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The role of osteopontin expression during malignant melanoma progression

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The Examination takes place at library of Department of Internal Medicine (Bldg. A), Faculty of Medicine, University of Debrecen at 11:00 on 17th April, 2024

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 13:00 on 17th April, 2024

INTRODUCTION

Melanoma is one of the most malignant forms of cancers and represents a significant public health problem globally, with several histopathological subtypes and varying incidence rates among different populations. The incidence of melanoma varies among different populations, primarily due to genetic factors and levels of sun exposure. The incidence of the disease has been steadily increasing worldwide over the past decades, particularly among light-skinned populations. In 2020, the International Agency for Research on Cancer (IARC) estimated that there were 324,635 cases of melanomas worldwide resulting in 57,043 deaths, however, the estimated age-standardized incidence (cases per 100 000 residents) varies widely. The development of melanoma is multifactorial, resulting from the interaction of genetic predisposition and environmental influences. Malignant melanoma is recognized as one of the most aggressive forms of skin cancer, known for its high mortality rate attributed to its increased metastatic potential and increased drug resistance. Early detection of the tumour and prompt removal are crucial for optimal management and improved outcomes.

Molecular background of malignant melanoma

In addition to its aggressive nature, malignant melanoma is also known for its heterogeneous biological behavior, with multiple signaling pathways involved in its molecular pathogenesis. In recent decades, numerous important molecular pathways have been discovered to be implicated in the initiation, proliferation, survival, progression, and invasion of the disease, including mitogen-activated protein kinase (MAPK) pathway,

phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT pathway, cell-cycle regulation pathway, epigenetic factors, and some other signaling pathways.

The role of biomarkers in cancer biology

During the last decades, important improvements cancer biology, genomics, and molecular technologies have led to the discovery of several new cancer biomarkers including human malignant melanoma. Common types of tumour biomarkers are classified as diagnostic, prognostic, and predictive. Diagnostic biomarkers are employed to detect and confirm the presence of tumours, enabling in the early detection of potential recurrence and guide treatment decisions. Prognostic biomarkers predict the probable course and possible outcomes of the disease, whereas predictive biomarkers are used to assess the probable response to a specific treatment.

Osteopontin and cancer progression

Osteopontin (OPN) also known as SPP1 (secreted phosphoprotein 1), or ETA-1 (Early T-lymphocyte Activation 1 protein) that was first identified in bone tissue. OPN is a multifunctional extracellular matrix protein comprising 314 amino acids and is encoded by the SPP1 gene, which is located on human chromosome 4. OPN is produced by various cell types and overexpressed in various types of cancer including breast, colorectal, gastric, liver, pancreas, lung, and melanoma. Extensive microarray analysis of melanoma has indicated that OPN has a serious role in melanoma progression. Published data suggests that OPN overexpression frequently associated with enhanced tumour invasion, metastasis and high-grade malignancies.

Several studies suggest that OPN would be a specific target for cancer therapy. Taken together, studies have demonstrated that OPN have exhibits antimetastatic and antitumorigenic effects in various cancers.

Current and novel targeted therapies in melanoma

Surgically removing early-diagnosed melanoma is a highly effective method for preventing disease recurrence. In the case of advanced-stage melanoma, the therapeutic standard of care used to be chemotherapy with dacarbazine (DTIC) and high-dose interleukin-2 (IL-2) were the therapeutic standard of care until the approval of the RAF inhibitor vemurafenib and the CTLA-4 antibody ipilimumab in 2011. The discovery of melanoma driver genes has played a crucial role in the development of targeted therapies.

Besides, the most important advances in molecular biology has been the discovery that small interfering RNAs (siRNAs) are able to regulate gene expression by RNA interference (RNAi). siRNA-based treatments can be hopeful gene-related therapeutics for cancer because many genes are upregulated at some stages in tumour initiation and contributed to cell proliferation and tumour progression. Targets of siRNA-based therapeutics are separated into three major groups in cancer treatment, including molecules that are participated in carcinogenesis, gene products that engaged in tumour-host interactions, and gene products that are involved in tumour resistance to chemotherapeutics.

