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Review

Molecular alterations in signal pathways of melanoma and new personalized treatment strategies: Targeting of Notch

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ABSTRACT

Despite modern achievements in therapy of malignant melanomas new treatment strategies are welcomed in clinics for survival of patients. Now it is supposed that personalized molecular therapies for each patient are needed concerning a specificity of molecular alterations in patient's tumors. In human melanoma, Notch signaling interacts with other pathways, including MAPK, PI3K-AKT, NF-κB, and p53. This article discusses mutated genes and leading aberrant signal pathways in human melanoma which are of interest concerning to their perspective for personalized treatment strategies in melanoma. We speculate that E3 ubiquitin ligases MDM2 and MDM4 can be attractive therapeutic target for p53 and Notch signaling pathways in malignant melanoma by using small molecule inhibitors. It is possible that restoration of p53-MDM2-NUMB complexes in melanoma can restore wild type p53 function and positively modulate Notch pathway. In this review we summarize recent data about novel US Food and Drug Administration approved target drugs for metastatic melanoma treatment, and suppose model for treatment strategy by targeting Notch.

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1. Introduction

Cutaneous melanoma is the most aggressive form of all skin cancers, frequently related with chemoresistance and worse patient prognosis. According to a World Health

Organization report, about 48,000 melanoma-related deaths occur worldwide per year [1]. The incidence of melanoma in Lithuania has doubled over the last decade and has amounted to 300 new cases per year [2]. Metastatic melanoma has a poor prognosis, with median survival for patients with stage IV melanoma ranging from 8 to 18 months after diagnosis,

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depending on the substage [3]. Despite several efforts in the treatment of malignant melanoma, surgery remains the standard of care. An excision of the primary tumor with prognosis adapted margins is recommended worldwide as a basic therapeutic approach. Now, 4 decades after its initial approval by FDA in 1975, dacarbazine (DTIC, antineoplastic drug frequently used in the treatment of various cancers) continues to be the standard of care for most patients with this disease [4]. High-dose interleukin-2 (HD IL-2), approved by the FDA in 1998 for metastatic melanoma, benefits a small subset of patients [4].

The good news is that now has effective drug treatments for metastatic melanoma. In 2011–2014 years several drugs, ipilimumab [5,6], vemurafenib [7], dabrafenib [8,9], trametinib [9,10] and PEG-interferon α -2b (PEG-IFN) [11–13] are approved by FDA. However, like all new cancer drugs, these drugs are expensive [14]. Vemurafenib, which targets the mutated BRAF oncogene present in 50% of melanomas, produces dramatic tumor regressions in most cases. However, the average duration of the response is just 6 months, with a median extension in overall survival of less than 4 months. Combined treatment with dabrafenib (drug for the treatment of cancers associated with a mutated version of the gene BRAF) and trametinib (mitogen-activated protein kinase [MEK] inhibitor drug with anti-cancer activity) significantly prolonged progression-free survival compared with dabrafenib alone (median 9.4 versus 5.8 months, hazard ratio (HR) 0.39, 95% confidence interval (CI) 0.25–0.62) and decreased dermatologic toxicity, manifested by squamous cell carcinoma (including keratoacanthoma), although the incidence of pyrexia was increased (71% versus 26%) [15,16]. Ipilimumab, a potent but nonspecific immunostimulant, rarely induces tumor regressions, but the disease is stabilized for 3 or more years in a subset of around 10% of patients. Interferon α -2b remains a controversial therapy. Toxicity is substantial with

neuropsychiatric, constitutional, and hepatic toxicity being the major issues [17]. Although it improves recurrence-free survival, adjuvant (postsurgery) treatment with PEG-IFN (pegylated interferon α -2b) adversely affects quality of life among patients with Stage III melanoma [13].

Now it is supposed that personalized molecular therapies for each patient are needed concerning a specificity of molecular alteration and mutation paternity in patient's tumors. One of such therapies can be restoration of wild type p53 function by using small molecular inhibitors of E3 ubiquitin ligases [18–20]. Several reports have demonstrated a role for aberrant Notch signaling in melanomagenesis and progression [21]. Thus, another possible strategy for melanoma treatment can be targeting of Notch pathway by using various inhibitors, for example, γ -secretase inhibitors [22,23], or combination of several target therapies according to molecular alteration in melanoma tumors. Fig. 1 summarizes knowledge about mutated genes and aberrant pathways in melanoma. So it looks like melanoma progression in patients is the result of a combination of deregulations of the various effectors acting the different molecular pathways. We suppose that the understanding of molecular alterations in pathogenesis of melanoma should suggest targets for personalized drug therapy.

2. Mutated genes and aberrant pathways in melanoma

Melanoma progression is associated with not only certain genetic alterations (mutations, deletions, amplifications, or translocations of genes) but also with epigenetic changes which modulating of transcription activities by methylations and chromatin reorganization. These changes as a consequence cause the aberrant signal pathways in melanoma. Next

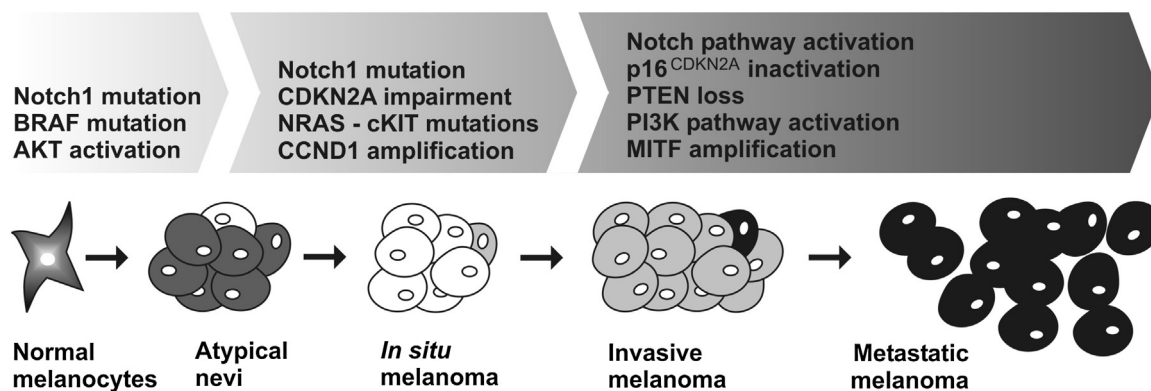


Fig. 1 – Molecular aberrations in melanoma progression. Notch1; Notch receptors; BRAF, human gene that makes a protein called B-Raf (serine/threonine-protein kinase); AKT; Serine/threonine-specific protein kinase; CDKN2A, cyclin-dependent kinase inhibitor 2A; NRAS; neuroblastoma RAS viral (*v-ras*) oncogene homolog; cKIT, receptor of tyrosine kinase (proto-oncogene); CCND1, G1/S-specific cyclin-D1; P16CDKN2A, tumor-suppressor gene, involved in the p16/cyclin-dependent kinase/retinoblastoma gene pathway of cell cycle control; PTEN, phosphatase and tensin homolog; MITF, microphthalmia-associated transcription factor; normal melanocytes, melanin-producing cells located in the bottom layer (the stratum basale) of the skin epidermis; atypical nevi, unusual benign moles that may resemble melanoma; in situ melanoma, earliest stage of melanoma. Cancer cells are in the top layer of skin (the epidermis); invasive melanoma, penetrated deeper into the skin and may have spread to other areas of the body; metastatic melanoma, when melanoma spreads to other places in your body, it's called metastatic, or advanced, melanoma.

