression pattern and functional roles of the *OPN* splice variants have been demonstrated to have tissue and tumor-specific characteristics.

Viktoria Koroknai and István Szász contributed equally to this work.

WHAT QUESTION DID THIS STUDY ADDRESS?

In this study, we aimed to investigate the expression of *OPN* splice variants in melanoma cell lines, and to characterize the association with clinicopathological parameters and the invasiveness, as well as to examine the potential correlation with the relative gene expression of *CD44* and integrin receptor genes.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

In melanoma, the high expression of *OPNa*, *OPNb*, and *OPNc* appears to be associated with poor prognosis, whereas *OPNc* is specifically linked to the invasive behavior of melanoma cells.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

The therapeutic potential of OPN is still undergoing intensive research, and further investigations are required to elucidate the role of *OPN* and its variants, particularly *OPNc*.

INTRODUCTION

Osteopontin (OPN) plays an important role in regulating various physiological processes, including bone metabolism, immune response, wound healing, cell adhesion, cell migration, and inflammation.[1–3] Many studies have described that OPN is involved in the pathogenesis of various diseases, including different types of cancer, cardiovascular diseases, and autoimmune disorders.[4–6] It has been demonstrated that OPN has a role in promoting tumor growth and facilitating metastasis formation by promoting angiogenesis and modulating the immune response.[7,8] OPN is encoded by the *SPP1* gene that is localized on chromosome 4q22.1, and consists of seven exons, from which exon 1 is untranslated, and exons 2–7 contain the coding sequences.[3] OPN protein has two terminal zones including C-terminal, which binds heparin molecules, such as CD44, and N-terminal, which binds integrin receptors.[9] At the transcript level, at least five isoforms are generated: OPNa (full-length), OPNb (lacking exon 5), OPNc (lacking exon 4), OPN4 (lacking exons 4 and 5), and OPN5 (alternative N-terminus upstream of exon 4). Besides these variants, four additional isoforms have been described for OPN5 (OPN5b, OPN5c, OPN5d, and OPN5e).[10–12] Exons 6 and 7 encode over 80% of the OPN protein, which contains the region responsible for interacting with integrin receptors. Integrins are cell-surface transmembrane heterodimer receptors, which are formed through noncovalent binding of an α and a β subunits, of which there are 18 and eight variants, respectively.[13,14] Integrins mediate interaction with the extracellular matrix and play an important role in cell migration, tumor initiation, and metastasis.[14,15]

OPN splice variants play crucial roles in cancer progression, as several studies have linked specific associations of these variants with the development and progression of cancer, as well as metastasis formation.[10] However, limited information is available regarding *OPN* isoforms in malignant melanoma. In our previous study, we focused on the gene expression of *OPN* variants in melanoma tissue samples and described that relative gene expression of *OPNa*, *OPNb*, and *OPNc* is significantly higher in metastatic tumors compared to primary tissues and observed an association between *OPNc* expression and Breslow thickness.[16] Notably, *OPNc* also exhibited a significant positive correlation with the presence of metastasis.

It has been described that OPN receptors include αv (β1, β3, or β5) and (α4, α5, α8, or α9) β1-integrins, receptor CD44, and epidermal growth factor receptor.[3,7] Among the various integrins through which OPN and its splice variants can bind, αvβ3 is the most frequently observed.[9,17] However, limited information is available regarding the variants and their receptors in different types of cancers. In the case of esophageal adenocarcinomas, it has been reported that OPNb acts through integrin-dependent signaling, whereas OPNc has a different mechanism.[18] In our previous work on melanoma, we found a positive correlation among the gene expression of *OPN4*, *OPN5*, and several integrin receptors. Conversely, the other variants (*OPNa*, *OPNb*, and *OPNc*) negatively correlated with *ITGA2* which results suggest that downregulation of *ITGA2* may be linked with tumor progression in malignant melanoma, and integrin α2 might function as a metastasis suppressor.[16] The decreased expression of the α2 receptor subunit was described in association with more advanced status and the presence of metastasis in breast cancer.[19]

Melanomas harbor an activating mutation in the *BRAF* oncogene (40%–50%) that constitutively activates the mitogen-activated protein kinase pathway; however, targeted inhibition of $BRAF^{V600E}$ gene is one of the most promising therapeutic strategies for patients with melanoma.[20,21] *OPN* expression levels have been reported in association with $BRAF^{V600E}$ mutation, but no data are available about the possible relationship between *OPN* variants and *BRAF* mutation.[22–24]

In this study, our objectives were to characterize the relative gene expression levels of *OPN* isoforms in different melanoma cell lines derived from primary and metastatic melanomas. We aimed to investigate the association with clinicopathological parameters and the invasive characteristics of the developed melanoma cell lines, as well as examine the potential correlation with the relative gene expression with *CD44* and seven integrin receptor genes (*ITGA2*, *ITGA3*, *ITGA5*, *ITGA6*, *ITGA9*, *ITGAV*, *ITGB1*, and *ITGB3*). On the other hand, we also aimed to investigate the possible association between the expression of the *OPN* variants and the $BRAF^{V600E}$ mutation.

