VILNIUS UNIVERSITY

Julija
MOZŪRAITIENĖ

New molecular targets among E3 ubiquitin ligases for the treatment of metastatic melanoma

SUMMARY OF DOCTORAL DISSERTATION

Medical and Health Sciences,
Medicine (M 001)

Vilnius 2020

The dissertation was prepared over the period from 2015 through 2020 at the Vilnius University. The research was conducted at the National Cancer Institute and the Life Sciences Center of Vilnius University.
The research was supported by the Research Council of Lithuania, doctoral studies were funded by the EU Structural Funds.

Scientific supervisor:
Dr. Živilė Gudlevičienė (National Cancer Institute; Medical and Health Sciences, Medicine – M 001);

This doctoral dissertation will be defended in a public meeting of the Dissertation Defence Panel:
Chairman – **Prof. Dr. Sonata Jarmalaitė** (Vilnius University, Natural Sciences, Biology – N 010).

Members:
Prof. Dr. Dainius Characiejus (Vilnius University, Medical and Health Sciences, Medicine – M 001);
Dr. Simona Kavaliauskienė (Oslo university hospital, Institute for cancer research, Natural Sciences, Biology – N 010);
Prof. Dr. Kęstutis Sužiedėlis (Vilnius University, Natural Sciences, Biochemistry – N 004);
Prof. Dr. Skaidra Valiukevičienė (Lithuanian University of Health Sciences, Medical and Health Sciences, Medicine – M 001).

The dissertation will be defended at a public meeting of the Dissertation Defence Panel at 13:00 on October 29, 2020 in the auditorium of prof. K. Pelcar of the National Cancer Institute. Address: Santariškių str. 1, Vilnius, Lithuania. Tel. +370 611 29056; e-mail: julija.mozuraitiene@nvi.lt

The text of this dissertation can be accessed at the library of Vilnius University (address: Universiteto str. 3, LT-01122, Vilnius, Lithuania) as well as on the website of Vilnius University: www.vu.lt/lt/naujienos/ivykiu-kalendorius

VILNIAUS UNIVERSITETAS

Julija
MOZŪRAITIENĖ

Naujų molekulinių taikinių metastazavusiai melanomai gydyti paieška tarp E3 ubikvitino ligazių

DAKTARO DISERTACIJOS SANTRAUKA

Medicinos ir sveikatos mokslai,
Medicina (M 001)

Vilnius 2020

Disertacija rengta 2015–2020 metais Vilniaus universitete.
Moksliniai tyrimai atlikti Nacionaliniame vėžio institute ir Vilniaus universiteto Gyvybės mokslų centre.
Mokslinius tyrimus rėmė Lietuvos mokslo taryba, doktorantūra buvo finansuojama ES struktūrinių fondų lėšomis.

Mokslinė vadovė:
dr. Živilė Gudlevičienė (Nacionalinis vėžio institutas; medicinos ir sveikatos mokslai, medicina – M 001).

Gynimo taryba:
pirmininkė – **prof. dr. Sonata Jarmalaitė** (Vilniaus universitetas, gamtos mokslai, biologija – N 010).

Nariai:
prof. dr. Dainius Characiejus (Vilniaus universitetas, medicinos ir sveikatos mokslai, medicina – M 001);
dr. Simona Kavaliauskienė (Oslo universitetinė ligoninė, Vėžio tyrimų institutas, gamtos mokslai, biologija – N 010);
prof. dr. Kęstutis Sužiedėlis (Vilniaus universitetas, gamtos mokslai, biochemija – N 004);
prof. dr. Skaidra Valiukevičienė (Lietuvos sveikatos mokslų universitetas, medicinos ir sveikatos mokslai, medicina – M 001).

Disertacija ginama viešame Gynimo tarybos posėdyje 2020 m. spalio mėn. 29 d. 13 val. Nacionalinio vėžio instituto K. Pelčaro auditorijoje. Adresas: Santariškių 1, Vilnius, Lietuva, tel. +370 611 29056; el. paštas: julija.mozuraitiene@nvi.lt

Disertaciją galima peržiūrėti Vilniaus universiteto bibliotekoje (Universiteto g. 3, LT-01122, Vilnius) ir VU interneto svetainėje adresu: *https://www.vu.lt/naujienos/ivykiu-kalendorius*

ABBREVIATIONS

BRAF– proto-oncogene *B-Raf*, serine/threonine-protein kinase *B-Raf*
c-Myc, c-Jun – transcription factors that activate the cell cycle
CI – confidence interval
DNA – deoxyribonucleic acid
DMSO – dimethyl sulfoxide
ERK/MAPK – an extracellular signal-regulated kinase involved in the MAPK/ERK signaling pathway.
FBW7 – protein 7 with a conserved F-sequence and WD repeat domain
HR – hazard ratio
KIT – receptor tyrosine kinase
MDM2 – protein ubiquitin ligase (mouse double minute 2 homolog)
MEK1/MEK2 – a dual-specificity mitogen-activated protein kinase 1/2 involved in the MAPK/ERK signaling pathway (MAP kinase/ERK kinase)
NRAS – proto-oncogene *NRAS* of the *RAS* gene family
p53/*TP53* – protein p53, a tumor suppressor gene encoding the p53 protein
PD-1 – programmed cell death protein-1 which regulates the immune response
PGR – a polymerase chain reaction
RITA – a low molecular weight compound that activates apoptosis in P53 and non-P53 cells (reactivation of p53 and induction of tumor cell apoptosis)
TMA – tissue microarrays

SUMMARY

1. INTRODUCTION

Skin melanoma is the most aggressive skin cancer among all forms of skin cancer. The incidence of melanoma is increasing every year. The global prevalence of melanoma has more than doubled over the last three decades. Although melanoma accounts for less than 5 % of all the cases of skin cancer, the mortality rate from melanoma is more than 70 % of all deaths from all skin tumors (Miller and Mihm, 2006). According to the Lithuanian Cancer Registry of the National Cancer Institute, the incidence of melanoma in Lithuania has doubled in the last decade and amounts to approximately 320 new cases per year (National Cancer Institute, Cancer Registry, 2012).

The main treatment for melanoma is surgical removal of the tumor, but in the presence of advanced melanoma and the occurrence of distant metastases, additional targeted therapy or immunotherapeutic treatment is required. Metastasis accounts for the majority of melanoma-related deaths (Avilés-Izquierdo *et al.*, 2016; Bowyer *et al.*, 2016). A decade ago, the median survival of patients with metastatic melanoma was 8-9 months, and the overall survival of three years was less than 15 % (Balch *et al.*, 2009). Significant progress has been made in recent years to understand the disease at the molecular level, and deepened knowledge of the molecular pathogenesis of melanoma has led to the search for new drugs to treat melanoma. Melanoma treatment has changed, classical melanoma therapy has been replaced by new selective drugs, and a new approach to personalized melanoma treatment has emerged.

Knowledge of oncoimmunology and research about signaling pathways and their interactions in melanoma cases have enabled the identification of new targets (signaling pathway inhibitors,

modulators of the immune response) for the effective treatment of melanoma. An extensive examination of patients and the identification of specific biomarkers and gene mutations allow to select a personalized, specific treatment for melanoma for a particular patient and thus achieve better treatment results. Several main groups of medical preparations that replace the classical melanoma therapy are target therapy: selective *BRAF* (protooncogene B-Raf, serine/threonine kinase) inhibitors (Vemurafenib, Dabrafenib, Encorafenib), specific MEK1/MEK2 (a dual-specificity mitogen-activated protein kinase) inhibitor (Trametinib) and immunotherapy: cytotoxic T-lymphocyte antigen 4 (CTLA-4) monoclonal antibody (Ipilimumab), programmed cell death protein-1 (PD-1) inhibitor antibody (Nivolumab, Pembrolizumab).

Since 2011, with the introduction of new medicines, the median overall survival of patients with inoperable or metastatic melanoma has increased from 6 to 8 months up to 2 years (Larkin *et al.*, 2014; Long *et al.*, 2015; Robert *et al.*, 2015). Although new treatment strategies are effective in many cases, the problem of developing the resistance to treatment remains, leading to poorer treatment outcomes. Therefore, research is needed to identify new therapeutic targets, to discover appropriate medicines and their combinations with other medications and other treatments for the treatment of metastatic melanoma. Knowing the gene mutations in a particular patient with melanoma, the changes in the expression of the proteins encoded by gene mutations, an effective (personalized) target therapy can be administered the advantage of which is using small molecules, inhibitors or activators, or antibodies to affect the mutated proteins that play a decisive role in the progress of the disease.

E3 ubiquitin ligases, which are specific enzymes of the ubiquitin-proteasome system, are attracting the attention of researchers as a target for medicines because they are related to the functions of many proteins and the interactions among them.

Various E3 ligase complexes may act as oncogenes or tumor suppressors in the course of the development of melanoma. These proteins regulate the functions of various signaling pathways and proteins such as p53 and Notch. According to the data obtained by diverse studies, the decreased expression of E3 ubiquitin ligase FBXW7 is associated with a poorer five-year survival rate in melanoma patients (Aydin *et al.*, 2014), the increased c-Myc expression is associated with distant metastases in melanoma and poor prognosis (Lin *et al.*, 2017), and the decreased MDM2 expression is associated with better survival in melanoma patients (Manfredi, 2010). Individual researchers have made an attempt to identify gene products whose expression changes as the disease progresses, which could be both new, potential molecular targets in the treatment of melanoma, and new prognostic markers for anticipating the progress of the disease (Aydin *et al.*, 2014; Morrow *et al.*, 2015). In the case of melanoma, the increased Notch signaling pathway expression and the decreased p53 expression have been found (Sczaniecka *et al.*, 2012; Palmieri *et al.*, 2015).

The disruption of these signaling pathways in melanoma is related to changes in the expression and activity of the E3 ubiquitin ligases FBXW7 and the MDM2 oncogene as the regulator of p53. Nutlin-3 and RITA have been identified as the new generation inhibitors of the E3 ubiquitin ligases that disrupt the MDM2-p53 interactions in tumors and restore the apoptotic function of p53 (Panayi *et al.*, 2013). Oridonin activates FBXW7 and thus provides the arrest of the cell cycle and the apoptosis of tumor cells (Huang *et al.*, 2012). The study aimed at the determination of the dependences of the above-mentioned E3 ubiquitin ligases and the expression of their substrates p53 and c-Myc proteins on the stages of melanoma that would help to evaluate their significance as potential targets for the treatment of melanoma, while the MDM2-p53 interaction inhibitors nutlin-3 and RITA as well as oridonin, the activator of E3 ubiquitin ligases FBXW7, will be able to induce the apoptosis in metastatic melanoma cells and arrest the growth and progress of the tumor, in this way helping to model a

personalized mode in melanoma treatment. Therefore, our dissertation is devoted to the search for new molecular targets for the treatment of melanoma among E3 ubiquitin ligases.

1.1. The aim of the dissertation

The aim of this dissertation was to determine the role of the expression of E3 ubiquitin ligases FBXW7 and MDM2, and their substrates p53 and c-Myc in the development of metastatic melanoma and to evaluate possible new molecular targets for the treatment of this disease.