it will be discussed mutated genes and leading aberrant pathways in human melanoma which are of interest concerning to their perspective for new personalized treatment strategies in melanoma (Table).

2.1. The RAS/RAF/MEK/ERK pathway

The RAS/RAF/MEK/ERK pathway has been reported to be activated in over 80% of all cutaneous melanomas. This signaling pathway is regulated by receptor tyrosine kinases, cytokines, and heterotrimeric G-protein-coupled receptors. The small G protein RAS (HRAS, KRAS, and NRAS in humans) downstream activates RAF (ARAF, BRAF and CRAF in humans) followed by sequential activation of MEK and ERK, and this signal is finally transduced to regulation of transcription in the nucleus [24]. This pathway is constitutively activated by growth factors (epidermal growth factor [EGF], platelet-derived growth factor [PDG], vascular endothelial growth factor [VEG], stem cell factor [SCF], fibroblast growth factor [FGF], hepatocyte growth factor [HGF], and glial-cell-derived neurotrophic factor [GDNF]) [24,25]. When RAS is activated, it can form complex with RAF. This activated complex leads to the phosphorylation of mitogen-activated protein kinases (MAPK also known as ERK) via activation of MEK. MAPK, when phosphorylated, can directly enter the nucleus and in that way effect expression of genes. This eventually leads to the changes of the control of cellular proliferation [25].

Activating mutations of BRAF are seen in 50%–60% of melanomas [26]. Among the BRAF mutations observed in melanoma, over 90% are at codon 600, and among these, over 90% are a single nucleotide mutation resulting in substitution of valine to glutamic acid (BRAFV600E). The less common mutation is BRAFV600K, e.g. substituting valine to lysine, that represents 5%–6%. However, the prevalence of BRAFV600K has been reported as higher in some populations [27]. BRAFV600K mutation activates BRAF and induces constitutive MEK-ERK signaling in cells. Activated BRAF also participates in the control of cell cycle progression [28]. All these events are led by constitutively active MAPK pathway leading to promotion of proliferation, survival, invasion, and angiogenesis of melanoma. It is of interest that BRAF mutations also occur at high frequencies (>80%) in melanocytic nevi, suggesting that these somatic alterations occur early in melanomagenesis [29]. However, most nevi do not transform into malignant melanoma. This implies that BRAF mutation may be necessary but not sufficient to induce malignant transformation. It is suggested that BRAFV600E induced checkpoint mechanisms may produce a senescence-like state in the absence of additional genetic or molecular events that promote tumorigenesis [29,30]. It is supposed that other mutations along with BRAFV600E may be necessary for tumor initiation and progression, especially in melanomas arising in association with pre-existing nevus [31].

In human melanomas, mutations in NRAS are most common and involve approximately 15%–30% of cases. Among all NRAS mutations most frequent are RASQ61K/R- substitutions of glutamine at position 61 by a lysine or an arginine [32]. 81% of congenital melanocytic nevi harbor RASQ61K/R mutations. Forced expression of oncogenic RASQ61K/R in normal melanocytes triggers a senescence phenotype via growth

arrest. It is known that nevi can remain arrested in growth for decades [29]. Oncogenic expression of NRAS and BRAF triggers promotion of proliferation, survival, invasion, and angiogenesis of melanoma through the activation of the MAPK pathway [25]. Also, NRAS/BRAF activation mediates an epithelial-to-mesenchymal transition (EMT) switch in a late-stage melanoma. EMT is an independent factor of poor prognosis in melanoma patients [29]. NRAS/BRAF signaling pathway is potential target for anticancer therapy respecting to its high frequency of mutations and its important role in melanoma disease [29].

However, in addition to NRAS and BRAF some other MAPK pathway components frequently also are mutated in human melanoma cell lines and melanoma samples. Stark et al. [33] have established mutations in MAP3K5 and MAP3K9. Recently, noncanonical BRAF mutations have been identified, resulting in constitutive ERK phosphorylation and higher resistance to MEK inhibitors. Screening a larger cohort of individuals with melanoma revealed the presence of recurring somatic MAP2K1 and MAP2K2 mutations, which occurred at an overall frequency of 8% [34]. Although it is possible that the MEK1/2 mutations activate ERK, the presence of these alterations in the face of oncogenic BRAFV600E lesions suggests that other signaling effects may be occurring, and at least some MEK1/2 mutations may also confer resistance to RAF inhibition [35].

2.2. CDKN2/CDK4 tumor suppressive pathway

The cyclin-dependent kinase inhibitor 2A (CDKN2A) is the major gene involved in melanoma pathogenesis and predisposition. It is located on chromosome 9p21 and encodes two proteins, p16INK4a (Inhibitor of Kinase a) and p14ARF (translated in alternative reading frame), both known to function as tumor suppressors [29]. Loss-of-function mutations in CDKN2A locus are the most frequent genetic anomalies in familial melanoma (around 40% of familial melanoma cases). These mutations in patients without any history are relatively rare (around 8.2%) [26,28]. The inactivation of CDKN2A is mostly due to deletion, mutation or promoter silencing (through hypermethylation) [28]. These mutations can affect p16INK4a, p14ARF, or both proteins. p16INK4a interacts specifically with CDK4 and cell division protein kinase 6 (CDK6) and blocks their association with D-type complexes. Thus, loss-of-function of p16INK4a are detected in 50% of melanoma cases and promotes CDK4 and CDK6 activation, resulting in hyperphosphorylation of retinoblastoma protein (pRB), and activation of the transcription factor E2F1 by promoting of cell proliferation. It is also established that p16INK4a loss-of-function promotes melanocyte immortalization [28,29]. p14ARF is mainly known to function by preventing tumor suppressor p53 degradation by the E3 ubiquitin-protein ligase MDM2 and is also frequently inactivated in melanoma [29]. MDM2 negatively regulates p53, and MDM2 function is regulated by p14ARF. Although direct mutations in p53 are infrequent in melanoma (about 5%–10% of humans), inactivation of p53 pathway occurs more via CDKN2A and loss-of-function of its product p14ARF. Therefore, loss-of-function of p14ARF also leads to increased growth and proliferation [18,26].

