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MUTATIONS AND MOLECULAR SIGNATURES IN HUMAN MELANOMA

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To my son Mihailo

ABSTRACT

Cutaneous melanoma is a disease which results from a complex mixture of various extrinsic and intrinsic factors, with some major players still unknown. Although primary tumors are highly curable with surgical excision, particularly those which are non-ulcerated and have a thickness of less than 1 mm, poor survival rates are observed in advanced disease. This has been associated both with the biological nature of melanoma, as well as with the limited efficacy of current treatments of metastases. With this work, we wanted to obtain further insights into the biological background of skin melanoma in different stages of progression. We focused on commonly altered pathways and genes, *NRAS*, *BRAF* and *CDKN2A*, and their effects on activation and expression of downstream genes in different stages of melanoma development.

For the first two studies, we used primary melanoma tumors that had either sporadic or hereditary origin, respectively. Samples used in the analyses were formalin-fixed, paraffin-embedded tissue blocks. For the other two studies we focused on melanoma metastases originating from sporadic melanoma cases. Fresh frozen tumor specimens were used in these two analyses.

Results obtained showed that *NRAS* and *BRAF* mutations in sporadic primary melanomas were observed at 26% and 39%, respectively. Approximately a two-fold increase in mutation frequencies were observed, for both genes, in the group of patients that had shorter overall survival. The presence of ulceration and lack of cytoplasmic activation of ERK were independent prognostic markers. *BRAF* mutations were markers of worse survival in the presence of ulceration. In familial primary melanomas, *NRAS* and *BRAF* mutations were found in 3 samples (16%) and 7 samples (37%), respectively. Three samples harbored coexisting alterations in both genes on the background of a germline *CDKN2A* L32P mutation.

Metastatic melanoma samples with *BRAF* mutations had higher expression of genes when compared to tumors with *NRAS* alterations. The majority of observed genes were found to be located on chromosome 7. Additionally, this overexpression pattern was accompanied with copy number increase of chromosome 7 in *BRAF*-mutated tumors. Results obtained through the last study showed that the majority of differentially genes show significantly decreased expression which paralleled *CDNK2A* allelic loss. These

differences were more evident in samples with bi-allelic loss, when compared to those with intact *CDKN2A*. Finally, pathways analysis showed that differentially regulated genes, that showed lower expression in respect to *CDKN2A* gene dosage, were linked to immune response in metastatic melanoma samples.

In conclusions, *NRAS* and *BRAF* mutations are observed at similar frequencies in sporadic and familial melanomas. Although both N-Ras and B-Raf convey signals along the same pathway, signatures in our analysis showed differences between these two effectors. In metastatic melanomas with *BRAF* mutation the great majority of observed changes included overexpression and amplification of genes located on chromosome 7, in samples with *BRAF* mutation. The differences in gene expression between *BRAF* and *NRAS* mutated tumors may be caused both by direct effects on cell signaling by activated *BRAF* as well as effects related to overexpression and gene copy number gains on chromosome 7, where the *BRAF* gene is located. Finally, in the last study, loss of the *CDNK2A* gene correlated mainly with lower expression of genes in metastatic melanomas with a majority of these linked to immune response.

LIST OF PUBLICATIONS

I. **Jovanovic B**, Kröckel D, Linden D, Nilsson B, Egyhazi S, Hansson J. Lack of cytoplasmic ERK activation is an independent adverse prognostic factor in primary cutaneous melanoma. *J Invest Dermatol.* 2008 Nov;128(11):2696-704. Epub 2008 May 29.

II. **Jovanovic B**, Egyhazi S, Eskandarpour M, Ghiorzo P, Palmer JM, Bianchi Scarrà G, Hayward NK, Hansson J. Coexisting *NRAS* and *BRAF* Mutations in Primary Familial Melanomas with Specific *CDKN2A* Germline Alterations. *J Invest Dermatol.* 2010 Feb;130(2):618-20. Epub 2009 Sep 17.

III. **Jovanovic B**, Ploner A, Huang F, Egyhazi S, Reeves K, Perkins NA, Pawitan Y, Ringborg U, Hansson J. *BRAF* mutation is associated with a distinct molecular signature and chromosome 7 amplifications in metastatic melanomas. *Submitted for publication.*

IV. **Jovanovic B**, Xia H, Huang F, Egyhazi S, Hansson J. Gene expression in human melanoma tumors - association with *CDKN2A* deletions. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

ALM	Acral lentiginous melanoma
ARF	Alternative reading frame
BRAF	V-raf murine sarcoma viral oncogene homolog B1
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin dependent kinase inhibitor 2A
CDKN2B	Cyclin dependent kinase inhibitor 2B
CGH	Comparative genomic hybridization
CMM	Cutaneous malignant melanoma
CPD	Cyclobutane pyrimidine dimmer
DNS	Dysplastic nevus syndrome
ERK	Extracellular signal-regulated kinase
FAMM	Familial atypical mole melanoma syndrome
FFPE	Formalin-fixed, paraffin-embedded
GTP	Guanosine triphosphate
INK4	Inhibitor of cyclin-dependent kinase 4
IRS	Immunohistochemical score
LCM	Laser capture microdissection
Limma	Linear models for microarray analysis
LMM	Lentigo maligna melanoma
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin 1 receptor
MEK	MAPK/ERK kinase
NM	Nodular melanoma
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol-3-kinase
PIP3	Phosphatidylinositol tri-phosphate
PTEN	Phosphatase and tensin homolog
Rb	Retinoblastoma
RGP	Radial growth phase
RTK	Receptor tyrosine kinase
SSCP	Single strand conformation polymorphism
SSM	Superficial spreading melanoma
UV	Ultraviolet
VGP	Vertical growth phase

BACKGROUND

Melanoma is a malignant tumor of melanocytes, cells that are derived from the neural crest. Most melanomas arise in the skin however these tumors are also observed in other parts of the body, to which neural crest cells migrate during embryonic development (e.g. the uvea of the eye, mucosal surface of the mouth, vulva etc.). Melanocytes synthesize melanin, which is a pigment that provides protection against the damaging effects of ultraviolet radiation.

Cutaneous malignant melanoma (CMM) is a good example of a multifactorial disease since both environmental and host factors have been linked in development of this neoplasm. The etiology of cutaneous melanoma is incompletely understood although the exposure to solar ultraviolet (UV) radiation is the major environmental risk factor. Host factors that have been related to this disease are family history of melanoma, the presence of large numbers of nevi, skin pigmentation and genetic changes in melanoma susceptibility genes.

Prognosis of this tumor is generally good if diagnosed in early stages however, there is no effective cure for metastatic disease. Previously, melanoma thickness and presence of ulceration were identified as the main prognostic markers in early stages of melanoma. In the advanced disease, number of metastatic lymph nodes, the site of distant metastases and the presence of elevated serum lactic dehydrogenase were linked to worse prognosis (1). However, the finding of comprehensive molecular signatures related to different stages of melanoma might give us better understanding of this disease, ultimately leading in discovery of new therapeutic targets for this disease.