METHODS

Cell culture

During our experiments, we used 11 melanoma cell lines originated from primary melanoma and eight cell lines derived from melanoma metastasis. Cell lines were purchased from the American Type Culture Collection, Coriell Institute for Medical Research, and Rockland Immunochemicals. The HT199 (origin: primary melanoma), HT168, and HT168-M1 (origin: melanoma metastases) cell lines were developed and characterized at Semmelweis University, Budapest, Hungary.[25,26] The clinicopathological characteristics of the cell lines are summarized in Table 1. Cell culture medium (RPMI 1640: Lonza

TABLE 1 Characteristics and invasiveness of human melanoma cell lines.

Cell line	Growth phase	Histologic subtype	BRAF mutation status	NRAS mutation status	Invaded cells/field (mean ± SD)[a]
Primary tumor derived cell lines					
WM35	RGP	SSM	V600E	WT	0.0 ± 0.0
HT199	RGP	NM	V600E	WT	15.0 ± 3.3
WM793B	RGP/VGP	SSM	V600E	WT	4.7 ± 0.9
WM3211	RGP/VGP	SSM	WT	WT	77.3 ± 25.5
WM1361	VGP	SSM	WT	Q61L	0.0 ± 0.0
WM39	VGP	NM	V600E	WT	0.0 ± 0.0
WM278	VGP	NM	V600E	WT	244.0 ± 15.1
WM983A	VGP	NM	V600E	WT	5.7 ± 2.1
WM1366	VGP	SSM	WT	Q61L	376.0 ± 26.4
WM3248	VGP	n.d.	V600E	WT	0.0 ± 0.0
Mel1617	n.d.	n.d.	V600E	WT	0.0 ± 0.0
Melanoma metastasis originated cell lines					
WM983B	–	–	V600E	WT	1.8 ± 1.3
A2058	–	–	V600E	WT	30.0 ± 3.6
HT168[b]	–	–	V600E	WT	26.7 ± 5.2
M24	–	–	WT	Q61R	27.0 ± 4.9
M24met[c]	–	–	WT	Q61R	74.0 ± 5.4
Melur	–	–	n.d.	n.d.	31.5 ± 4.2
SK-Mel-28	–	–	V600E	WT	n.d.
HT168-M1[b]	–	–	V600E	WT	52.3 ± 9.4

Abbreviations: E, glutamic acid; K, lysine; L, leucine; n.d., no data; NM, nodular melanoma; Q, glutamine; R, arginine; RGP, radial growth phase; SSM, superficial spreading melanoma; V, valine; VGP, vertical growth phase; WT, wild-type.

[a]Data are presented as the mean ± SD of three independent invasion assay experiments.

[b]HT168 and HT168-M1 cell line originated from the A2058 cells after subcutaneous injection in immunosuppressed mouse.[26]

[c]M24met originated after in vivo injection of M24 cells into nude mice.[48]

Group Ltd) was supplemented with 10% FBS (Gibco), and cells were cultured at 37°C, 5% CO_2.

In vitro invasion assay

The invasive properties of the cell lines were determined as we have described before.[27,28] Briefly, we used BD Biocoat Matrigel invasion chambers (pore size: 8 μm, 24 wells; BD Biosciences) and followed the manufacturer protocol. Shortly, the upper chamber of the insert was filled with cell suspension (in serum-free medium; 500 μL; 5×10^4 cells/well), and the lower chamber contained medium supplemented with 10% FBS as a chemoattractant. Each melanoma cell line was incubated for 24 h at 37°C. After incubation, the invaded cells in the lower layer were fixed with 100% methanol and stained with hematoxylin–eosin for microscopic observation. The average number of invaded cells was counted under a light microscope (200× magnification); seven different visual fields were evaluated in the case of all cell lines. The data are presented as the means ± SD of three independent experiments. Cell lines with more than five invaded cells/fields were characterized as invasive cell lines. We used this cutoff value for the reason that we wanted to avoid the loss of specific gene expression pattern belonging to the invasive cells. During the invasive experiments, our aim was to detect invasive property and gene expression signature in parallel to define the current expression pattern and characterize the phenotype. However, if the heterogeneous cell population includes only a few invasive cells, the gene expression of the invasive cells possibly suppressed by the major cell type in that population. Melanoma cells have a unique feature that they can switch between invasive and proliferative phenotype, and this switch is coincident with an exchange in gene expression pattern from proliferative to invasive and vice versa.[29] Many studies have identified gene signatures associated with reversible proliferative or invasive states in melanoma, where genes that were normally expressed in the proliferative state were often inversely correlated in cells in the invasive state and vice versa.[30,31]