Table – Mutated genes and aberrant pathways associated with melanoma progression.

Pathway	Gene	Mutation	Frequency (%)	Progression stage	Consequence	References
RAS/RAF/MEK/ERK	N-RAS	Gain-of-function RASQ61K/R	81 15–30	Congenital melanocytic nevi Melanoma	Triggers a senescence phenotype in nevi. Constitutively active MAPK pathway. Increased survival and growth.	[24,25]
		BRAF	Gain-of-function BRAFV600E	82 50	Benign nevi Melanoma	Triggers a senescence phenotype in nevi and transforms immortalized melanocytes. Constitutively active MAPK pathway. Increased survival and growth.
	MEK	Gain-of-function MEK1/2		Melanoma	Constitutive ERK phosphorylation and higher resistance to MEK inhibitors.	[31–33]
	MAPK	Gain-of-function MAP3K5 and MAP3K9	67–85 8	Metastatic melanoma Melanoma	Loss of MAPK kinase functions; decreased MAP3K pathway activity maybe can lead to chemoresistance in melanoma. Constitutive ERK phosphorylation and higher resistance to MEK inhibitors.	[34,35]
CDKN2A/CDK4	CDKN2A	Loss-of-function CDKN2A	40 8.2	Melanoma (familial)	Mutations affect p16INK4a, p14ARF, or both proteins by deletion, mutation or promoter silencing. Uncontrolled cell proliferation.	[36]
		Loss-of-function CDKN2A	56	Melanoma		
		Loss-of-function CDKN2A (CNV)		Melanoma		
	p16INK4a	Loss-of-function p16INK4a	50	Melanoma	CDKN2A loss-of-function. CDK4 and CDK6 activation and promoting of cell proliferation.	[36]
	p14ARF	Loss-of-function p14ARF	90–95	Melanoma	Loss-of-function of p53. Promoting of p53 degradation. Uncontrolled cell growth and proliferation.	[36]
	CDK4	CDK(R24C) Gain-of-function CDK4(CNV)	37	Melanoma (familial) Melanoma	Make p16INK4a unable to inhibit D1/CDK4 complex. Cell cycle progression.	[36]
PI3K/AKT	AKT3	Amplification AKT3	35	Melanoma	Negative regulation of PI3K/AKT pathway. Uncontrolled cell proliferation.	[37–39]
	PTEN	Loss-of-function PTEN	40–60	Melanoma	Negative regulation of PI3K/AKT pathway. Constitutive AKT activation. Uncontrolled cell proliferation.	[37–39]
	PI3K	PI3K	5	Melanoma	Negative regulation of PI3K/AKT pathway. Constitutive AKT activation. Uncontrolled cell proliferation.	[37–39]
Wnt/ β -catenin	β -catenin	β -catenin stabilizing mutation in exon 3	1.5–3	Melanoma	Accumulation of β -catenin protein in cytoplasm and nucleus, upregulation of mitogenic proteins like Myc and Cyclin D1. Uncontrolled cell growth and proliferation.	[40,41]
	Dickkopf-1, 2, 3	Reduced or lost expression of Dickkopf-1, 2, 3		Melanoma	Uncontrolled cell growth and proliferation.	[42,43]
	WIF-1	Reduced or lost expression of WIF-1		Melanoma	Uncontrolled cell growth and proliferation.	[44]

Table (Continued)

Pathway	Gene	Mutation	Frequency (%)	Progression stage	Consequence	References
Notch pathway	Notch1	Haplo insufficiency or gain-of-function of Notch1		Melanoma	High expression level of Notch1, enhanced activation of Notch pathway. Upregulated β -catenin level. Uncontrolled cell growth.	[45-58]
p53 pathway	P53	Mutations or deletions of TP53	1-5 11-25	Primary melanoma Metastatic melanoma	G1/S cell cycle arrest, inactivated p53 pathway; uncontrolled cell proliferation.	[59,60]
	MDM2	Amplification Highly expressed	5 50	Melanoma Melanoma	G1/S cell cycle arrest, enhanced p53 degradation; uncontrolled cell proliferation.	[59,60]
	MDM4	Highly expressed	65		Direct suppression of p53 transactivation; uncontrolled cell proliferation;	[59,60]

Cyclin dependent kinase 4 (CDK4A) is located at 12q13.6 and encodes a protein interacting with the *p16INK4a* gene product. Germline mutations in this gene have been identified in a very small percentage of familial melanoma. The mutation of arginine at position 24 into cysteine or histidine (CDK4R24C/H) renders the protein insensitive to regulation by *p16INK4a* but preserves interaction between CDK4 and cyclin D1 leading to constitutive activation of the complex and aberrant proliferation, through retinoblastoma protein inactivation and E2F activation [28,29].

Recently, Young et al. in a cohort of 143 patients with primary invasive melanoma detected gene copy number variations (CNVs) in *CDK4*, *CCND1*, and *CDKN2A*. It was shown that CNVs were common in melanoma, with gain of *CDK4* or *CCND1* in 37% and 18% of cases, respectively, and hemizygous or homozygous loss of *CDKN2A* in 56% [36].

2.3. PI3K/AKT pathway

The PI3K/AKT pathway is one of the most important signaling networks in cancer. Numerous studies have shown that the activation of this pathway plays a significant role in melanoma, frequently in the setting of concurrent activation of RAS/RAF/MEK/ERK signaling pathways [37]. PI3K/AKT pathway is activated by growth factors or mitogenic stimuli such as insulin-like growth factor 1 (IGF1) and/or RAS. PI3K catalyzes the phosphorylation of phosphatidylinositol (PI) into phosphatidylinositol-3 phosphate (PIP3) which activated serine/threonine kinase AKT [25]. Phosphatase and tensin homologue phosphatidylinositol phosphate phosphatase (PTEN) negatively regulates the PI3K pathway by dephosphorylating and inactivating PI3K. PTEN is frequently inactivated in human cancers. Inactivated PTEN cannot inhibit PI3K; as a result mitogenic proteins are activated by driving their upstream regulator AKT3 [25,29,31]. AKT3 is the form of serine/threonine kinase that preferentially expressed in human melanomas and their activation by gene amplification is found in about 60% of sporadic melanomas (35% of the cases) or by inactivation of *PTEN* (40%-60% of the cases). These mutations negatively regulate the PI3K/AKT pathway. Mutations of *PI3K* have been identified in only 5% of the cases [25,38]. Approximately 30% of metastatic melanomas with

monoallelic losses of *PTEN* show abnormally low transcript and protein levels, suggesting that epigenetic regulation may be involved in tumor development. Recent studies have revealed the correlation between epigenetic silencing of *PTEN* and poorer disease outcome [25,28,29,31].

