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UNRAVELLING MOLECULAR MECHANISMS UNDERLYING THERAPY RESISTANCE IN CUTANEOUS MELANOMA

Alireza Azimi

Karolinska Institutet

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UNRAVELLING MOLECULAR MECHANISMS UNDERLYING THERAPY RESISTANCE IN CUTANEOUS MELANOMA

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Alireza Azimi

Principal Supervisor:
PhD Suzanne Egyházi Brage
Karolinska Institutet
Department of Oncology and Pathology

Co-supervisor(s):
Professor, MD Johan Hansson
Karolinska Institutet
Department of Oncology and Pathology

PhD Carolina Hertzman Johansson
Karolinska Institutet
Department of Physiology and Pharmacology

Opponent:
Professor, Daniel S Peeper
Netherlands Cancer Institute (NKI)
Department of Molecular Oncology

Examination Board:
Professor, Nico Dantuma
Karolinska Institutet
Department of Cell and Molecular Biology

Associate Professor,MD Ada Girnita
Karolinska Institutet
Department of Oncology and Pathology

Associate Professor, Jochen Schwenk
The Royal Institute of Technology (KTH)
Department of Biotechnology

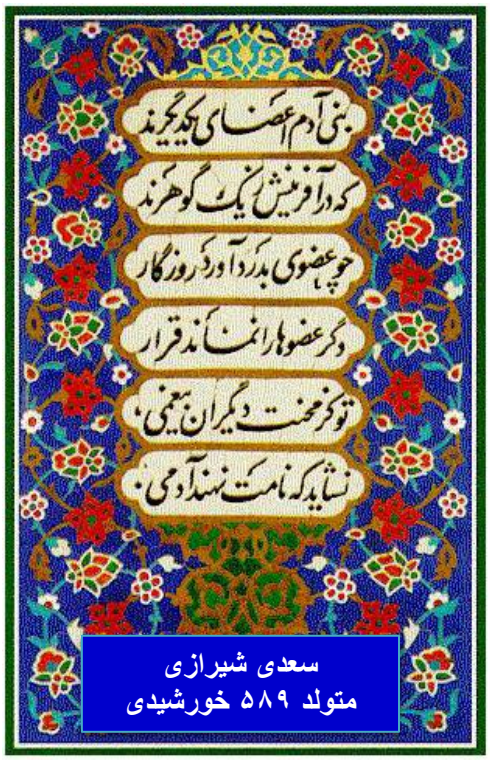
Human beings are members of a whole

In creation of one essence and soul

If one member is afflicted with pain

Other members uneasy will remain

Saadi (Persian poet 1210-1291)



To my family

ABSTRACT

For many years the standard treatment of advanced metastatic melanoma with chemotherapeutic agents, including temozolomide (TMZ) and dacarbazine (DTIC), has been unsuccessful. The paradigm shift in melanoma treatment occurred with the identification of mutations in the *BRAF* gene that leads to a constitutively active BRAF V600E protein. This resulted in the development of BRAF mutant targeted therapies with small molecule inhibitors and showed favorable response in patients harboring BRAF mutations. However, most patients relapse due to acquired resistance to the inhibitors and biomarkers that can predict the therapy response is still lacking.

In the first study we assessed the protein expression of melanosome related proteins in tumor biopsies from melanoma patients with different response to DTIC or TMZ. We found that expression of MITF and GPR143 was significantly higher in tumor samples from patients that did not respond to chemotherapy.

In the second study we performed whole proteome profiling utilizing mass spectrometry based proteomics on pretreatment biopsies from melanoma patients receiving DTIC/TMZ. Our data showed a significant association between high expression of S100A13 protein and resistance to chemotherapy.

Third study focused on identification of mediators of resistance to BRAF inhibitors. We therefore, established mutant BRAF inhibitor resistant sublines of BRAF V600E mutated melanoma cell line A375. By performing mass spectrometry based proteomics we identified several overexpressed proteins in the resistant sublines. We found two novel resistance mediators, aminopeptidase N (ANPEP/CD13) and FLI1 as well as the previously known receptor tyrosine kinase EPHA2 to be overexpressed and demonstrated to mediate vemurafenib resistance in our resistant sublines. Finally, we suggest that combination of vemurafenib with the multi kinase inhibitor dasatinib can overcome resistance in the melanoma cell lines.

In the fourth study the efficiency of combining BRAF inhibitor PLX4720 with TMZ was evaluated in melanoma cell lines with variable sensitivity to BRAF inhibitors. We observed a schedule dependency in the response to the combination of PLX4720 with TMZ and further investigations indicated involvement of DNA damage response activation after PLX4720 treatment. Depletion of DNA repair protein MGMT by lomeguatrib abrogated the schedule dependency effect. Moreover, inhibition of ATR or disruption of the MDM2-p53 interaction by ATR inhibitor or nutlin-3, respectively, synergized with PLX4720 in induction of apoptosis.

This thesis highlights some potential key molecular markers mediating resistance to chemo and targeted therapies in cutaneous malignant melanoma and emphasizes on the importance of using drug combination modalities as a way to overcome or bypass innate or acquired resistance.

LIST OF SCIENTIFIC PAPERS

I. Carolina Hertzman Johansson, **Alireza Azimi**, Marianne Frostvik Stolt, Seyedmehdi Shojaee, Henning Wiberg, Eva Grafström, Johan Hansson and Suzanne Egyházi Brage. Association of MITF and other melanosome-related proteins with chemoresistance in melanoma tumors and cell lines. Melanoma Research 2013, 23:360–365

II. **Alireza Azimi***, Maria Pernemalm*, Marianne Frostvik Stolt, Johan Hansson, Janne Lehtiö, Suzanne Egyházi Brage and Carolina Hertzman Johansson. Proteomics analysis of melanoma metastases: association between S100A13 expression and chemotherapy resistance. British Journal of Cancer (2014) 110, 2489–2495 | doi: 10.1038/bjc.2014.169

III. **Alireza Azimi**; Rainer Tuominen; Fernanda Costa Svedman; Stefano Caramuta; Maria Pernemalm; Marianne Frostvik Stolt; Lena Kanter; Pedram Kharaziha; Janne Lehtiö; Carolina Hertzman Johansson; Veronica Höiom; Johan Hansson; Suzanne Egyházi Brage. CD13/ANPEP, FLI1 and ligand independent EPHA2 activation mediate vemurafenib resistance in human melanoma cells. Manuscript

IV. **Alireza Azimi**, Rainer Tuominen, Hanif Rassool Zadeh, Samaneh Ghashghaei, Marianne Frostvik Stolt, Marianne Farnebo, Carolina Hertzman Johansson and Suzanne Egyházi Brage. BRAF inhibition induces DNA damage response in melanoma cells. Manuscript

*Authors contributed equally

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LIST OF ABBREVIATIONS

53BP1	p53 binding protein 1
AKT	Protein kinase B, Serine/Threonine kinase
ANPEP	CD13, aminopeptidase N
ARAF	A-Raf Proto-Oncogene, Serine/Threonine Kinase
ATM	Ataxia-telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia-telangiectasia and Rad3-related protein
BRAF	Braf, B-Raf proto-oncogene, serine/threonine kinase
CDK	Cyclin-dependent kinase
CDK4/6	Cyclin dependent kinase 4/ cyclin dependent kinase 6
CDKN2A	p16INK4A and p14ARF ,Alternative Reading Frame
CMM	Cutaneous malignant melanoma
CRAF	RAF1 proto-oncogene serine/threonine-protein kinase
CREB1	cAMP responsive element binding protein 1
CSTB	Cystatin B
DDR	DNA damage response
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase, catalytic subunit
DSB	Double-strand break
DTIC	dacarbazine
EGFR/ERBB1	Epidermal growth factor receptor
EPHA2	Ephrine receptor A2
ERK	Extracellular-signal regulated kinase
ETS	E26 transformation-specific
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin embedded
FLI1	Fli-1 proto-oncogene, ETS transcription factor
GDP	Guanosine diphosphate

GTP	Guanosine triphosphate
H2AX	H2A histone family, member X
HGF	Hepatocyte growth factor
HR	Homologous recombination
IGF1R	Insulin-like growth factor 1 receptor
IHC	Immunohistochemistry
KIT/CD117	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
LC/MS-MS	Liquid chromatography coupled tandem mass spectrophotometry
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin-1 receptor
MDM2	mouse double minute 2 homolog
MEK1/MAP2K1	Mitogen-activated protein kinase kinase 1
MEK2/MAP2K2	Mitogen-activated protein kinase kinase 2
MET	hepatocyte growth factor receptor
MGMT	O-6-methylguanine-DNA methyltransferase
MITF	Microphthalmia-associated transcription factor
MMR	Mismatch repair
NFκB	nuclear factor kappa B
NHEJ	Non-homologous end joining
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
NRTK	Non-receptor tyrosine kinase
pAkt	Phosphorylated-Akt
PDGFR	platelet-derived growth factor receptor
pERK	Phosphorylated-ERK
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol 3, 4, 5-trisphosphate
PTEN	Phosphatase and tensin homolog
RAF	Rapidly accelerated fibrosarcoma