Real-time quantitative polymerase chain reaction analysis

RNeasy Plus Mini Kit (Qiagen GmbH) was used for RNA isolation following the manufacturer's protocol. RNA concentration and quality were measured using NanoDrop (Agilent Technologies) as described before.[32] Only RNA samples with a 260/280 ratio greater than or equal to 1.8 were involved in further analyses. High Capacity cDNA Archive Kit (Applied Biosystems) was applied for reverse transcription of total RNA (1000 ng) according to the manufacturer's protocol.

The gene expression levels of all five OPN isoforms and eight integrins, as well as the *CD44* gene, were determined by real-time polymerase chain reaction (RT-PCR). We used Xceed qPCR Probe 2× Mix (Institute of Applied Biotechnologies) and a LightCycler 480 Instrument II (Roche Diagnostics Nederland BV). The RT-PCR analyses were performed as we described previously.[16] Briefly, the RT-PCR settings were the following: pre-activation: 95°C for 1 min, followed by 45 cycles of 95°C for 5 s (denaturation), annealing at 55–62°C for 10 s (specific annealing temperature and sequence of each primer are summarized in Table S1), extension at 72°C for 15 s, cooling at 40°C for 30 s; and finally, melting curve analysis. Primers were purchased from Life Technologies. For analysis of quantitative RT-PCR (qRT-PCR) data, for internal control gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was used, and data are presented as the average of $2^{-\Delta Ct}$ values from three independent experiments.

Statistical analysis

Statistical analyses were done using SPSS 19.0 (SPSS). The normality of the data was assessed using the Shapiro–Wilk test. Pearson's correlation coefficient was applied to calculate the correlation between the relative expression data of *OPN* variants and receptors. Mann–Whitney test was used to compare the relative expression profile of primary tumors and melanoma metastasis originated cell lines, invasive and noninvasive cell lines, *BRAF* mutated, and wild type cell lines. Kruskal–Wallis test was used to compare the qRT-PCR data of superficial spreading melanoma (SSM), nodular melanoma (NM), and metastatic melanoma (MM) tumor derived cell lines. Any $p \leq 0.05$ was considered statistically significant.

RESULTS

Gene expression pattern of *OPN* splice variants and receptors in association with clinicopathological subgroups of melanoma cell lines

The qRT-PCR analyses were performed to investigate the expression patterns of the *OPN* variants of primary

($n=11$) and MM ($n=8$) originated cell lines (Figure 1). Our results revealed a higher relative gene expression of the *OPNc* variant in metastasis-derived cell lines compared to the primary cells, however, this difference was not significant ($p=0.083$).

The primary tumor-originated cell lines were divided based on the histological type. We observed significantly higher expression of all *OPN* variants in NM ($n=4$) compared to SSM ($n=5$; Figure 2). Furthermore, all five variants exhibited a significant increase in gene expression in MM-derived cell lines compared to cell lines originating from the SSM subtype of primary melanoma. In case of the receptor genes, we observed that cell lines originated from NM had the highest expression level compared to SSM or MM cell lines (Figure S1). Cell lines derived from metastatic tumors displayed lower mRNA levels than NM samples, which was a significant difference in *ITGAV* ($p=0.041$).

Comparison of the gene expression of *OPN* isoforms and receptors in invasive and noninvasive melanoma cell lines

To investigate the association between the gene expression of the *OPN* variants and the invasive potential, an in vitro invasion assay was performed on melanoma cell lines. The results are summarized in Table 1. Cell lines with more than five invaded cells per field were classified as invasive, whereas those with fewer than five were considered noninvasive. Based on our data, 11 cell lines exhibited invasive behavior (WM278, WM983A, HT199, WM1366, WM3211, A2058, HT168, HT168-M1, M24, M25met, and Melur), whereas seven cell lines were noninvasive (WM35, WM793B, WM3248, WM39, WM1361, Mel1617, and WM983B). One cell line (SK-MEL-28) failed to attach to the membrane and was excluded from further invasion analysis.