Functional experiments have demonstrated important roles of the PI3K/AKT pathway in both melanoma initiation and therapeutic resistance. The availability of many inhibitors against the PI3K/AKT pathway is rapidly leading to the development of trials that will ultimately determine its clinical significance in this disease [37]. In addition, Chi et al. recently have established that insulin attenuates the therapeutic efficacy of DTIC and PLX4720 in melanoma cells, which is mediated by activation of the PI3K/AKT pathway and can be overcome by PI3K inhibitors [39].

2.4. WNT/ β -catenin pathway

Alterations in Wnt/ β -catenin signaling pathway are involved in numerous abnormalities of development, growth and homeostasis [40]. WNT proteins include various secretion glycoproteins which join to Frizzled receptors and low density lipoprotein receptor-related protein, in order to stabilize the critical β -catenin protein. β -catenin is a multifunctional protein that binds to E-cadherin and α -catenin at the plasma membrane to assist in cell-cell adhesions. β -catenin is also detected in the cytoplasm or in the nucleus where it acts as a transcriptional co-factor with TCF and LEF proteins. In the absence of Wnt-Frizzled signaling free β -catenin is bound by the inhibitor of the Wnt signaling pathway (Axin), glycogen synthase kinase 3 beta (GSK3b), and APC in a complex that direct phosphorylated β -catenin for proteasome degradation [29,40]. β -catenin is activated when WNT ligands bind to the Frizzled cell-surface receptors. This binding is important for melanoma formation through their downstream inhibitory effects on Gsk3b. β -catenin gene mutations in exon 3 (approximately 1.5% of melanomas) inhibit its phosphorylation by Gsk3b by resulting in its accumulation in the cytoplasm and translocation into the nucleus where it binds and activates transcriptional factor partners TCF and LEF. This leads to upregulation of mitogenic proteins like Myc and Cyclin D1 [29,31]. Also it was established that expression of negative

regulators of canonical WNT signaling pathway such as Dickkopf-1, 2, 3 (Dkk-1, 2, 3) and WNT inhibitory factor-1 (WIF-1) is strongly reduced or lost, both in melanoma cell lines and tumor samples [29]. Recent studies have shown that activation of the Wnt/ β -catenin pathway decreases tumor growth and cooperates with ERK/MAPK pathway inhibitors to promote apoptosis in melanoma [41]. Recently, Conrad et al. by screening siRNA data identified a protein, FAM129B, as a potential regulator of WNT/ β -catenin signaling. It was demonstrated that siRNA-mediated knockdown of FAM129B in A375 and A2058 melanoma cell lines inhibits WNT3A-mediated activation of a β -catenin-responsive luciferase reporter and inhibits expression of the endogenous WNT/ β -catenin target gene, AXIN2. It was also shown that FAM129B knockdown inhibits apoptosis in melanoma cells treated with WNT3A. These experiments support a role of FAM129B in linking WNT/ β -catenin signaling to apoptosis in melanoma [42].

It has been established that ICAT, another inhibitor of β -catenin, and TCF inhibit β -catenin transcription by competing with T-cell factor/lymphoid enhancer factor [43]. Human melanoma cells with high ICAT levels are frequently characterized by deregulated β -catenin signaling. High ICAT levels correlated with formation of metastases in nude mice. Ectopic expression of ICAT in melanoma cells did not affect their proliferation but increased cell motility and invasion of metastatic cells [43].

It has recently been shown that elevated nuclear β -catenin level is associated with improved survival in melanoma patients. Patients with higher nuclear β -catenin in their tumors did not exhibit the survival advantage previously observed in molecularly-unselected melanoma patients who did not receive BRAFi. Also, activation of Wnt/ β -catenin signaling is markedly inhibited in cultured melanoma cells treated with long-term BRAFi. These observations suggest that long-term treatment with BRAFi can impact the interaction between BRAF/MAPK and WNT/ β -catenin signaling to affect patient outcomes [44]. So, understanding of WNT/ β -catenin pathway interactions will be necessary for melanoma patients to facilitate individualized therapies and therefore prolonged their survival.

2.5. Notch pathway

It is known that aberrant Notch signaling leads to skin cancer, but the regulation of the Notch system members in the pathogenesis of human skin tumors are not yet completely understood. Notch is a cell-surface receptor that transduces short-range signals by interacting with transmembrane ligands such as Delta (termed Delta-like in humans) and Serrate (termed Jagged in humans) on neighboring cells. Ligand binding leads to cleavage of Notch receptor and release of the Notch intracellular domain (NICD). Released NICD travels to nucleus and regulates transcriptional complexes containing DNA-binding protein CBF1/RBPjk/Su(H)/Lag1 (CSL). Components of transcription machinery (MAML1, HAC), are recruited to the NICD-CSL complex, leading to the transcriptional activation of Notch target genes (Fig. 2) [45]. Ligands and receptors of Notch pathway are modified by various post-translational events that regulate their quantity, quality or activation processes. These post-translational regulations

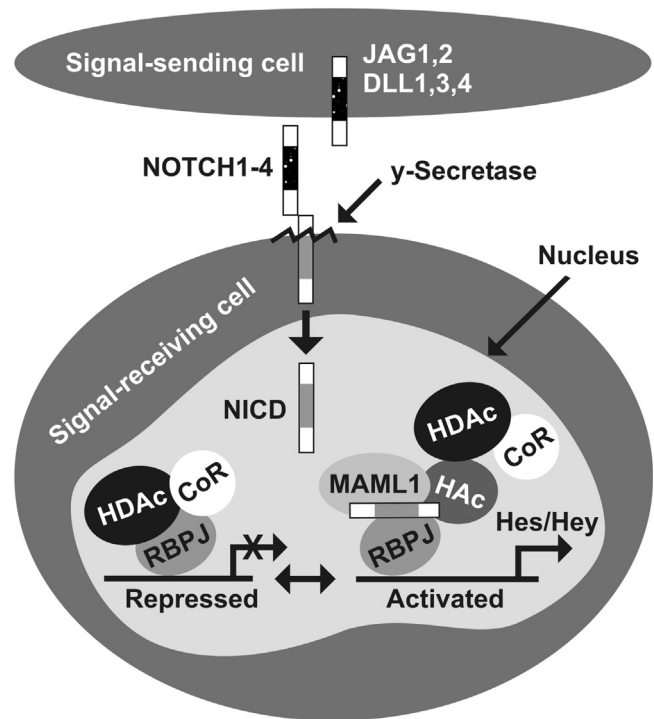


Fig. 2 – Notch signaling pathway. JAG1, 2, Notch ligands; DLL1, 3, 4, Notch ligands; NOTCH1, 2, 3, 4, Notch receptors; γ -secretase, multi-subunit protease complex; NICD, Notch intracellular domain; HDAC, histone deacetylases; HAC, histone acetyltransferases; CoR, CoR corepressors; RBPJ, recombining binding protein suppressor of hairless; MAML1, mastermind-like protein 1; Hes/Hey, transcription factors HES1/Hey (hairy and enhancer of split-1).