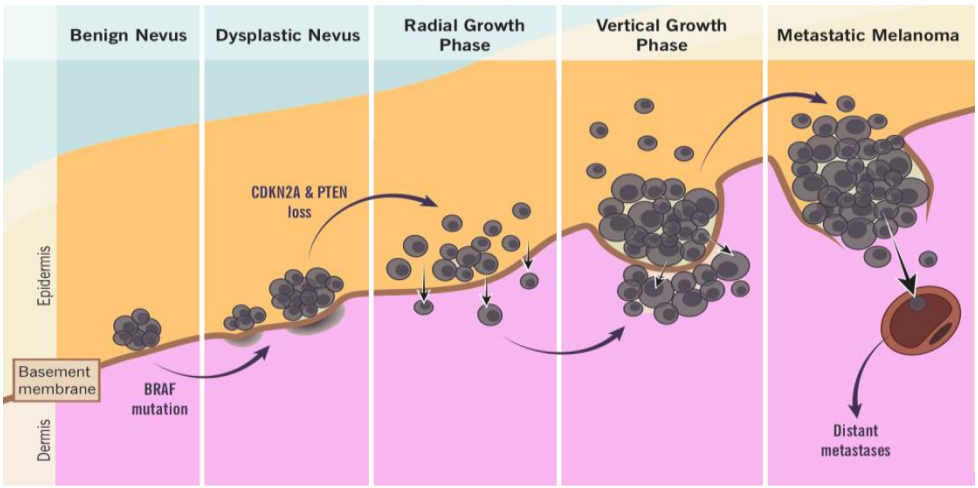
RAS	Rat sarcoma virus
Rb	Retinoblastoma
RNA	Riboneucleic acid
RTK	Receptor tyrosine kinase
S100A13	S100 Calcium-Binding Protein A13
siRNA	Silencing RNA
SRC	SRC proto-oncogene, non-receptor tyrosine kinase
STAT	Signal transducer and activator of transcription
TMZ	temozolomide
TP53	P53, tumor protein p53
TYR	Tyrosinase
UV	Ultraviolet
γH2AX	Phosphorylated H2AX

1. INTRODUCTION

1.1 Cutaneous malignant melanoma

Although cutaneous malignant melanoma (CMM) only accounts for 5% of all skin cancers, it is responsible for 75% of skin cancer related deaths. In advanced disease with distant metastasis the median survival is between 6-12 months. Melanoma arises from the melanin producing cells, melanocytes, and may appear throughout the skin. The malignant transformation of melanocytes to advanced melanoma (Figure1) may occur in four steps: 1. Benign melanocytic nevi (controlled proliferation in normal melanocytes) to atypical/dysplastic nevi (pre-malignant nevi with aberrant proliferation), 2. Radial growth phase (horizontal proliferation and spread to epidermis), 3. Vertical growth phase (vertical invasion through basement membrane), and 4. Metastasis (spread of malignant melanocytes to lymph nodes and other tissues). During vertical growth phase and metastasis, melanoma cells may undergo phenotype switches similar to epithelial to mesenchymal transition (EMT) [1].

Figure1. Melanoma development and progression



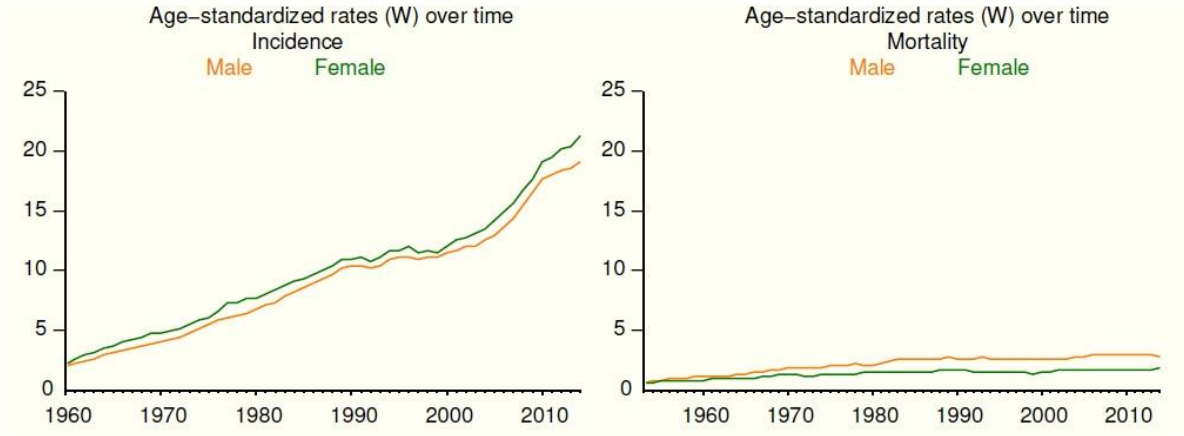
JAMA. 2004 Dec 8;292(22):2771-6. Fitzpatrick's Dermatology in General Medicine,7e. Chapter 124 (modified)

Major causal factors for developing CMM are environmental factors, UV irradiation from sun or exposure to artificial UV light from tanning beds. Light skin color and genetic susceptibility, especially with a history of CMM in the family, are some risk factors [2-4]. The Caucasian population with light skin is particularly at high risk for CMM development especially for subjects with red hair who often have a poor tanning response [5]. A minor part of all melanoma cases appears in a familial setting, with genetic germ-line alterations

increasing the risk for CMM development. The *CDKN2A* gene is found to be altered by mutations in the germline in a proportion of families with hereditary CMM. *CDKN2A* is translated using two alternate first exons into two separate tumor suppressor proteins, p16INK4A and p14ARF; using two partially overlapping transcripts with different reading frames, and somatic alterations have been observed in sporadic CMM including inactivating mutations, deletions and transcriptional silencing [6].

The incidence of CMM has been rapidly increasing globally over the past years rendering it the fastest increasing cancer among all solid tumors, with similar trend between males and females [7] with a yearly increase in incidence of over 5% in Sweden. Figure 2 shows the incidence and mortality rate of skin melanoma in Nordic countries.

Figure2. Statistics for skin melanoma incidence and mortality in Nordic countries: Number of new cases and related deaths in 100,000 individuals, age 0-85^{+}, between years 1950/60-2014 (NORDCAN database)



The Association of Nordic Cancer Registries

2. COMMON ABERRATIONS IN CMM

2.1 Genetic alterations and signaling pathway activation

a. RAS/RAF/MEK/ERK pathway

The RAS/RAF/MEK/ERK pathway is also known as the MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) pathway (Figure 3). MAPK/ERK is the major pathway in control of cell proliferation, growth, survival, senescence and differentiation in melanoma. Compromised regulation of the MAPK pathway in oncogenic transformation of melanoma, results in un-controlled growth of melanoma tumors [8-10].

Main regulators and components of the MAPK/ERK pathway are three RAS proteins (HRAS, KRAS and NRAS), three RAFs (ARAF, BRAF and CRAF), two MEKs (MEK1 and MEK2) and two ERKs (ERK1 and ERK2). RAS proteins are members of the small GTPase family of proteins. Switch of GDP (guanosine diphosphate) to GTP (guanosine triphosphate) activates RAS (RAS-GTP) proteins. The RAS pathway is normally activated by extracellular signals and through interaction of receptor tyrosine kinases with their cognate ligands [11, 12] (tyrosine kinases such as EGFR, MET, KIT, VEGFR, IGF1R, FGFR and EPHA2). Signaling of activated, GTP-bound RAS leads to activation of downstream MAPK pathway either as a response of extracellular, mitogenic signaling molecules or due to constitutive activation of RAS by a mutation (Figure 3). Downstream of RAS are the serine/threonine RAF kinases. BRAF (chromosome 7q34) is fully activated by phosphorylation of amino acids T600 and S602 but for activation of ARAF and CRAF phosphorylation of the N-terminus region of the protein is required, in addition to the kinase domain [13, 14]. Nevertheless, BRAF shows higher kinase activity compared to the other RAFs. Alternative splicing of BRAF results in several different BRAF isoforms from 70 to100 kDa. Relatively high expression levels of BRAF is observed in melanocytes and neural crest cells [15]. Active RAS/RAF signaling leads to ERK activation and to translocation of ERK to the nucleus and activation of downstream targets such as transcription factors c-jun, c-myc etc. [16].

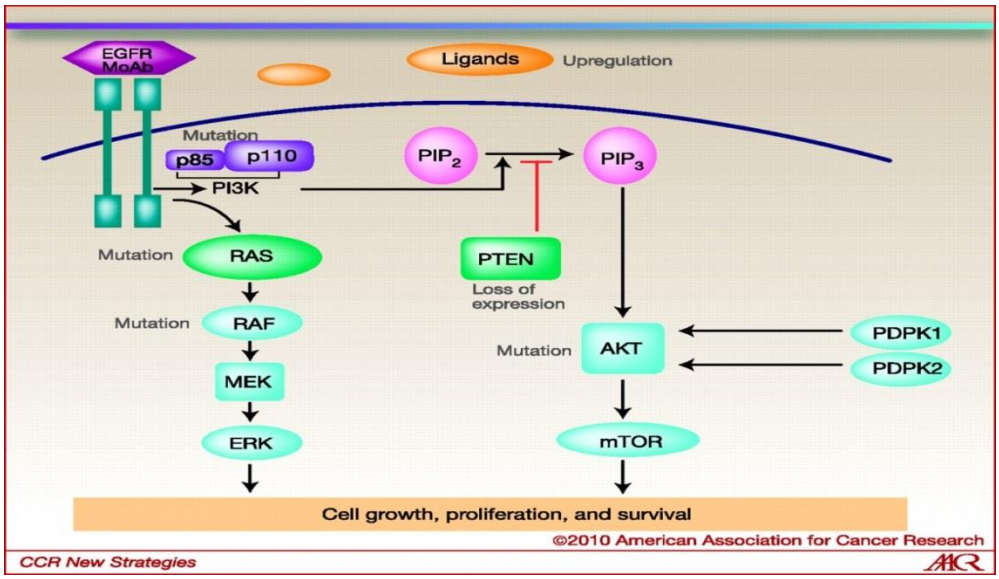
RAS genes are mutated in 15-20% of all human cancers [17, 18] and *BRAF* is mutated in 15% of all human cancers [19, 20]. In CMM, activating mutation in *RAS* and *BRAF* result in activation of MAPK/ERK signaling [9]. A majority of the RAS mutations occur in codon 61 but also in codons 12 and 13 with lower frequency [21]. A majority of *BRAF* mutations occur in exon 15, among those, the most frequent *BRAF* activating mutation occurs in codon 600 (BRAFV600E) that substitutes valine by glutamic acid [18]. Other mutations in BRAF are (BRAFV600K) which substitutes valine with lysine and accounts for less than 20% of melanoma cases with BRAF mutation [22, 23]. BRAF V600R (substitution of valine by arginine) or BRAF V600D (substitution of valine by aspartic acid) are less common BRAF mutations. Another rare *BRAF* mutation occurs in exon 11 leading to BRAF G468A (glycine to alanine substitution) [18]. In CMM the prevalence of NRAS (chromosome 1p13.2) mutations (codon 61) is up to 30% [24-29] and >50% of the melanoma tumors carry BRAF mutations [18, 30] while mutations in ARAF and CRAF are uncommon. NRAS and BRAF mutations in CMM have a clear tendency toward mutual exclusivity (TCGA database: http://cancergenome.nih.gov/).