OBJECTIVES

The focus of this study was to deeper understand the role of OPN expression during melanoma progression. We aimed was to examine OPN expression in a series of primary tumour tissues with distinct biological behavior. Subsequently, we investigated the effect of inhibition of OPN expression using small interfering RNA (siRNA) on cell proliferation and invasion, aiming to elucidate the molecular mechanisms underlying OPN's involvement in cutaneous melanoma progression.

The specific objectives of the recent study were the followings:

1. Determine OPN expression at both mRNA and protein levels of various subtypes of primary melanoma tissues using immunohistochemistry on tissue microarrays, and quantitative real-time polymerase chain reaction (qRT-PCR). Additionally, we aimed to correlate the expression patterns with the clinical-pathological parameters of the melanoma patients.

2. Assess the mRNA and protein levels of OPN expression in melanoma cell lines derived from primary and metastatic tissues.

3. Inhibit OPN expression using siRNA in specific melanoma cell lines that exhibit high OPN expression. Subsequently, assess the impact of transfection on cell proliferation and invasion.

4. Investigate the protein expression patterns of the parental and siRNA silenced melanoma cell line pairs that were developed form the same

patients primary and metastatic tumours. The investigation will be carried out using proteome profile analysis.

MATERIALS AND METHODS

Melanoma tissue samples

Melanoma tissues were obtained from the Department of Dermatology, University of Debrecen, Hungary. This study received approval from the Regional and Institutional Ethics Committee of the University of Debrecen (Hungary) and the Medical Research Council (Hungary). The research was conducted in compliance with all relevant regulations and guidelines (DE RKEB/IKEB: 2836/2008; RKEB/IKEB: 4820-2017; TUKEB (MRC): 25364-1/2012/EKU (449/PI/12). Written informed consent was obtained from all participating patients. Lesions were diagnosed based on formalin-fixed paraffin-embedded (FFPE) tissue sections stained with haematoxylin-eosin (H&E) and the classification was performed according to the latest edition of TNM staging system. It is important to note that the examination of OPN expression in melanoma tissues was not conducted simultaneously; therefore, the studied set of melanoma samples did not completely overlap.

Melanoma cell lines

The experiments were conducted using primary melanoma cell lines derived from the following sources: WM35, WM793B, WM3211, WM902B, M35/01, WM1361, WM1366, HT199, WM39, WM3248, WM278, WM983A. Metastatic melanoma cell lines used were: WM1617, WM983B,

SK-MEL-28, A2058, HT168-M1, M24, M24met, Melur. The cell lines were cultured in RPMI 1640 medium (Lonza Group Ltd, Basel, Switzerland) or MCDB153-L15 medium (Sigma-Aldrich Co. LCC, St Louis, Missouri, USA) and supplemented with 10% foetal bovine serum (Gibco, Carlsbad, California, USA) at 37°C in an atmosphere containing 5% CO2.

Detection of BRAF and NRAS mutation

The majority of melanoma cell lines had their BRAF and NRAS mutation status had previously tested and provided by ATCC or Coriell Institute for Medical Research. For six specific cell lines (A2058, HT168-M1, M24, M24met, HT199 and WM902B) the mutations in BRAF codon 600 and NRAS codon 61 were determined by melting curve analysis using fluorescent probes on the LightCycler real time PCR System (Roche Diagnostics GmbH, Mannheim, Germany). Primers and probes were obtained from TIB Molbiol GmbH (Berlin, Germany). The reaction procedure was carried out according to the previously described method.

RNA extraction and qRT-PCR analysis

The RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany) was used to isolate total RNA from melanoma cell lines following the manufacturer's protocol. The concentrations of the RNA samples were measured using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription (RT) was carried out on 600 ng total RNA using the High-Capacity cDNA Reverse Transcription Kit, following the manufacturer's instructions (Life Technologies Corporation, Carlsbad, California, USA).