include proteolysis (furin-processing of the receptor in the trans-Golgi network (TGN), or successive cleavages by ADAM and gamma-secretase of the Notch receptor upon activation), unusual glycosylation of the receptor during its maturation, trafficking and ubiquitination [46,47]. According to recent data, ubiquitination of Notch pathway plays an important role in regulation of its activity and aberrant ubiquitination enzymes often are related with cancer development, including melanoma [46,48]. Deltex and Nedd4E3 ubiquitin ligases and proteins NUMB and α -adaptin regulate steady-state levels of Notch receptor at the cell surface. Neur and MIB E3 ubiquitin ligases regulate ligand activation by ubiquitylating its intracellular domain [46]. NICD is degraded by the SCF Fbw7 E3 ubiquitin ligase complex via the ubiquitin-proteasome system. Skeletrophin (also known as mind bomb homolog 2) is a RING-finger E3 ubiquitin ligase for the Notch ligands, Jagged2 and Delta. Skeletrophin adds poly-ubiquitin chains to Delta, leading to endocytosis but not degradation. This modification by Skeletrophin positively regulates Notch signaling. The expression of Skeletrophin is suppressed in melanomas by promoter hypermethylation [46,48]. Recently, there were established interactions between NUMB, Notch and p53. As mentioned released NICD enters into to the nucleus where it binds to the nuclear transcription factor CSL and stimulates the transcription of the Notch target genes. NUMB acts as a docking protein for NICD, preventing NICD from translocation

to the nucleus, thereby inhibiting intracellular Notch signaling. At the same time, NUMB interacts with the p53 regulating protein MDM2 (murine double minute 2). MDM2 inhibits p53 function by blocking its transcriptional activity, favors its nuclear export and stimulates its degradation through poly-ubiquitination. It has recently been shown that NUMB forms a trimeric complex with p53 and MDM2, thereby regulating the stability of p53 [49,50]. Interestingly, *MDM2* gene amplification or enhanced gene expression is common event in melanoma [18]. Activated *MDM2* expression must be one of important factors in melanoma, because it is related with regulation of at least two cell signaling pathways (Notch and p53). It is of interest that most of E3 ubiquitin ligases involved in Notch regulation are silenced in melanoma. These findings suggest that MDM2 can be an attractive target for melanoma therapy.

In the last few years, there has been huge interest in the role of the Notch signaling pathway in healthy and diseased skin. Recent studies have shown that Notch signaling plays an important role in epidermal development; however, the underlying molecular mechanisms should be clarified. Notch receptors and ligands are differentially expressed in the different cell layers of the viable epidermis [51]. In healthy skin all four Notch receptors are expressed. Notch signaling seems also to affect the regulation of melanocyte lineage development. Notch is able to determine cell localization and to regulate cell terminal differentiation. Also, Notch signaling is decreased in hyperproliferating skin conditions, including psoriasis vulgaris [51].

Deregulated Notch signaling (haplo insufficiency or gain-of-function of Notch, or Notch-related genes) is frequently observed in a variety of human cancers and is related with poorer outcomes for patients. Notch can act as either an oncogene, or a tumor suppressor depending on both cellular and tissue contexts [52].

Recent studies have shown that the absence of Notch1, Delta1, and Jagged1, missing or decreased Notch signaling lead to disorder in epidermal differentiation and proliferation and promotes formation of basal-cell carcinomas (BCCs) [51]. Notch signaling is also reported to promote the development of cutaneous squamous cell carcinoma (SCC) [53]. It can be assumed that Notch seems to function in the skin as a tumor suppressor. Activation of Notch1 signaling enhanced primary melanoma cell growth in vitro and in vivo and enabled primary melanoma cells to gain metastatic capability. Also, it was shown that oncogenic effect of Notch1 on melanoma cells was mediated by β -catenin, which was upregulated following Notch1 activation. Moreover, inhibiting of β -catenin expression can sustain Notch1-enhanced tumor growth and metastasis [54]. It was found that the expression of proteins Notch1, Notch2, Jagged1, Jagged2, Delta-like 1 is upregulated in dysplastic nevi and melanomas as compared with common melanocytic nevi. These results indicate that the activation of Notch may represent an early event in melanocytic tumor growth and upregulation of Notch signaling may sustain tumor progression [55]. Pinnix et al. [56] have shown that Notch1 alone is sufficient to transformation of human melanocytes. Also, Notch1 enhances vertical growth phase by the activation of the MAPK and AKT pathways; inhibition of either the MAPK or PI3K-AKT pathway reverses the tumor cell growth induced by Notch1 signaling [57]. It was established

also that Notch4 is specifically required for expression of Nodal in aggressive cells, and plays a vital role in cell growth and in maintenance of aggressive phenotype [58].

Recent studies provide evidence that active Notch signaling maintains the cancer stem-cell pool, induces epithelial-mesenchymal transition and promotes chemoresistance. These studies imply that pharmacological inhibition of Notch signaling may refine control of cancer therapy and improve patient survival. Gamma secretase inhibitors (GSIs) are drugs that inhibit Notch signaling and may be successful in controlling cancer cell growth in conjunction with standard chemotherapy, but substantial side effects have hampered their widespread use. Recent efforts have been aimed at the development of antibodies against specific Notch receptors and ligands with the hope of limiting side effects while providing the same therapeutic benefit as GSIs. Together, studies characterizing Notch signaling and modulation have offered hope that refined methods targeting Notch may become powerful tools in anticancer therapeutics [52].

Future studies aimed at identifying new targets of Notch1 signaling will allow the assessment of the mechanisms underlying the crosstalk between Notch1, MAPK, PI3K-AKT, NF- κ B, and p53 pathways.