Mutations in BRAF V600 and NRAS Q61 are early events during melanoma development. In melanocytes presence of these mutations appears to induce oncogene induced senescence. To induce malignant transformation of melanocytes to melanoma, additional alterations in tumor suppressors such as *CDKN2A* gene (inactivation of p16INK4A), loss of normal function of p53 and/or loss of phosphatase and tensin homologue (PTEN) are required [31].

b. PI3K/AKT (phosphatidylinositol 3-kinase/AKT) pathway

Similar to the MAPK/ERK pathway, PI3K/AKT is activated by signals from the receptor tyrosine kinases and also RAS signaling. This pathway is activated (Figure 3) in CMM. Upon activation of the PI3K pathway, AKT proteins (AKT_{1-3}) are phosphorylated by PDK1 and mTORC2 and thereafter activate downstream targets. PI3K/AKT activation is counteracted by the tumor suppressor PTEN [32]. *PTEN* mutations, deletions or promoter methylation results in PTEN loss and consequently leads to AKT activation [33]. The tumor suppressive activity of PTEN functions through dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), inhibiting phosphorylation of serine/threonine AKT and inactivating the pathway [34]. PTEN loss (approximately in 10% of CMMs) is correlated to decreased overall survival in patients with BRAF V600E mutated tumors and to increased invasive capacity of the CMMs [35].

Figure3. Schematic figure of PI3K/AKT and MAPK/ERK pathway activation



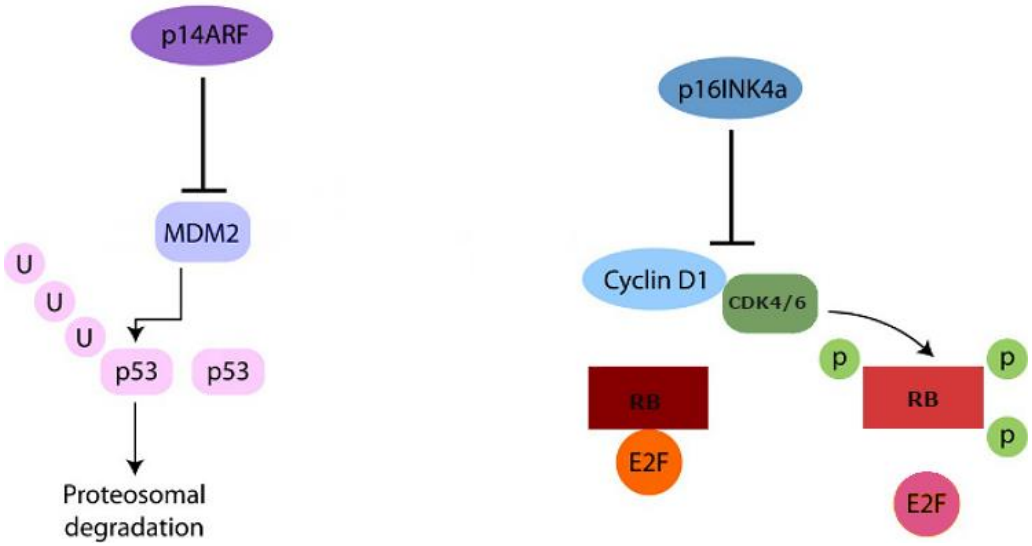
Arvind Dasari, and Wells A. Messersmith Clin Cancer Res 2010 [36] (reproduced with permission)

c. CDKN2A inactivation

CDKN2A is a tumor suppressor gene located on chromosome 9p21 with two reading frames that result in two distinct tumor suppressors, p16INK4A and p14ARF. Genomic alterations such as deletions, mutations and copy number alteration of *CDKN2A* are reported in 44% of CMM (TCGA database: http://cancergenome.nih.gov/). As already discussed, germ-line mutations in the *CDKN2A* gene are found in some kindred with familial CMM [37]. Recently, mutated *CDKN2A* in CMMs, both familial and sporadic cases, was shown to be correlated to decreased patient survival [38]. P16INK4A inhibits cell cycle progression by inhibiting the cyclin dependent kinases CDK4 and CDK6. Retinoblastoma protein RB is phosphorylated by cyclin D1 and CDK4/6 kinases (Figure 4) and, once phosphorylated, is inactivated and released from the E2F transcription factor. E2F actively transcribes its target genes leading to cell progression from G1 to S-phase [39, 40]. Co-occurrence of sustained expression of mutated BRAF and induction of p16INK4A induces cell cycle arrest and senescence in melanocytes [41]. Thus, inactivation of p16INK4A induces uncontrolled cell cycle progression and abolishes cellular senescence.

P14ARF is the result of transcription of *CDKN2A* in a second alternative reading frame and is an inhibitor of MDM2 (Figure 4) by disrupting the MDM2-p53 interaction. Loss of p14ARF inhibits p53 induced apoptosis and in cooperation with *NRAS* mutation drives malignant transformation of melanocytes [42].

Figure4. Schematic picture of the two *CDKN2A* gene products' roles in cellular processes



http://www.intechopen.com/books/recent-advances-in-the-biology-therapy-and-management-of-melanoma/aberrant-death-pathways-in-melanoma (modified) [43]

d. NF1

Neurofibromin 1 (NF1, chromosome 17q11.2) is a negative regulator of the RAS signaling pathway, by enhancing RAS-GTPase activity and converting RAS-GTP to inactive RAS-GDP. Mutations in *NF1* are found in around 26% of CMMs with wild type BRAF or NRAS [44]. While co-occurrence of loss of function mutation in NF1 together with BRAF or NRAS mutation is observed at a much lower frequency of about less than 10% [45].

2.2 Kinases

Protein kinases (PK) constitute a large family of regulatory proteins. They are responsible for phosphorylation of other proteins that usually leads to activation of the modified protein. Two of the major subfamilies of kinases are serine/threonine kinases (STK) and protein tyrosine kinases (PTK).

a. Serine/threonine kinases (STK)

This group of kinases has enzymatic activity that catalyzes the phosphorylation of the OH group of serine or threonine side chains of proteins. Altered expression of these kinases is a common phenomenon in cancers, often following one of three patterns: a) Overexpressed in tumors while absent in normal tissues. b) Generally expressed in normal tissues while overexpressed in tumors and c) Under-expressed in tumors versus normal tissues [46]. The most frequently altered serine/threonine kinase in melanoma is the BRAF protein, which harbors V600 mutations in approximately 50% of CMM patients [18].

b. Protein tyrosine kinases (PTK)

This large family of proteins is involved in post-translational modifications of almost 30% of the human proteome by phosphorylating proteins on tyrosine residues [47]. Two main subgroups of PTKs are receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (NRTKs). RTKs form the largest family of protein oncogenes. They have aberrant expression and activation in many cancer types and are widely regarded as potential therapeutic targets in cancers [48, 49].

<u>NRTKs</u>

Thirty two out of the 90 known PTKs are NRTKs, which are grouped into 10 families based on the intron-exon structure [50]. NRTKs play several cellular and molecular roles such as signaling, migration, and differentiation and cytoskeletal structure. Unlike RTKs, they lack

the receptor features and are mainly localized in cytoplasmic regions of the cells [51, 52]. NRTK families are listed in table 1.