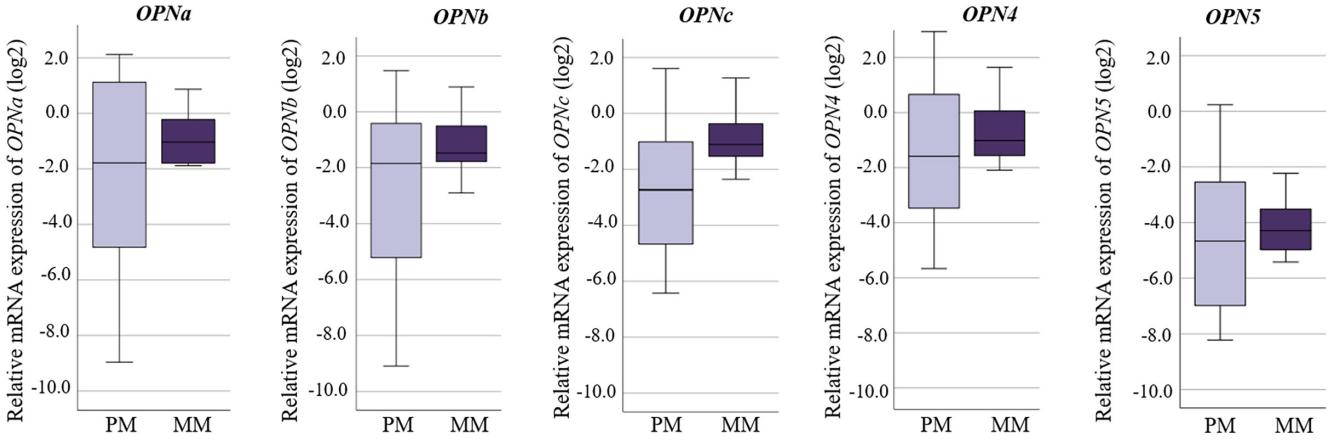

FIGURE 1 Relative mRNA expression levels of *OPN* isoforms (*OPNa*, *OPNb*, *OPNc*, *OPN4*, and *OPN5*) in PM ($n=11$) and MM ($n=8$)-originated cell lines. *OPNc* showed higher relative expression in MM compared to PM, however, this difference was not significant ($p=0.083$; Mann–Whitney test). MM, metastatic melanoma; OPN, osteopontin; PM, primary melanoma.

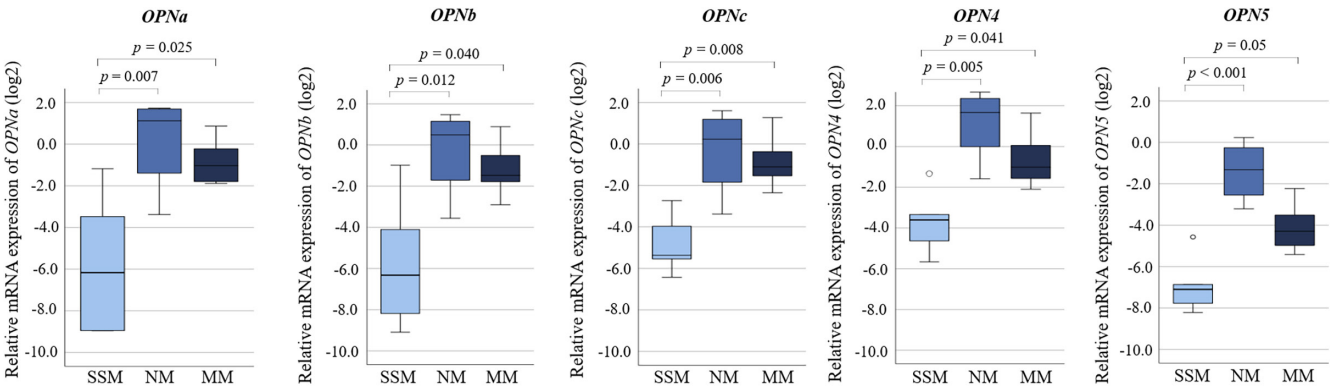

FIGURE 2 Relative mRNA expression levels of *OPN* isoforms (*OPNa*, *OPNb*, *OPNc*, *OPN4*, and *OPN5*) in melanoma cell lines compared by the histological subtypes. SSM ($n=5$); NM ($n=4$); and MM ($n=8$) originated cell lines. Reported *p* values were calculated with the Kruskal-Wallis test. MM, metastatic melanoma; NM, nodular melanoma; OPN, osteopontin; SSM, superficial spreading melanoma.