2.6. p53 pathway

p53 regulates positively or negatively many genes involved in cell cycle regulation (CDKN1A), induction of autophagy, senescence, and apoptosis (NOXA, PUMA, and BAX), as well as genes involved in the DNA repair or cellular metabolism [29,59]. p53 protein is activated through tetramerization, which allows p53 to recognize sequence specific binding sites on target genes and stimulate their activation [59]. Although mutations of p53 are found in approximately 50% of human cancers, but only 1%–5% primary melanomas and 11%–25% metastatic melanoma harbor mutated p53 [18,29]. Mutations in p53 itself remain less frequent in melanoma, therefore it is suggested, that functional anti-tumor properties of p53 can be repressed by many mechanisms. It is established that the MDM2 functions as a negative regulator of p53. Its ability to inhibit p53 is regulated by a negative feedback loop in which activated p53 leads to the transcription and translation of MDM2, and this results in inhibition of p53 [20]. MDM2 interacts with the transactivation domain of p53 via a p53-interacting domain on the N-terminus of MDM2. Binding of MDM2 to p53 prevents p53 from binding to its transcriptional co-activators and subsequently prevents p53 from activating target genes [20].

Although MDM2 has been found to be highly expressed in half of invasive primary and metastatic melanomas, amplification of the *MDM2* locus is infrequent [18,19]. In patient follow-up studies, decreased MDM2 expression was associated with higher rates of survival [18]. This could be explained by the auto-regulatory loop between p53 and MDM2. Increased expression of MDM2 is also possible due to the loss of its repressor p14ARF, a common mutation or deletion seen in melanoma. Without its repressor, MDM2 is constitutively active. These events can be determining of the apoptotic resistance in melanoma despite the largely wild-type status of p53 [18]. It is supposed that targeting of p53-MDM2 regulatory

feedback loop with small molecular inhibitors can be appropriate treatment strategy for patients with wild type p53 metastatic melanoma [20].

In addition to the commonly mutated genes *BRAF*, *NRAS*, *PTEN*, *p53*, and *p16*, new candidate genes have been identified, including *GRIN2A*, *ERBB4*, and *MMP8* (mutated in 30%, 19%, and 7% of melanoma cases, respectively). More recently, were established new melanoma driver genes such as *PREX2*, *PPP6C*, and *RAC1* [29,60].

3. Present treatment of aggressive melanoma

Surgical excision is the standard treatment for localized melanoma. In patients with high-risk factors such as tumor thickness (depth greater than 4 mm), ulceration, high mitotic rate or regional node involvement the risk of developing metastases can be very high (30%–80%) [61]. Still nowadays systemic therapy is the main stay of therapy for most patients with stage IV melanoma and it includes cytotoxic chemotherapy, immunotherapy, or a combination approach such as biochemotherapy [4]. Before, for patients with surgically resected, thick (≥ 2 mm) primary melanoma with or without regional lymph node metastases, the only effective adjuvant therapy was interferon- α (IFN- α) [4]. However, because of the limited benefit upon disease-free survival and the smaller potential improvement of overall survival, the indication for IFN- α treatment remains controversial [17]. Systemic approaches that have been evaluated to date for metastatic disease include cytotoxic chemotherapy as single agents and in multi-drug combinations, including DTIC, temozolomide and platinum agents (carboplatin, paclitaxel, and protein-bound paclitaxel), and immunotherapy, including the cytokines interferon- α (IFN- α) and interleukin-2 (IL-2) [62]. For more than 30 years, standard recommended therapy for patients with stage IV metastasis according to the American Joint Committee on Cancer (AJCC) was single DTIC [63]. Treatment with DTIC alone or in combination has resulted in low response rates, rare durable responses, and no impact on survival. Though response rates for treatment with IL-2 alone have been low, but treatment with this drug had attracted some attention concerning to reports about durable responses in complete responders [64]. The combination of chemotherapy with immunotherapy (biochemotherapy) resulted in increased response rates as observed in numerous phase III trials [65,66]. However, survival benefit has not been demonstrated while toxicity was significantly increased [66].

The increased knowledge about the molecular pathogenesis of melanoma has opened the door to a personalized approach to the treatment of melanoma. The several leading groups of agents that are changing the classical melanoma therapy are the highly selective *BRAF* inhibitors (vemurafenib, dabrafenib), highly specific inhibitor of MEK1/MEK2 (trametinib) and the monoclonal antibody of anti-cytotoxic T-lymphocyte antigen 4, CTLA-4, (ipilimumab).

Vemurafenib is an oral tyrosine kinase inhibitor of the oncogenic BRAFV600 protein kinase. Vemurafenib is recommended for the treatment of adult patients with unresectable or metastatic melanoma and with positive BRAFV600 mutation [7]. In a phase III trial 675 patients with unresectable stage

IIIC/stage IV melanoma with the BRAFV600E mutation were treated. According to results, vemurafenib was associated with statistically significantly improved overall survival (OS) and progression-free survival (PFS) compared with DTIC [7].

Results from the December 2010 data cut-off of the BRIM-3 trial showed that treatment with vemurafenib led to a statistically significant reduction in death (HR = 0.37; 95% CI, 0.26–0.55; $P < 0.001$). At 6 months, overall survival was 84% (95% CI, 78%–89%) in the vemurafenib group and 64% (95% CI, 56%–73%) in the dacarbazine group. People treated with vemurafenib also had a statistically significant reduction in tumor progression (HR = 0.26; 95% CI, 0.20 to 0.33; $P < 0.001$). The estimated median progression-free survival (evaluated in 549 patients) was 5.32 months (95% CI, 4.86–6.57) in the vemurafenib group and 1.61 months (95% CI, 1.58–1.74) in the alternative dacarbazine group [7].

Results based on the February 2012 data cut-off, with patients who switched over from DTIC to vemurafenib and other BRAF inhibitors, showed that treatment with vemurafenib led to a statistically significant progression-free survival benefit (HR = 0.38; 95% CI, 0.32–0.46; $P < 0.001$) compared with DTIC. Median overall survival was 13.6 months in the vemurafenib group and 10.3 months in the dacarbazine group (uncensored HR = 0.76; 95% CI, 0.63–0.93; $P < 0.01$) [7].

The most commonly reported adverse events (grade 2 or more) associated with vemurafenib treatment in the BRIM3 study were cutaneous events, arthralgia and fatigue (December 2010 cut-off based on 618 patients). A total of 61 people (10%) treated with vemurafenib experienced grade 3 cutaneous squamous-cell carcinoma, keratoacanthoma or both, and were treated with simple excision [67]. Molecular studies indicate that development of squamous cell carcinomas and keratoacanthomas during the treatment with BRAF inhibitors are related with paradoxical activation of the mitogen activated protein kinase MAPK pathway that bypasses the inhibition of BRAFV600 [68].