TaqMan one-step RT-PCR was employed to quantify the mRNA level of the OPN gene in 28 primary melanoma tissues. Each reaction was run in triplicate on ABI-PRISM 7000 instrument (Applied Biosystems, Carlsbad, CA, USA). PCR Master Mix and assay (Hs00167093_m1 OPN targeting the exon 3-4 boundary) were obtained from Life Technologies.

In our subsequent studies, the relative expression level of the OPN gene was assessed through quantitative real-time PCR using the LightCycler® 480 Real-Time PCR System (Roche Diagnostics, GmbH, Mannheim, Germany). This analysis was conducted on 34 primary and 12 metastatic melanoma tissues, 12 primary melanoma- and 8 melanoma metastasis originated melanoma cell lines. The qPCR reactions were performed using SYBR premix Ex Taq (Takara Holding Inc., Kyoto, Japan) master mix.

The raw PCR data were analyzed using the Livak method ($2^{-\Delta\Delta Ct}$), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control gene. Pooled nevi (n=8) and cultured melanocyte were utilized as the calibrator samples.

Immunohistochemistry on melanoma Tissue Microarray

Immunohistochemistry was performed on 93 FFPE melanoma tissue sections. Four µm tissue sections of the original melanoma blocks were initially stained with haematoxylin-eosin and then examined by pathologists. The pathologists selected the specific area to be automatically punched using the fully automated tissue microarray (TMA Master, Carl Zeiss, Jena, Germany). Tumour cores measured 1 mm (triplicate) were obtained from each melanoma tissue block. Serial sections of 4 µm were sliced from the

TMA blocks and used for immunohistochemical investigations. Microarray sections were stained with haematoxylin-eosin and validated the sample spots for tumour cell content under a microscope. After deparaffinization, endogenous peroxidases were blocked by treating the samples with 1% H_2O_2 for 10 min at RT. Non-specific binding sites were subsequently blocked. Antigen retrieval was performed by heating the samples in 1 mM boiling citrate buffer (pH 6.0) for 3 minutes. The primary antibody, rabbit polyclonal anti-osteopontin IgG (Thermo Scientific, Waltham, Massachusetts, USA) was added at a 1:20 dilution and incubated for 1 hour at room temperature. Following the primary antibody incubation with, the samples were stained using the peroxidase/DAB based Envision detection system (DAKO Inc., Carpinteria, CA, USA) and VIP peroxidase substrate kit (Vector Laboratories LTD, Peterborough, UK). Nuclei were counterstained with methyl green (Vector Laboratories LTD). Negative controls were prepared by omitting the primary antibody. The HeLa cervical cancer cell line served as the positive control in this study. The tissue microarray slides were digitized using MiraxScan slide scanning device (Carl Zeiss). Digital images were viewed using MiraxViewer software (Carl Zeiss) and evaluated by two dermatopathologists who were blinded to the tissues clinical-pathological characteristics. We implemented two scores for tissue characterization: 1.) tumour cell proportion score and 2.) intensity staining score. The proportion score determined the fraction of positively stained tumour cells based on the following criteria: 0 = none, 1+ = <5%, 2+ = 5-50%, 3+ = >50%. The estimated average staining intensity of the positive tumour cells was expressed as follows: 0 = none, 1+ = weak, 2+ = moderate, 3+ = strong.

Indirect immunofluorescence

Melanoma cells were cultured on 8-well Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL) in RPMI 1640 medium (Lonza Group Ltd, Basel, Switzerland) containing 10% FBS (Gibco, CA, USA) overnight. The cells were washed twice with PBS, followed by fixation with acetone for 10 minutes and air drying. Subsequently, the cells were rinsed with PBS (15 minutes) and blocked with 5% BSA in PBS at room temperature for 30 minutes. Primary antibodies, including anti-OPN antibody diluted to 1:500 (Rockland Immunochemicals Inc., Limerick, PA) and anti-NF-κB p65 antibody diluted to 1:50 (Cell Signaling Technology Inc., Danvers, MA), were then incubated with the cells overnight at 4°C in a humidified container. After rinsing with PBS, the cells were incubated with DyLight488-conjugated anti-rabbit or DyLight594-conjugated anti-rabbit IgG (Vector Laboratories Ltd, Peterborough, UK) for 1 hour at room temperature. Following another round of PBS washing, the cell nuclei were stained with 4'6-diamidino-2-phenylindole (DAPI, Vector Laboratories Ltd, Peterborough, UK). Images were analyzed using a confocal laser scanning microscope (Zeiss LSM700, Carl Zeiss).