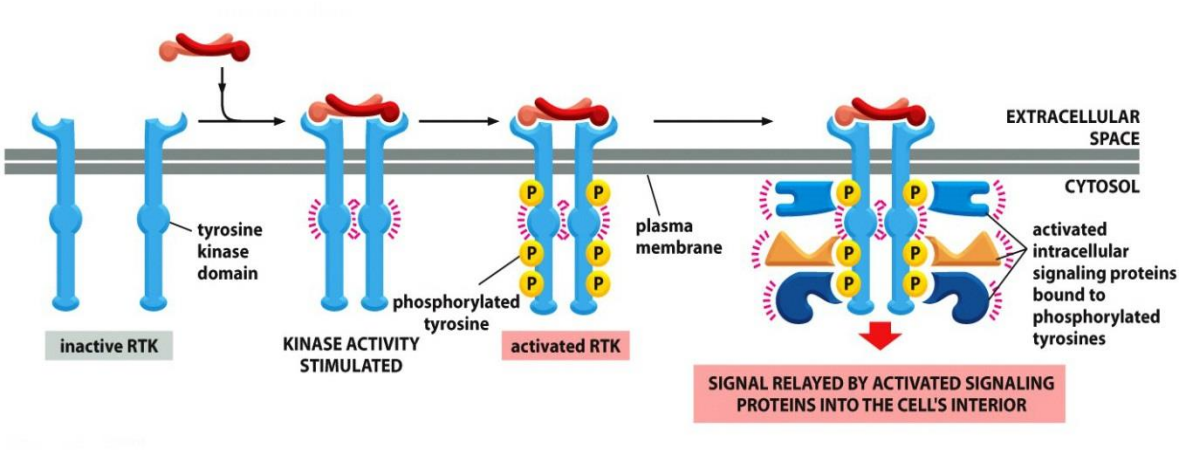
Table1. Non-receptor tyrosine kinases are grouped into 10 families

1. ABL: Abelson murine leukemia viral oncogene homolog 1
2. ACK: Activated CDC42 kinase 1
3. CSK: C-terminal Src kinase
4. FAK: Focal adhesion kinase
5. FES: feline sarcoma oncogene/Fujinami avian sarcoma viral oncogene
6. FRK: Fyn-related kinase
7. JAK: Janus kinase
8. SRC: SRC kinase
9. TEC: Tyrosine-protein kinase Tec
10. SYK: Spleen tyrosine kinase

RTKs

RTKs consist of 58 members divided in 20 families. They all share similar structure that includes a single transmembrane helix, an extracellular domain that functions as ligand binding part, intracellular regulatory and kinase domains [53]. Except for the insulin receptor which is present as a dimer on the cell surface, the other RTKs have been thought to appear as monomers in their inactive forms. Upon ligand triggering they form dimers and become active (Figure5). The ultimate RTK homo- or heterodimer activation involves receptor autophosphorylation of the intercellular residues. Recent structural studies have shown that some of the RTKs are present as an inactive pre-form but in dimers [54]. Overexpression of the RTKs has been correlated to CMM progression, as blocking receptors such as IGF-1R by antibodies inhibits cell proliferation in melanoma cell lines [55, 56]. Activation and dimerization of RTKs are shown in Figure 5.

Figure 5. Summary figure of RTK signaling pathways



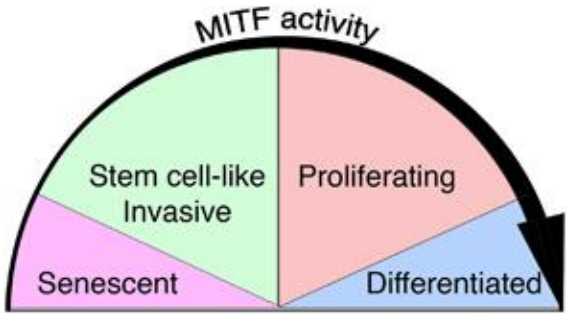
http://oregonstate.edu/instruction/bi314/fall11/signalingtwo.html (modified)

2.3 Transcription factor (TF) dysregulation

Transcription factors are a family of upstream regulatory proteins and alterations of them can lead to dysregulation of several downstream pathways. During progression of CMM the following families of transcription factors have been shown to play pivotal roles: The microphthalmia-associated transcription factor (MITF), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), activating protein 1 and 2α (AP-1), activating enhancer-binding protein 2 alpha (AP-2α), Notch, C-terminal-binding protein (CtBP1), cAMP response element-binding protein (CREB), E26 transformation-specific (ETS), high mobility group box 1(HMGB1), LEF/TCF/β-catenin, Paired Box 3 (PAX3), SKI, Zinc finger protein SNAI1 (Snail) and signal transducer and activator of transcription (STAT family) [57]. In paper I we investigated the role of MITF and other melanosomal related proteins in association with chemotherapy resistance in CMM patients [58].

MITF was identified as a transcription factor for the *tyrosinase* gene. Tyrosinase is a melanocyte-specific essential enzyme for the biosynthesis of melanin [59, 60]. *MITF* has been suggested as an oncogene in melanoma and is amplified in 10-20% of melanoma tumors. MITF amplification is more prevalent in metastatic CMMs and correlates with shorter survival of patients [61]. MITF has a paradoxical role in melanoma: low MITF levels can efficiently induce tumors and evade senescence in melanocytes; melanoma and melanoma stem cells, whereas high MITF regulate genes involved in S-phase progression and mitosis [62-64]. Figure 6 illustrates that differential MITF expression levels are correlated to divergent forms of cellular behavior.

Figure 6. Schematic depiction of how MITF level affects melanoma. Low MITF induces cell cycle arrest in G1, invasiveness and stem cell phenotype while high MITF levels induce differentiation.



Oncogene (2011) 30, 2304–2306 [62] (reproduced with permission)

The ETS family of proteins is one the largest among the transcription factor families, consisting of 29 human TFs grouped in 12 subfamilies. Ets1 is one of the members of this family that was shown to be overexpressed in malignant melanoma and to regulate vascularization and tumor cell invasion trough induction of matrix degrading proteins [65]. FLI1 is another member of the family that was shown to be positively correlated with Ki67 (a cell proliferation marker) and to be highly expressed in tumor samples from patients with metastatic melanoma [66].

NFκB is a protein complex that controls transcription of several genes and their products are involved in a cascade of cellular stress response. The NFκB complex is aberrantly regulated in several cancers including CMM. As thoroughly reviewed by Ueda and Richmond, in melanoma versus melanocytes, NFκB activation has switched the pro-apoptosis behavior of the NFκB complex to anti-apoptosis [67].

STAT is a family of proteins with seven members involved in transcriptional activation and signal transduction. STAT proteins are activated by tyrosine phosphorylation and consequently form homo or heterodimers [57]. One of the members of this family, STAT3, was demonstrated to be activated by SRC kinase activity in melanoma cell lines. Moreover, in the same melanoma cell lines, inhibition of either SRC kinase activity or STAT3 phosphorylation induced apoptosis. This indicates the involvement of SRC induced STAT3 activation in CMM cell survival [68].

2.4 DNA damage response

Among many DNA lesions occurring every day only a very low proportion is double strand breaks (DSBs). If not properly repaired, these lesions may have consequences such as cell death, mutations, or in more severe cases, lead to cancer, immunodeficiency or neurodegenerative diseases [69]. Besides endogenous causes for DNA damage (such as enzymatic activity, DNA replication or oxidative respiration), environmental factors such as UV irradiation can induce DNA damage. The cellular defenses against DNA damage are termed DNA damage response (DDR) and include mechanisms both to counteract and repair DNA damage and to maintain genomic integrity. Several DNA repair pathways have been identified. Mismatch repair (MMR) is activated when a mismatched base occurs during DNA replication/recombination. The mismatched base is recognized by a protein complex including "Mut" proteins. Following this, the single, affected, strand of DNA is incised and resynthesized by a DNA polymerase [70]. Damage to single bases is corrected by base excision repair (BER) and UV light induced helix-distorting base is repaired by nucleotide excision repair (NER). Two main repair pathways are activated in response to double strand breaks, homologous recombination (HR) and non-homologous end joining (NHEJ). During HR, a stretch of nucleotides is exchanged between identical DNA molecules to repair DSBs while in NHEJ the ends of DNA are ligated without the use of a homologous template.

In DDR several cascades of events are activated, including phosphorylation of the apical phosphatidylinositol 3-kinase-related kinases, ataxia-telangiectasia (ATM), Rad3-related protein (ATR) and DNA-dependent protein kinase catalytic subunit (DNA-PKs) kinases. When DDR is activated, one of the early events is serine 139 phosphorylation of histone variant H2AX (γH2AX) by ATR, ATM or DNA-PKs [71, 72]. Phosphorylation of H2AX is contributory in accumulation of repair proteins at the site of DNA damage [73]. Upon DNA damage, checkpoint kinase 1 (CHEK1) is activated through kinase activity of ATR which further activates p53, implying the link between ATR and p53 [74]. Activation and stabilization of tumor suppressor p53 occurs through several post transcriptional modifications. Activated p53 functions as a transcription factor for regulators of cell cycle regulation, induced cell death and senescence [75-77].

DNA alkylating drugs such as temozolomide deliver methyl group to purine bases of DNA (O6-guanine; N7-guanine and N3-adedine). The O6-methylguanine (O6-MeG) DNA lesion raised DDR and is removed by methylguanine methyltransferase (MGMT) protein or through MMR mechanism [78].

3. CMM TREATMENT

For early stage CMM surgical excision of the primary tumor is the standard treatment with a good prognosis, while the prognosis for advanced metastatic disease is very poor. Surgery of advanced metastatic disease (if performed) often has a palliative role e.g. to remove obstruction in the bowel, etc. and instead systemic drug treatment is used [79, 80]. Radiotherapy has also a therapeutic role in advanced CMM but generally as palliative therapy in metastatic disease. However, targeted therapy with BRAF inhibitors has significantly increased median overall survival and median progression-free survival compared to dacarbazine [81]. Immunotherapy with check-point inhibitors such as ipilimumab has also improved overall survival in CMM patients [82].

3.1 Chemotherapy: For several decades the standard therapy for disseminated CMM has been mono- or combination therapy with chemotherapeutic agents using: DNA alkylating drugs, dacarbazine (DTIC), temozolomide (TMZ) or nitrosoureas (such as fotemustine); platinum compounds (such as cisplatin or carboplatin); antimicrotubular agents; vinca alkaloids (microtubule assembly inhibitors such as vindesine and vinblastine) and taxanes (inhibitors of microtubule disassembly such as paclitaxel). However, these therapy regimens result in therapy response rates of 5-12% with a median overall survival of less than one year [83, 84]

3.2 Immunostimulants: This group includes immune system stimulators like interleukin 2 (IL-2) and interferon alpha (IFNα). As reviewed by Bhatia et al., several studies compared combination of chemotherapy with IL-2 and low dose IFNα versus chemotherapy alone and, despite moderately improved objective response rate with combination therapy, they have increased toxicity without improving overall survival [83].

