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MOLECULAR STUDIES IN DIFFERENT TYPES OF MELANOMA TUMORS - CORRELATIONS TO CLINICAL DATA

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Institution för onkologi-pathologi

MOLECULAR STUDIES IN DIFFERENT TYPES OF MELANOMA TUMORS - CORRELATIONS TO CLINICAL DATA

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ABSTRACT

Approximately 90% of melanomas arise from skin sites (known as cutaneous malignant melanoma; CMM), whereas the non-cutaneous melanoma (mucosal and ocular melanomas) are rare, accounting for about 10%. Familial melanoma accounts for up to 10% of patients diagnosed with CMM. Both genetics (e.g. *CDKN2A* and *CDK4* germline mutations, as well as polymorphisms in *MC1R* and other genes) and environmental factors (ultraviolet radiation) contribute to the induction of melanoma. The MAPK and the PI3K are the two most commonly activated signaling cascades in melanomas. Activation of these two pathways occurs frequently through alterations in *BRAF*, *NRAS* and *KIT* oncogenes. The involvement of these oncogenes in common CMM subtypes is well-studied. However, the frequency of mutations in *BRAF*, *NRAS* and *KIT* and also *PTEN* has not been well-characterized in the other rare melanoma subtypes, at least not in Caucasian populations. The overall aim of this thesis was to better define the molecular genetic alterations of *BRAF*, *NRAS* and *KIT* in different subtypes of melanomas and to correlate the mutation status with the histopathological features of the tumors and with the clinical parameters of the patients.

For the first project, Formalin-fixed paraffin embedded samples of primary familial and sporadic CMMs were collected from eight centers in Europe and Australia. The overall aim was to better define the frequencies of *BRAF* and *NRAS* mutations in familial melanoma with and without germline *CDKN2A* mutations. Overall, 89 tumors from patients with germline *CDKN2A* mutations, 46 from patients without germline *CDKN2A* mutations, and 50 sporadic melanomas were analyzed for *BRAF* exon 15 and *NRAS* (exon 2) mutations using direct DNA sequencing. The tumors were also evaluated for pERK and pAkt expression by immunohistochemistry. The *BRAF* and *NRAS* mutation frequencies detected in familial melanomas were 43% and 11%, respectively. These frequencies did not differ significantly between tumors from germline *CDKN2A* mutation carriers and non-carriers. The frequency of *BRAF* mutation (41%) and *NRAS* mutation (12%) in the sporadic melanomas did not differ significantly from that identified in the familial melanomas. Expression of pERK and pAkt was observed in 65% and 46% of the familial melanomas, respectively. Similar frequencies of pERK and pAkt expressions were observed in the sporadic melanomas.

In the second project, we analyzed a large number of a rare subtype of melanoma; sinonasal mucosal melanoma. In total, 56 primary tumors were screened for mutations in *KIT* (exons 11, 13 and 17), *NRAS* (exons 1 and 2) and *BRAF* exon 15 using direct sequencing. Twelve of the 56 (21%) tumors contained one mutation in these oncogenes; 2 tumors harbored *KIT* mutations, another 2 harbored *BRAF* mutations and 8 had *NRAS* mutations. The mutations were more frequently detected in tumors originated from the paranasal sinuses than from the nasal cavity (p=0.045). Patients with melanoma in the paranasal sinuses had a worse overall survival than patients with melanoma in the nasal cavity (p=0.027).

In the third project, primary and metastatic acral lentiginous melanomas were investigated for mutations in *BRAF* (exons 11 and 15), *NRAS* (exons 1 and 2), *KIT* (exons 9, 11, 13, 17 and 18) and *PTEN* (exons 1, 3-6 and 10-12) by direct sequencing. The data showed an identical mutation frequency of 15% (13 out of 88) of both *KIT* and *NRAS*, whereas *BRAF* mutations were found in 17% (15 out of 88) of the primary tumors. Of the 25 cases evaluated for *PTEN* mutations, only one tumor contained a mutation (4%). The *BRAF*, *NRAS* and *KIT* mutation status in 16 metastases was similar to that identified in the matched primaries. In comparison with *BRAF* wild-type tumors, *BRAF* mutated tumors were more commonly diagnosed in young individuals (p=0.028) and significantly associated with tumor location on the feet (p=0.039) and female gender (p=0.039). The anatomical site was an independent prognostic factor with better overall survival for patients with tumors on hand or subungual areas than those with tumors on the feet or under toenails (p=0.025).

In the fourth project, we evaluated 124 primary and 76 metastatic (73 were matched metastases) CMMs for $\text{BRAF}^{\text{V600E}}$ expression by immunohistochemistry using VE1 antibody. Overall, 55% (110 out of 200) tumors displayed a positive homogenous staining. There was a consistency in $\text{BRAF}^{\text{V600E}}$ staining between the matched primaries and metastatic CMMs. In 28 tumors a discrepancy was observed between the VE1 staining and the mutation analysis methods. Re-analysis of 25 tumors of the discrepant cases by pyrosequencing revealed a new $BRAF^{V600E}$ mutation in three cases, supporting the results seen with VE1 staining. In the remaining 22 tumors the results of the pyrosequencing and the initial mutation methods were similar. Overall sensitivity and specificity with VE1 antibody staining were 97% and 80%, respectively.