Cell proliferation assay during the siRNA experiment

To assess cell proliferation as an indicator of undesirable RNAi activity, the WST-1 Cell Proliferation Reagent (Roche Magyarország Kft., Budaörs, Hungary) was utilized according to the manufacturer's instructions. The measurements were performed 78 hours after siRNA transfection. Absorbance was recorded at 450 nm using a NanoDrop ND-1000 UV-Vis

Spectrophotometer (NanoDrop Technologies), with the reference absorbance was set at 700 nm.

In vitro invasion assay

Melanoma cell lines invasive capacity was determined using BD Biocoat Matrigel invasion chambers (pore size: 8 μm, 24-well; BD Biosciences, Bedford, Massachusetts, USA). The upper chamber of the insert was filled with 500 μl of cell suspension in serum-free media (5×10^4 cells/well), while the lower chamber contained medium with 10% FBS as a chemoattractant. The cells were incubated for 24 hours at 37°C. Non-invading cells were removed with a cotton swab, cells with invasive potential attaching to the lower layer of the membrane were fixed with methanol and stained with haematoxylin–eosin. The average number of invaded cells was determined in 7 different visual fields (at 200X magnification using a light microscope). The data are presented as the mean ± SD of three independent experiments.

siRNA experiments

The siRNA targeting human secreted phosphoprotein 1 (SPP1; OPN-targeting siRNA: ID: SI02757615) and the AllStars Negative Control siRNA (NC-siRNA ID: 1027281) were purchased from Qiagen GmbH (Hilden, Germany).

One day prior to transfection, cells were seeded in 24-well plates at a density of $5x10^4$ cells per well and cultured in 500 μl of growth medium in each well. Importantly, the growth medium did not contain antibiotics because it can interfere with the transfection process or affect viability. Cells were allowed

to grow until cells reached a confluence level of 30-50%. This growth state is often preferred for transfection experiments to ensure that the cells are actively dividing and capable to effectively taking up the transfected genetic material. The transfection complexes were prepared using siRNA (at a concentration of 5 nM) and Lipofectamine 2000 transfection reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) resulting in a final volume of 100 µl, according to the manufacturer's protocol. The complexes were added to each well of the culture plate. After the 3 hours' incubation period, the medium was replaced with fresh medium in each well. The cells were harvested 48 hours after transfection for subsequent analysis, and gene silencing efficacy was evaluated by qRT-PCR.

Proteome array analysis

Cells were harvested in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific Inc. Waltham, Massachusetts, USA) containing Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific Inc. Waltham). The total protein concentration in the supernatants was measured using a Quick StartTM Bradford Protein Assay (Bio-Rad Hungary Ltd. Budapest, Hungary) following the manufacturer's protocol.

Protein expression was assessed using a Proteome Profiler Human XL Oncology Array Kit (R&D Systems, Abingdon, United Kingdom) according to the manufacturer's instructions. This membrane-based antibody array allows for the detection of 84 cancer-related proteins between samples. The array spots were analyzed using ImageJ Lab 1.51 Software and normalized to the signal intensities of the positive control (1.51a, NIH, Bethesda,

Maryland, USA) and evaluated by subtracting the background. The density of the postitive control was considered as 100%.

The protein – protein functional associations were analyzed using STRING (Search Tool for the Retrieval of Interacting Genes) software, specifically utilizing database version 11.0 (http://string-db.org).