3.3 Targeted therapy: The term "targeted therapy" refers to therapeutic small molecules that are designed to inhibit specific molecules in the cells that are driving aberrant proliferation and growth in cancer cells; therefore, they may be more efficient with fewer side effects compared to conventional cytotoxic chemotherapies. For BRAF mutated CMMs, two inhibitors of the mutated BRAF protein have been approved by drug regulatory authorities (the US FDA and the European EMA): vemurafenib (Zelboraf®) in (2011 and 2012 respectively) and dabrafenib (Tafinlar®) (FDA approved in 2013). Studies comparing DTIC with the two BRAF inhibitors (BRAFi), vemurafenib and dabrafenib, have shown improved progressions free survival (PFS) as well as overall

survival (OS) with the targeted therapies [81, 85]. For the wild type MEK protein two inhibitors have been approved: trametinib (Mekinist®) and cobimetinib (Cotellic®). The MEK inhibitors are used for patients with mutated BRAF as follows: Trametinib is used in combination with dabrafenib and cobimetinib is approved for use in combination with vemurafenib. Side effects for therapy with the targeted drugs are generally milder than those for chemotherapies. Commonly observed and reported side effects of treatment with BRAFi are reviewed by Welsh and Corrie [86] and include: vemurafenib induced moderate to severe photosensitivity, rash, keratoacanthoma, cutaneous squamous cell carcinoma (SCC) arthralgia, diarrhea and fatigue [87]. Despite sharing common side effects, photosensitivity is rare in patients treated with dabrafenib compared to those treated with vemurafenib.

3.4 Immunotherapy: In parallel with targeted drugs, novel immune therapies have been developed for CMM. In 2011, ipilimumab (Yervoy), a monoclonal blocking antibody against CTLA-4 was approved for treatment of unresectable CMMs. CTLA-4 is a protein receptor on the surface of cytotoxic T-lymphocytes and negatively regulates immune response in detection and destruction of cancer cells [88]. Blocking the CTLA-4 receptor has shown improved overall survival in melanoma patients with advanced disease [89]. Interaction of programmed death ligand-1 (PDL-1) and its receptor (PD-1), expressed on activated T-cells, B-cells and myeloid cells, is another target for immune therapy. The interaction of the ligand-receptor suppresses immune response and is inhibited by two FDA approved antibodies pembrolizumab (KEYTRUDA®) and nivolumab (OPDIVO®). In a phase I clinical trial, CMM patients refractory to ipilimumab were treated with pembrolizumab (anti PD-1) resulting in an overall response rate of 26% and only 3% severe adverse effects [90]. Nivolumab treatment has significantly improved overall survival in BRAF wild type CMM patients compared to DTIC and has superior response rate in comparison to DTIC and ipilimumab [91, 92].

3.5 Autologous T-cell therapy: This treatment refers to an approach of collection, *ex vivo* expansion and reinfusion of tumor infiltrating T-cells (TILs). For the purpose, TILs are collected by enzymatic digestion of surgical tumor material [93, 94] and expanded in vitro. Propagated TILs are re-infused to the patient, usually after an intense conditioning treatment with chemotherapy that depletes the patient's bone marrow. This autologous adoptive T-cell transfer targets the cancer cell antigen specifically and has shown antitumor activity in advanced CMM patients [95].

4. THERAPY RESISTANCE IN CMM

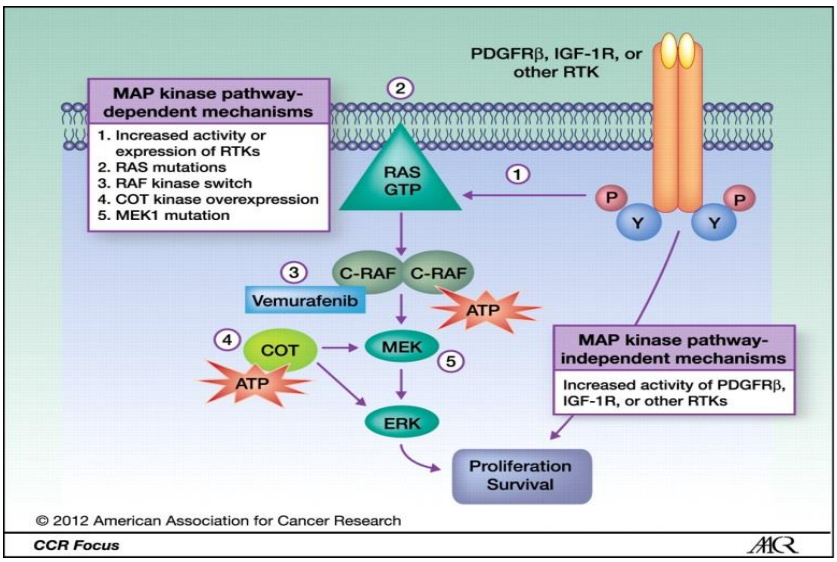
Resistance to therapies is a major problem for CMM treatment. For several decades, chemotherapy with DTIC and TMZ has been largely unsuccessful both due to innate and to acquired resistance to treatment [96]. Drug resistance in solid tumors has been vastly investigated with regards to epigenetic and genetic alterations such as mutations, deletions and gene amplification. Changes in drug uptake, metabolism or export of the drug from the cells are also suggested to play roles in drug resistance. Tumor microenvironment and extracellular matrix (ECM) interaction with cancer cells, tumor hypoxia and acidity and abnormal tumor vasculature and inefficient blood flow inside tumor (affects drug delivery to the tumors cells) are important effectors in drug response in solid tumors [97].

4.1 Chemotherapy resistance mechanisms: For many years investigations have been performed to unravel resistance mechanisms to conventional chemotherapies. Some general mechanisms involved in chemoresistance are: a) classical multi drug resistance in which cell membrane efflux pumps are increased and eliminate and excrete cytotoxic agents, b) induction of certain enzyme systems e.g. for detoxification of alkylating agents by conjugating the chemotherapy agents to glutathione through activity of glutathione-S-transferases (GST) and c) disruption of drug-target interactions possibly due to alterations in the targets which results in reduced binding affinity [98, 99]. Alteration in drug distribution inside cells is an important mechanism in induction of resistance to chemotherapies [100]. One way to alter distribution of the drug is through sequestration of drugs in subcellular organelles such as melanosomes (cellular organelles responsible for synthesis, storage and trafficking of melanin), which have been shown to have a role in cisplatin (CDDP) resistance [101]. Biogenesis, accumulation, and structural integrity of melanosome and melanosomal related proteins such as microphthalmia-associated transcription factor (MITF), G-protein coupled receptor 143 (GPR143), pre-melanosome gp100 (gp100/PMEL), MLANA (MART1), tyrosine related protein 1 (TYRP1) and melanosome trafficking related protein RAB27a are associated with chemotherapy resistance in melanoma cells [58, 102]. Targeting protein trafficking and melanosome formation enhanced cytotoxic effects of anticancer therapies with CDDP, dacarbazine and TMZ in melanoma cells [102, 103]. Anti-apoptotic pathways and enhanced DNA repair in cancer cells also play roles in unresponsiveness to chemotherapy in CMM [104]. As previously discussed, MGMT is a DNA repair protein inducing resistance to DNA damaging effect of DNA alkylating drugs e.g. TMZ, by removing the drug induced

alkyl adducts. Depletion of MGMT, with the pseudo-substrate O6-benzylguanine and lomeguatrib, inhibits the removal of O6-MeG lesions and sensitizes tumors to alkylating drugs [78]. In patients with advanced CMM, inactivation of MGMT through promoter methylation is associated with better TMZ response and prolonged progression free survival [105]. Depletion of MGMT with regards to tolerability and efficacy, alone or in combination with DTIC, has been the focus of some studies in melanoma patients [106, 107].

4.2 Targeted therapy resistance mechanisms: Despite a rapid response to targeted drugs such as vemurafenib, dabrafenib and trametinib, in the majority of patients relapse eventually occurs. Resistance to the kinase inhibitors is due to multiple mechanisms; some of the resistance mechanisms to BRAF inhibitors are shown in Figure 7 and briefly mentioned below: genetic alterations such as gene amplification (e.g. amplification of *BRAF*) , novel or secondary mutations (e.g. secondary *NRAS* or novel MEK1 mutations), presence of abnormal protein isoforms (e.g. BRAF), hetero- and homodimerization of proteins (e.g. BRAF-CRAF heterodimers) activation of alternative signaling pathways (e.g. PI3K/AKT pathway activation and PTEN loss), signaling bypass (e.g. MAPK reactivation by overexpression of COT and CRAF), tumor microenvironment derived resistance (e.g. hepatocyte growth factor HGF induced activation of the MET receptor, upregulation of PDGFRβ), histological transformation (e.g. epithelial to mesenchymal transition or vice-versa), RTK overexpression (e.g. AXL, IGF1R, MET, EGFR, EPHA2) and aberrant expression of transcription factors (e.g. MITF downregulation) [108-120].