1.2. The objectives of the dissertation:

1. To determine the effect of the E3 ubiquitin ligase FBXW7 activator oridonin and E3 ubiquitin ligase MDM2 and the tumor suppressing protein p53 (MDM2-p53) interaction suppressors nutlin-3 and RITA and their combinations (nutlin-3 and RITA; oridonin and RITA; oridonin and nutlin-3, oridonin, nutlin-3 and RITA) on the viability of the human metastatic melanoma SK-MEL-26, FM-94 and FM-3 cell lines.
2. To determine the effect of E3 ubiquitin ligase FBXW7 activator oridonin and E3 ubiquitin ligase MDM2 and tumor suppressor protein p53 (MDM2-p53) inhibitors nutlin-3 and RITA on *NUMB, FBW7, MDM2, MDM4, NOTCH1, NOTCH2, NOTCH2, NOTCH BBC3, p53, c-MYC, Jun, Parp, CCNE1, CCND1, JAG, LNX1* gene expression in human metastatic melanoma SK–MEL–26 and FM–94 cell lines.
3. To determine the expression of E3 ubiquitin ligases FBXW7 and MDM2 and their substrates p53 and c-Myc in the postoperative material of patients with dysplastic moles and

melanoma and to compare them with clinical and pathological characteristics of melanoma.

4. To evaluate the significance of the investigated molecular biomarkers as potential targets for the treatment of metastatic melanoma.

1.3. Scientific novelty and practical significance of the dissertation

Over the period from 2012 through 2015, an increased interest in the use of E3 ubiquitin ligases and potential inhibitors of these enzymes in the treatment of cancer was observed globally. The researchers aimed to identify gene products whose expression changes as the disease progresses. Nutlin-3 and RITA are known to inhibit the development of melanoma cells, but until 2015, little was known about the effects of oridonin on metastatic melanoma cells. There were also no data on the effects of nutlin-3/RITA and oridonin and their combination on melanoma cells. According to individual authors, on the one hand, the increased expression of the *MDM2* gene in melanoma cases leads to an increased p53 degradation, Notch signaling pathway activation and tumor cell proliferation (Manfredi, 2010), on the other hand, nutlin-3 may adversely affect MDM2-p53 interactions and thus have an inhibitory effect on melanoma. However, the results of studies on the changes in *MDM2* expression and the function after treatment with nutlin-3 are not yet available (Pishas *et al.*, 2014).

In the course of this study, after carrying out *in vitro* experiments using human metastatic melanoma cell lines, based on the data available to us, for the first time in scholarly world, we have comprehensively evaluated the impact of MDM2-p53 interaction inhibitors nutlin-3 and RITA, E3 ubiquitin ligase FBXW7 activator oridonin and their combinations (nutlin-3 and RITA; oridonin and RITA; oridonin and nutlin-3; oridonin,

nutlin-3 and RITA) on the viability of melanoma cells depending on the inhibitors and activator, the concentration of their combinations and the exposure time. The effects of these inhibitors (nutlin-3, RITA) and the activator (oridonin) on the expression of genes involved in the regulation of p53 and MDM2 and affecting the cell cycle, proliferation and death pathway in human metastatic melanoma cells were also investigated.

In an *in vivo* study using postoperative material from patients with melanoma or dysplastic moles, the expression of the E3 ubiquitin ligases FBXW7 and MDM2 and their substrates p53 and c-Myc was determined at the protein level.

The association between the changes in the expression of the E3 ubiquitin ligases FBXW7 and MDM2 and their substrates p53 and c-Myc and in the clinical and pathological characteristics of patients was evaluated. A univariate and multivariate *Cox* regression analysis was performed to evaluate the significance of changes in the expression of the studied proteins, clinical-pathological characteristics and their interaction for the risk of death in patients.

Low expression levels of the E3 ubiquitin ligase FBXW7 were found to be associated with melanoma depth (pT3 and pT4) and the morphological melanoma type (nodular melanoma type) and could therefore be a potential molecular target for the treatment of melanoma. After examining the metastatic melanoma cells, oridonin was found to be the most effective in suppressing the melanoma cell viability, therefore oridonin, as an activator of FBXW7, could be a potential therapeutic factor in the treatment of melanoma.

2. MATERIALS AND METHODS

2.1. Characteristics of the study group

The human melanoma cell lines and the samples from patients (postoperative material) were used in this study.

Melanoma cell lines. The human metastatic melanoma cell lines SK-MEL-26, FM-94 and FM-3 used in the study were obtained from the Latvian Center for Biomedical Research and Studies. The SK-MEL-26 cell lines confirmed *BRAF* mutation and wild-type *NRAS and TP53*. The FM-94 and FM-3 cell lines confirmed *BRAF, NRAS* mutations and wild-type *TP53*. The cells of the FM-94 line were obtained from a male patient with metastatic malignant skin melanoma, and FM-3 and SK-MEL-26 were taken from a female patient.

Samples from patients. Prior to conducting the study, the permission for biomedical research was received from the Vilnius Regional Biomedical Research Ethics Committee (No. 158200–16–878–387, 2016–12–13). The patients who participated in the study, had signed the Personal Information and Informed Consent forms.

The results of the study were obtained by using TMA for examining the operative biological material of 100 patients. In all, 16 patients diagnosed with dysplastic moles, 16 – *in situ* melanoma, 17 – stage pT1 melanoma, 17 – stage pT2, 17 – stage pT3 and 17 – stage pT4 melanomas were included as the subjects of the study. All the samples were evaluated for FBWX7, c-Myc, MDM2, and p53 protein expression by points ranging from 0 to 3 and as percentages based on nuclear staining intensity. Subsequently, the protein expression was divided into high and low by combining the immunohistochemical staining intensity scores and the nuclear staining intensity percentages. Of 100 cases under analysis, 39 were men and 61 – women, with the mean age of 54.1 years. According to the morphological type of melanoma, 48 cases of superficial

melanoma, 27 cases of nodular melanoma and 9 cases of *lentigo maligna* were detected. In 77 % of cases, the tumor was diagnosed in sun-exposed areas. Of all melanoma cases analyzed: 26 had melanoma depths of less than 1.0 mm while 42 cases – larger than 1.1 mm. Of the 68 melanomas ranging from stages pT1 to pT4, 22 were ulcerated. The clinicopathological characteristics of the subjects are presented in Table 1.

The following clinical characteristics of patients and pathological skin structures were collected and evaluated from case histories: gender, age of patients, tumor stage by depth (pT), morphological tumor type, ulceration, and localization.

Table 1. Clinicopathological characteristics of the study group.

Characteristics		**n**
Total cases		**100**
Gender	Men	39
	Women	61
Age	≤58	44
	>58	56
Tumor stages by depth (pT)	Dysplastic mole	16
	Melanoma *in situ*	16
	pT1	17
	pT2	17
	pT3	17
	pT4	17
Morphological type of tumor	Superficial spreading melanoma	48
	Lentigo maligna melanoma	9
	Nodular melanoma	27
	Dysplastic mole	16
Depth of tumor invasion	0.1– 1.0 mm	26
	1.1 mm +	42
	Without invasion	32
Tumor localization	Sun non-exposed skin	13
	Sun exposed skin	77
	Unknown	10
Melanoma ulcer (pT1–pT4)	Total	68
	Without ulceration	46
	With ulceration	22

2.2. Research methods

2.2.1. Sample preparation

- SK-MEL-26, FM-94 and FM-3 human metastatic melanoma cell lines were cultured in DMEM (*Dulbecco's modified eagle medium*) medium with 10 % of FBS (*fetal bovine serum*), 2 mML – glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, under conditions of 37°C, 5 % of CO_2 and 95 % humidity in a CO_2 incubator. The cells were seeded every 2-3 days. The number of seed cells was determined using a Neubauer hemocytometer chamber (*HEINZ HERENZ Medizinalbedarf GmbH*, Germany).
- The cells were incubated in a CO_2 incubator for 24 hours. Later the cells in the wells were treated with different concentrations of inhibitors: nutlin-3 (30 μM, 20 μM, 16 μM), RITA (1.6 μM, 1 μM, 0.5 μM), oridonin activator (120 μM, 60 μM, 30 μM, 30 μM, 15 μM, 2.5 μM) and combinations (20 μM nutlin-3 + 0.5 μM RITA, 30 μM oridonin + 0.5 μM RITA, 30 μM oridonin + 20 μM nutlin-3, 30 μM oridonin+20 μM nutlin-3 + 0.5 μM RITA). After exposure, the cells were incubated for 24, 48, and 72 hours.
- The postoperative material was selected from 100 patients who over the period from 2013 through 2018 were diagnosed with dysplastic moles or melanoma that were surgically removed at the National Cancer Institute (NCI) and morphologically confirmed at the State Pathology Center (SPC) of Vilnius University Hospital Santara Clinics. Tissue microarrays (TMA) preparation, histological and immunohistochemical (IHC) analysis, and visual pathologist evaluation of the scanned images were performed at SPC. In all samples, the expression of FBXW7, c-Myc, MDM2, and p53 proteins was scored on a scale of 0 to 3 and in percent according to the intensity of nuclear staining.

- Tissue microarrays were constructed from 10 % paraffin-impregnated tissue blocks fixed in buffer formalin solution. Tissue paraffin blocks were reviewed and selected by microarrays by a physician pathologist. The samples of 2 mm in diameter (1 per patient) were taken from the tumor sites marked by the pathologist. In this way, 2 TMAs were constructed using a tissue microarray preparation apparatus (*3DHISTECH, TMA Master*, Budapest, Hungary).

2.2.2. Determination of cell viability

Cell viability was assessed after exposure with the inhibitors (nutlin–3/RITA) and the activator (oridonin) using MTT dye (3- [4,5-dimethylthiazol-2-yl] -2,5-diphenyl tetrazol) and *Vybrant® MTT* cell proliferation analysis tool (*Molecular Probes*, USA). Depending on the amount of NAD(P)H in the cell, the color indicator – tetrazol salt MTT of many dehydrogenases is reduced to a strong chromophore – an insoluble compound of formazan. The amount of formazan is proportional to viable cells. Its concentration is determined spectrophotometrically by measuring the optical densities of all the samples. An optical density at 540 nm wavelength is measured with a spectrophotometer (Mindray MR-96A, China). The obtained optical densities of the samples were processed using the *Microsoft Office Excel 2016* program.

2.2.3. Quantitative PCR

RNA was isolated from cells using the *GeneJET RNA Purification Kit (Thermo Scientific*, Lithuania). The total amount of RNA was determined by spectrophotometer (*Thermo Scientific Nanodrop 200c*, Lithuania) and integrity was found by 2 % agarose gel electrophoresis. kDNA synthesis was performed using the *RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific*, Lithuania) and the synthesis kit.

cDNA synthesis was performed on an automated thermocycler (*Eppendorf Thermomixer*, Germany) according to the manufacturer's recommendations. RNA obtained by treating SK-MEL-26, FM-94 cells with a control solution, inhibitors of nutlin-3/RITA and activator oridonin was used for cDNA synthesis. The synthesized cDNA was used as a template for the quantitative PCR reaction (Table 8).