Statistical analysis

Statistical analysis was performed using SPSS 22.0 (Statistical Package for the Social Sciences 22.0) software (SPSS Inc., Chicago, IL, USA). Fisher's exact and Mann-Whitney-Wilcoxon tests were employed to compare the OPN expression levels and the clinical-pathological data of primary melanomas and melanoma cell lines. Survival analysis was conducted using Cox regression analysis to evaluate predictors of survival. Logistic regression was employed to analyze association between prognostic factors and formation of metastasis. The Kruskal-Wallis test was used to determine significant differences among OPN gene expression in melanoma subgroups (SSM, NM and melanoma metastasis). Student's t-test was performed for the statistical analysis of the experimental siRNA data. A p value ≤ 0.05 was considered statistically significant.

RESULTS

OPN mRNA expression in primary melanoma tissues

Gene expression analysis of OPN was performed in 28 primary melanomas. A greater than twofold increase in the OPN mRNA levels compared to

controls was found in 93% (26/28) of these melanomas. We determined the relationship between gene expression levels and clinical-pathological parameters. OPN mRNA expression was significantly increased in thicker melanomas (greater than 4.00 mm) and ulcerated lesions; furthermore, *OPN* expression was present mainly in late stage tumours (Clark levels IV and V). Fourteen overlapping samples were used to compare the gene and protein expression levels in the same tumour samples. Increased OPN mRNA expression levels were detected in the majority of primary melanoma samples and were associated with different degrees of protein expression. We did not observe any correlation between the OPN protein levels and mRNA expression.

Association between OPN protein expression and clinical-pathological characteristics in primary melanomas

We examined OPN protein expression levels in 93 primary melanomas. In total, 87% (81/93) of the melanoma samples were positive for OPN. The remaining 12 samples showed no OPN positivity. Increased OPN protein level (2+ and 3+) were detected in 71% of primary melanoma samples. Melanoma tissues, in which at least 50% of the tumour cells exhibited high OPN expression were categorized as 3+. High protein positivity (3+) was found in 40% of melanoma samples associated with unfavourable prognosis (nodular subtype, advanced stage, >4.01 mm Breslow thickness, ulcerated surface, presence of metastasis and <5-year survival). OPN positivity exhibited a significant correlation with Breslow tumour thickness and metastasis. Melanoma samples with poor clinical outcome showed stronger OPN staining. We analysed the relationship between the OPN expression

levels and clinical-pathological parameters of the primary tumours. Increased OPN expression was significantly associated with increasing tumour Breslow thickness. We assessed the effect of OPN expression and prognostic factors on metastasis formation using multivariate logistic regression analyses. These results indicate that melanomas with OPN expression exhibited a 1.39-fold increased risk of metastasis, but this association was not significant. However, Breslow thickness and ulceration were significantly associated with metastasis.

Multivariate Cox regression analyses of survival were performed regarding OPN expression levels and various prognostic factors such as ulceration, Breslow thickness and metastatic potential. In this study, we demonstrated that high OPN expression, tumour thickness and the presence of metastasis were significantly associated with reduced melanoma patient survival.

***OPN* gene expression in melanoma**

OPN expression was determined by qRT-PCR in melanoma cell lines (n=20). The cell lines originated from primary melanomas (n=12) and melanoma metastases (n=8). The relative *OPN* mRNA expression was significantly enhanced in the metastatic tumour originated cell lines. The *OPN* expression in the $BRAF^{V600E}$ mutant cell lines (n=14) were significantly higher compared to cell lines with wild-type BRAF (n=6).

We also determined the relative mRNA expression of the *OPN* in primary and metastatic tumour tissues. Our data showed significant differences between melanoma subgroups. We observed the highest relative gene expression in a melanoma metastasis (84.5). For 8 of the 12 metastatic samples the range was between 13.1-84.5. Three primary tumours of the

aggressive nodular subtypes (n=10) also showed elevated relative *OPN* expression (17.6-61.8), while samples from the less aggressive SSM subtypes (n=24) exhibited a more uniform pattern with low *OPN* gene expression (range between 0.1-9.14).

OPN gene expression and NF-κβ p65 protein level in melanoma cell lines

We aimed to determine the subcellular distribution of both OPN and NF-κB p65 protein. We observed that in the cell line, which originated from early lesion of a primary tumour without metastatic potential, the expression of OPN and NF-κB p65 protein was observed in the cytoplasm. On the other hand, in the cell line which is a tumorigenic primary melanoma cell line with competence for metastasis, expression of both OPN and NF-κβ p65 protein was observed in both the cytoplasm and nuclei.