HISTORY

History teaches us that knowledge about melanoma has been re-invented repeatedly, and by doing this, suggesting the complexity of this disease and lack of progress in substantially changing its dreadful course. Most early evidence about melanoma in humans comes from Peruvian mummies, originating from the 4^{th} century BC, which were found to have diffuse bone metastases, in the skull and extremities, as well as rounded melanotic masses in the skin (2). Probably the first recognized descriptions of

melanoma dates back in 5th century BC, mentioned by Hippocrates (2, 3), which were followed by another Greek physician of ancient times, Rufus of Ephesus, some 500 years later (2). No decisive information related to this disease is observed during almost the next 1,500 years, until the 17th and 18th century when several prominent Europeans referred to this illness as "fatal black tumors with metastasis and black fluid in the body" (2). The first known description of melanoma as disease entity was done by René Laennec, in the beginning of the 19th century (4). What followed was that the hereditary basis of melanoma was suggested in 1820 by Norris (3), whereas Carswell used the medical term "melanoma" to describe pigmented malignant lesions of the skin, in 1838 (2). Between 1857 and 1858, Norris and Pemberton advocated wide and deep radical excision of melanoma (2, 4), which became an accepted surgical technique only hundred years after their original ideas. At the beginning of the 20th century, in 1907, Handley suggested possible lymphatic spread of disease (4), whereas in the following year, Pringle advocated "en bloc" excision of melanoma with wide margins based on Handley's concept of melanoma spread through lymphatic permeation (2). Again intriguingly, Handley's advocacy of elective lymph node dissection is still being debated, almost one hundred years later after his original proposal (4). In the 1930s, Darier suggested that nevi were likely to have an inherited basis (5), a concept that was fully proposed almost 50 years later, separately, by Lynch and Clark and was originally named "BK mole syndrome" (6, 7). This "dysplastic nevus syndrome (DNS)/ familial atypical mole melanoma syndrome (FAMM)" which they described in families with a genetic susceptibility to melanoma has been studied intensively (8-10). This resulted in genetic linkage of this syndrome to the short arm of chromosome, 9p21 (11). Subsequently, this resulted in the identification of germline alterations in the *CDKN2A* gene located at this locus (12-15). The new millennium started with the findings of a high rate of somatic mutations in the *BRAF* gene, suggesting its possible important role in the melanoma neoplastic process (16). However, a later study showed an even higher percent of these mutations in nevi indicating that *BRAF* mutations are early events in the development of melanocytic neoplasms, which occur already before the malignant transformation (17).

EPIDEMIOLOGY

There has been an increasing incidence of melanoma throughout the world over the last 40 years, mainly in white-skinned population (18), although recently at a slower rate

than seen in the past in some countries (19). Nevertheless, the mortality rates show alternation depending on observed world regions, of which some follow rates of new reported cases, whereas some are opposing (19). Current literature show that the highest incidence rate in the world is found in Queensland (Australia), averaging 55.8/100,000/annum for males and 41.1/100,000/annum for females (20, 21). In Sweden, an estimated 2,600 new cases of melanoma were diagnosed in 2008 (22), which were equally distributed among females and males. Almost 5% of all new reported cancers are melanoma, which rank this tumor as the 6th most common new neoplasm in the Swedish population. The age standardized incidence was 28.8 and 27.6 new cases per 100,000 per year in 2008 in males and females, respectively. The rates were mostly increasing among older population, above 54 years. However, the mortality rates in Sweden were stabilized during 1990's, which were attributed to early detection practice and subsequent surgical therapy of thin melanomas (20).

A likely explanation for the increase in melanoma incidence during this time is the altered behavior in the general population related to exposure to natural or artificial ultraviolet radiation. Increase in life standard of the middle class population in the 1960s, that followed the rapid industrial development of that time, gave people an opportunity to have vacations in easy accessible holiday destinations, in many cases involving extensive sun exposure. These altered habits are likely to contribute to the increased melanoma incidence since previously, brief and intermittent sun exposure, followed by burning episodes, has been identified as a major melanoma risk factor (23).

RISK FACTORS

Both extrinsic (environmental) and intrinsic (host) factors are recognized as the important risk factors for developing melanoma in humans.

Extrinsic

Ultraviolet light exposure is the only known extrinsic risk factor in melanoma. Mainly, increased melanoma incidence has been attributed to altered sun exposure habits in the general population. Chronic repeated exposures to sunlight from childhood are epidemiologically shown to increase the risk of melanoma, especially if accompanied with sunburn episodes. However, this relationship is still being investigated because of

paradoxes surrounding it (24). As an example, people working outdoors tend to have lower incidence of melanoma when compared to those working indoors. This interesting observation could be explained by the notion that untanned skin is more melanoma prone to sunburns than skin that is repeatidly sun exposed (25).

UV light is an electromagnetic radiation with wavelength range between 10-400 nm. This span is shorter than visible light but longer than x-rays. Three major UV bands are emitted by the sun, UVA (320-400 nm), UVB (280-320 nm) and UVC (100-280 nm), however almost 99% of all sun-emitted UV radiation is blocked by the earth's ozone layer. Of the UV irradiation reaching terrestrial ground, approximately 95% is UVA (26), depending on the earth's latitude and solar angels measurements. Although it has the smallest amount of energy, this waveband has been linked to DNA damage and induction of mutations by formation of reactive oxygen species via photosensitized reactions, which have been confirmed both in humans and in cell cultures (27). However, epidemiological and clinical studies that link UVA to melanoma are still debated (27). Likewise, up to 5% of the total UV light reaching earth's surface is a UVB (26). This waveband is absorbed by DNA more efficiently than UVA (28) and it is therefore assumed to induce more damage and thus to be more carcinogenic. It has been shown that UVB light can harm skin by inducing mutations, mainly cyclobutane pyrimidine dimers (CPD), by immunosuppresion or by up-regulating expression of different genes through intracellular signal transduction pathways that promote tumor formation (29). Interestingly, experiments show that UVB induces CPD photolesions in skin more efficiently than UVA light (28). However, both UVA and UVB can induce dipyrimidines, of which some are found in the non-coding sequence opposite of the *BRAF*^{V600E} mutation, the most common alteration observed in this gene, which is thus possibly associated with UV damage (26). Whether these two wavebands, UVA and UVB, alone or in cooperation are responsible for mutagenic effect in melanoma is still questionable and are pending better and more extensive experiments. Finally, UVC has the shortest wavelength but highest energy of ultraviolet wavebands emitted by the sun. Numerous studies demonstrate the DNA damaging effect of UVC (30, 31) but since almost all of this UV light is absorbed by the ozone layer and not reaching earth's surface, the damaging effect of this waveband is discarded.

Intrinsic

Different epidemiological studies have identified several host factors that contribute to increased melanoma risk. Of these, the most recognized include family history of melanoma, number and types of nevi, skin type, pigmentation type and alterations in melanoma susceptibility genes.

Family history of melanoma

Familial history of melanoma has been implicated in increased melanoma risk with approximately 5-10% of all melanoma cases arising in the context of familial hereditary predisposition (32). However, there still some controversies regarding definition of family history of melanoma since different studies used different methodology. Most common approach is either to have more than two first degree-relative with melanoma or at least three relatives that are affected by this disease. Since these kindreds are followed-up on regular basis, thin melanomas are most commonly observed and, usually, at an earlier age (33, 34). Additionally, these family members affected by melanoma are more prone toward developing multiple melanomas and certain other malignancies, notably pancreatic carcinoma in carriers of *CDKN2A* germline mutations (see below) (35, 36). Interestingly, hereditary melanomas have similar prognosis and are histologically indistinguishable from sporadic cases (37).