Regarding the gene expression results of *OPN* splice variants, the relative mRNA level of all five isoforms was higher in invasive melanoma cell lines than in noninvasive ones. However, this difference was statistically significant only for the *OPNc* variant (Figure 3).

We also compared the relative gene expression levels of the integrin and *CD44* receptors. *ITGB3*, *ITGAV*, *ITGB1*, and *ITGA6* displayed lower expression in invasive cell lines compared to noninvasive cells, however, these differences were not significant (Figure S2).

Association of *OPN* isoforms gene expression with *BRAF*^{V600E} mutation

We had a possibility to compare the gene expression level of the splice variants of 14 *BRAF*^{V600E} mutant melanoma cell lines with four *BRAF* wild-type (WT) cell lines. Based on our analysis, we observed that all five variants were significantly upregulated in *BRAF* WT cells compared to *BRAF* mutant cells (Figure 4).

Correlation of the relative expression of *OPN* isoforms, integrins, and *CD44* receptor

To explore potential associations between the expressions of *OPN* variants and their potential receptors, we assessed the relative gene expression of the *CD44* receptor and the following integrin receptor: *ITGA2*, *ITGA3*, *ITGA5*, *ITGA6*, *ITGA9*, *ITGAV*, *ITGB1*, and *ITGB3* genes (refer to Table S1). The results of correlation analysis (Pearson's correlation) between the relative gene expression data ($\log_2$) are presented in Figure 5 and Table S2.

A significant positive correlation was observed between *ITGB3* and *ITGA9* with all five *OPN* variants. Additionally,

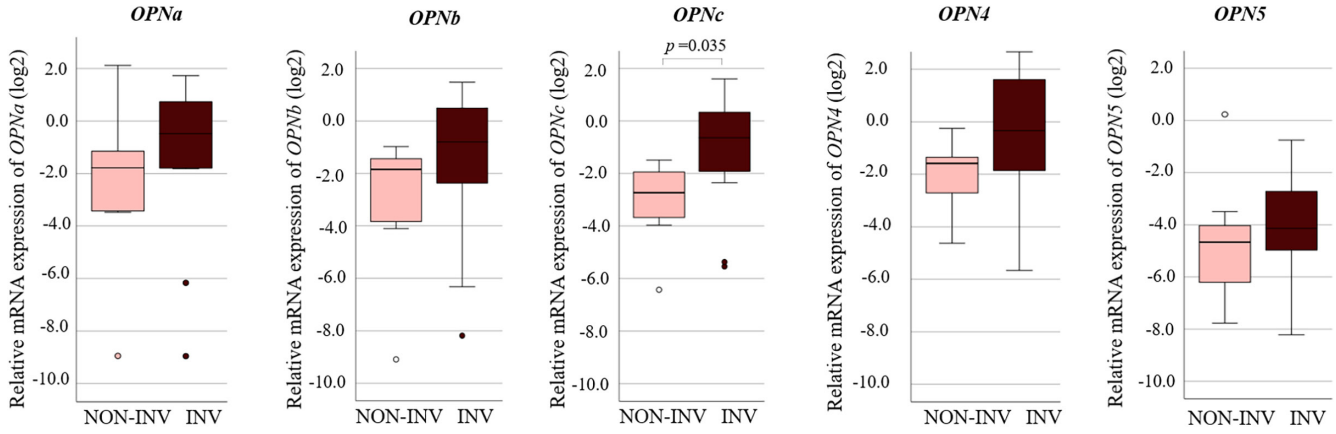

FIGURE 3 Relative mRNA expression levels of *OPN* isoforms (*OPNa*, *OPNb*, *OPNc*, *OPN4*, and *OPN5*) according to the invasion capacity of cells. NON-INV ($n = 7$); and INV ($n = 11$). Reported p value was calculated with the Mann–Whitney test. NON-INV: noninvasive cell lines; INV, invasive cell lines; OPN, osteopontin.