Dabrafenib is another BRAF kinase inhibitor that has demonstrated significant activity in patients with advanced melanoma compared with DTIC chemotherapy. Dabrafenib was approved by the US Food and Drug Administration (FDA) in May 2013 for the treatment of patients with advanced melanoma that contains the BRAFV600E mutation [8]. Results of the pivotal phase III trial (250 patients with unresectable stage III or stage IV melanoma who had the BRAFV600E, have that treatment with dabrafenib significantly increased PFS compared with DTIC (median 5.1 versus 2.7 months; HR = 0.33, 95% CI, 0.20–0.54) [69]. Based upon the independent review of the data, the PFS was similarly increased (6.7 versus 2.9 months; HR = 0.35; 95% CI, 0.20–0.61). Objective responses, as assessed by the independent review committee, were seen in 93 of 187 patients treated with dabrafenib (50%), including six cases (3%) with a complete response. Among those treated with DTIC there were four partial responses in 63 cases, for an overall response rate of 6%. Overall survival was updated at the 2013 ASCO meeting [70]. With a median follow-up of 15 and 13 months for the two groups, overall survival favored patients treated with dabrafenib (HR = 0.76; 95% CI, 0.48–1.21), but was not statistically significant [70].

Treatment with dabrafenib was generally well tolerated. Like vemurafenib, the most frequent grade 2 or greater

toxicities of dabrafenib were dermatologic. Other grade 2 or greater toxicities observed in between 5% and 15% of cases included arthralgia, fatigue, headache, and fever [69].

About 50% of patients treated with dabrafenib or vemurafenib develop disease progression 6–7 months after starting treatment [70]. Multiple mechanisms of acquired resistance have been described including elevated expression of the kinases CRAF, COT1 or mutant BRAF [71–73], activating mutations in NRAS, MEK1, or AKT1 [35,74], aberrant splicing of BRAF [75], activation of phosphatidylinositol-3-OH kinase (PI3K) via the loss of PTEN [76] and persistent activation of receptor tyrosine kinases, including PDGFR β , IGF-1R, and EGFR [74,77,78]. The relative frequency of these resistance mechanisms and correlation with clinical outcome to BRAF inhibitor therapy is poorly understood yet. No single study has analyzed all known mechanisms of resistance in a single patient cohort nor correlated them with clinicopathologic features or outcomes.

Trametinib is a potent, highly specific inhibitor of MEK1/MEK2. Trametinib was originally approved for the treatment of patients who had previously been treated with a BRAF inhibitor for advanced melanoma that contained a BRAFV600 mutation [10]. This approval was based upon prolongation of overall survival using trametinib as a single agent in patients who had not received prior treatment with a BRAF inhibitor. Subsequently, trametinib was approved by the FDA for use in combination with dabrafenib as the initial targeted therapy for patients whose melanoma contained a BRAFV600E or BRAFV600K mutation [15]. This extended approval was based upon the demonstration of an improvement in the duration of progression-free survival from 5–6 months to over 9 months associated with a higher response rate [79]. However, improvement in disease-related symptoms or overall survival relative to dabrafenib alone or vemurafenib has not yet been demonstrated for the combination of dabrafenib and trametinib [79].

The efficacy of trametinib as a single agent was demonstrated in the phase III METRIC trial, with 322 patients with advanced melanoma [15]. All patients had either the BRAFV600E or BRAFV600K mutation in their melanoma (87% and 13%, respectively). One third of patients had received prior chemotherapy and 30 percent had received prior immunotherapy, but prior BRAF inhibitor therapy was not allowed. Crossover to trametinib was permitted in patients who progressed on chemotherapy. According to the results, free survival was significantly increased with trametinib compared with chemotherapy (median 4.8 versus 1.5 months; HR = 0.47; 95% CI, 0.34–0.65). Overall survival was significantly improved with trametinib (6 month survival rate 81% versus 67%; HR = 0.54; 95% CI, 0.32–0.92), even though 47% of patients who progressed on chemotherapy received secondary treatment with trametinib. The improvements in PFS and overall survival were present in all patient subsets, including those with brain metastases or other visceral metastases [15].

To delay the development of resistance to treatment and to minimize the toxicity associated with BRAF inhibition, there was study in which trametinib has been combined with dabrafenib [15]. A phase I/II study demonstrated significantly prolonged progression-free survival compared with dabrafenib alone (median 9.4 versus 5.8 months; HR = 0.39; 95% CI,

0.25–0.62), and significantly increased the proportion of patients alive and progression-free at one year (41% versus 9%). Dermatologic toxicity, manifested by squamous cell carcinoma (including keratoacanthoma) was decreased in both combination dose levels (5% versus 19%), although the incidence of pyrexia was increased (71% versus 26%) [15].

Ipilimumab is a monoclonal antibody that specifically blocks the inhibitory signal of cytotoxic T lymphocyte antigen 4 (CTLA-4), resulting in T cell activation, proliferation and lymphocyte infiltration into tumors, leading to melanoma cell death [5]. Ipilimumab was approved by the FDA for the treatment of unresectable metastatic melanoma in March 2011.

In two large phase III trials, ipilimumab significantly prolonged survival in patients with advanced melanoma. In a placebo-controlled phase III trial, 676 patients were randomly assigned in a 3:1:1 ratio to ipilimumab plus a glycoprotein 100 (gp100) vaccine, ipilimumab alone, or gp100 alone [5]. All patients were HLA-A*0201 positive and had unresectable metastatic melanoma. All patients had received prior systemic treatment for advanced disease with either cytotoxic chemotherapy or IL-2. Results of this trial showed that overall survival was significantly increased in patients given ipilimumab (ipilimumab plus gp100 versus gp100, median 10.0 versus 6.4 months, HR for death 0.68; ipilimumab alone versus gp100 alone 10.1 versus 6.4 months, HR 0.66). Overall survival rates for the ipilimumab plus gp100, ipilimumab alone, and gp100 alone were 44%, 46%, and 25% at 12 months and 22%, 24%, and 14% at 24 months, respectively. The objective response rate was significantly improved in both groups of patients treated with ipilimumab compared to gp100 alone (5.7% and 10.9% versus 1.5%, respectively). Responses to ipilimumab, either alone or in combination with gp100, continued to improve more than 24 weeks after initiation of therapy.