Two genes conferring susceptibility to melanoma have been identified within high-risk families, *CDKN2A* and *CDKN2B* (15, 38) and are further discussed below.

Number and types of nevi

Increased number of common nevi confers high melanoma risk (37). Although the influence of different types of nevi (common versus dysplastic) on melanoma risk were not commonly evaluated in published studies, the presence of dysplastic nevi showed elevated risk (39, 40). Furthermore, number of nevi was associated with increased risk in familial melanoma (41).

Skin and pigmentation type

Almost all epidemiological studies that looked at the impact of pigmentation as a risk factor for developing melanoma showed that fair-skinned individuals were more prone

toward developing melanoma by comparison to darkly pigmented individuals (42). Mainly, this has been correlated to the frequent alterations found in the melanocortin-1 receptor gene (*MC1R*) that is involved in the production of melanin (eumelanin or pheomelanin). These genetic variants were frequently found in melanoma patients and a two-fold melanoma risk was calculated for some of these (43). Interestingly, this increased risk of melanoma in carriers of *MC1R* variants may be partly independent of skin type, which could be associated either to some changes distinct from skin type or that these individuals are not able to synthesize enough of eumelanin, which is related to the photoprotective effect in the skin (43).

Alterations in melanoma susceptibility genes

Alterations observed in *CDKN2A* and *CDK4* genes confer susceptibility to melanoma. Genetic studies in melanoma-prone families resulted in identification of 9p21 locus, with the *CDKN2A* and *CDKN2B* gene centered in this, as a result of co-segregation with melanoma susceptibility in melanoma kindred (15, 38). Additionally, 9p21 homozygous deletions in *CDKN2A* are frequently observed in melanoma cell lines (44). These results suggested the importance of gene products $p16^{INK4A}$ and $p14^{ARF}$ (from the *CDKN2A* gene) and $p15^{INK4B}$ (from the *CDKN2B* gene) as crucial for melanoma development.

Approximately 40% of all melanoma-prone families carry *CDKN2A* germline alterations. Distinctive variants of this gene have been observed in different populations (45) with some of these being referred as founder mutations (46, 47). For example, in Swedish melanoma kindreds, a *CDKN2A* insertion of 3 base pairs (p.R112_L113insR) is the most common alteration observed (46, 48, 49), which is also acknowledged as founder mutation (46). Similarly, germline mutations of *CDK4* are observed in kindred of different populations but at smaller rates (50, 51).

Apart from mutations in these two high-risk genes, alterations observed in the *MC1R* gene, which has a critical role in the type of melanin produced, as discussed above, have been associated with minor increases in melanoma risk (52-54).

CLASSIFICATION

There are several different classifications used in melanoma nomenclature which has been a source of controversy (55). According to the organ of the primary melanoma origin, there are skin-, mucosal-, uveal- and primary melanomas of internal organs (sometimes mucosal melanoma is also referred as a skin form). Skin, or cutaneous, melanoma is the most common form found in humans and can be further subdivided into superficial spreading melanoma (SSM), acral lentiginous melanoma (ALM), lentigo maligna melanoma (LMM) and nodular melanoma (NM). Even this categorization is flawed, since it is a mixture of several different parameters used in this classification. Thus, the premises for this division are based either on the body site at which melanoma is observed (acral – periphery or the apex of the limb), or to different growth phases that are observed in melanoma samples (radial growth phase, RGP, in SSM or vertical growth phase, VGP, in NM), or to the clinical presentation of melanoma (lentigo - oval pigmented macula). RPG is usually observed in early stages of skin invasion, Clark levels of I and II, whereas VGP tumors are those that penetrate the skin more deeply, Clark levels III-V (sometimes tumors with Clark level II of invasion can also be recognized as VGP; see below). Superficial spreading melanoma is the most common form of skin melanoma which is usually observed on parts of the skin that are intermittently exposed to sun, especially on the back in men and the lower limbs of females (55). A typical feature of this form is a prolonged radial growth phase during tumor development. Acral lentiginous melanoma is found on the palms, soles and areas under the nails i.e. mainly on body surfaces that are not related to sun exposure (55, 56). This form is more common in people with dark pigmented skin types. Lentigo maligna melanoma is usually found on chronically sun exposed skin, i.e. the face or forearms, and observed in elderly people (55, 57). Nodular melanoma is a form that is characterized by rapid vertical growth phase that tends to invade dermis early (55). Apart from these, rare forms of melanoma that are infrequently mentioned in the literature include desmoplastic melanoma, verrucous melanomas, polypoid melanoma, spitzoid melanoma and malignant blue nevus (55).

Other forms of melanoma which do not originate in skin include mucosal and uveal melanoma. Mucosal melanoma arises in mucosal membranes and is most commonly located in the mouth and nasal region as well as in the vulva and anus. Uveal melanoma arises from melanocytes that are found in the uveal layer of the eye (iris, ciliary body, pars plana and choroid). Primary melanomas of internal organs are sporadically mentioned in the literature, most of which are accidently observed during clinical

diagnostic evaluation of some other tumor formation or as a result of a search for the tumor of unknown primary lesion.

PROGRESSION

Melanomas are predominantly occurring in adults with more than half of reported cases arising in apparently normal areas of the skin. Melanomas develop either from epidermal melanocytes or from melanocyte precursor cells. Melanocytes originate from the neural crest and migrate to the skin, in the greatest percent, during embryonic development (58). Melanoma progression from normal melanocytes is a stepwise process with relatively well defined clinicopathological lesions observed during development (Figure 1). These progression stages include common nevi, dysplastic nevi, melanoma in situ, RGP melanoma, VGP melanoma and metastases. Additionally, this can also be further refined as a 3-stage process of tumor progression, as Clark previously described (59, 60).

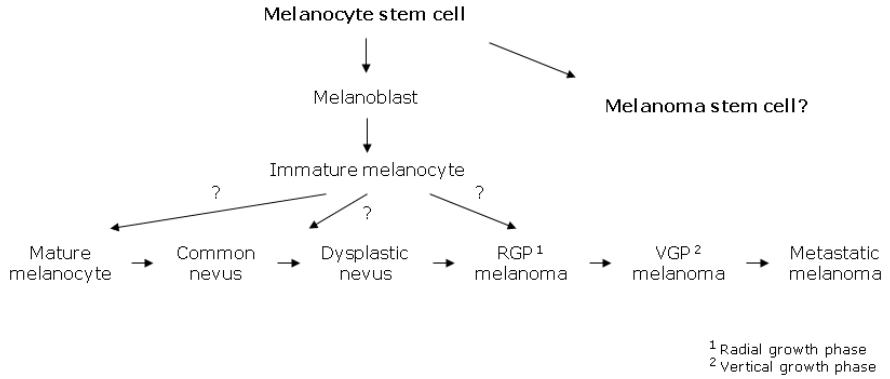

Figure 1: Melanoma progression. Adapted from: "Melanoma" in Encyclopedia of life sciences, 2001.