LIST OF PUBLICATIONS

I. **Zebary A**, Omholt K, van Doorn R, Ghiorzo P, Harbst K, Hertzman Johansson C, Höiom V, Jonsson G, Pjanova D, Puig S, Scarra GB, Harland M, Olsson H, Egyhazi Brage S, Palmer J, Kanter-Lewensohn L, Vassilaki I, Hayward NK, Newton-Bishop J, Gruis NA, and Hansson J. Somatic *BRAF* and *NRAS* Mutations in Familial Melanomas with Known Germline CDKN2A Status: A GenoMEL Study. *J Invest Dermatol*. 2013 Jun 14; doi:10.1038/jid.2013.270.

II. **Zebary A**[*], Jangard M[*], Omholt K, Ragnarsson-Olding B, and Hansson J. *KIT*, *NRAS* and *BRAF* mutations in sinonasal mucosal melanoma: a study of 56 cases. *Br J Cancer*. 2013 Aug 6; 109:559-64.

III. **Zebary A**, Omholt K, Vassilaki I, Höiom V, Lindén D, Viberg L, Kanter-Lewensohn L, Hertzman Johansson C, and Hansson J. *KIT*, *NRAS*, *BRAF* and *PTEN* mutations in a sample of Swedish patients with acral lentiginous melanoma. *J Dermatol Sci.* 2013 Aug 8; 72:284–289.

IV. Eriksson H[*], **Zebary A**[*], Vassilaki I, Omholt K, Ghaderi M, and Hansson J. $BRAF^{V600E}$ protein expression in primary cutaneous melanoma and paired metastases. Submitted for publication.

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LIST OF ABBREVIATIONS

ALM	Acral lentiginous melanoma
BAP1	BRCA-1 associated protein 1
BRAF	v-RAF murine sarcoma viral oncogene homolog B1
CDK	Cyclin-dependent kinase
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CMM	Cutaneous malignant melanoma
CSD	Chronic sun-damage
ERK	Extracellular-signal regulated kinase
FAMMM	Familial atypical multiple mole melanoma syndrome
FDA	Food and Drug Administration
GDP	Guanosine diphosphate
FFPE	Formalin-fixed paraffin embedded
Grb2	Growth factor binding protein 2
GTP	Guanosine triphosphate
IHC	Immunohistochemistry
LMM	Lentigo maligna melanoma
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin-1 receptor
MEK	MAP kinase extracellular signal regulated kinase
MITF	Microphthalmia-associated transcription factor
MMM	Mucosal malignant melanoma
NM	Nodular melanoma
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
pAkt	Phosphorylated-Akt
pERK	Phosphorylated-ERK
PIP3	Phosphatidylinositol 3, 4, 5-trisphosphate
PI3K	Phosphatidylinositol 3-kinase
PTEN	Phosphatase and tensin homolog
RAF	Rapidly accelerated fibrosarcoma
RAS	Rat sarcoma virus
Rb	Retinoblastoma
RGP	Radial growth phase
RTK	Receptor tyrosine kinase
SNMM	Sinonasal mucosal melanoma
SOS	Son of sevenless
SSM	Superficial spreading melanoma
TYR	Tyrosinase
TYRP	Tyrosinase-related protein
UV	Ultraviolet
VGP	Vertical growth phase
α-MSH	α-melanocyte stimulating hormone

1 INTRODUCTION

Melanoma is believed to have been known since the fifth century BC were it was first reported by Hippocrates, and in the 17th century melanoma was described as "fatal black tumor" (Rebecca *et al.*, 2012). In the beginning of the 19th century, the term "melanosis" was used to describe the melanoma as a disease entity and at the same time it was postulated that there is a familial predisposition for melanoma development (Laennec, 1812; Norris 1820). The actual term "melanoma" was coined in 1838 by pathologist Sir Robert Carswell (Rebecca *et al.*, 2012).

It is well-established that melanoma originates from malignant transformation of a specialized type of cell called the melanocyte. During embryogenesis, melanocyte precursors, the melanoblasts, migrate from the neural crest to their final destination in various parts of the body where they differentiate to melanocytes. In addition, it has been shown that a subset of skin melanocytes are derived from Schwann cell precursors (Adameyko *et al.*, 2009). The major function of melanocytes is the production of melanin pigments, which give the skin and other pigmented tissues their color and serve as a protection from ultraviolet (UV) radiation. The melanocytes are present in the basal layer of the epidermis of the skin, within hair follicles, the uveal layer of the eye, the mucosal membrane lining the respiratory, gastrointestinal and genitourinary tracts and the leptomeninges of the central nervous system. Since the majority of the melanocytes reside in the skin, cutaneous malignant melanoma (CMM) is by far the most common form of melanoma, accounting for about 90% of all diagnosed melanomas (Chang *et al.*, 1998). Melanomas originating from other body sites such as mucosal and uveal tract melanomas, as well as melanomas of unknown primary origin are rare. The vast majority of melanomas (~90%) are considered sporadic, whereas only 5 to 10% are familial melanomas (Hansson, 2008; Hayward, 2003). The average age of patients diagnosed with melanoma is around 65 years; however, melanoma can affect individuals of all age groups and, indeed, melanoma is one of the most common cancers among young adults (D'Orazio, 2011).