Figure 7. Schematic illustration of mechanisms of resistance to BRAFi in CMM



Alexander M. Alcalá, and Keith T. Flaherty Clin Cancer Res 2012 [113] (reproduced with permission)

4.3 Prediction of response to therapy: Most CMM patients experience either innate or acquired resistance to therapeutic agents. Unlike for targeted therapies, only a small subpopulation of patients responds to chemotherapies. Therefore, identification of reliable biological markers which can predict long-term therapy response would enable oncologists to find those that would benefit from the treatment. This approach may help to further individualize the treatments based on the therapy response prediction. Moreover, biomarker-based identification of possible non-responders enables the use of alternative treatment options such as combination of two targeted drugs, or combination of immunotherapeutic agents. As previously discussed, epigenetic and genetic (such as deletion/amplification/mutation) alterations, tumor vascularization, tumor hypoxia and acidity, tumor-microenvironment interactions, RTK overexpression and MAPK reactivation are predictors of therapy response in CMM [121]. Epithelial to mesenchymal transition-like phenotype switching in melanoma, is also predictive of drug response especially with regards to BRAFi treatment [122]. Besides EMT molecular markers, such as loss of E-cadherin and induction of N-cadherin and osteonectin (SPARC), aminopeptidase N (ANPEP/CD13) is a surface receptor that is found to be expressed on melanoma cell surface and correlate to high angiogenesis and metastatic capacity of the cells [1, 122, 123] and may also predict treatment response in CMM. High levels of calcium binding S100 protein was shown to correlate to increased tumor angiogenesis, metastatic capacity and immune evasion in different cancers [124]. S100A13 is an angiogenic marker that positively correlates with high vascular endothelial growth factor (VEGF)-A protein expression in advanced melanoma patient tumors [125]. Serum level of S100B protein can be used as a marker in predicting and monitoring drug response in CMM patients treated with chemoimmunotherapy [126]. High expression of MITF and other melanosomal proteins correlate with poor chemotherapy response, while for mutant BRAF inhibitors low MITF expression is predictive of unresponsiveness [58, 120, 127].

5. AIMS OF THE THESIS

The research projects presented in the current thesis, primarily aim at identification of therapy resistance mechanisms in CMM and finding novel predictive biomarkers for therapy response. Moreover, a project investigates drug combination schedules (simultaneous or concomitant) that might abrogate/bypass the therapy resistance or delay the onset of resistance.

Specific aims for the papers I-IV

Paper I: Investigate the correlation between chemotherapy resistance and melanosomal related proteins; specifically MITF

Paper II: Identify predictive biomarkers for response to chemotherapy in pre-treatment; fresh frozen tumor biopsies from CMM patients, utilizing a mass spectrometry based proteomics based approach

Paper III: Uncover novel and confirm known resistance mechanisms to BRAFi in melanoma cell lines with acquired resistance to BRAFi, using mass spectrometry based proteomics, gene expression analysis and targeted next generation sequencing.

Paper IV: To investigate the efficiency of combining targeted therapy (BRAFi) with conventional chemotherapy (TMZ) in an *in vitro* model.

6. MATERIALS AND METHODS

6.1 CMM tumor samples:

Paper I: For this study, pretreatment tumors from 52 CMM patients; treated with DTIC/TMZ, including 34 men and 18 women, were collected. The mean age was 58 (25-82) years. A majority of the tumors were lymph nodes metastases and the others were skin metastases. The tumors were formalin-fixed and paraffin embedded. Eighteen patients were responders and 34 were non-responders to chemotherapy.

Note: World Health Organization WHO criteria, defines response to the therapy as 50% reduction in tumor size (by measure of the sum of products of two perpendicular tumor diameters).

Paper II: In this study a collection of 14 fresh frozen lymph node CMM metastases were selected from two groups, responders (n=5) and non-responder (n=9) to chemotherapy with DTIC/TMZ. The selection was made based on presence of at least 50% tumor cells in the lymph nodes (majority >70%). Five responders and five non-responders were matched for age and sex and the rest of the samples were selected since they had been previously analyzed by gene expression microarray. For validation of the findings an extended set of formalin-fixed paraffin-embedded pretreatment tumors from 16 responders and 34 non-responders to DTIC/TMZ were selected for IHC analysis. The selected samples are overlapping with those used for the study in paper I.

Paper III: Samples from three patients with metastatic CMM (stage M1c) who received BRAF inhibitor (vemurafenib or dabrafenib) were selected. Tumors were collected before treatment and after relapse. All samples were formalin-fixed paraffin embedded.

For all of the patient tumors used in the studies I-III, ethical permits were approved by Regional Ethics Committee of Stockholm and patients gave informed consent.

6.2 *In vitro* cell line models and sublines with acquired drug resistance

For all four studies, a set of melanoma cell lines (SKMEL24 and SKMEL28 and A375) were purchased from American type culture collection (ATCC). The pigmented MNT-1 cell line was kindly provided by Dr. Pier Giorgio Natali, Instituto Regina Elena; Rome, Italy.

Induction of BRAF inhibitor resistance: A375 cells were consecutively treated with either vemurafenib or PLX4720 (non-clinical analogue of vemurafenib) in a dose escalating manner. Three BRAF inhibitor resistant sublines were established over a course of two months treatment. A375PLX4720R1 (A375PR1; resistant to PLX4720), A375vemuR3 (A375VR3; resistant to vemurafenib) and A375vemuR4 (A375VR4; resistant to vemurafenib) were established. Sensitivity to BRAF inhibitors was tested by MTS

proliferation assay. STR profiling of A375 cells and all the BRAFi resistant sublines was performed to confirm the authenticity of the cell lines using the AmpFLSTR™ Identifiler™ PCR Amplification Kit (Thermo Fisher Scientific).

6.3 Proteome profiling using LC/MS-MS

Liquid chromatography (LC) coupled to tandem mass spectrometry (MS-MS) is a powerful technique with high sensitivity in analytical chemistry. Liquid chromatography separates molecules based on the physical property and mass spectrometry separates ions based on their mass to charge ratio (m/z) in a gas phase. Utilizing the LC/MS-MS technique, the cell proteome can be thoroughly analyzed in a quantitative manner. The process of LC/MS-MS proteome profiling is briefly described below:

a. Sample preparation and protein extraction: Human tissue disruption was performed using tissue homogenizer or Mixer mill MM200 (Retsch, Hann, Germany). For this purpose, Teflon cylinders were precooled in liquid nitrogen before transferring $1mm^3$ of frozen tissues to the cylinder and the disrupting the tissues into powder by shaking. Then each sample was dissolved in 20mM HEPES solution and immediately frozen in -80^oC. For the analysis the tissue suspension was thawed and mixed with 1mM of DTT and 3.75% SDS. The mixture was heated at 90^oC 5min followed by sonication for 5min in room temperature (RT, FASP protocol). Finally, after centrifugation of the samples at 10,000g for 10min the supernatants were collected for protein concentration measurement. For acetone precipitation, 120mg protein from each sample was mixed with four volumes of ice-cold acetone and incubated at 4^oC for 2hrs to form the flocculent. Another centrifugation at 10,000g (10min) was performed to remove the supernatant and collect the precipitated proteins. The protein pellet was air dried at room temperature. This final step was only performed for the MS-MS part but not for the immunoblotting confirmation. For the similar analysis of the cell line protein profiling for the BRAF inhibitor resistance analysis (project III) the protein extraction buffer was a complete RIPA buffer system purchased from Santa Cruz biotechnology Inc.

b. Protein digestion and iTRAQ labeling: Each sample was trypsin digested (1:20 trypsin to protein) and labeled with a unique iTRAQ label according to the manufacturer´s protocol (Thermo Fisher Scientific, Waltham, MA, USA). Eight iTRAQ labels were available and therefore, seven samples and one internal standard were pooled (each 100µg) for the MS analysis. The other seven protein samples were assigned to the second pool and were analyzed separately due to limited number of iTRAQ labels. Pooled samples were then cleaned to remove excess unbound labels by applying them through 1ml Strata X-C 33mM polymeric strong cation exchange (SCX) microcolumns (Phenomenex, Torrance, CA, USA).

c. Narrow-range IEF: Samples mixed with low pH (pH 3.3) buffer were loaded on narrow range isoelectric focusing strips (pH 3.4-4.7; GE healthcare, Uppsala, Sweden). Then the loaded peptides were separated accordingly to their isoelectric points, and eluted to 72 predefined fractions. Each fraction was separately eluted by milliQ water and dried in speedVac for MS analysis.

d. Mass spectrometry: Each of the 72 fractions with a specific pH was injected into HPLC/MS (Agilent Technologies, Santa Clara, USA/ Thermo Fischer Scientific, San Jose, USA; LTQ-Orbitrap Velos). For detailed procedural explanation see reference [128].

e. Database search: Data from the Orbitrap mass spectrometer were aligned against Uniref100 protein sequence database using Proteome Discoverer 1.1 software (Thermo Fisher Scientific).

6.4 Additional methods: The main methods used in this thesis are briefly described below.

Immunoblotting: To validate the candidate proteins' expression and evaluate efficiency of gene expression modulation, human melanoma cells or human tissues were lysed and protein was extracted using RIPA buffer system or HEPES-DTT-SDS buffer (FASP protocol; explained below). The protein concentration of samples was measured using Pierce™ BCA Protein Assay Kit (Thermo Fischer Scientific, USA). Equal concentration of the samples were loaded on NuPAGE Novex Bis-Tris Gel (Life Technologies, Carlsbad, CA, USA) and then transferred to PVDF membranes (Thermo Scientific, Rockford, IL, USA), according to the manufacturer's standard protocol. Chemiluminescent method was used to visualize the protein expression.