Melanocytic nevi, apart from congenital nevi, commonly appear after the birth and, histologically they are classified as junctional, compound and intradermal nevi, depending on growth patterns (61). In junctional nevus, nests of nevus cells are present in the epidermis in contrast to intradermal nevus which compromises of nests present only in dermis of the skin. A melanocytic nevus with cells that are found in the epidermis and in the dermis is termed a compound nevus. Common nevi are patches of hyperpigmented cells found in epidermis. These cells differ from ordinary melanocytes by three distinct characteristics: cells are more rounded, they tend to retain pigment and have a propensity to form nest of cells. All of these characteristics are in contrast to normal melanocytes which tend to have dendritic processes, transfer pigment to keratinocytes and be located as single cells in the basal layer of the epidermis. Clinically, common nevi are symmetrical and well delineated from normal skin. Although they are regarded as precursors of dysplastic nevi and melanoma, the great majority will go into senescence and regress after four or five decades.

Dysplastic nevi were first recognized in familial melanoma as a key characteristic of the DNS/FAMM syndrome (6, 7). These are benign lesions that tend to have cytologic and architectural atypia when compared to common or congenital nevi (62). The main clinical characteristic is that they are moderately larger by comparison to common nevi and have irregular, asymmetric, and indistinct borders. Parts of dysplastic nevus may be raised above the skin surface but in most cases they are flat. Additionally, color variations are observed, and ranges from pink to dark brown. Histologically, the presence of lymphocytes is a common feature.

Nevi and dysplastic nevi have been suggested to be direct precursors of melanoma. This comes mainly from histological studies that showed that around 30% of melanomas were associated with preexisting nevi, as well as that there is a significantly higher risk of developing melanoma from nevus (63, 64).

“Melanoma in situ” is a RGP melanoma but is still a big diagnostic challenge because it is often being misdiagnosed as a nevus. RPG melanomas are distinguished by the lack of melanoma cells present in the dermis and lack of mitoses observed in the dermal component of the melanoma. These melanomas can easily be cured with surgical excision suggesting that these lesions are still lacking ability of tumorigenic growth and development of metastasis (65, 66). Conversely, VGP primary melanomas, which

invade the dermis with tumorigenic growth contain genetic abnormalities that allow cells to grow invasively and also provides metastatic capability (Clark level III or deeper). These two main histologic features that distinguish VGP from RGP are the presence of dermal nests that should be larger than any epidermal or junctional nest of tumor cells and increased mitotic rates observed in dermal component of the tumor.

Metastasis is a consequence of altered survival and outgrowth of cell subpopulations that are independent of growth factors. These cells show higher level of genetic instability compared to the primary neoplasm. Frequently melanoma metastases are found in adjacent skin or lymph node closest to the primary lesion. However, distant metastases are not uncommon and are usually observed in brain, gastrointestinal tract, lung and liver.

PATHWAYS COMMONLY ALTERED IN MELANOMA

Numerous regulatory pathways have been implicated in the control of cell proliferation, survival, viability and invasiveness during melanoma initiation and progression (67, 68). The most common ones that have been extensively studied are Ras-Raf-MAPK, Ras-PI3K-Akt, p16-Rb and p14-p53, which were the basis of the research and experiments performed for this thesis.

Ras-Raf-MAPK

A large body of evidence has implicated hyperactivity of Ras-Raf-MAPK as an early event in melanoma pathogenesis, contributing to the induction of uncontrolled cell proliferation (Figure 2). Initiation of this cascade is a result of either activation of receptor tyrosine kinases (RTKs) by a variety of ligands, or by activating mutations found in *NRAS* or *BRAF* genes. It was suggested that interactions between Ras and Raf induces a conformational change in the latter, which is followed with anchoring of the Raf protein to the plasma membrane. After this transient binding, Raf activity becomes independent of Ras, which is subsequently followed by the signal transmission to the nucleus through downstream effectors. ERK, which is one of these effectors, has been reported to be activated in melanoma (69). This is an early event in tumor progression since 54-100% of primary melanomas (70-73) showed activation of this protein. This activation has been shown in several melanoma models. Moreover, knockdown of

mutant *NRAS* by RNA interference was followed with reduction of activated ERK levels and subsequent induction of apoptosis in cells carrying oncogenic *NRAS* mutations (74). Nevertheless, previous studies also identified a subgroup of melanomas lacking mutations in *NRAS* and *BRAF* genes, for which it was speculated that ERK activation occurs independently of these two genes (75-77).

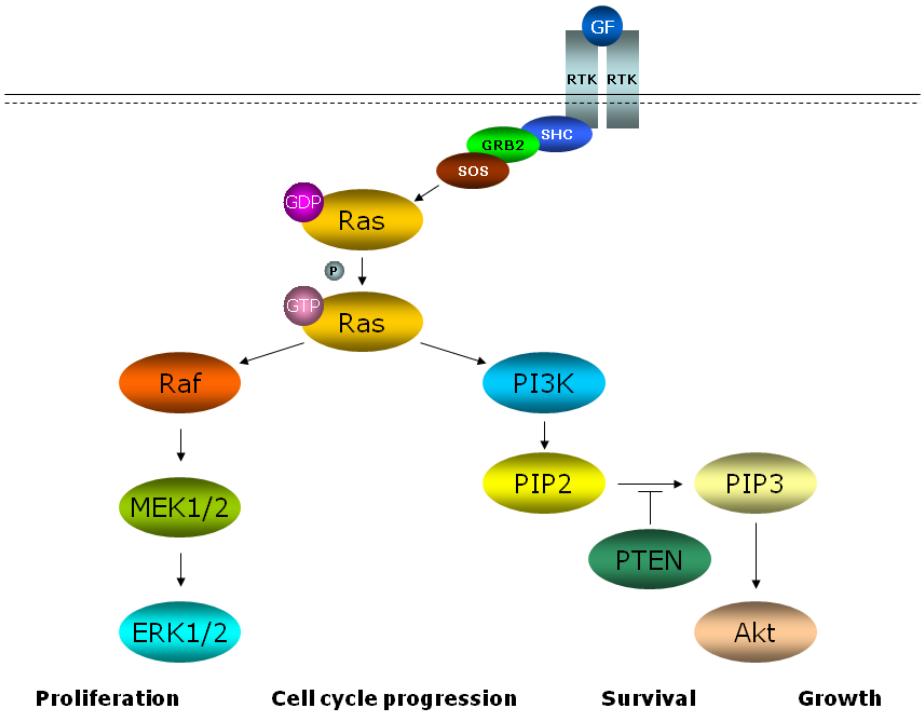

Figure 2: The Ras-Raf-MAPK and Ras-PI3K-Akt pathways. Adapted from: Dahl C, Guldberg P. The genome and epigenome of malignant melanoma. APMIS 2007; 115:1161-76.

NRAS

H-Ras, N-Ras and K-Ras are small guanine-nucleotide binding proteins that are members of the Ras family (17). In the melanocyte lineage, N-Ras is the most frequently altered Ras protein with activating mutations found in up to 56% of congenital nevi (78, 79) and between 4-53% of melanomas (80). Mainly, these activating *NRAS* mutations have been correlated with areas of the body that are sun exposed as well as to the NM subtype (81, 82). However, mutations in this gene are rarely observed in dysplastic nevi (79, 81). Activation of H-Ras is commonly observed

in Spitz nevi and rarely in melanoma (83, 84) whereas *KRAS* mutations infrequently have been described in human melanocytic lesions (83, 85). These mutation patterns point to distinct biological activities of different Ras family members in melanocyte lineage. There are three main effectors of Ras: Raf kinase, Ral-GEFs and PI3K, which are all activated upon binding to Ras.