Table 8. Direct and reverse primers of the selected genes used for quantitative PCR reaction.

Gene	Primer	Nucleotide sequence
HPRT1	direct	5'-CTGAAGAGCTATTGTAATGACCAG-3'
	reverse	5'-CCTGACCAAGGAAAGCAAAG-3'
TBP	direct	5'-GTACCGCAGCTGCAAAAT-3'
	reverse	5'-CTCATGATTACCGCAGCAAA-3'
p53	direct	5'-CTCACTCCAGCCACCTGAA-3'
	reverse	5'-AAGTGGAGAATGTCAGTCTGAGTC-3'
NUMB	direct	5'-TGCAGTGCCAGAAGTAGAAGG-3'
	reverse	5'-AGCAGATGAGAAGGGGTCCT-3'
MDM4	direct	5'-TGCATTCTTGCCTAGTTTTCC-3'
	reverse	5'-AGCACAATTAAAAAGTATTAAAAGCA-3'
MDM2	direct	5'-GACCCTGGTTAGACCAAAGC-3'
	reverse	5'-TGTTGCTTCTCATCATATAATCGTT-3'

Table 8. Continued

Gene	Primer	Nucleotide sequence
NOTCH1	direct	5'-GCAGAACAACAGGGAGGAGA-3'
	reverse	5'-CAGCAGCCTCACGATGTC-3'
NOTCH2	direct	5'-GGAGGAGGCGACCGAGAA-3'
	reverse	5'-GCCTTCTGGACATTTGCAGTATC
NOTCH3	direct	5'-CCTGTGGCCCTCATGGTATC-3'
	reverse	5'-CATGGGTTGGGGTCACAGTC -3'
FBXW7	direct	5'-ATGTTTCAGAGCTGGAGTGGA-3'
	reverse	5'-TGCCAACTCTTTAGGGAGCA-3'
LNX1	direct	5'-GGAATTACCACGGTGCTTGTAT-3'
	reverse	5'-TGTATGCTGGTGTTCCTTCAAC-3'
BBC3	direct	5'-GGAGACAAGAGGAGCAGCAG-3'
	reverse	5'-GCACCTAATTGGGCTCCATC-3'
c-Myc	direct	5'-CAGCGACTCTGAGGAGGAAC-3'
	reverse	5'-GCTGTGAGGAGGTTTGCTGT-3'
JUN	direct	5'-CCAATTTGGAATCTTCTCTTTGA-3'
	reverse	5'-TTTTGGTATTTGAATACATTTATTGTG-3'
CCNE1	direct	5'-CTTGGATTTGCTGGACAAAG-3'
	reverse	5'-GCTCTGCTTCTTACCGCTCT-3'
CDKN1A	direct	5'-GCATGACAGATTTCTACCACTCC-3'
	reverse	5'-AAGATGTAGAGCGGGCCTTT-3'
CCND1	direct	5'-TGTGCCACAGATGTGAAGTT-3'
	reverse	5'-CGGGTCACACTTGATCACTC-3'
CDKN2A	direct	5'-CAGACATCCCCGATTGAAAG-3'
	reverse	5'-TTTTCTAAATGAAAACTACGAAAGC-3'
PARP9	direct	5'-GGATGGACTTTTCCATGGTG-3'
	reverse	5'-TCCCCTTTCTCCACTGAGG-3'
JAG2	direct	5'-CAGCTGGACGCCAATGAGT-3'
	reverse	5'-CCCGGGATGCAATCACAGTA-3'

The amplification product was detected using *Sybr Green PCR Mix* (2x) reagents (*Thermo Scientific*, Lithuania). The relative change in gene expression was calculated by the comparative critical threshold value method using a real-time PCR system (*Eppendorf Mastercycler epgradient S*, USA). *HPRT1* and *TBP* "housekeeping" genes were amplified to normalize the expression of the studied genes.

Transcript counts of the test genes – changes in gene expression (by times) that are comparable to the number of transcripts in cells untreated with reagents (control) were calculated according to the formula (1):

$$k = 2^{-\Delta\Delta Ct}$$

In the formula, the degree indicator is expressed by the equation (2):

$$\Delta\Delta Ct = (Ct\ gene - Ct\ HPRT1\ or\ TBP)\ after\ exposure\ to\ reagents - (Ct\ gene - Ct\ HPRT1\ or\ TBP)\ control.$$

Ct (*threshold cycle*) is a value that indicates how many amplification cycles were required to capture the signal of the test sample.

The threshold value from which a significant increase/decrease in gene expression is observed is considered when the value of the change in gene expression (by times) is ≥ 2 or ≤ 2.

2.2.4. Immunohistochemical (IHC) analysis of tissue microarrays

The TMAs were constructed from 10 % buffered formalin-fixed paraffin-embedded (FFPE) tissue blocks; 2 mm diameter cores were punctured from the tumor block as randomly selected by a pathologist (1 core per patient), thus producing 2 TMAs constructed using the tissue arraying instrument (3DHISTECH, TMA Master). The TMA blocks for immunohistochemistry were cut into 2 μm-thick sections

and mounted on TOMO adhesion glass slides (Matsunami Glass, Osaka, Japan). The sections were deparaffinized, dehydrated and antigen retrieval for p53 and c-Myc was performed using DAKO PTLink system with EnVision FLEX Target buffer (pH 8.0) at 95°C for 20 min (Dako; Agilent Technologies, Inc.), and for FBXW7 and MDM2, Ventana Benchmark Ultra system was used with Cell Conditioning solution (pH 8.5) at 100°C for 36 min. (Ventana Medical Systems). The sections were subsequently incubated with p53 (cat. no. DO-7; Dako; Agilent Technologies, Inc.; 1:200) and c-Myc (cat. no. Y69; Abcam; 1:40) at room temperature for 30 min using a DAKO EnVision FLEX system. Incubation with FBXW7 (cat. no. OT16F5; Invitrogen; 1:50) and MDM2 (IF2; Invitrogen; Thermo Fisher Scientific, Inc.; 1:250), was performed using Ventana Ultraview DAB detection kit at 37°C for 32 min. Finally, the sections were developed using DAB, counterstained using Mayer's hematoxylin at room temperature for 10 min. each and mounted. Negative controls were performed by omitting the application of the primary antibody. The IHC slides were digitized at x20 objective magnification (0.5 μm resolution) using a bright field ScanScope XT Slide Scanner (Leica Aperio Technologies, Vista, CA, USA).

2.2.5. Statistical data analysis

Student's test was applied to FM-94 and FM-3 melanoma cell viability to assess how significantly the results obtained (percentage cell viability) differ from control (DMSO). Three tests were performed with the appropriate concentrations of inhibitors (nutlin-3/RITA) or the activator oridonin and their combinations to record the optical density values of the samples. An arithmetic mean was derived from all the values of the three tests. The mean value was compared with the control mean value and the difference was expressed by percentage. The standard deviation was also estimated to evaluate how

much the obtained values differed from the mean value. Data normality was assessed using the Kolmogorov–Smirnov test.

One-way analysis of variance (ANOVA) was performed to compare the values of the viability of SK-MEL-26 line cell after exposure to oridonin. The Holm-Sidak test was used to compare the paired values.

Changes in the expression of the genes under analysis (by times) were calculated using the Ct formula (described in Section 2.2.3. Quantitative PCR).

The threshold value from which a significant increase/decrease in the gene expression is observed is considered when the value of the change in gene expression (by times) is ≥ 2 or ≤ 2.

The statistical analysis of protein expression findings in patients' postoperative material was performed using the STATA 11.2 (*StataCorp LP*, TX, USA) program. The intensity of protein immunostaining was scored as 0-3: 0, Negative; 1, weak; 2, moderate and 3, strong. The percentage of nuclear staining was graded in 4 categories: 0-25 %, 1; 26-50 %, 2; 51-75 %, 3; and 76-100 %, 4. The combined score was graded as: 0-6, Low; and 7-12, high staining. The χ^2 (Chi-square) test was used to assess the association between the changes in protein expression and clinical and pathological characteristics of patients.

The sample size was calculated using a program developed by the University of Düsseldorf http://www.gpower.hhu.de/. The sample size was calculated so that at a significance level of 0.05, the power of the criterion would be sufficient to detect statistically significant changes. It was estimated that at a significance level of α=0.05 (β=0.20) for an analytical study of a single-group, the sample size should consist of 85 samples. In all, 100 samples were used in this study. When comparing the two groups (32 non-invasive tumors and 68 melanomas) at α=0.05 (β=0.20, standard deviation SD=1), the calculated power for the study was 80 % and the effect size 0.593. To check for the differences in individual subgroups (by pT stages), at a significance level of 0.05 (β=0.20, SN=0.5), 17 samples in each group are sufficient.

Univariate and multivariate *Cox* regression analysis was performed to assess which of the studied protein expression changes, clinical-pathological characteristics, and their interactions are relevant to patients' risk of death.

The study evaluated the hazard ratio (HR), adjusted HR and 95 % confidence intervals. p value <0.05 was considered statistically significant. When the value of p ranged from 0.05 to 0.1, the differences were considered biased.

The experiments with cell lines were performed at the Life Sciences Center.[1]

[1]We would like to extend our sincere gratitude to dr. Violeta Jonušienė, dr. Danutė Labeikytė, Vilius Vilčinskis and Aistė Petrovaitė. Ieva Vincerževskienė acted as consultant for statistical calculations.

3. RESULTS

3.1. Evaluation of the effect of MDM2-p53 interaction suppressors nutlin-3 and RITA and E3 ubiquitin ligase FBXW7 activator oridonin on cell viability in human metastatic melanoma SK-MEL-26 cell line

Effects of MDM2-p53 interaction suppressors nutlin-3 and RITA on the viability of the SK-MEL-26 cell line. After 48 hours of incubation, both the suppressors of MDM2-p53 interaction (nutlin-3 and RITA**)** did not have a significant effect on the viability of the cells of the SK-MEL-26 line – the cell viability decreased only to 85 %. Later the effect of higher exposure (after 72 h of incubation) on the cells was evaluated.

The cell viability did not change after 72 hours like after 48 hours. After exposure to the MDM2-p53 interaction suppressor nutlin-3 at its concentration 12 μM SK-MEL-26 cell viability remained high (98.73 %), while after exposure to the combination of MDM2-p53 interaction suppressors RITA (0.5 μM) and nutlin-3 (6 μM), the viability of the cells of the SK-MEL-26 line lightly reduced (up to 81.15 %).

The data obtained from the study showed that the MDM2–p53 interaction suppressors nutlin-3 and RITA had no significant effect on cell viability of the SK-MEL-26 line after 48 and 72 hours of incubation.

Effect of E3 ubiquitin ligase FBXW7 activator oridonin on viability of SK-MEL-26 cell line. After 24 hours of incubation the cell viability of the SK-MEL-26 line was most effectively affected by 60 μM and 120 μM oridonin concentrations – cell viability decreased to respectively 30 % and 24 %.

After 48 hours and 72 hours of incubation the same trend in the viability of the tested SK-MEL-26 line cells were observed, i. e. higher oridonin concentrations (60 μM and 120μM) had the most effect on the cell viability – the cell viability decreased to 18 % (Fig. 1).