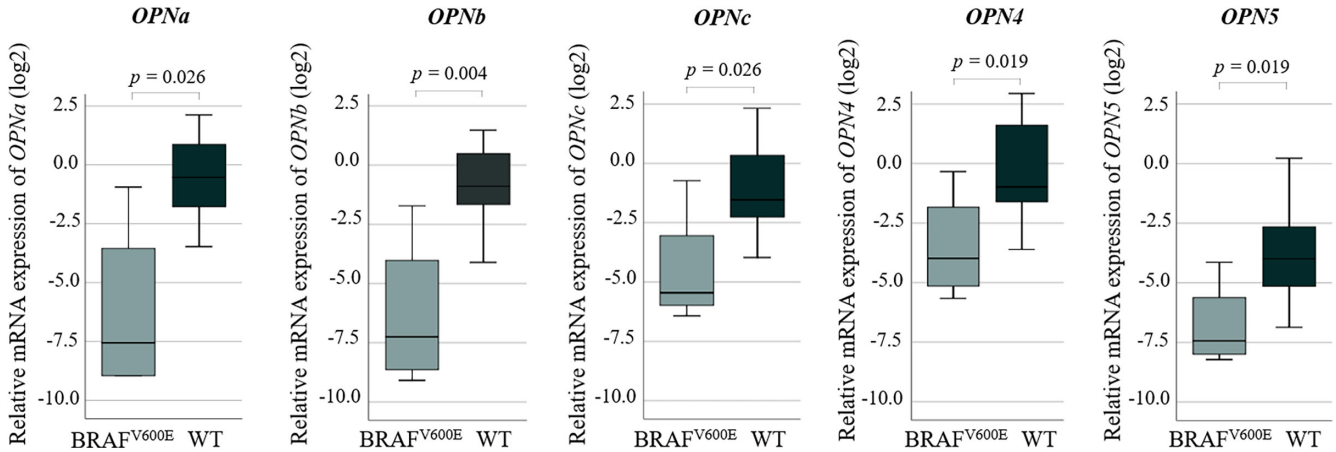

FIGURE 4 Relative mRNA expression levels of *OPN* isoforms (*OPNa*, *OPNb*, *OPNc*, *OPN4*, and *OPN5*) in *BRAF*^{V600E} mutant ($n = 14$) and *BRAF* WT ($n = 5$) melanoma-derived cell lines. Reported p values were calculated with the Mann–Whitney test. OPN, osteopontin; WT, wild type.