Commonly observed single point mutations in *RAS* are observed in codons 12, 13, 59 and 61. The most frequent changes in the *NRAS* gene are observed in codon 61, mainly c.181C>A or c.182A>G (p.Q61K or p.Q61R), which have been related to impaired GTP hydrolysis. When present, these mutations are capable of locking the Ras protein in the GTP-bound state leading to recruitment of Raf to the plasma membrane and phosphorylation-driven activation of the Ras-Raf-MAPK pathway.

BRAF

There are three different Raf serine-threonine-specific, protein kinases in humans: A-Raf, B-Raf and C-Raf (86). Of these, mutations in the *BRAF* isoform are almost the only ones observed. Current reports show that activating *BRAF* mutations have been detected in a variety of tumor types (87, 88), with the highest incidence found in melanoma, between 20-80% (80). These point mutations observed in melanoma, mainly clustered in the kinase domain of this protein, with almost 90% of reported mutations being the c.1799T>A, p.V600E, change. When present these alterations confer constitutive activation of the Ras-Raf-MAPK cascade (89). The activating *BRAF* alteration is known to be an early event since it is already found in common and dysplastic nevi (17, 90-92), which indicates that the mutation by itself is not sufficient for malignant initiation.

High *BRAF* mutation frequencies are usually observed in melanomas arising in skin with intermittent sun exposure when compared to acral and mucosal melanomas and. intriguingly, melanomas from chronically sun-exposed areas (93-95). These results suggest that some uncharacterized factors, related to the sun exposure, could be responsible for the onset of the *BRAF* mutation. Additionally, the presence of the *BRAF* mutation has been associated with *MC1R* germline variants, indicating an influence of constitutive genotype on the acquisition of specific mutations during melanoma

development (96). Finally, it is worth noting that mutations in *NRAS* and *BRAF* are almost always present in a mutually exclusive pattern.

Ras-PI3K-Akt

The Ras-PI3K-Akt pathway is an established regulator of cell survival in many human cancers (97-99) Activation of Akt is commonly observed in melanoma but not in nevi (100, 101). *AKT3*, an Akt isoform, is commonly altered in melanomas, and DNA copy gains of this gene were correlated with melanoma progression. (102). Additionally, a decrease in *AKT3* expression promoted apoptosis in melanoma cells (102). In relation to this pathway, the loss of *PTEN*, which opposes the activity of *PI3K* (103), is considered to be frequently involved in melanoma pathogenesis, in cooperation with mutated *BRAF* (77).

PI3K

PI3K triggers the activation of AKT by conveying signal transduction through *PIP3* (104). Mutations in the *PIK3CA* gene, the catalytic subunit of *PI3K*, occur at high frequencies in some human cancers (105) but are rarely observed in melanoma (106). These alterations are capable of inducing Akt activation.

PTEN

PTEN is a suppressor of extracellular growth signals transmitted through the PI3K-Akt cascade. By dephosphorylating *PIP3*, *PTEN* is capable of suppressing *PI3K* activity, which subsequently decreases the signal transduction toward Akt.

Mutations in the *PTEN* gene are infrequently observed in melanoma tumors, but at increased rates found in melanoma cell lines, 8-60% (107-109). This suggests that in tumors, additional genetic events might be involved in Akt activation. Interestingly, *NRAS* and *PTEN* mutations are mutually exclusive in melanoma specimens, whereas *BRAF* and *PTEN* mutations are found to be coexisting (77, 93, 110).

Additionally, homozygous deletions of the 10q23–24 locus, where *PTEN* is located, are commonly observed in a number of human cancers (111) including 30–40% of

melanoma cell lines and 5–15% of uncultured melanoma specimens (77, 107, 109, 110, 112).

p16-Rb and p14-p53

Two pathways, p16-Rb and p14-p53, have been related to cell cycle regulation and are frequently altered in numerous cancers (Figure 3).

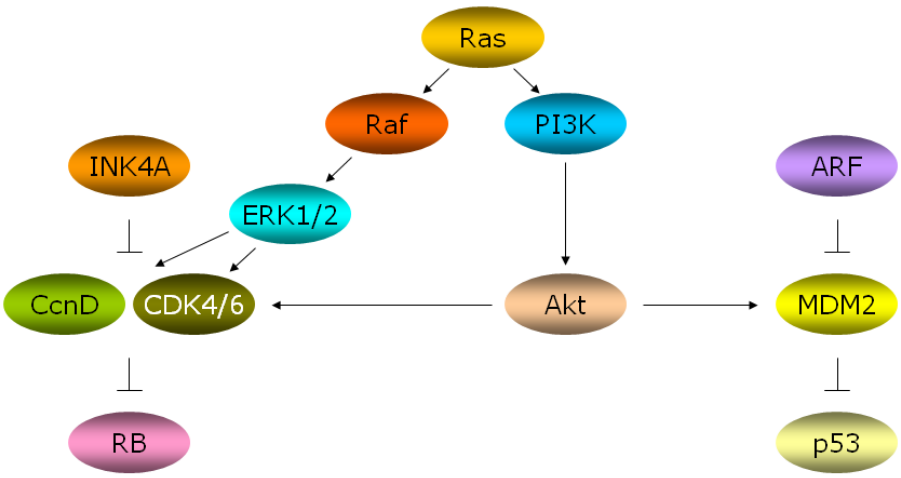

Figure 3: The p16-Rb and p14-p53 pathways. Adapted from: Dahl C, Guldberg P. The genome and epigenome of malignant melanoma. APMIS 2007; 115:1161-76.

CDKN2A (p16^{INK4A} and p14^{ARF})

The *CDKN2A* gene induces transcription of two different proteins, p16^{INK4A} and p14^{ARF} (Figure 4), through the use of alternative promoters (113). Loss of p16^{INK4A}, which is a CDK4-6/cyclin D inhibitor, induces inactivation of the Rb protein which results in unrestrained cell cycle progression (114). Loss of p14^{ARF} induces p53 degradation,

through loss of inhibition of MDM2-mediated ubiquitination, which is followed by the loss of cell cycle control and deregulation of DNA repair (115).

In sporadic melanoma, the most common mechanisms of somatic inactivation of $p16^{INK4A}$ are mutations, promoter hypermethylation and deletions.

Homozygous deletions of the *CDKN2A* region have been observed in 11-25% of melanoma biopsies with higher frequency observed in melanoma cell lines. These deletions frequently involve large parts of the 9p21 locus that harbors both the *CDKN2A* and the *CDKN2B* genes (Figure 4). The latter encodes $p15^{INK4B}$ which is another CDK4/cyclin D inhibitor (116).