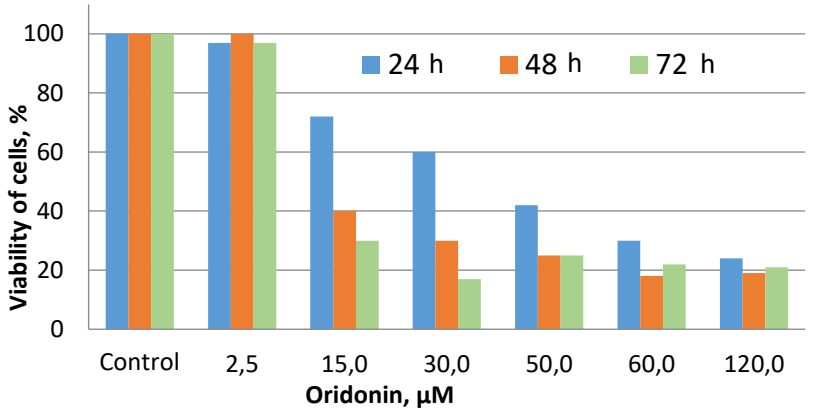

Figure 1. Viability of SK-MEL-26 line cells after exposure to oridonin (2.5 μM, 15 μM, 30 μM, 60 μM, and 120 μM) after 24, 48, and 72 hours of incubation

The data obtained from this study show that the FBXW7 activator oridonin was effective in suppressing the viability of SK-MEL-26 cells dependent on the time of exposure and the dose, oridonin at a concentration of 60 μM was most effective after 48 hours of incubation.

In order to more accurately assess the effect of MDM2-p53 interaction suppressors nutlin-3 and RITA and E3 ubiquitin ligase FBXW7 activator oridonin on melanoma cell viability, the research was continued with different cell lines by treating the cells with higher concentrations of MDM2-p53 interaction suppressors and FBXW7 activator, as well as their combinations.

3.2. Evaluation of the effect of MDM2-p53 interaction suppressors nutlin-3 and RITA and E3 ubiquitin ligase FBXW7 activator oridonin and their combinations on the viability of FM-3 and FM-94 lines

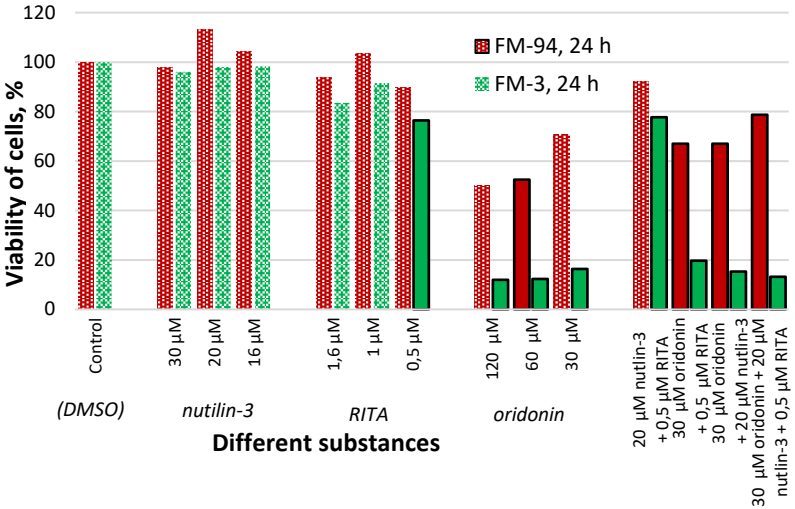

Figure 2. Viability of FM-94 and FM-3 cells after exposure to combinations of nutlin-3 (30 μM, 20 μM, 16 μM), RITA (1.6 μM, 1 μM, 0.5 μM), oridonin (120 μM, 60 μM, 30 μM) inhibitors and activator: nutlin-3 (20 μM) and RITA (0.5 μM); oridonin (30 μM) and RITA (0.5 μM); oridonin (30 μM) and nutlin-3 (20 μM); oridonin (30 μM), nutlin-3 (20 μM) and RITA (0.5 μM) (after 24 h of incubation). The highlighted bars show a statistically significant decrease in cell viability.

Following 24 hours of incubation (Fig. 2):

- All the concentrations of the MDM2-p53 interaction suppressor nutlin-3 did not significantly affect the cell viability of FM-94 and FM-3 lines.
- Cells of the FM-94 and FM-3 lines were resistant to RITA, an inhibitor of the MDM2-p53 interaction.

- After exposure to E3 ubiquitin ligase FBXW7 activator oridonin (120 μM, 60 μM, 30 μM), cell viability was significantly reduced in both lines. The viability of FM-94 (according to the order of the indicated concentrations) was 50.24 %, 52.46 % and 70.82 %, and FM-3 cells – 11.95 %, 12.30 % and 16.34 %.
- The combination of MDM2-p53 interaction suppressors nutlin-3 (20 μM) and RITA (0.5 μM) had no significant effect on cells in both lines.

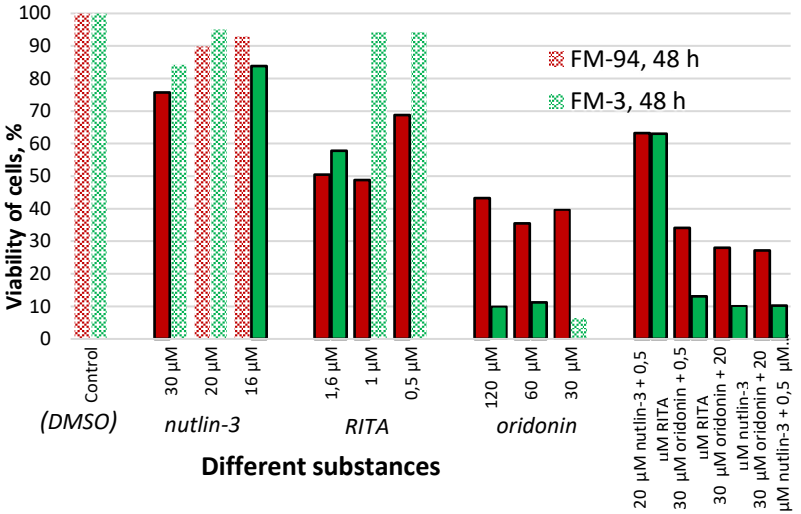

Figure 3. Viability of FM-94 and FM-3 cells after exposure to nutlin-3 (30 μM, 20 μM, 16 μM), RITA (1.6 μM, 1 μM, 0.5 μM), oridonin (120 μM, 60 μM, 30 μM) combinations of suppressors and activator: nutlin-3 (20 μM) and RITA (0.5 μM); oridonin (30 μM) and RITA (0.5 μM); oridonin (30 μM) and nutlin-3 (20 μM); oridonin (30 μM), nutlin-3 (20 μM) and RITA (0.5 μM) (after 48 h of incubation). The highlighted bars show a statistically significant decrease in cell viability.

Following 48 hours of incubation (Fig. 3):

After 48 hours of incubation, cell viability of both lines showed a larger decrease than after 24 hours of incubation.

- After treatment of FM-94 and FM-3 line cells with MDM2-p53 interaction suppressor nutlin-3 (30 μM, 20 μM, 16 μM) after 48 hours as after 24 hours of incubation, cells in both lines were sufficiently resistant and cell viability remained high.
- Due to the effect of the MDM2-p53 interaction suppressor RITA (1.6 μM, 1 μM, 0.5 μM) after 48 hours, as after 24 hours, the cell viability of both lines was slightly reduced: by the order of the indicated concentrations, respectively 50.47 %, 48.81 % and 68.78 %, FM-3 – 57.81 %, 94.30 % and 94.22 %.
- The effect of all concentrations of FBXW7 activator oridonin (120 μM, 60 μM, and 30 μM) on cell viability in both lines was more effective than after 24 hours. The cell viability of the FM-3 line (according to the indicated concentrations, respectively) was 9.93 %, 11.22 %, 6.37 %, and the cell viability of the FM-94 line was 50.24 %, 52.46 % and 70.82 %.
- The combination of MDM2-p53 interaction suppressors nutlin-3 (20 μM) and RITA (0.5 μM) had a small effect on cell viability of both lines, but showed a larger decrease in cell viability compared to that observed after 24 hours of incubation.

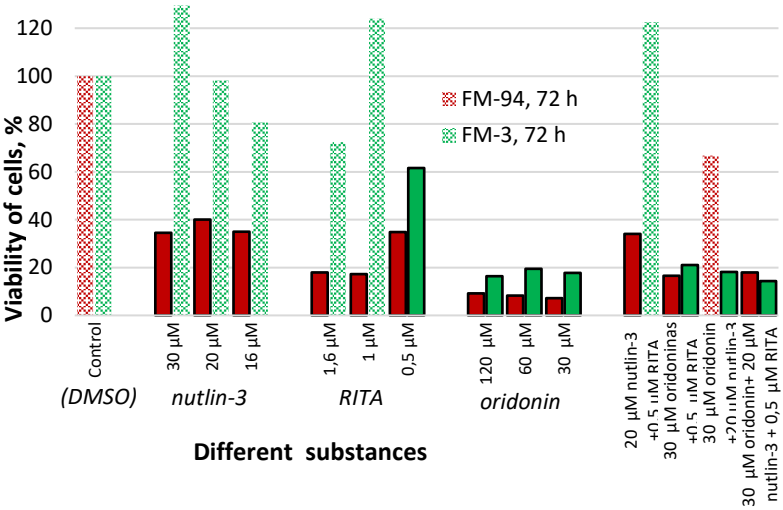

Figure 4. Cell viability of FM-94 and FM-3 lines exposed to nutlin-3 (30 μM, 20 μM, 16 μM), RITA (1.6 μM, 1 μM, 0.5 μM), oridonin (120 μM, 60 μM, 60 μM, 30 μM), combinations of suppressors and activator: nutlin-3 (20 μM) and RITA (0.5 μM); oridonin (30 μM) and RITA (0.5 μM); oridonin (30 μM) and nutlin-3 (20 μM); oridonin (30 μM), nutlin-3 (20 μM) and RITA (0.5 μM) (after 72 h of incubation). The highlighted bars show a statistically significant decrease in cell viability.

Following 72 hours of incubation (Fig. 4):

After 72 hours of incubation the viability of FM-94 line cells decreased and that of FM-3 line – increased unlike after 24 hours and 48 hours of incubation.

- After 72 hours of inhibition MDM-p53 interaction suppressor nutlin-3 (30 μM, 20 μM, and 16 μM) reduced the viability of FM-94 line cells more effectively than after 24 and 48 hours of incubation. However, at a concentration of 30 μM of nutlin-3, the opposite effect occurred – the

viability of FM-3 line cells increased and reached 129.47 %.