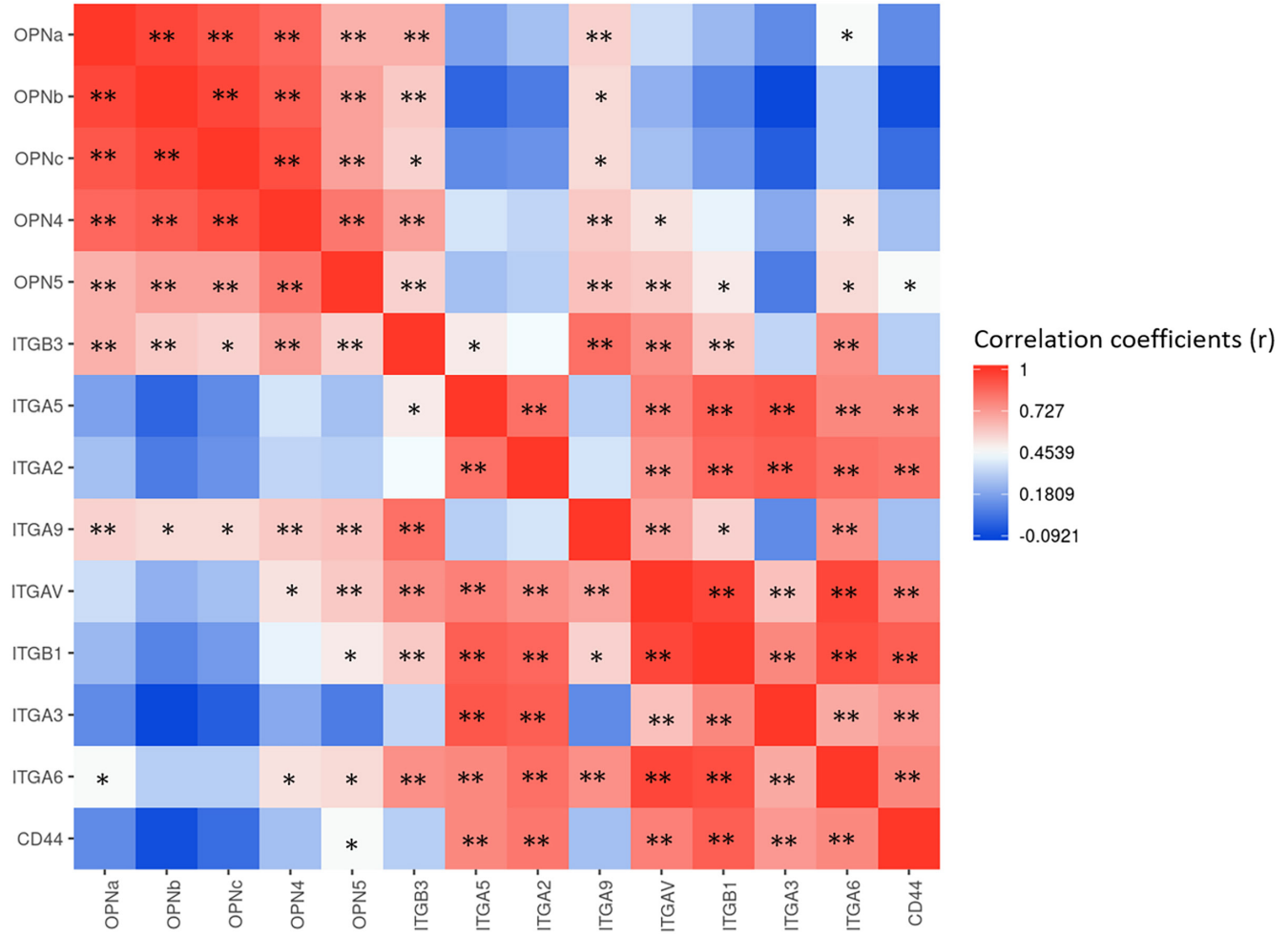

FIGURE 5 Pairwise correlation matrix of relative expression data ($\log_2$). The Histogram panel shows Pearson's correlation coefficients (r); red indicates a strong correlation, white indicates a weak correlation, and blue indicates no correlation. Significant correlations are indicated by asterisks (*$p \leq 0.05$; **$p \leq 0.01$). The Histogram panel was generated using www.heatmapper.ca[47] accessed on June 12, 2023.

ITGAV and *ITGA6* exhibited significant correlations with *OPN4* and *OPN5*. Specifically, *ITGA6* showed a positive correlation with the *OPNa* variant, whereas *ITGB1* correlated with *OPN5*. *CD44* displayed only a weak correlation with *OPN5*.

DISCUSSION

Identifying clinically applicable biomarkers for cancer progression remains a significant focus of intensive research. One potential candidate for such biomarkers is OPN, a ubiquitous protein produced by a wide range of cell types and tissues. It is abundant in body fluids, such as blood, milk, and urine.[1,10] In the context of cancer, *OPN* gene products are typically not mutated in transformed cells but exhibit aberrant expression or splicing.[10,33] *OPN* splice variants have been widely studied in various tumor models, revealing their tumor and tissue-specific roles. A recent comprehensive meta-analysis of studies on *OPN* variants highlighted the association of specific splice variants with tumor grade, stage, or patient survival in various cancers.[11] However, in the case of MM, the only available study to date is our previous investigation focusing on five *OPN* splice variants (*OPNa*, *OPNb*, *OPNc*, *OPN4*, and *OPN5*) and their correlation with clinicopathological features of patients' tumor tissues.[16] The present study aimed to analyze the relative gene expression levels of *OPN* isoforms in cell lines derived from primary and MM tissues and explore the association with clinicopathological parameters and invasive capacity. Additionally, we aimed to examine the potential correlation between the *OPN* isoforms and the expression of genes encoding different receptor genes (*ITGA2*, *ITGA3*, *ITGA5*, *ITGA6*, *ITGA9*, *ITGAV*, *ITGB1*, *ITGB3*, and *CD44*).

First, we examined the relative gene expression differences between primary and metastasis melanoma-derived cell lines. Our findings unveiled a notable increase in the expression of *OPNc* in metastatic cells relative to primary cells. Additionally, when primary melanoma cell lines

were categorized based on their histological type (SSM and NM), we observed significantly higher expression of *OPNa*, *OPNb*, *OPNc*, *OPN4*, and *OPN5* in more advanced stages, such as primary NM and metastatic cell lines compared to the cell lines derived from primary SSM. These results align with the association between *OPN* variants and melanoma progression we previously reported for *OPNa*, *OPNb*, and *OPNc* isoforms.[16] The relationship between *OPN* splice isoforms and tumor stage in various cancers is well-established; however, most studies focus on *OPNa*, *OPNb*, and *OPNc* isoforms.[10,18,34,35] The high expression of *OPNa*, *OPNb*, and *OPNc* appears to be associated with poor prognosis in melanoma.