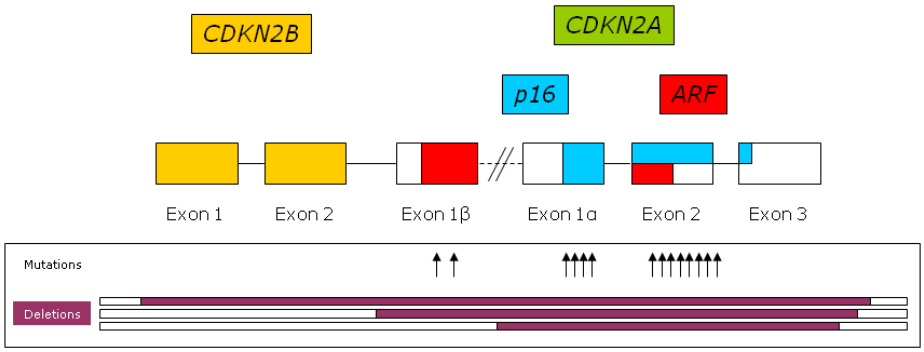

Figure 4: The $p16^{INK4A}$ and $p14^{ARF}$ locus. Adapted from: Haber DA. Splicing into senescence: the curious case of p16 and p19ARF. Cell 1997; 91:555-8.

CHROMOSOMAL ABERRATIONS FREQUENTLY FOUND IN MELANOMA

An abundance of chromosomal aberrations has been identified in melanoma tumors suggesting heterogeneous nature of this disease. Most commonly observed structural chromosomal aberrations in melanomas were found in chromosomes 1, 6, 7, 9 and 10 (93, 117, 118). With recent advances in genome-wide technologies it is now possible to identify these changes with high resolution techniques. The non-random nature of some of the chromosomal alterations found in melanoma most likely determines the outcome of this disease. Identification of specific patterns related to these alterations could help researchers in distinguishing melanoma tumors resulting in new therapeutic targets for this disease (67).

With this approach, it was possible to identify genes that were differentially expressed during melanoma progression, from nevi to primary and metastatic melanoma (119). Two distinct groups were found, obviously in respect of the stage of the disease, suggesting two key events that were linked with most significant expression patterns, in *in situ* melanoma and VGP melanoma. In a similar study, that looked at genes that are differentially expressed between primary and metastatic melanomas, a subset of cell cycle regulating genes were indicated as important (120). Additionally, a recent genome-wide CGH study identified specific signatures in melanomas arising in different anatomic sites and with different histories of UV exposure (93).

Several other studies focused on correlating different genetic aberrations commonly observed in melanoma with differences in gene expression. The most interesting finding was observed in tumors harboring *BRAF* mutation, which correlated with specific signatures in primary melanomas (121). This was in agreement with previous reports that associated *BRAF* mutation with frequent gains in chromosome 7 (93, 122, 123).

AIMS OF THESIS

The overall aim of the thesis was to obtain a better molecular understanding of mutation rates and the roles of frequently altered genes, *NRAS*, *BRAF* and *CDKN2A*, in activation and expression of downstream genes or on downstream signaling in different stages of melanoma development.

Specific aims of the thesis were:

- To investigate the mutation frequency of *NRAS* and *BRAF* genes in sporadic primary melanomas and activation of their downstream proteins, Akt and ERK in early stages of melanoma (tumor thickness ≤ 2 mm), with different clinical outcome. Additionally, to correlate the effects of these gene alterations and protein activations with previously established clinicopathological factors of importance for survival (Paper I).

- To determine *NRAS* and *BRAF* mutation frequency in primary tumors from patients with familial melanoma with different *CDKN2A* germline alterations (Paper II).

- To identify differentially expressed genes by evaluating gene expression profiles of human metastatic melanomas that harbor either *NRAS* or *BRAF* mutation or are without these. Furthermore, to determine DNA copy variations in *BRAF*-mutated tumors compared to those with *NRAS* mutation by performing a single nucleotide polymorphism analysis (Paper III).

- To detect genes that are differentially expressed between human metastatic melanomas that either retain both *CDKN2A* alleles or have either mono- or bi-allelic deletions of this gene that affect both gene products, $p16^{INK4A}$ and $p14^{ARF}$ (Paper IV).

RESULTS

Paper I

Prognosis of primary melanomas with a thickness equal or less than 2.0 mm is generally good, but there is a subset of patients that have a worse survival and for which identification of additional prognostic markers are needed. Therefore, we analyzed the impact on survival of *NRAS* and *BRAF* mutations and activation of Akt and ERK in T1-2 primary cutaneous melanomas, in relation to previously established clinicopathological factors.

A cohort of 57 primary cutaneous T1-2 melanoma tumors (with tumor thickness ≤ 2 mm) was studied. These represented two distinct groups differing in overall survival: 42 (74%) patients who lived more than five years and 15 (26%) who survived less than five years after diagnosis of primary tumor. A higher ulceration frequency in the short survival group (p=0.003) was the only significant difference regarding clinicopathological parameters between the groups. All biopsy samples were formalin-fixed, paraffin-embedded (FFPE) tissue blocks. LCM technique was used for enrichment of samples with melanoma cells from dissected slices of tissue. Mutation analysis of *NRAS* (exons 1 and 2) and *BRAF* (exon 15) was performed using PCR amplification, SSCP and nucleotide sequence analyses. Each mutation was confirmed by two independent PCR/SSCP analyses followed by sequence analysis performed in both directions. Immunohistochemistry was performed using a rabbit anti-phospho-Akt (Ser473) and anti-phospho-p44/42 MAPK antibody. IRS was calculated by multiplying quantity score (positively stained cells either in cytoplasm or nucleus over the whole tumor area) with the intensity score (color intensity of the stained antibody).

Results showed that the mutation frequency for both genes was 61% (*NRAS*-26% and *BRAF*-39%, with 4% of coexisting mutations found in both genes). In a univariate analysis, shorter overall survival was associated with the presence of ulceration (p=0.001) and *BRAF* exon 15 mutations (p=0.005) as well as the absence of nuclear activation of Akt (p=0.022) and of cytoplasmic activation of ERK (p=0.003). Surprisingly, ulceration was a significant adverse prognostic factor only in melanomas with *BRAF* mutations, whereas there was no effect of ulceration on

overall survival in tumors with wild type *BRAF*. A multivariate analysis showed that significant independent adverse survival prognostic markers were: absence of cytoplasmic activation of ERK (p=0.007) and ulceration (p=0.008), whereas *BRAF* exon 15 mutation status showed a non-significant trend (p=0.066).

Paper II

Mutation frequencies of *NRAS* and *BRAF* genes in sporadic primary cutaneous melanomas are observed between 4-50% and 25-80%, respectively (80). However, there is still limited information on *NRAS* and *BRAF* mutations in familial melanoma. Previously, a high frequency of *NRAS* mutations (95%) has been reported in Swedish familial melanoma cases with germline *CDKN2A* alterations (124), whereas the association of *BRAF* somatic mutations with *MC1R* germline variants was demonstrated (96). Therefore, we sought to identify their mutation frequency in melanomas from patients with different *CDKN2A* germline alterations.

NRAS and *BRAF* mutations were analyzed in 19 FFPE primary tumors from familial melanoma cases that harbored eight different *CDKN2A* germline mutations. Tumor biopsy samples originated from Brisbane, Australia (16 samples from 15 patients) and Genoa, Italy (3 samples/patients). LCM, DNA extraction, PCR, SSCP and nucleotide sequence analyses of *NRAS* exon 2 and *BRAF* exon 15 were carried out as previously described (Paper I).