- Due to the effect of the MDM2-p53 interaction suppressor RITA (1.6 μM, 1 μM, 0.5 μM), the viability of FM-94 line cells (according to RITA concentrations, respectively) decreased respectively by 17.94 % and 17.25 % and 34.82 %.
- The effect of all concentrations of FBXW7 activator oridonin (120 μM, 60 μM, and 30 μM) on cell viability of both lines was largest and more effective than after 24 and 48 hours of incubation. The study found that cells of the FM-94 line were more sensitive to the effects of the activator oridonin (120 μM, 60 μM, and 30 μM), and the concentration of 30 μM oridonin was most effective in reducing the viability of FM-94 line cells (up to 7.21 %).
- Exposure of FM-94 cells to combinations of suppressors and activator reduced cell viability more than after 24 and 48 hours of incubation.

Thus, summarizing the obtained cell viability data for FM-94 and FM-3 lines after 24, 48, and 72 hours of incubation, it was found that the cell viability of both lines (FM-94 and FM-3) was most effectively affected by oridonin, the activator of E3 ubiquitin ligase FBXW7. The viability of FM-3 line cells was most effectively suppressed by oridonin concentrations of 120 μM (after 24, 48 and 72 h), while FM-94 line cells were most effectively suppressed by 30 μM oridonin concentration after 72 hours of incubation. Cell viability of FM-94 line after 72 hours of incubation was also well suppressed by MDM2-p53 interaction suppressors nutlin-3 (20 μM) and RITA (0.5 μM), oridonin (30 μM) and RITA (0.5 μM) and oridonin (30 μM), nutlin-3 (20 μM) and RITA (0.5 μM) combinations.

3.3. Analysis of changes in gene expression

To determine which genes are active or inactivated by treatment of FM-94 and SK-MEL-26 cells with MDM2–p53 interaction suppressors nutlin-3 (30 μM), RITA (1.6 μM) or E3 ubiquitin ligase FBXW7 activator oridonin (7.5 μM), the analysis of changes in the expression of the selected genes was performed. The threshold value from which a statistically significant increase or decrease in gene expression was observed when the value of the change in gene expression (by times) was ≥2 or ≤2.

The analysis of gene expression of FM-94 line cells after exposure to the MDM2-p53 interaction suppressors nutlin-3 (30 μM) and RITA (1.6 μM) and E3 ubiquitin ligase FBXW7 activator oridonin (7.5 μM). The exposure of FM-94 line cells to the MDM2-p53 interaction suppressor nutlin-3 (30 μM) resulted in the largest increase in *MDM2* gene expression – almost 8-fold compared to *HPRT1* and 6-fold compared to *TBP* (Fig. 5).

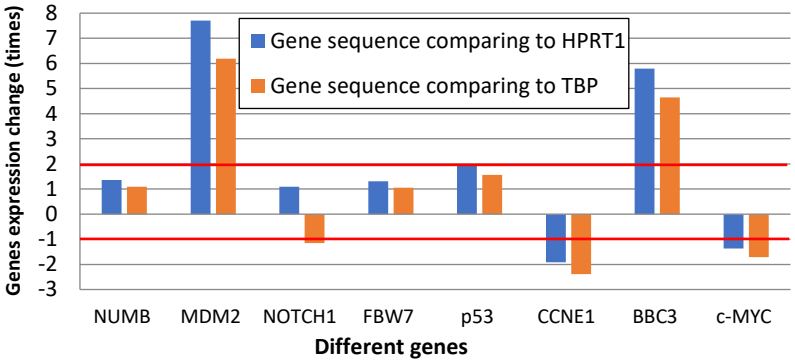

Figure 5. Changes in gene expression of FM-94 line cells compared to "housekeeping" genes *HPRT1* and *TBP* after exposure to MDM2-p53 interaction suppressor nutlin-3. The red lines indicate the threshold value at which statistically significant changes in gene expression were observed.

The expression of the *BBC3* gene was also sufficiently high, increasing almost 6-fold compared to the expression of the *HPRT1* gene and almost 5-fold compared to the expression of the *TBP* gene. *p53* expression increased almost 2-fold compared to both "housekeeping" genes. *CCNE1* and *c-MYC* gene expression was reduced by almost 2-fold.

The analysis of changes in gene expression of FM-94 cell cells under the influence of MDM2-p53 interaction suppressor RITA (1.6 μM) showed a significant decrease in *c-MYC* gene expression by 7-fold compared to *HPRT1* gene expression and by almost 10-fold compared to *TBP* gene expression. There was also a statistically significant decrease in the expression of the *FBW7* gene – almost 6 and 7 times compared to the "housekeeping" genes, respectively. There was a statistically insignificant decrease in the expression of *MDM2*, *NUMB* and *NOTCH1* genes, while the *p53* gene expression increased (Fig. 6).

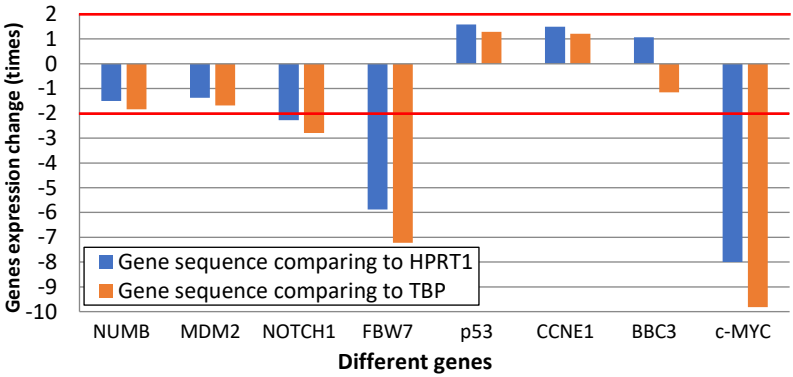

Figure 6. Changes in gene expression of FM-94 line cells compared to the “housekeeping” genes *HPRT1* and *TBP* when exposed to the MDM2-p53 interaction suppressor RITA. The red lines indicate the threshold value at which statistically significant changes in gene expression were observed.

The exposure of FM-94 line cells to E3 ubiquitin ligase FBXW7 activator oridonin (7.5 μM) showed a statistically significant increase in the *CCNE1* gene expression compared to the *TBP* gene – 2.5-fold; *BBC*3 – 2-fold, while *c-MYC* gene expression decreased 2.5-fold compared to *HPRT1* gene expression. *NOTCH1* and *FBW7* gene expression slightly decreased compared to *HPRT1* gene expression (Fig. 7).

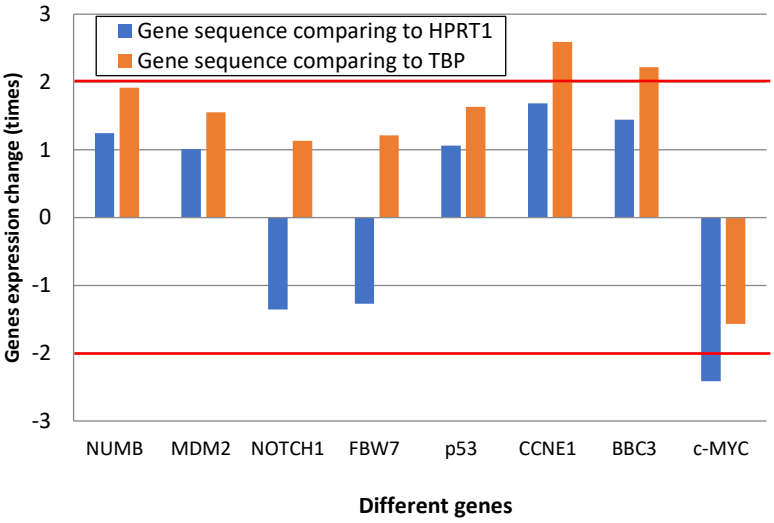

Figure 7. Changes in the gene expression of FM-94 line cells compared to the "housekeeping" genes *HPRT1* and *TBP* upon exposure to E3 ubiquitin ligase FBXW7 activator oridonin. The red lines indicate the threshold value at which statistically significant changes in gene expression were observed.

Thus, the activator of E3 ubiquitin ligase FBXW7 oridonin effectively inhibits cell viability of the FM-94 line by activating the proapoptotic *BBC3* gene and inhibiting the cell cycle activator *c-MYC*.

The analysis of the gene expression of SK-MEL-26 line cells after exposure to MDM2-p53 interaction suppressors RITA and nutlin-3. When SK-MEL-26 line cells were exposed to the MDM2-p53 interaction suppressor RITA, most of the changes in the gene expression were not detected. Only small fluctuations in changes in the expression of *NUMB, FBW7, CDKN1A, p53, c-MYC, CCND1*, and *Parp* genes were found, which may be related to the weak effect of the suppressor RITA on cell viability. Only the expression of the *Jun* gene decreased statistically significantly – almost 9 times compared to the expression of the *TBP* gene and almost 8 times compared to the expression of the *HPRT1* gene. Similar results were obtained by treating the cells of the SK-MEL-26 line with the MDM2-p53 interaction suppressor nutlin-3.

Thus, the changes in the gene expression of the SK-MEL-26 cell line showed that a single effect of the MDM2–p53 interaction suppressors RITA and nutlin-3 had a weak effect on the expression of the studied genes, except for the expression of the *Jun* gene.

The analysis of the gene expression of the SK-MEL-26 cell line after exposure to the E3 ubiquitin ligase FBXW7 activator oridonin. The exposure of the SK-MEL-26 line cells to E3 ubiquitin ligase FBXW7 activator oridonin resulted in a statistically significant increase in the *NOTCH1* gene expression 3-fold, *NOTCH2* – 5-fold, *NOTCH3* – 8.5-fold and *LNX1* almost – 4-fold, *FBXW7* – 2-fold, and *JAG* gene expression decreased 3-fold

compared to the expression of “housekeeping” genes (Fig. 8).

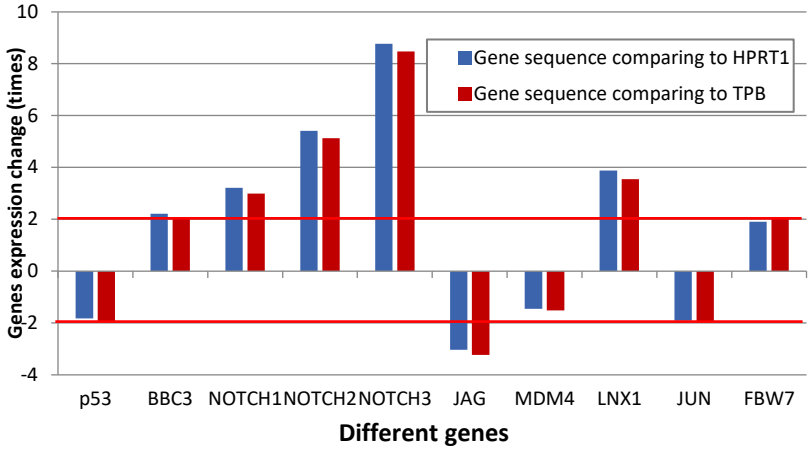

Figure 8. Changes in the gene expression of the cells in the SK-MEL-26 cell line compared to “housekeeping” genes *TBP* and *HPRT1* upon exposure to FBXW7 activator oridonin. The red lines indicate the threshold value at which statistically significant changes in gene expression were observed.

Thus, oridonin, the activator of E3 ubiquitin ligase FBXW7, effectively suppressed the cell viability of the SK-MEL-26 line by activating the E3 ubiquitin ligases and the Notch signaling pathway.