Effect of RNAi on OPN expression

In order to determine the effectiveness of OPN-siRNA silencing, we chose a primary and metastatic cell line pair (WM278 – WM1617) that were originated from the same patient. Both cell lines had $BRAF^{V600E}$ mutation and were wild type for RAS gene. We effectively inhibited OPN expression in melanoma cell lines with high OPN expression using validated OPN-specific siRNA. At the same time, the proliferation of the transfected cells was significantly reduced compared to the negative control treated and untreated control cells.

Effect of RNAi on the invasive behaviour of melanoma cell

In order to define the invasive potential of OPN-siRNA silenced cells, invasion assay was performed after OPN knockdown. We found that invasion was significantly lower in silenced cells compared to control cells, suggesting that downregulated OPN expression is associated with reduced invasion.

Protein array analysis of the original and OPN siRNA-transfected cells

To determine protein expression differences between the original and OPN siRNA-transfected cell lines, we used a Proteome Profiler Human XL Oncology Array, which detects 84 cancer-related proteins. Numerous differentially expressed proteins were observed in the original cell lines compared to their transfected counterparts.

According to our protein interaction analysis, the proteins formed multiple clusters, one is a cluster of extracellular matrix organization, the other is a cluster of regulation of angiogenezis, cell death and cell migration, and the final one is a cluster of PI3K-Akt, MAPK and focal adhesion signaling pathway.

Besides OPN, altered expression of five proteins were detected in both transfected cell lines. In addition to OPN, we found significantly decreased expression of the OPN, EGFR, tenascin C and survivin. Galectin -3 and enolase 2 were significantly downregulated in the WM278 OPN-siRNA primary tumour originated cell line, in contrast, the expression of these two proteins were increased in the OPN-siRNA silenced metastasis originated WM1617 cells.

DISCUSSION

OPN is considered as one of the key molecules in the tumorigenesis, progression and metastatic dissemination of different malignancies, including melanoma. Several investigations of different tumour types have documented that OPN expression is related to metastatic potential and it is a useful diagnostic and therapeutic biomarker for different types of cancer.

The aim of our study was to better understanding the molecular mechanism of OPN expression during melanoma progression. Studying the specific molecular mechanisms involved during the progression of melanoma, can provide crucial insights into the diagnosis and treatment of the disease. Understanding these mechanisms may lead to the development of targeted therapies that can specifically inhibit or modulate OPN, thereby potentially slowing down or preventing the development of melanoma metastasis. Our work group using a high throughput Affymetrix gene expression array found that *OPN* had the highest expression levels in melanoma tissue samples which was associated with less favourable clinical outcome. Therefore, firstly we validated the aforementioned microarray results by qRT-PCR. In the analysis, we found increased level of *OPN* mRNA that was significantly associated with the later stage melanomas (Clark IV-V), with ulcerated surface and higher thickness (more than 4 mm). We extended our studies into the protein levels of OPN and therefore, we fabricated tissue microarray platforms in n=93 primary melanomas. By performing immunohistochemistry on the primary melanoma specimens, we observed the higher level of OPN protein in samples with poor prognosis. Although, our protein and gene expression results did not show clear relationship in all

samples, in average, we observed that the high level of *OPN* mRNA was associated with strong antigen positivity. The one of the reason for this phenomenon might be due to the relatively limited number of melanoma specimens. Furthermore, variations in the interactions between individual mRNA molecules and proteins can lead to differences in correlation. In melanoma, the significant heterogeneity within tumour tissues, aberrations in signalling pathways and some epigenetic changes may also influence the difference between mRNA and protein levels.

We also aimed to examine the effect of OPN expression and prognostic factors on metastasis formation. We found that increase of formation of metastasis in melanomas with higher thickness. Furthermore, we observed that high osteopontin expression emerged as a predictor of survival after thickness and formation of metastasis. Patients with high OPN expression (score of 2 or 3) had a 2.02-fold higher risk of death from disease. Elevated OPN expression is associated with advanced stage, tumour invasion and metastasis in multiple cancers.