Immunohistochemistry (IHC): IHC was performed on formalin-fixed paraffin-embedded tissues (3-4µm thick samples). First, sections were heated in pressure cooker in citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase was blocked with 10 minutes incubation in 3% H_2O_2 and after rinsing in 1x TBS buffer, they were incubated in 2,5% blocking buffer (1x TBS+ horse serum) to minimize the risk of unspecific antibody binding. Then sections were incubated with an optimized concentration of primary antibody (diluted in blocking buffer) at 4^oC overnight. Sections were rinsed in 1x TBS and a secondary, biotinylated antibody together with streptavidin/ peroxidase (Vectastain Universal Quick Kit, Vector) was added at room temperature for 1hr. Developing and nuclear counter staining were performed by incubating the sections for 10 minutes in 3,3′-diaminobenzidine (DAB kit, Vector Laboratories Inc., Burlingame, CA, USA) and 45 seconds in Mayer's haematoxylin. Each

section was mounted and sealed by a coverslip (Histolab, Sweden). Evaluation of protein expression in each tissue section was performed blinded with regard to the clinical data.

DNA and RNA extraction: DNA and RNA were extracted from cell lines with and without treatments using the extraction kits according to the product manual; RNeasy kit (cat no. 74104) and DNA mini kit (cat no. 51304) both from Qiagen.

Gene silencing: To study the role of selected genes in therapy response, we silenced the gene of interest using silencing RNA (siRNA) in the melanoma cell lines and evaluated the treatment response. Non-targeting siRNA was used a negative control for these experiments.

Gene expression analysis (qPCR): Quantitative real-time PCR was used to quantify mRNA expression of selected genes in cell lines with DMSO versus drug treatment or non-targeting si-control and gene specific siRNA to ensure efficient gene silencing.

Gene copy number analysis (CNA): Copy number analysis of the gene of interest was performed using custom TaqMan Copy Number Assays (Applied Biosystems, USA).

Next generation sequencing (NGS): For identification of any mutations involved in BRAF inhibitor resistance we performed NGS sequencing (Agilent HaloPlex technology followed by next generation sequencing; Illumina Hiseq) on a selected set of 120 genes known to be relevant in CMM. The findings were then confirmed by standard Sanger sequencing (BigDye Terminator v.3.1 system in ABI 3700 capillary electrophoresis system (both Applied Biosystems, Carlsbad, CA).

Cell proliferation assay: For evaluation of base line and treatment effect of siRNA or targeted/chemotherapeutic agents, treated and untreated cells were subjected to measurement of proliferation inhibition or induction using Celltiter96 AQueous one solution cell proliferation assay (MTS; Promega, USA).

Apoptosis/necrosis assay: End point effect for many of the targeted or chemotherapeutic agents is apoptosis induction. Therefore, we performed flow cytometry based apoptosis and necrosis analysis using Annexin V and propidium iodide (PI) staining. Annexin V binds to externalized phosphatidylserine and indicates apoptotic cells and PI intercalates with DNA indicating disrupted cell membrane in necrotic cells.

Scratch assay (wound healing assay): To study the proliferation/gap filling capacity of the A375 cell line and its corresponding vemurafenib resistant subline A375VR4 we performed a scratch assay. Cells were seeded in 6-well plates and grown to confluent state; then with

the tip of a pipette tip a radial scratch was made. Cells were also grown with human recombinant Ephrin-A1 (EFNA1; 10882-H03H a cognate ligand of EPHA2) to evaluate the EPHA2 degradation effect on the gap filling rate (EPHA2 is degraded upon ligand treatment).

Colony forming assay: In 4 cm plates, 200 cells were plated and 24h after seeding they were treated with vemurafenib or siRNA against EPHA2. The number of colonies formed were fixed in crystal violet and counted 14 days after seeding. Culture media for the cells was changed every 3 days but the treatment was done only once.

Confocal microscopy: Twenty five thousand cells were seeded on coverslips and after 24h incubation in 37 °C CO_2 incubator; the cells were treated for 48h with BRAF inhibitor and fixed in 4% paraformaldehyde. Cell membrane was permeabilized and for minimizing the risk of unspecific binding, cells were treated with blocking buffer containing 2% bovine serum albumin (BSA). Then cells on coverslip were incubated overnight with two primary antibodies against pAb anti-53BP1 (100904 Novous diluted 1:400 in blocking buffer) and γH2AX (Ser139) (05-636 Merck Millipore diluted 1:1000 in blocking buffer) for 1h at room temperature. Then cells were incubated with goat anti-Rabbit IgG (H+L) secondary antibody, Alexa Fluor® 488 conjugate and donkey anti-Mouse IgG (H+L) secondary antibody, Alexa Fluor® 594 conjugate were added to cells and incubated for 1 hour at room temperature in the dark. Lastly, coverslips were sealed by adding mounting medium containing DAPI. The images were captured by a LSM700 confocal microscope (Zeiss), mounted on Axio observer Z1 (Zeiss) equipped with a Plan-Apochromat 63X/1.4 oil immersion lens.

7. RESULTS AND DISCUSSION

Paper I:

Results: In this project we investigated the role of six melanosome-related proteins involved in chemotherapy resistance to the alkylating agents TMZ/DTIC in CMM. These proteins, MITF, G protein coupled receptor 143 (GPR143), premelanosomal protein gp100/PMEL, Melan-A (MLANA), Rab27A and tyrosinase-related protein 1 (TYRP1), have previously been suggested to play a role in resistance to cisplatin in melanoma cell lines [101]. Therefore, we investigated the expression of these proteins by immunohistochemistry in a set of 52 formalin-fixed paraffin-embedded pretreatment tumor biopsies from patients with metastatic CMM. MITF and GPR143 expression levels were significantly higher (Fisher's exact test; $p<0.05$) in tumor samples from non-responders than responders. Since gp100/PMEL has previously been associated with resistance to cisplatin and paclitaxel, we studied the effect of transient knockdown of gp100/PMEL by siRNA in a pigmented MNT-1 melanoma cell line and observed increased sensitivity to cisplatin and paclitaxel, but no effect on DTIC or TMZ sensitivity.

Discussion: Metastatic CMM has a low response rate to chemotherapeutic agents. Our data confirms the previous hypothesis of involvement of MITF and other melanosomal related protein in chemoresistance in CMM. Melanosomes are suggested to decrease the efficacy of chemotherapy through sequesteration of the drug in them [102]. Higher expression of the melanosomal proteins such as GPR143, MLANA, TYRP1, RAB27A etc. that are transcriptional targets of MITF [129] could correlate to unresponsiveness to chemotherapy and may serve as markers for lack of therapy response. Inhibiting or downregulated expression of melanosomal pathway effectors may be a way to improve the chemotherapy effect.

Paper II:

Results: In this study we performed proteome profiling of fresh frozen CMM lymph node metastases from five responders and nine non-responders to DTIC/TMZ based chemotherapy. We performed mass spectrometry based proteomics. Among the identified proteins with differential expression levels in responders and non-responders, we selected four protein candidates for technical validation by immunoblotting. The four candidate proteins were calcium binding S100A13, cystatin B (CSTB), coagulation factor A1 (F13A1) and inositol-3-Phosphate synthase 1 (ISYNA1). We further evaluated

the expression of CSTB and S100A13 proteins in an extended set of 50 formalin fixed paraffin embedded CMM tumors from 16 responders and 34 non-responders by IHC. Despite high expression of CSTB in most of the tumors, it did not show significant difference between the two groups. High levels of S100A13 expression were significantly associated with unresponsiveness to DTIC or TMZ.

Discussion: A majority of the patients with metastatic CMM are unresponsive to chemotherapies but there is a small subpopulation among the patients that are long-term responders to the treatment. In this study we aimed at identifying a set of potential predictive biomarkers for response to chemotherapy with DTIC or TMZ, to have a higher probability to identify patients that may benefit from DTIC or TMZ. Proteomics analysis using mass spectrometry is a powerful tool in the field of biomarker discovery. The technique enables simultaneous analysis of biological samples with high throughput. Utilizing LC/MS-MS technique we investigated proteome alterations in fresh frozen tumor biopsies from CMM patients with metastatic disease. We identified that high expression of the S100A13 protein correlated with resistance to chemotherapy with DTIC/TMZ in the patients. S100A13 has previously been suggested to be involved in angiogenesis in CMM as well as in regulating several signaling pathways such as NFκB and high mRNA levels of S100A13 has been demonstrated to correlate with higher risk of relapse of CMM [125, 130]. Finally, high expression of S100A13 protein in tumor tissues may be suggestive of highly invasive and treatment unresponsive disease.

Paper III:

Results: To investigate resistance mechanisms to BRAFi and devising strategies to overcome resistance, we established BRAFi resistant sublines of melanoma A375 cells. We then performed whole proteome profiling using liquid chromatography coupled to mass spectrometry LC/MS-MS. Several protein families were identified to be differentially expressed in parental A375 cells compared to the BRAFi resistant sublines such as receptor tyrosine kinases, cell surface receptors, transcription factors, etc. We then validated the already known BRAFi resistance mediators as well some novel protein candidates for mediating resistance to BRAFi. Overexpression of hepatocyte growth factor receptor (MET), Ephrine receptor A2 (EPHA2), aminopeptidase N (CD13/ANPEP) and transcription factor FLI1 were observed in the BRAFi resistant cells. Silencing of EPHA2, FLI1 and targeting ANPEP by blocking antibody resulted in elevation of apoptosis or sensitization to BRAF inhibitor vemurafenib. More specifically, a blocking antibody against ANPEP as monotherapy induced massive cell death not only

in the resistant cells with high CD13 expression but also in the sensitive A375 cells. Knocking down EPHA2 or FLI1 sensitized the resistant cells to vemurafenib. Due to overexpression of several receptor tyrosine kinases, we combined vemurafenib with the multi kinase inhibitor dasatinib (inhibiting phosphorylation of EPHA2, SRC, etc.) and observed an induction of massive cell death compared to any of the drugs alone.