On the other hand, our previous findings have shown a significant increase in *OPNc* expression associated with thicker melanoma tissue samples.[16] Elevated levels of *OPN* expression have been linked to cell adhesion, migration, and invasion of various cancers, including melanoma, and have been recognized as a key prognostic marker.[36–39] Therefore, we investigated the association between the gene expression of the *OPN* variants and the invasive capacity of the cell lines. Our results revealed a significant elevation of *OPNc* expression in invasive cell lines compared to noninvasive cells. Although the correlation between *OPNc* and invasion has been reported in different types of cancers, our present study described this association for the first time in melanoma. It has been pointed out that *OPNc* overexpression promotes aggressive behavior and metastasis formation in breast cancer. However, low expression of *OPNc* was reported in association with poor prognosis of gastric cancer.[34 40,41] Zhang et al. have found that high levels of *OPNc* are closely related to invasion and tumor stage in esophageal squamous cell carcinomas, whereas Tang et al. has been reported that higher *OPNc* expression is related to lymph node metastasis, deeper local invasion, and advanced TNM stage in gastric cancer. Because the *OPNc* variant lacks exon 4, which appears necessary for cell adhesion, this *OPN* isoform remains soluble and can support invasiveness.[41,42]

As it has been hypothesized that some different *OPN* variants acts through different integrin-dependent signaling, we also aimed to analyze the possible relationship between the expression of *OPN* splice variants and different receptor genes, including *CD44* and specific integrins. OPN is an essential ligand of CD44, whose binding promotes clonal growth, invasion, and metastasis.[43] Sun et al.[44] described the association between OPNa, integrin β3, and CD44 in lung cancer promoting the growth in lung cancer cells. Moreover, it has been demonstrated in glioma cells that cellular invasion induced by *OPNa* and *OPNc* is mediated by the PI-3K/AKT/NF-kB pathway through ligation of αvβ3 integrin.[42]

Similar to these previous findings, the gene expression of *ITGB3* showed a significant positive correlation with all the five *OPN* variants in this study. In contrast, whereas *ITGAV* showed a correlation with *OPN4* and *OPN5*. The expression of *CD44* had only a weak correlation with *OPN5*. On the other hand, we found a significant positive correlation of *ITGA9* with all the five *OPN* variants, whereas *ITGB1* correlated with *OPN5*. It has been reported that when binding to α9β1, OPN activates ERK signaling, promoting melanoma tumor cell migration.[45] It is possible that these associations between the expression of *OPN* splice variants and the integrin receptors could have a role in the progression of melanoma; however, further investigations are needed to clarify these effects.

Interestingly, $BRAF^{V600E}$ mutated cell lines displayed lower gene expression of *OPN* variants than WT cell lines. However, *OPN* could serve as a target for screening patients who respond to immune checkpoint inhibitor therapy.[46] Moreover, our previous publication revealed that OPN expression is downregulated in BRAF inhibitor (PLX4720)-resistant melanoma cells; however, this is the first study to relate *OPN* variants to *BRAF* mutation.[23]

Further studies investigating the expression levels of *OPN* isoforms in serum samples of patients with melanoma would help validate the utility of *OPN* variants in diagnostic approaches. *OPN* variants, in especially *OPNc*, shows a promise as potential diagnostic marker for identifying patients with melanoma with poor outcome. Detecting of *OPNc* levels could be utilized to assess the risk of metastasis in patients with melanoma.

AUTHOR CONTRIBUTIONS

V.K. and M.B. wrote the manuscript. V.K. and M.B. designed the research. K.J. and I.S. performed the research. V.K., I.S., and K.J. analyzed the data.

FUNDING INFORMATION

This research was funded by the National Research Development and Innovation Fund (grant number K-135752), the European Regional Development Fund (GINOP-2.3.2-15-2016-00005), the Hungarian Academy of Sciences (TK2016-78), and the Eötvös Loránd Research Network (TKCS-2021/32). The work was also supported by the ÚNKP-23-4 New National Excellence Program of the Ministry for Culture and Innovation from the Source of National Research, Development, and Innovation Fund (ÚNKP-23-4-II-DE-229; ÚNKP-23-II-DE-283).

CONFLICT OF INTEREST STATEMENT

The authors declared no competing interests for this work.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Koroknai V, Szász I, Jámbor K, Balázs M. Expression pattern of osteopontin isoforms in malignant melanoma cell lines. *Clin Transl Sci*. 2024;17:e13694. doi:10.1111/cts.13694