3.4 Investigations of the protein expression of the E3 ubiquitin ligases FBXW7 and MDM2 and their substrates p53 and c-Myc in postoperative material inpatients with dysplastic moles and melanoma

When conducting research of the patient samples, one of the objectives was to determine the expression of proteins in E3 ubiquitin ligases FBXW7 and MDM2 and their substrates p53 and c-Myc in samples obtained from patients with dysplastic moles and melanoma and compare them with the clinical and pathological characteristics of melanoma.

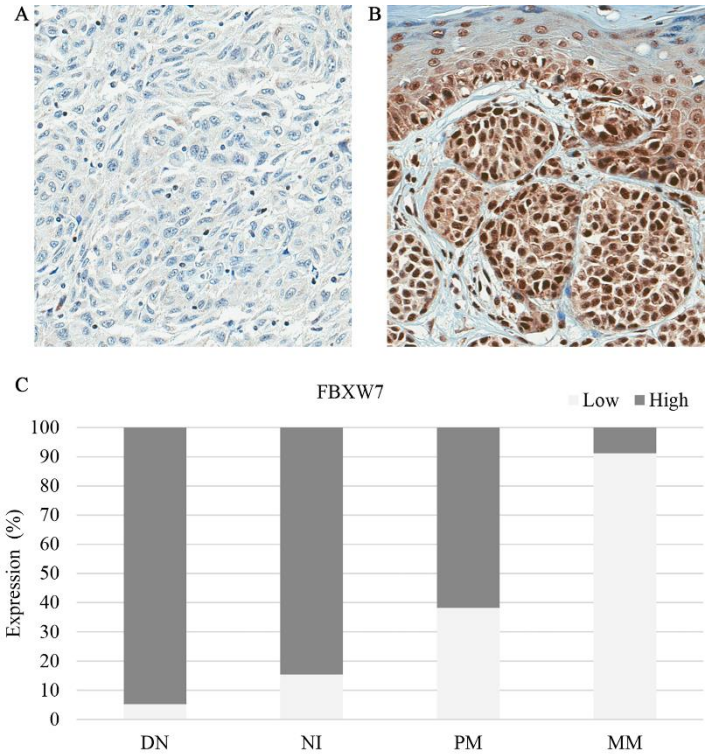

Figure 9. The expression of FBWX7 protein in skin melanoma. Negative expression of FBXW7 by immunohistochemical staining in metastatic melanoma (**A**) and high expression of FBXW7 in primary melanoma (**B**). **C** – low expression of FBXW7 is associated with melanoma progression. DN – dysplastic nevus, NI – non-invasive melanoma (melanoma *in situ*).

The FBXW7 expression is associated with the progression of melanoma, the depth of melanoma invasion and the morphological type of the tumor. A high expression of FBXW7 was observed in 53 % of samples, and a low expression – in 47 % of cases. There was a significantly lower level of FBXW7 expression in melanoma progression (pT3/pT4) compared with dysplastic nevus, melanoma *in situ* and stage I melanoma ($P<0.001$; Fig. 9).

Additionally, a statistically significant association was found between the FBXW7 expression and the morphological type of the tumor ($P<0.001$); the high FBXW7 expression was observed in 93.7 % of dysplastic nevus tissues, in 77.8 % of lentigo maligna cases and in 54.2 % of cases of superficial spreading melanoma, whereas in 81.5 % of nodular melanomas, the low FBXW7 expression was observed. There was a statistically significant association between the FBXW7 expression levels and the depth of the tumor ($P<0.001$), at a depth of <1 mm, in 76.9 % of cases the increased expression of FBXW7 was observed, where as in 90.5 % of cases with melanoma invasion >1.1 mm, the decreased FBXW7 expression was found. There was no statistically significant association between the FBXW7 expression and the patients' sex, age or tumor localization (Table 2).

Table 2. Associations between the FBXW7 protein expression and clinicopathological characteristics

Clinicopathological characteristics		n	FBXW7 expression		p value
			high n (%)	low n (%)	
All cases		100	53 (53.0)	47 (47.0)	–
Gender	Men	39	23 (54.8)	16 (45.2)	0.34
	Women	61	30 (49.2)	31 (50.8)	
Age	≤58	44	25 (56.8)	19 (43.2)	0.50
	>58	56	28 (50.0)	28 (50.0)	
Stage by tumor depth (T)	Dysplastic mole	16	15 (93.7)	1 (6.25)	**0.001**
	Tis	16	14 (73.7)	2 (12.5)	
	T1	17	15 (88.2)	2 (11.8)	
	T2	17	6 (35.3)	11 (64.7)	
	T3	17	1 (5.9)	16 (94.1)	
	T4	17	2 (11.8)	15 (88.2)	
Depth of invasion	0.1–1.0 mm	26	20 (76.9)	6 (23.1)	**0.001**
	1.1 mm +	42	4 (9.5)	38 (90.5)	
	Non-invasive	32	29 (90.6)	3 (9.4)	
Morphological type	Superficial spreading	48	26 (54.2)	22 (45.8)	**0.001**
	Lentigo maligna	9	7 (77.8)	2 (22.2)	
	Nodular	27	5 (18.5)	22 (81.5)	
	Dysplastic mole	16	15 (93.7)	1 (6.5)	
Localiza-tion	Sun unexposed	13	5 (38.5)	8 (61.5)	0.50
	Sun exposed	77	42 (54.5)	35 (45.5)	
	Unknown	10	6 (60.0)	4 (4.0)	

P53 expression is associated with the depth of melanoma invasion and the morphological type of the tumor. There were no statistically significant associations of p53 expression and melanoma progression by stages. Almost equal proportions of high and low levels of p53 expression were observed in dysplastic nevus and melanoma *in situ*. High p53 expression was found when comparing p53 expression in dysplastic nevus and melanoma *in situ* tissues with stage pT1/pT2 and

pT3/pT4 melanoma (Fig. 10). High p53 expression was detected in 70-80 % of melanomas ranging from stage pT1 to pT4, but the expression was not associated with the stage (p=0.08). Additionally, there was no association of p53 expression with patients' sex, age and tumor localization.

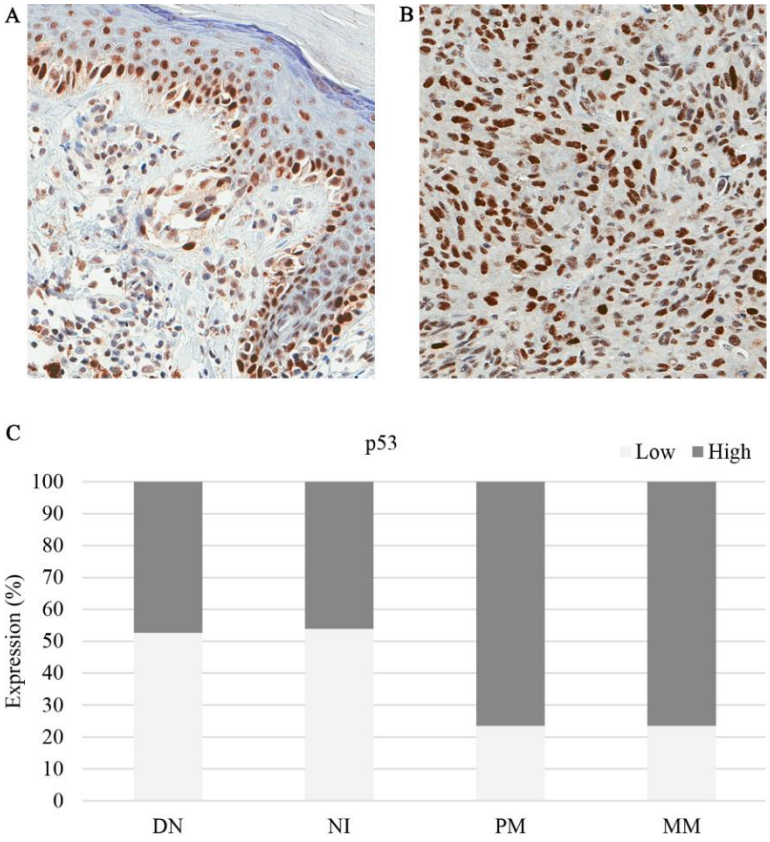

Figure 10. p53 protein expression in the case of skin/cutaneous melanoma. Low expression of p53 by immunohistochemical staining in the case of *in situ* melanoma (**A**) and high expression of p53 in the case of metastatic melanoma (**B**). **C** – changes in p53 expression in DN – dysplastic nevus, NI – non-invasive melanoma (melanoma *in situ*).

There was a statistically significant increase in the expression of p53 associated with the increasing depth of melanoma invasion; when melanoma depth was <1.0 mm and >1.1 mm, p53 expression was observed in 76.9 % and 76.2 % of cases, respectively, whereas a significantly lower level of p53 protein expression was detected in 53.1 % of non-invasive tumors (p=0.01). There was also a statistically significant association between the p53 expression levels and the morphological type of the tumor, the high p53 expression was observed in 85.2 % of nodular melanomas and 68.8 % high levels of p53 expression were found in surface spreading melanomas. The levels of p53 expression in lentigo maligna tissues were low (p=0.01; Table 3).

Table 3. Associations between the expression of p53 protein and clinicopathological characteristics.

Clinicopathological characteristics		n	p53 expression high n (%)	p53 expression low n (%)	p value
All cases		100	67 (67.0)	33 (33.0)	–
Gender	Men	39	23 (59.0)	16 (41.0)	0.17
	Women	61	44 (72.1)	17 (27.9)	
Age	≤58	44	31 (70.5)	13 (29.5)	0.51
	>58	56	36 (64.3)	20 (35.7)	
Stage by depth of tumor (T)	Dysplastic mole	16	8 (50.0)	8 (50.0)	0.08
	Tis	16	7 (43.7)	9 (56.3)	
	T1	17	14 (82.3)	3 (17.7)	
	T2	17	12 (70.6)	5 (29.4)	
	T3	17	12 (70.6)	5 (29.4)	
	T4	17	14 (82.3)	3 (17.7)	
Depth of invasion	0.1–1.0 mm	26	20 (76.9)	6 (23.1)	**0.01**
	1.1 mm +	42	32 (76.2)	10 (23.8)	
	Non-invasive	32	15 (46.9)	17 (53.1)	
Morphological type	Superficial spreading	48	33 (68.8)	15 (31.2)	**0.01**
	Lentigo maligna	9	3 (33.3)	6 (66.7)	
	Nodular	27	23 (85.2)	4 (14.8)	
	Dysplastic mole	16	8 (50.0)	8 (50.0)	
Localization	Sun unexposed	13	11 (84.6)	2 (15.4)	0.33
	Sun exposed	77	49 (63.6)	28 (36.4)	
	Unknown	10	7 (70.0)	3 (30.0)	

c-Myc protein expression is not associated with melanoma progression. The examination of c-Myc protein expression levels showed a high expression in 36 % of cases and a low expression in 64 % of cases. c-Myc protein expression was not significantly associated with melanoma progression. Similarly, c-Myc expression was not associated with any clinicopathological parameters, including

sex, age, morphological type of the tumor, and depth or localization of invasion.