Previously, published data support that OPN binding to integrin activates the phosphorylation cascade of the NF-κβ pathway, thus inducing increased nuclear translocation of the p50 and p65 subunits of NF-κβ to promote cell proliferation, survival, angiogenesis, tumour growth and metastasis. Using immunohistochemistry, we observed nuclear translocation of the NF-κβ p65 protein in a melanoma cell line (WM278) with high *OPN* mRNA expression, suggesting that OPN induces NF-κβ pathway activation. Melanoma cell lines with low OPN expression did not show NF-κβ p65 nuclear localization. This result strongly supports the hypothesis that the integrin-binding domain of OPN is crucial for metastasis formation in melanoma cells.

In the last decade, RNAi, which is a post-transcriptional mechanism for inhibiting gene expression, has shown promising results in molecular-targeting gene therapy for different types of cancer. In this study, we also aimed to inhibit osteopontin expression by RNAi in selected cell lines, which are characterized by high OPN expression. In addition, we examined the effect of transfection on the cells. We found that OPN expression can be downregulated using OPN-specific siRNA in primary and metastatic melanoma cell lines. We observed a decrease in cell proliferation and cell migration after effectively silencing the OPN gene.

Because silencing a gene can induce changes in the expression of different proteins, we used a Proteome Profiler Human XL Oncology Array to define the expression levels of 84 cancer-related proteins. Comparing the expression patterns of the original and transfected cell lines, we detected a number of differentially expressed proteins. The OPN protein expression levels decreased in both OPN-silenced cell lines (primary tumour-and metastasis-derived). Altered expression of other proteins included EGFR, tenascin C, survivin, galectin-3 and enolase 2. Marked reductions in the expression of OPN and the antiapoptotic protein survivin were detected in association with BRAF inhibitor resistance by us and others. Decreased tenascin C and survivin protein expression in the OPN-siRNA transfected cell lines are in very good agreement with the OPN expression levels, which play important roles in various metastasis-associated mechanisms, including cell proliferation, apoptosis, invasion and migration. The expression of tenascin C has been shown to be essential in cellular invasion and migration and is important during the development of metastasis. According to recent studies, there is a significant association between OPN and EGFR expression

in clear cell renal cell carcinoma. Similar to our findings, OPN inhibition led to decreased EGFR expression and increased apoptotic cell death. Apoptosis was significantly enhanced in OPN knockout mice and was accompanied by EGFR downregulation. Furthermore, we found that decreased OPN expression was associated with lower galectin-3 and enolase 2 protein levels in the primary tumour-derived transfected cell line; however, the expression of these proteins was increased in the metastatic cell line after OPN silencing. Galectin-3 expression is a marker and promoter of progression and metastasis in many tumours; interestingly, galectin-3 and OPN were proposed as potential targets (or at least predictors) in future personalized antiaging therapies. Galectin-3 and enolase 2 overexpression were detected in the transfected metastatic cell lines, and we assume that both play an important role in promoting the aggressive phenotype of melanoma cells. The simultaneous expression of these proteins and their role in tumour progression have not been previously described in melanoma.

We assume that the proteins altered in the OPN-siRNA silenced cells are involved in the following biological processes: extracellular matrix binding (galectin-3 and OPN) and protein dimerization (EGFR, galectin-3, enolase 2 and survivin). It was shown that down-regulation of galectin-3 and the other proteins were associated with decreased migration, invasion and reduced tumour growth. Nevertheless, galectin-3 has a regulatory role in cancer stemness related pathways, beside other pathways, the EGFR/FGFR pathway is also involved, and it was published that OPN induced migration and invasion is strongly associated with activation of different EGF receptors. Consequently, decreased level of OPN and other proteins might contribute to the less aggressive phenotype. Additionally, three of the altered

proteins (EGFR, OPN and tenascin C) are related to the PI3K-Akt signalling pathway. On the other hand, EGFR, galectin-3 and OPN potentially influence the extracellular signal-regulated RAF/MEK/ERK pathway, and both pathways are fundamental in melanoma tumorigenesis. EGFR, OPN and tenascin C have crucial role in the focal adhesion pathway, the multiprotein focal adhesion complexes silenced by OPN-siRNA can promote the connection between the extracellular matrix and cytoskeleton, and functionally control cell proliferation, differentiation, and motility. Alterations of these pathways are significantly important in the pathogenesis of melanoma by affecting tumorigenesis, cellular growth, chemoresistance, invasion and migration.