In a second phase III trial, 502 patients with metastatic melanoma were randomly assigned to ipilimumab plus DTIC or to placebo plus DTIC [6]. Approximately one-fourth of patients had received prior adjuvant therapy, but those previously treated for metastatic disease were not eligible. The trial showed that overall survival was significantly increased in patients assigned to ipilimumab plus DTIC compared with placebo plus DTIC (median 11.2 versus 9.1 months). Survival rates at one, two, and three years consistently favored treatment with ipilimumab (47% versus 36%, 29% versus 18%, and 21% versus 12%, respectively). The overall incidence of grade 3 or 4 toxicity was significantly higher with ipilimumab plus DTIC compared with DTIC alone (56% versus 28%). Overall, grade 3 or 4 immune-mediated adverse reactions were significantly more common with the ipilimumab combination (38% versus 4%). Hepatic toxicity was significantly more common with the combination than with DTIC alone (overall incidence of transaminase elevation 29%–33% versus 6%). Furthermore, the incidence of hepatic toxicity was much higher compared with that observed in the phase III trial when ipilimumab was given without DTIC or in prior phase II trials in which ipilimumab administered at this dose and schedule. The increase in hepatic toxicity may be due to its combination with DTIC, which is also known to be hepatotoxic. The incidence of other immune related toxicities (colitis, rash, and hypophysitis) was less than that seen in prior studies with ipilimumab alone,

suggesting that DTIC may have blunted these toxicities and/or the higher incidence of hepatotoxicity may have pre-empted or altered the immune toxicity profile [6].

Another data suggest a potentially synergistic benefit to combining vemurafenib and ipilimumab [80]. It has been observed that non-specific inhibitors of the MAPK pathway, such as MEK inhibitors, may reduce T-cell function, and treatment with vemurafenib has been shown to increase melanoma differentiation, antigen expression, and improve antigen-specific T-cell recognition [80].

As mentioned above, a number of other molecular targets have been identified in melanoma patients. These include C-kit protein (c-KIT), BRAF, MEK, NRAS, PI3K, AKT, mTOR, and GNAC. Several pharmacological inhibitors targeting mutated signal transduction molecules are being explored in clinical trials.

However, despite success of BRAF inhibitors during initial treatment of melanoma patients, was also observed high toxicity of these drugs and resistance, according to novel bypassing mutation in RAS/RAF/MEK/ERK pathway. Also, ipilimumab, a potent but nonspecific immunostimulant, rarely induces tumor regressions. Although, after treatment with ipilimumab disease stabilizes for 3 or more years in a subset of around 10% of patients, but this subset cannot yet be presumptively identified by biomarkers or other tests, so most treated patients do not benefit. Take together these facts, can be concluded that therapy targeting only one molecular target cannot be sufficient to treatment response for great number of patients. So, development of other treatment strategies is required for successful personalized therapy of melanoma patients. Certainly potential target is Notch signaling pathway concerning to its important role in melanomagenesis and its relation with other aberrant pathways in melanoma.

4. Targeting of Notch pathway in melanoma

Notch signaling is a complex pathway able to regulate multiple aspects of the biology of melanoma and of many other cancers. Notch signaling in melanoma cells interacts with additional pathways involved in tumorigenesis, including MAPK, PI3K-AKT, NF- κ B, and p53 [81]. Therefore we could speculate that the targeting several such cascades might be a better approach against melanoma progression.

Thus, given the key role Notch signaling plays in melanoma growth and progression, the targeting of the Notch pathway represents a valuable approach in melanoma therapy. According to recent data [48], E3 ubiquitin ligases can be potential targets for cancer treatment and possible prognostic biomarkers. MDM2 is known as a main regulator of p53 tumor suppressor protein. p53 and MDM2 interact to form an auto-regulatory loop, where increased p53 transcriptionally activates MDM2 and the latter in turn decreases the level of p53 [20]. p53 oncoprotein is overexpressed in many human tumors that retain the wild type p53 allele, including melanoma [19]. The most common approaches used for target validation have been aimed at disrupting MDM2-p53 interactions. According to research data, nutlins are potent and selective inhibitors of p53-MDM2 interaction and effective anticancer agents in vitro and in vivo [82].

Although p53 is often structurally preserved, but functionally crippled, by CDKN2A/ARF loss in melanoma, MDM2 can be attractive target for p53 and Notch signaling pathways in malignant melanoma by restoration of wild type p53 function. MDM2 is overexpressed in malignant melanomas [18]. Recently it has been shown evident relationship between MDM2 expression and tumor thickness and invasion in primary cutaneous malignant melanoma [83].

MDM2 plays a role in Notch signaling: first by upregulating the ubiquitination of NUMB, leading to NUMB degradation, and thus indirectly to an increase of Notch signaling; second by directly targeting Notch 1, resulting in stabilization and activation of NICD. It is thus possible that p53-MDM2-NUMB complexes coordinate the regulation of both the p53 and the Notch pathways [49,50,84].

Another negative regulator of the p53 is MDM4. MDM4 forms a heterocomplex with MDM2 that potentiates the ubiquitination and degradation of p53. Unlike MDM2, MDM4 is not a transcriptional target of p53. Amplification of MDM4 is seen in many tumors, including melanoma, and, interestingly, amplification of MDM4 appears to correlate with both p53 WT status and an absence of MDM2 amplification. MDM4 is upregulated in a substantial proportion (~65%) of stage I-IV human melanomas [19]. Recently were developed stapled SAH-p53 peptides, which inhibit MDM4. Inhibition of the p53-MDM4 interaction restored p53 function in melanoma cells, resulting in increased sensitivity to cytotoxic chemotherapy and to inhibitors of the BRAFV600E oncogene. MDM4 is a key determinant of impaired p53 function in human melanoma and designate MDM4 as a promising target for antimelanoma combination therapy [19].

Thus, MDM2 and MDM4 can be attractive target for p53 and Notch signaling pathways in malignant melanoma by restoration of interaction, including p53-MDM2 (nutlins), MDM2-NUMB (stapled peptide under development), p53-MDM4 (stapled SAH-p53) according to heterogeneity of melanoma tumors and specify of molecular aberrations. Also, delineating the precise interactions between Notch and other signaling cascades in each tumor may significantly improve responses to GSI-based therapies or therapies with BRAF inhibitors.

5. Conclusions

Knowledge of various molecular alterations in signal pathways of melanoma suggest potential targets for new drugs for personalized therapy of melanoma patients. In recent years, the FDA approved several new drugs for patients with unresectable metastatic melanoma. This article summarized present data about molecular mechanisms of signaling pathways of melanoma (including Notch pathway) and approved drug treatment of melanoma molecular targets. We suppose that promising strategy for personalized treatment of melanoma patients can be targeting of Notch.

Conflict of interest

The authors state no conflict of interest.

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