The low c-Myc expression was observed in 56 % of melanoma cases, and in 71.4 % of these cases with low expression, the high expression was observed at melanoma depths >1.1 mm. There were no changes in c-Myc expression in dysplastic nevus, as both the high and low expression was found in each of 50 % of cases. A slight decrease in c-Myc expression was found in cases with melanoma *in situ* and stage I melanoma, and low c-Myc protein expression levels were observed in 70.6 % of melanoma cases in stages pT2, pT3 and pT4. However, there was no statistically significant association between the c-Myc expression levels and the stage of melanoma ($p=0.77$).

The MDM2 expression levels are not associated with melanoma progression. The present study showed low MDM2 expression levels in 97 % of all the investigated tumors; therefore, the examination of MDM2 expression did not show any statistically significant association with melanoma progression or any other clinicopathological parameters.

The low FBXW7 protein expression levels are associated with low c-Myc protein expression levels. A χ^2 test was performed to evaluate the relationship between the changes in FBXW7, c-Myc, MDM2 and p53 expression levels. There was a statistically significant association between the FBXW7 and c-Myc expression levels. The results showed that in 76.6 % of cases with the low FBXW7 expression levels, the low c-Myc expression levels were also observed and this finding was statistically significant ($p=0.01$; Table 4).

Table 4. Associations between the changes in the expression of FBXW7, c–Myc, MDM2, and p53 proteins.

Protein	Protein expression	p53 high n (%)	p53 low n (%)	p	c-Myc high n (%)	c-Myc low n (%)	p	MDM2 high n (%)	MDM2 low n (%)	p
c-Myc	**high**	27 (75.0)	9 (25.0)	0.20						
	low	40 (62.5)	24 (37.5)							
MDM2	**high**	2 (66.7)	1 (33.3)	0.99	2 (66.7)	1 (33.3)	0.26			
	low	34 (35.0)	36 (65.0)		34 (35.0)	36 (65.0)				
FBXW7	**high**	33 (62.3)	20 (37.7)	0.28	25 (47.2)	28 (52.8)	**0.01**	2 (3.8)	51 (96.2)	0.63
	low	34 (72.3)	13 (27.7)		11 (23.4)	36 (76.6)		1 (2.1)	46 (97.9)	

A univariate *Cox* regression analysis was used to determine which of the clinicopathological indicators under analysis may be associated with survival. The analysis showed that the patients' sex, age, morphological type and the localization of tumors were not associated with mortality. However, tumor ulceration was found to increase the risk of death 2.79 times, although this rate was not statistically significant (p=0.06). Patient survival was most notably influenced the stage of the tumor where the risk of death increased by 5.6 times (p=0.03).

There was no statistically significant association between the changes in the FBXW7, c-Myc, MDM2 and p53 expression levels and the risk of death. (Table 5).

Table 5. Assessment of risk posed by different factors of protein expression and other clinicopathological factors in patients with melanoma (univariate *COX* regression analysis).

Characteristics (n=68)	HR (95 % CI)	p
Gender (men *vs* women)	2.01 (0.56–7.22)	0.29
Age (≤58 *vs*>58)	1.16 (0.39–3.49)	0.79
Depth of the tumor (T1/T2 *vs* T3/T4)	**5.60 (1.24–25.2)**	**0.03**
Morphology (superficial spreading *vs* others)	2.48 (0.83–7.43)	0.10
Ulceration (present *vs* absent)	2.79 (0.95–8.08)	0.06
Localization (sun unexposed *vs* sun exposed)	0.57 (0.16–2.11)	0.40
p53 expression (low *vs* high)	2.48 (0.83–7.43)	0.10
c-Myc expression (low *vs* high)	0.36 (0.08–1.63)	0.19
FBXW7 expression (low *vs* high)	0.16 (0.02–1.23)	0.08

4. DISCUSSION OF THE RESULTS

Previous studies have identified a new generation of the suppressors of E3 ubiquitin ligases: nutlin-3 and RITA, which may disrupt the MDM2-p53 interactions in tumors and thus restore the apoptotic function of p53 (Panayi *et al.*, 2013) while the activator of E3 ubiquitin ligase FBXW7 oridonin may activate FBXW7 and thus ensure the cell cycle arrest and the apoptosis of tumor cells (Huang *et al.*, 2012).

In this study, the first experiments were performed using the human metastatic melanoma SK-MEL-26 line cells which were exposed to different concentrations of MDM2-p53 interaction suppressors nutlin-3 and RITA. Only a slight effect on cell viability was observed with respect to the dose and the time of exposure. Oridonin acted most effectively on these cells. The analysis of the

gene expression of SK-MEL-26 cell line after exposure to MDM2-p53 interaction suppressors nutlin-3 and RITA did not show significant changes. The only relevant and statistically significant change was in the expression of the *Jun* gene, the expression of the which decreased by almost 9 times in comparison with the "housekeeping" genes with a constant pattern of expression.

The *Jun* gene is thought to be involved in carcinogenesis and its increased expression is most commonly found in many malignancies (Davis, Welcker and Clurman, 2014). The inhibition of *Jun* results in the decrease of the *MDM2* gene expression (Wang *et al.*, 2015). Taking into account that p53 interacts with MDM2 to form a negative autoregulatory loop, MDM2 expression should decrease with the increasing p53 expression. It can be assumed that in the present study, after the exposure of SK-MEL-26 line cells to MDM2-p53 interaction suppressors and after a decrease in the potential for p53 ubiquitination, *MDM2* expression decreased concomitantly and through a decrease in *Jun* transcription factor expression.

During our research, when the SK-MEL-26 line cells were exposed to the E3 ubiquitin ligase FBXW7 activator oridonin, a statistically significant increase in the *NOTCH1, NOTCH2* and *NOTCH3* gene expression was observed in comparison with the “housekeeping” gene expression, while a 3-fold decrease in NOTCH target *JAG* gene expression was found. NOTCH proteins are a substrate for FBXW7 and may act as an oncoprotein or as a tumor suppressor, depending on the type of tissue. FBXW7 ubiquitinates and negatively regulates the Notch signaling pathway (Öberg *et al.*, 2001). In melanoma, Notch pathway expression is increased, which may be associated with mutation or inactivation of *FBXW7* (Öberg *et al.*, 2001; Aydin *et al.*, 2014; Davis, Welcker and Clurman, 2014). The intracellular domain of Jagged-1 (JAG1) has also been found to interact with the cellular domain of NOTCH1 and stimulate its degradation via the FBXW7-dependent

proteasomal pathway (Kim *et al.*, 2011). The decreased *JAG* expression was found in our study, and this may affect the increased expression of *NOTCH1*.

After exposure of the SK-MEL-26 line cells to oridonin, the gene expression of ubiquitin ligases *LNX1* and *FBXW7* was also increased. Although the expression of the *FBXW7* gene was only slightly increased (2-fold), this indicates that oridonin partially activated the function of *FBXW7* in the cells of the SK-MEL-26 line. The expression of the *LNX1* gene increased almost 4-fold. LNX1 is a ubiquitin ligase that binds to the NUMB protein and directs it to proteosomal degradation. Thus, LNX1 can activate the Notch signaling pathway by cleaving NUMB (Nie *et al.*, 2002). Based on the results obtained, it can be assumed that the altered Notch pathway activity resulting from exposure to oridonin important for the survival of melanoma cells, and increased expression of *NOTCH* genes may be associated with the increased expression of *LNX1* and insufficiently restored *FBXW7* function, which should inhibit the Notch signaling pathway.

Summarizing the obtained results of cell viability of FM-94 and FM-3 lines, it should be noted that, as in the case of SK-MEL-26 line cells, all concentrations of MDM2-p53 interaction suppressors nutlin-3 and RITA had the least effect on cell viability. The viability of both cell lines was most effectively reduced by the E3 ubiquitin ligase FBXW7 activator oridonin.

In the study, the exposure of FM-94 line cells to the MDM2-p53 interaction suppressor nutlin-3 resulted in an almost 2-fold increase in p53 gene expression compared to "housekeeping" (*HPRT1, TBP*) genes, but a larger increase in *MDM2* and *BBC3* gene expression, while *CCNE1* and *c-MYC* gene expression was reduced by almost 2-fold. The results of the research showed that the effect of the MDM2-p53 interaction inhibitor was ineffective in inhibiting the viability of FM-94 line cells, which may have led to high expression of the *MDM2* gene. The *BBC3* gene (*BCL2 binding component 3, PUMA*) encodes BCL-2 family proteins that cause

the p53-mediated apoptosis of cells (Akhter, Sanphui and Biswas, 2014). The increased expression of the *BBC3* gene in the study after exposure to the MDM2-p53 interaction suppressor nutlin-3 could indicate that p53-induced cell apoptosis was activated.

The *CCNE1* (*cyclin E1*) gene encodes proteins belonging to the cyclin family. Cyclins act as regulators of CDK kinases and regulate the cell cycle. c-MYC, a transcription factor that regulates cell proliferation, apoptosis, metabolism, and differentiation, increases as melanoma progresses. Nutlin-3 is known to suppress cell cycle and proliferation (Vassilev, 2007), therefore, the results of a study showing that exposure to FM-94 cells in nutlin-3 decreases the expression of CCNE1 and c-MYC genes and cell viability may be associated with the ability of nutlin-3 to suppress the proliferation of these cells.

Thus, the effect of the MDM2-p53 interaction suppressor nutlin-3 on FM-94 line cells may have led to the decreased cell cycle activator*CCNE1* expression and the increased expression of the apoptosis-inducing gene *BBC3*.

The exposure of FM-94 line cells with the MDM2-p53 interaction suppressor RITA resulted in a statistically significant decrease in *c-MYC* and *FBW7* gene expression. c-Myc is one of the major targets of FBXW7, and the decreased expression of FBXW7 results in the increased expression of c-Myc. Our study showed that the exposure of FM-94 cells to the suppressor RITA significantly reduced the expression of the *FBXW7* gene, therefore it can be assumed that RITA as the suppressor of the MDM2-p53 interaction does not affect the expression of the *FBW7* gene. However, as FBXW7 expression decreases, c-Myc expression is expected to increase, but this was not found in our study using FM-94 line cells.

The analysis of the changes in the expression of genes obtained from the exposure of the FM–94 line cells to FBXW7 activator oridonin revealed the increased *CCNE1* and *BBC3* gene expression. As mentioned, the exposure of FM-94 line cells to the MDM2-p53 interaction inhibitor nutlin-3, the increased *BBC3* gene expression

may have been associated with the increased p53 expression and p53-induced cell apoptosis. However, the exposure of FM-94 line cells with FBXW7 activator oridonin did not increase the expression of p53 gene, suggesting that oridonin could have activated the *BBC3* expression in a manner independent from p53.

Cyclin E is encoded by the *CCNE1* gene, so in the presence of the decreased FBW7 expression, the expression of both, cyclin-E and *CCNE1* gene should decrease. However, the exposure of FM-94 line cells to the FBXW7 activator oridonin increased the *CCNE1* gene expression, and knowing that oridonin did not activate *FBW7* in FM-94 cells, it can be concluded that the increase in the *CCNE1* expression may have been impacted by other factors that together inhibited *FBW7*.