In summary, we have shown that tumour OPN expression is a strong predictor of poor prognosis. Multivariate analysis confirmed that OPN was a significant predictive factor for primary melanoma patient survival. Furthermore, we showed that a high expression level of OPN is associated with a more aggressive phenotype in melanoma. This study is the first to reveal protein expression differences between the untreated and OPN-siRNA-transfected melanoma cell lines. Our results suggest that silencing the OPN gene can promote the proliferation and invasion of melanoma cells by effecting EGFR, tenascin C, survivin, galectin-3 and enolase 2 expression.

In conclusion, we observed nuclear translocation of the NF-κβ p65 protein in a melanoma cell line with high *OPN* mRNA expression, suggesting that OPN induces NF-κβ pathway activation. We inhibited OPN expression using siRNA in melanoma cell lines and found decreased cell proliferation and invasion in vitro. Our findings suggest that OPN overexpression plays an

important role in the progression of melanoma and that it is possible to successfully inhibit OPN expression in melanoma cells; therefore, OPN overexpression may be a promising target for melanoma therapy.

MAIN STATEMENTS AND RESULTS

The main purpose of the doctoral thesis was to deeper understand the role of OPN expression during melanoma progression.

Investigation of OPN expression at the mRNA level in different subtypes of primary melanoma tissues and melanoma metastases:

- mRNA expression analysis of *OPN* showed an increased *OPN* level in advanced stage melanomas, with ulcerated surface and greater thickness.
- We found a significant increase in *OPN* mRNA levels during melanoma progression.

Analysis of the OPN protein expression in primary melanomas:

- OPN protein expression was associated with Breslow thickness and metastasis formation.
- We revealed that metastasis formation is more characteristic on primary melanomas with higher Breslow thickness.
- We examined the association between OPN expression levels and survival and found that high OPN expression was significantly associated with reduced survival in melanoma patients.

Determination of OPN gene expression patterns in melanoma cell lines derived from primary and metastatic tissues:

- We observed that the relative *OPN* mRNA expression significantly higher in the cell lines with metastatic potential.
- Comparing the relative osteopontin expression with BRAF mutational status of cell lines, we found that *OPN* expression was

significantly higher in BRAF mutant cells compared to wild-type BRAF cells.

Examining the effect of RNAi on selected cell lines which are characterized with high OPN expression:

- We successfully inhibited *OPN* mRNA and protein expression in the selected melanoma cell lines.
- We observed a reduction in cell proliferation and cell migration following effective silencing the OPN gene.

Protein array analysis of the parental and siRNA silenced melanoma cell line pairs:

- Our study is the first to reveal differences in protein expression between untreated melanoma cell lines and those transfected with OPN-siRNA.

In conclusion, we showed that a high expression level of OPN is associated with a more aggressive phenotype in melanoma. Our results revealed for the first time that silencing the OPN gene influences proliferation and invasion of melanoma cells by effecting EGFR, tenascin C, survivin, galectin-3 and enolase 2 expression. Taken together these data suggest that OPN might be an ideal target for drug development and therapies.

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Registry number: DEENK/17/2023.PL
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Candidate: Tímea Kiss
Doctoral School: Doctoral School of Health Sciences
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List of publications related to the dissertation

1. **Kiss, T.**, Jámbor, K., Koroknai, V., Szász, I., Bárdos, H., Mokánszki, A., Ádány, R., Balázs, M.: Silencing Osteopontin Expression Inhibits Proliferation, Invasion and Induce Altered Protein Expression in Melanoma Cells.
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