Discussion: We are reporting for the first time that FLI1 mediates vemurafenib resistance. In addition we show that CD13/ANPEP is overexpressed in our melanoma cell line with acquired resistance to vemurafenib and it could serve as a novel target for therapy. CD13 is a cell surface aminopeptidase involved in tumor progression and cell proliferation [131]. It has variable expression throughout melanoma progression with low levels in melanocytes and high in melanoma cells [132]. As shown in a study, high expression levels of CD13/ANPEP correlates to low grade of differentiation and reduced melanocytic markers gp100, MART-1 and S100B [133]. Another of our identified novel BRAFi resistance protein candidates, FLI1, is a member of the large ETS transcription factor family [134] and has been associated with an increased proliferation, differentiation and evasion of apoptosis in human cancer cells [135, 136]. Aberrant FLI1 activation induces dysregulated cell division and malignant transformation [137, 138]. In endothelial cells, ETS transcription factor phosphorylation via the RAS/MAPK pathway is required for CD13 induction [139]. Our vemurafenib resistant A375VR4 cells, overexpress FLI1 at protein and mRNA levels, and silencing with FLI1 siRNA re-sensitized the cells to the drug, suggesting that FLI1 contributes to vemurafenib resistance. The effect was also shown in another melanoma cell line, SKMEK24, with moderate expression of FLI1. Besides increased apoptosis in vemurafenib treated A375VR4/SKMEL24 cells, silencing of FLI1 also inhibited proliferation of these cells and the parental A375 cells. We also showed that the combination of vemurafenib with the multi-kinase inhibitor dasatinib could overcome vemurafenib resistance in cells with EPHA2 receptor overexpression. Also, blocking CD13/ANPEP diminished or abolished phosphorylation of EPHA2 in the vemurafenib resistant A375VR4 and SKMEL24 cells indicative of its role in oncogenic signaling activation of EPHA2.

Paper IV:

Results: Combination of BRAFi PLX4720 with the conventional chemotherapeutic agent TMZ gave additive effects in cell proliferation inhibition. For this analysis A375 melanoma cell line with the V600E BRAF mutation and its BRAF inhibitor (BRAFi: PLX4720) resistant subline A375PR1 was used. Concomitant or alternating drug scheduling showed different cell growth inhibitory or viability effects. The additive effect of the combination was achieved only when TMZ treatment was added prior to BRAFi but not when BRAFi was added prior to or simultaneously with TMZ. We investigated the cell cycle

effect of BRAFi and TMZ: Despite an increased proportion of G1 arrest in cells after BRAFi treatment, neither of the treatment schedules ere significantly different in their effects on cell cycle distribution, or specifically G1 arrest. This indicates that when treatment schedules starts with either BRAFi or TMZ, cell cycle distribution did not differ significantly. Then, we found that the DNA damage response marker γH2AX appears after BRAFi treatment and peaks at 48h post-treatment, while after TMZ, γH2AX is observed and peaks at later time points. To bypass the effect of DNA damage signaling effectors we depleted the MGMT using lomeguatrib. Upon MGMT depletion, the schedule dependency of the treatment was abrogated indicating that induction of MGMT by BRAFi treatment could confer resistance to TMZ. Moreover, we inhibited ATR by specific ATR inhibitor or disrupted MDM2-p53 interaction by nutlin-3 which stabilized p53 (and also induced Ser15 phosphorylation in p53) and observed additive effects in induced cell death when each of them was combined with the BRAFi.

Discussion: In many patients resistance to vemurafenib occurs rapidly after the start of treatment (6-8 months after the start of treatment) [140] and combination of a targeted therapeutic agent with another targeted drug is significantly more efficient compared to monotherapy. For example, BRAFi combined with MEKi increases progression free and overall survival in patients. Here we speculated whether the conventional standard chemotherapy with TMZ would be useful to delay the occurrence or overcome the resistance to BRAFi. Efficiency of the combination of BRAFi (PLX4720) with TMZ showed a schedule dependency. When BRAF inhibitor was added prior to TMZ an additive effect of the combination was not observed. It has been shown that BRAF inhibition by vemurafenib elevates reactive oxygen species (ROS) and nitric oxide (NO) level in A375 melanoma cells [141]. We have also observed that in response to BRAF inhibition, as indicated by increased γH2AX, DNA damage response (DDR) was activated. Therefore, we speculated that ROS and NO induction upon BRAFi treatment might induce DNA damage. On the other hand, induction of DDR in the cells exposed to BRAFi reduces the DNA damaging effect of TMZ possibly through activating DNA repair proteins. To overcome the counteracting effect, we added MGMT inhibitor lomeguatrib to the BRAF-TMZ combination which led to abrogation of schedule dependency. Thus, when DNA repair protein MGMT was depleted by lomeguarib, concomitant treatment with BRAFi and TMZ showed additive effect on apoptosis induction regardless of treatment order. To confirm the involvement of MGMT in unresponsiveness to TMZ, the SKMEL24 melanoma cell line which lacks MGMT expression due to *MGMT* promoter hypermethylation, was treated with TMZ and

BRAFi. No effect of treatment order was observed in SKMEL24 cells when combining BRAFi with TMZ whether BRAFi or TMZ was added first. Moreover, to validate the importance of DDR activation in therapy response, we inhibited ATR by a specific inhibitor and observed additive effect in apoptosis induction when combined with BRAFi. Similarly, disrupting MDM2-p53 interaction through nutlin-3 treatment elevated apoptotic response in combination with BRAFi. The findings highlight the importance of considering the cell death escape mechanisms in cancer cells when designing therapy regimens e.g. activation of DDR in response to BRAFi monotherapy. Furthermore, we suggest that combination therapies may be scheduled in a manner that allows bypassing or delaying the occurrence of drug resistance.

8. CONCLUSIONS

Paper I: Sequestration of the drugs by melanosomes was previously suggested as a mechanism of resistance to chemotherapy in CMM. Therefore, we performed IHC on pre-treatment metastatic lymph node biopsies from CMM patients with variable response to chemotherapy. We confirmed a significant association between high expression of MITF and GPR143 and drug resistance supporting the role of melanosomal proteins in chemotherapy resistance.

Paper II: Reliable biomarkers for therapy response in CMM are still crucially needed. Conventional methodologies such as immunoblotting are not suitable for identification of commonly altered protein expression patterns mainly due to low throughput. Advanced methods capable of analyzing deep proteome alterations in several samples in parallel are versatile for the purpose. We applied liquid chromatography coupled to mass spectrometry (LC/MS) based proteomics on fresh frozen pretreatment lymph node metastases from CMM patients. By comparing the whole proteome of biopsies from chemotherapy responders to non-responders, we found several protein candidates to be overexpressed in non-responder samples. S100A13 and CSTB were two proteins selected for further validation on larger sample sets. We found that a significantly higher expression of the S100A13 protein correlated with unresponsiveness to the alkylating chemotherapeutic agents DTIC and TMZ.

Paper III: Targeted therapies with mutated BRAFi have shown favorable response in CMM patients harboring the mutation. However, acquired resistance is a major problem in patients receiving the treatment. We established BRAFi resistant sublines of the BRAFV600E mutant A375 melanoma cell line and performed LC/MS based proteomics to evaluate proteome alterations among the resistant sublines compared to the proteome of the parental A375 cells. We identified that overexpression of the surface receptor amino peptidase N (ANPEP/CD13) and the transcription factor FLI1 mediate resistance to the BRAF inhibitor vemurafenib. Moreover, several receptor tyrosine kinases including previously known candidates, EPHA2 and MET, are overexpressed in our BRAF inhibitor resistant cell lines. We could overcome vemurafenib resistance by combining vemurafenib with either multi kinase inhibitor dasatinib or siRNA against FLI and through single treatment with blocking antibody against ANPEP/CD13

Paper IV: Combination treatment is an alternative therapy strategy to overcome or delay the occurrence of drug resistance towards BRAFi. We found a schedule dependency in the response to combination of BRAFi PLX4720 with TMZ. Optimal combination efficiency was achieved when adding TMZ before the BRAFi. By investigating the mechanisms underlying schedule dependency, we found that the DDR is activated by BRAFi treatment, leading to increases in levels or activities of DDR related proteins. MGMT is a repair protein that counteracts TMZ-induced DNA alkylation and we found that it is increased at the mRNA level by BRAF inhibitor treatment. Depletion of MGMT by lomeguatrib abolished the schedule dependency of the BRAFi-TMZ combination. Moreover, inhibition of ATR by a chemical inhibitor or p53 stabilization by nutlin-3 treatment enhanced BRAFi treatment efficacy.

9. REMARKS AND FUTURE PERSPECTIVES

Resistance to therapies in CMM might be reverted by simultaneous or concomitant combination of BRAFi with other inhibitors or chemotherapeutic agents. Despite frequent innate or acquired resistance to treatments, especially for chemotherapy, there is still a small subpopulation of patients that respond to the treatment. Therefore, detection of this group requires reliable predictive biomarkers for response to therapy, which are yet lacking in the field of melanoma. Unraveling mechanisms underlying resistance to therapies may also reveal novel drugable targets or alternative treatment strategies to overcome or delay the onset of drug resistance. Further investigations to uncover drug resistance mechanisms and relevant therapy response predictive biomarkers, is crucial for improvement of therapies and therapy regimens for patients with advanced CMM. For this purpose, mass spectrometry based proteomics is a powerful method to both study molecular pathways regulating therapy resistance and for predictive biomarker discovery.

Application of the constantly improving –omic (genomics, transcriptomics, proteomics and metabolomics) methodologies as standard clinical procedures, may further personalize the treatments and significantly enhance the therapy outcomes.

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