In summary of the cell viability studies and comparing them with the gene expression results, it can be concluded that the activator oridonin of E3 ubiquitin ligase FBXW7 is effective in inhibiting cell viability by activating proapoptotic proteins and inhibiting the cell cycle activator *c-MYC*.

In the second stage, the research focused on examination of E3 ubiquitin ligases FBXW7 and MDM2 and their substrates p53 and c-Myc protein expression in samples from dysplastic moles and melanoma patients.

FBXW7 is a tumor suppressor that controls the protein expression levels of several oncogenes. However, little is known regarding the regulation of FBXW7 itself in tumors. The regulation of FBXW7 expression may occur at the transcriptional level, protein level or as a result of post-translational modifications, such as phosphorylation (Min *et al.*, 2012). p53 molecules, NUMB4, NF-κB1, microRNA-27 and microRNA-223 and others are known to be important in the FBXW7 regulation (Wang *et al.*, 2014).

Previous studies have shown a reduced FBXW7 activity in melanoma cells (Cheng *et al.*, 2013; Aydin *et al.*, 2014). In the present study, it was shown that FBXW7 expression was significantly lower in primary melanoma compared with dysplastic nevus. FBXW7 levels

in metastatic melanoma were lower compared with primary melanomas, and its reduced expression was associated with a less favorable 5-year survival rate. Further *in vitro* studies demonstrated that FBXW7 suppressed the migration of melanoma cells via mitogen-activated protein kinase/extra cellular signal-regulated kinase signaling pathway. Thus, suppression by FBXW7 in melanoma cells results in the increased cell migration and stress fiber formation (Cheng *et al.*, 2013).

The results of the present study also showed that FBXW7 expression levels decreased as melanoma progressed, and a statistically significant negative association was found between the high expression of FBXW7 levels and the increasing stage of melanoma.

Previous studies showed that the reduced FBXW7 expression levels were associated with melanoma progression and the accumulation of c-Myc protein (Yada *et al.*, 2004; Cheng *et al.*, 2013; Aydin *et al.*, 2014). c-Myc is one of the major targets of FBXW7, and c-Myc regulates the expression of >15 % of the genes involved in the processes of differentiation, proliferation, protein synthesis, metabolism, and apoptosis, thus, the impaired c-Myc function may underlie the formation of a tumor (Welcker and Clurman, 2008; Davis, Welcker and Clurman, 2014). FBW7α promotes the ubiquitination of Myc in proteasomes, whereas FBW7γ ubiquitinates Myc in the nucleus and thus suppresses the ability of Myc to promote cell growth (Welcker *et al.*, 2004, Yada *et al.*, 2004; Bonetti *et al.*, 2008; Grim *et al.*, 2008). Thus, a decrease in the FBXW7 expression results in the increased expression of c-Myc (Wang *et al.*, 2014).

In the present study, 17 cases of stage pT2 melanoma, 17 cases of stage pT3 melanoma and 17 cases of stage pT4 melanomas were examined, and the expression of c-Myc protein was detected in 12 cases (70.6 %) in each group. The comparison of these groups with melanoma *in situ* and stage I melanoma showed no statistically significant changes in c-Myc protein expression. In addition, there was a strong direct association observed between the changes in the

FBXW7 and c-Myc expression with a decrease in the FBXW7 expression, and a decrease in c-Myc expression was also observed. These results differ from those obtained in previously published studies (Welcker *et al.*, no date; Yada *et al.*, 2004; Reavie *et al.*, 2013); the results of the present study may be influenced by the sample size, in addition, melanoma is a heterogeneous tumour and its development and progression is affected by the interaction of multiple genes and various signaling pathways. c-Myc may, in certain types of cancer, acquire loss-of-function mutations or, in the majority of cases, c-Myc expression is upregulated (Cancer and Atlas, 2011; Curtis, C., 2012; Witkiewicz *et al.*, 2015; Kalkat *et al.*, 2017). In both cases, the altered c-Myc expression results in tumor formation through the disrupted transcription, translation or differentiation (Davis, Welcker and Clurman, 2014; Kalkat *et al.*, 2017).

Rajabi et al (Rajabi, Karimian and Heidarpour, 2012) showed that there was an association between the MDM2 expression levels with tumor thickness and the invasion in primary cutaneous malignant melanoma. In 50 % of melanoma cases, a strong expression of the MDM2 gene is detected, leading to the enhanced degradation of p53, thus resulting in tumor cell proliferation (Polsky *et al.*, 2001; Soengas *et al.*, 2001; Polsky, 2002). p53 and MDM2 interact and form a negative autoregulatory loop in which elevated p53 transcriptional levels activate MDM2, which in turn decreases the levels of p53 (Vassilev, 2007; Wade, Li and Wahl, 2013; Nag *et al.*, 2014). The present study showed that there was a decrease in the MDM2 expression in almost all cases assessed (97 %). Contrary to the published studies (Polsky *et al.*, 2001; Polsky, 2002; Muthusamy *et al.*, 2006; Rajabi, Karimian and Heidarpour, 2012), the results of the present study did not reveal a significant association between the levels of MDM2 expression and melanoma invasion; and there was no association between the decrease in the MDM2 expression and the p53 expression levels. In the present study, an upregulated p53 expression was observed in primary and invasive melanoma. In the cases of melanoma with a thickness >1 mm, the p53 expression was

significantly increased, whereas the FBXW7 expression decreased as melanoma progressed.

In conclusion, the present study showed that FBXW7 exhibited the most statistically significant prognostic value and associations with the progression of melanoma. As most of the FBXW7 substrates are oncoproteins, their degradation by FBXW7 may underlie the mechanism by which the decreased FBXW7 expression results in tumor progression, and highlight these proteins as potential targets for treatment of melanoma.

5. CONCLUSIONS

1. The viability of the human metastatic melanoma SK-MEL-26, FM-94 and FM-3 line cells are most impacted by oridonin, the activator of E3 ubiquitin ligase FBXW7.
2. *In vitro* gene expression studies have shown as follows:

– the p53 signaling pathway of the human metastatic melanoma FM-94 line cells is functional – the MDM2-p53 interaction suppressor nutlin-3 reduces the viability of these cells by suppressing the cell cycle and activating apoptosis;
– the activator oridonin not only activates E3 ubiquitin ligase FBXW7, suppresses the cell cycle, but also activates apoptosis.

3. The postoperative material of patients with melanoma has revealed:

– In nodular melanoma and at greater depths of melanoma invasion (pT3 and pT4 stages), the expression of E3 ubiquitin ligase FBXW7 is low;
– In nodular melanoma and at melanoma depths greater than 1.1 mm, the expression of the protein encoded by the p53 tumor suppressor gene is high;
– c-Myc expression in invasive melanoma (pT3 and pT4 stages) is regulated not only by FBXW7 ligase – even with low

expression of FBXW7 protein, c-Myc expression in tumors is also low.

4. The decreased expression of E3 ubiquitin ligase FBXW7 in metastatic melanoma confirms the potential of this protein as a molecular target for anticancer therapy; oridonin, as an activator of FBXW7, could be a potential therapeutic agent for the treatment of melanoma.

6. PUBLICATIONS AND PRESENTATIONS

Articles on the topic of the dissertation

1. Kristina Bielskienė, Lida Bagdonienė, **Julija Mozūraitienė**, Birutė Kazbarienė, Ernestas Janulionis. E3 ubiquitin ligases as drug targets and prognostic biomarkers in melanoma. Medicina, 2015, 1 (51), 1–9.
2. **Julija Mozūraitienė**, Kristina Bielskienė, Vydmantas Atkočius, Danutė Labeikytė. Molecular alterations in signal pathways of melanoma and new personalized treatment strategies: Targeting of Notch. Medicina, 2015, 3 (51), 133–145.
3. Andrė Lideikaitė, **Julija Mozūraitienė**, Simona Rūta Letautienė. Analysis of prognostic factors for melanoma patients. Acta Medica Lituanica. 2017. Vol. 24. No. 1. P. 25–34.
4. **Julija Mozūraitienė,** Živilė Gudlevičienė**,** Ieva Vincerževskienė, Aida Laurinavičienė, Justinas Pamedys. A. study of the expression of FBXW7 and MDM2 E3 ubiquitin ligases and their c-Myc and p53 substrates in patients with dysplastic nevus or melanoma. – Accepted for publication in the journal “Oncology Letters”.

Presentations at research conferences

1. **Julija Mozūraitienė,** Vilius Vilčinskas, Lida Bagdonienė, Danutė Labeikytė, Kristina Bielskienė, ”The evolution of melanoma treatment and new personalized treatment strategies: targeting of Notch”.International conference, ”Evoliucinė medicina: šiuolaikinių sveikatos problemų evoliuciniai mechanizmai ir dėsningumai“ (**“***Evolutionary medicine: pre-existing mechanisms and patterns of current health issues***“**). Vilnius, 2016 (Electronic presentation).
2. **Julija Mozūraitienė**, Aistė Petrovaitė, Kristina Bielskienė, Živilė Gudlevičienė, ”Effect of FBW7 activator oridonin and MDM2 inhibitors nutlin-3/RITA on human melanoma cell lines.” *Evolutionary medicine: health and diseases in changing environment.* Vilnius, 2018 (Electronic presentation).
3. **Julija Mozūraitienė**, Aistė Petrovaitė, Andrė Lideikaitė, Kristina Bielskienė, Živilė Gudlevičienė. ,“The expression of genes in FM 94 melanoma cell line after exposure with nutlin-3, RITA, oridonin and cisplatin“ EADV congress, Madrid. 2019 (Electronic presentation).

Curriculum vitae

Date of birth 6 February 1980
Residential address Svajonės st 6, Didžioji Riešė, Vilnius district
Telephone number +370 686 72127
E-mail:julija.mozuraitiene@gmail.com

Education:
1998-2004 – Faculty of Medicine of Vilnius University
2004-2005 – Internship, Vilnius University
2005-2009 – Residentship in Dermatovenerology, Vilnius University, Dermatovenerology Centre, Vilnius University Hospital Santaros Klinikos

Work experience:
2005-2011 – junior researcher, Institute of Oncology, Vilnius University (Cancer Prevention Department, National Cancer Institute
From 2011 – to date – doctor dermatovenerologist – oncologist, Institute of Oncology (present National Cancer Institute, Vilnius University)
From 2011-2016 – junior researcher, Institute of Oncology, Vilnius University, Laboratory of Carcinogenesis and Tumor Pathophysiology
From 2011 – to date – coach, Allergan Medical Institute
From 2013 – to date – doctor dermatovenereologist, Valakupiai Plastic Surgery Clinic, VitkusClinic
From 2013 – to date – doctor dermatovenereologist, Clinic of Laser Dermatology „Era Esthetic"
From 2013 – to date – doctor dermatovenerologist,“Jaunatvės namai”

Membership:
Member of the Lithuanian Society of Dermatovenereologists (LDVD)

NOTES

NOTES

Vilnius University Press
9 Saulėtekio Ave., Building III, LT-10222 Vilnius
Email: info@leidykla.vu.lt, www.leidykla.vu.lt
Print run 30