We found that 3 samples had *NRAS* mutations (16%) and 7 samples had *BRAF* mutations (37%). Three samples harbored alterations in both genes on the background of a germline *CDKN2A* L32P mutation.

The *NRAS* mutation frequency in this study was considerably lower than those reported previously in melanomas from Swedish families with germline *CDKN2A* mutations (124). This could possibly be attributed to different origins of studied cohorts and to different *CDKN2A* germline alterations evaluated in these two studies (the p.R112_L113insR founder mutation is predominant in Swedish families, which is in contrast to genotypes reported here), as well as to methodological differences. Intriguingly, all 3 tumors with *NRAS* mutations also had $BRAF^{V600E}$ mutations. Thus far, it is recognized that a mutation in either *NRAS* or *BRAF* is sufficient for activation

of the Ras-Raf-MEK-ERK pathway. The presence of both *NRAS* and *BRAF* mutations in the same lesions is contrary to the current consensus that such mutations are almost always mutually exclusive in melanomas. However, these concomitant mutations were previously observed in 9% of nevi (17) and was suggested that this outcome might be due to different clonal nests of cells within these tumors carrying distinct mutations. This possibility might also explain our findings obtained in this study.

Paper III

Primary melanomas are highly curable with surgical excision, however poor survival rates are observed with a diagnosis of metastases. This has been associated both with the biological nature of melanoma, as well as with the limited efficacy of current treatments for advanced disease. A step toward better understanding and management of metastatic melanoma would be the finding of comprehensive molecular signatures present in advanced disease. We performed a gene expression profiling study of 112 human metastatic melanoma tumors that either harbored *NRAS* or *BRAF* mutation or were without these mutations.

Total cellular RNA was isolated from dissected slices of tissue from fresh frozen metastatic melanoma samples, which was followed by oligonucleotide array hybridization of *in vitro* prepared transcripts, on Human Genome U133A Array Affymetrix GeneChips® (HG-U133A), according to manufacturer's instructions (Affymetrix, Santa Clara, CA, USA). Limma method was used to moderate standard errors of the estimated log-fold changes (Limma, Bioconductor; http://www.bioconductor.org). Microarray data was confirmed for selected genes by qRT-PCR analysis. For the single nucleotide polymorphism analysis, we randomly selected metastatic melanoma samples that harbored either *NRAS* (11 samples) or *BRAF* (10 samples) mutations from the study cohort.

Through microarray analysis, we identified 149 probe sets, representing 121 unique genes, which were differentially expressed between *BRAF*- and *NRAS*-mutated tumors. Two probe sets had distinct expression between *BRAF*-mutated and wild type tumors whereas no probe sets showed significant expression variation between *NRAS*-mutated and wild type tumors. Our results showed that almost all (93%) differentially expressed genes had higher expression in *BRAF*-mutated tumors versus those that harbored *NRAS*

mutation. The major difference was the overexpression of a large number of genes (72%) residing on chromosome 7, which is also the location of the *BRAF* gene itself in humans. Subsequently, we carried out hierarchical cluster analysis to assess expression patterns in samples with different mutation status. We found two clearly separated groups of tumors; the smaller cluster that mainly consists of only *BRAF*-mutated tumors (28 *BRAF*-mutated and 8 wt), whereas the larger cluster is a mixture of 34 *NRAS*-mutated, 26 *BRAF*-mutated, and 16 wt tumors. Repeated clustering for differentially expressed probe sets located on chromosome 7 showed that all *BRAF*-mutated samples cluster together, with the exception of 4 samples. Likewise, all but one *NRAS*-mutated samples cluster as a group for differentially expressed probe sets that do not reside on chromosome 7. Finally, a follow-up analysis of the study, single nucleotide polymorphism analysis, showed copy number increase of chromosome 7 in *BRAF*-mutated tumors, when compared to those with *NRAS* mutation.

We could not directly relate differentially expressed genes located at chromosome 7 to pathways specific for melanoma, however, the increased expression of genes obtained from our study suggests that mutated B-Raf protein has some distinct downstream effectors that are not fully characterized. Additionally, when results obtained with gene expression were compared to those from gene copy number changes, we found that the greatest part of chromosome 7 showed no correlation between regions harboring increased genes expression levels and copy numbers. It is possible that the presence of the activating *BRAF* mutations may lead to increased expression of the *BRAF* gene itself, as well as large number of other genes on the same chromosome, although the mechanism linking *BRAF* mutation to gene copy number increase still remains to be elucidated.

Paper IV

CDKN2A is involved in cell cycle regulation, inhibition of apoptosis and cellular senescence through the Rb and p53 pathways. Inactivation of this gene by deletions and other mechanisms are frequently observed in melanoma, however, little is known regarding the effects of these on gene expression in melanoma tumors. Therefore, we performed a gene expression profiling study on human metastatic melanoma samples in order to identify genes that are differentially expressed in tumors that either retained

both *CDKN2A* alleles or had either mono- or bi-allelic deletions of this gene that affected both gene products, $p16^{INK4A}$ and $p14^{ARF}$.

We selected 72 melanoma metastases with previously documented *CDKN2A* gene copy status (21 with intact *CDKN2A* as well as 30 and 21 that had mono- or bi-allelic deletions of this gene, respectively) from a cohort of 112 metastatic melanoma samples, as previously described (Paper III). The expression profiling data for all 72 metastatic samples on all 22,283 probe sets of the Affymetrix HG-U133A chips were used from this cohort of 112 melanoma patients. Differential expression analysis between the samples that harbored either *CDKN2A* mono- or *CDKN2A* bi-allelic deletions or were lacking these was done using Limma analysis. Subsequently, expression data from the 463 genes differentially expressed between the group with intact and the group with bi-allelic deletions of *CDKN2A* were uploaded into GeneGo MetaCore™ analytical suite (GeneGo, St. Joseph, MI, USA; http://www.genego.com) for determining candidate interactions between genes. Microarray data was confirmed for selected genes by qRT-PCR analysis.

We identified 719 probe sets that were differentially expressed between these three groups. Four hundred sixty-three probe sets differed in expression when comparing the group of tumors without *CDKN2A* deletions with the group that had bi-allelic deletions, 66 up-regulated and 397 down-regulated. One hundred and forty-seven probe sets differed in expression when comparing tumors without *CDKN2A* deletions with those that had mono-allelic deletion of this gene, 35 up-regulated and 112 down-regulated. Two genes, *CDKN2A* and *IGHA1*, showed significant expression differences in all three comparisons (with the highest expression in tumors with intact *CDKN2A* gene, reduced expression in tumors with mono-allelic deletions and lowest expression in tumors with bi-allelic *CDKN2A* deletions). Hierarchical cluster analysis based on expression across 27 topmost differentially expressed probe sets, ranked by raw p values, among comparisons within three *CDKN2A* cohorts showed two distinct groups of tumors. A smaller cluster that consists of 35 tumors (with majority of samples with both *CDKN2A* alleles present) and a larger cluster that consists of 37 tumors (with the majority having mono- and bi-allelic deletions of *CDKN2A*). Samples in smaller cluster generally shows higher expression of the probe sets when compared to the larger cluster. Finally, pathway analysis of 463 differentially expressed genes between the groups that had either intact *CDKN2A* alleles or bi-allelic deletions of *CDKN2A*

showed that the majority of networks were related to immune system response processes.

Our results showed that the majority of genes with expression differences present significantly reduced expression in parallel with *CDNK2A* allelic loss, either mono- or bi-allelic. These differences were more evident in samples with bi-allelic loss, when compared to those with intact *CDKN2A*, showing that a fraction of the genes were gradually affected by the gene dosage of *CDKN2A*. The *CDKN2A* gene itself was one of the two genes showing the most significant relation to *CDKN2A* gene copy number status, which supports the validity of the gene expression profiling data. Pathways analysis showed that the majority of genes differentially regulated in relation to *CDKN2A* gene dosage were related to immune response. Currently there is no known link between these pathways and *CDKN2A* expression and it is therefore not possible to conclude whether these effects on immune response-related gene expression are the results of loss of *CDKN2A* function or whether they are related to loss of other genes at the 9p21 locus, which may be co-deleted with *CDKN2A*.

CONCLUSIONS

Studies included in this thesis focused on analyzing mutation frequencies of commonly altered genes (*NRAS* and *BRAF*) in sporadic and familial melanoma (with specific *CDKN2A* alterations) as well as determining their role in activation of downstream targets and expressions of genes in different melanoma stages.

General conclusions

The general conclusions are that although both N-Ras and B-Raf convey signals along the same pathway, signatures in our analysis show differences between these two effectors. In primary melanomas, both sporadic and familial, *NRAS* and *BRAF* mutations are observed at frequencies similar to those that were reported previously in tumors with similar thickness. However, we found increased rates of coexisting mutations in both genes. Although this finding correlated with the presence of a germline $p16^{L32P}$ mutation in familial melanomas as possibility alteration that may contribute to cellular tolerance of these dual alterations, it is plausible to hypothesize the presence of some other uncharacterized biological entity that could be responsible for these results. Additionally, different clonal nests within these tumors could harbor mutations in each gene separately which could be a possible explanation for these results. In metastatic melanomas the great majority of observed changes included overexpression and amplification of genes located on chromosome 7 in samples with *BRAF* mutation. The differences in gene expression between *BRAF* and *NRAS* mutated tumors may be caused both by direct effects on cell signaling by activated *BRAF* as well as effects related to overexpression and gene copy number gains on chromosome 7, where the *BRAF* gene is located. Finally, loss of the *CDNK2A* gene correlated mainly with lower expression of genes in metastatic melanomas with a majority linked to immune response.

Specific conclusions

Paper I

- The overall observed rate of *NRAS* mutations in sporadic primary melanomas was 26%, with the majority found in exon 2 (80% of total number). The *BRAF*

mutation rate was 39% with all located in the exon 15 (95% of total number were V600E). Coexisting *NRAS* and *BRAF* mutations were found at rates of 4%.

- Almost two-fold higher mutation frequencies for both genes were observed in the group of patients that had shorter overall survival.
- The presence of ulceration and lack of cytoplasmic activation of ERK were independent prognostic markers in primary sporadic melanoma.
- In patients with *BRAF* exon 15 mutations, presence of ulceration had an impact on survival.
- Cytoplasmic immunohistochemical score of p-ERK could be used as a prognostic marker for thin melanoma survival (with tumor thickness ≤ 2 mm).

Paper II

- *NRAS* and *BRAF* mutation rates in primary hereditary melanomas were 16% and 37%, respectively.
- Coexisting *NRAS* and *BRAF* mutations were observed in 16% of the tumors, on the background of a specific *CDKN2A* L32P germline mutation.
- The L32P germline mutation might contribute to cellular tolerance of these dual *NRAS* and *BRAF* mutations.

Paper III

- Almost all differentially expressed genes in evaluated melanoma metastases had higher expression in *BRAF*-mutated tumors versus those that harbored *NRAS* mutation.
- The major difference was the higher expression of a large number of genes residing on chromosome 7, which is also the location of the *BRAF* gene in humans.
- This overexpression pattern was accompanied with copy number increase of chromosome 7 in *BRAF*-mutated tumors.
- Our results suggest involvement of additional downstream signaling pathways apart from the Ras-Raf-MAPK pathway, in *BRAF*-mutated melanomas.

- Metastatic melanomas showed gradually decreased gene expression pattern which was in concordance with *CDNK2A* allelic loses.
- The majority of the discovered genes had lower expression and was found in the group with bi-allelic loss of *CDKN2A* when compared to the group with intact *CDKN2A* gene.
- Expression pattern obtained through this microarray analysis mainly correlated to immune response.
- A possible link between melanoma aggressiveness and immune response is suggested and plausible in metastatic disease.

FUTURE PERSPECTIVES

With this work, we wanted to obtain further insights into the biological background of skin melanoma in different stages of progression. Furthermore, we focused on finding some specific prognostic marker, which could be used in predicting outcome in this disease, and identifying therapeutic targets, which could be used for clinical trials. We think that our findings could be used as a starting point for extended studies that would address unresolved issues. Some of these are listed.

It would be of interest to perform a larger study to address the correlation between *BRAF* mutation and ulceration in primary melanomas. Results from our first study suggest that these might be connected through, as we speculated, programmed necrotic cell death pathway (125, 126). If confirmed, this could be the first major step in understanding the ulceration initiation in melanoma, which could lead toward specific targets for this biological entity. Furthermore ulceration seemed to impart a poor prognosis particularly in melanomas with *BRAF* mutations. It would be of interest to investigate whether this can be confirmed in a separate validation study.

Lack of cytoplasmic ERK activation in primary melanomas was showed to be an adverse prognostic marker in T1-2 melanomas, which is opposing current view in the field. We speculated that there might be an additional pathway that could be responsible for worse survival, which is simultaneously activated in tumors that are lacking ERK activation. Recently, similar activation effects of ERK have been found in prostate and endometrial cancers, suggesting that this might be more common feature found in different tumor types (127, 128). Additionally, lack of nuclear activation of Akt might also be common feature in some primary melanomas as this has been confirmed previously (129) as well as vaguely hinted in another publication (130). Thus, larger confirmatory studies might shed a light on this, eventually ending these controversies.

The presence of coexisting mutations in *NRAS* and *BRAF* genes are opposing current observations that these two are almost always mutually exclusive in melanoma and other tumor types. However, we found that these two are present at rates that could be suggestive that their occurrence could be of some biological relevance. These have already been found in nevi (17) but also in colorectal carcinomas at increased rates

(131). Intriguingly as reported in the latter study, these dual mutations in both genes were either preserved or eventually acquired during tumor progression of this cancer. As s first step, future study should try to determine whether these dual mutations are present in same cell and if this is confirmed, followed by functional studies that will address the significance of these coexisting mutations.

Data obtained through the first gene expression profiling study, where we compared tumors according to their *NRAS* and *BRAF* mutation status, are extensive. However, our present results are suggestive on focusing on results related with chromosome 7. Since our understanding of the role of overexpression and amplification of genes residing on chromosome 7 in association with *BRAF* mutation, including their role in melanoma progression is still limited, further work is needed.

Finally, the correlation identified between *CDKN2A* deletion status and immune response genes should be further validated. Loss of immune response related functions may be associated with the adverse prognosis previously demonstrated in metastatic melanoma with *CDKN2A* deletions (132).

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