CMM is one of the most lethal skin cancers. Although CMM accounts for a small fraction (<5%) of malignant skin tumors, it is responsible for the majority of the skin neoplasm-related deaths (Miller and Mihm, 2006). Exposure to environmental factors,

such as solar or artificial UV radiation and also inheritance of germline alterations in the *cyclin dependent kinase inhibitor 2A* (*CDKN2A*) and *cyclin dependent kinase* (*CDK4*) genes significantly increase the risk of melanoma development. Polymorphisms in the *melanocortin-1 receptor* (*MC1R*) gene are common especially in European populations and are associated with increased risk of melanoma formation. Other host factors, for instance positive family history of melanoma, presence of large numbers of nevi, dysplastic nevi and light complexion also increase the susceptibility to CMM. Therefore, the incidence of melanoma is much higher in light skinned people compared with dark skinned individuals.

Melanoma represents a heterogeneous disease in terms of genetic background and phenotypic features. Melanoma is characterized by activation of several signaling pathways, most importantly, the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways, which play a crucial role in the development of melanomas by regulating both proliferation and survival of melanoma cells. Activation of these pathways occurs most commonly through activating mutations in the *BRAF*, *NRAS* and *KIT* oncogenes, which interestingly occur in a mutually exclusive manner (Carvajal *et al.*, 2011; Omholt *et al.*, 2011).

In most cases, early detection of melanoma, *i.e.*, when the melanoma cells are confined to the epidermis, can be cured by surgical excision; however, the treatment options become very limited once the disease has metastasized. The 5-year survival for patients with thin localized melanoma is about 95%, whereas for those with distant metastatic disease is below 20% (Balch *et al.*, 2009). Conventional chemo- and immunotherapy with, for example, dacarbazine or interleukin-2, has for decades remained the main systemic therapy for patients with disseminated disease. Several small molecules targeting proteins in the aforementioned pathways, and immunotherapy in the form of human monoclonal antibodies are under investigation and some (vemurafenib, dabrafenib, trametinib and ipilimumab) have recently been approved for treatment of patients with metastatic or unresectable melanomas (Chapman *et al.*, 2011; Hauschild *et al.*, 2012; Hodi *et al.*, 2010). These agents significantly improve the overall and progression-free survival, as compared with the standard chemotherapy.

2 EPIDEMIOLOGY

The worldwide incidence of CMM continues to rise, especially among western Caucasian populations, with an annual increase of 3-7% (Bloethner *et al.*, 2009; Erdmann *et al.*, 2013; Garbe and Leiter, 2009; Godar, 2011; Linos *et al.*, 2009; Mansson-Brahme *et al.*, 2002). In the Unites States (US), the lifetime risk of melanoma in 1935 was 1 in 1500 while the current lifetime risk is approximately 1 in 50 persons (Rigel, 2010). The rate of increase in the CMM incidence is more rapid than for most other cancers (Linos *et al.*, 2009). Moreover, the incidence of childhood and adolescent melanoma has also increased during the last few decades (Wong *et al.*, 2013). In fact, melanoma is the second most common cancer in individuals aged 15-29, accounting for 11% of all malignancies diagnosed in this age group (Gandini *et al.*, 2011). The increase in the incidence of melanoma might be attributed to several different factors, such as an increase in the diagnosis of thin melanomas (Breslow ≤1 mm), changes in sun exposure behavior, public awareness and skin screening programs. On the other hand, it is believed that the mortality rates from melanoma has remained stable or not increased at the same pace as the incidence rates of melanoma (Erdmann *et al.*, 2013). Recently, however, there has been data suggesting that the incidence of melanoma may be leveling off, or even decreasing in several countries (Erdmann *et al.*, 2013).

The incidence of CMM varies with age, gender, ethnicity and country of residence (Erdmann *et al*., 2013; Linos *et al*., 2009). In 2012, there were more than 3.4 million new cases of cancer in Europe, of which more than 100 000 were CMMs, accounting for about 3% of all diagnosed malignancies (Ferlay *et al.*, 2013). The estimated total number of cancer death was 1.75 million, of which more than 22 000 (1.3%) were due to CMMs (Ferlay *et al.*, 2013). In Europe, the highest incidence rates of melanoma are those reported in the Scandinavian courtiers, Denmark, Norway and Sweden (Garbe and Leiter, 2009). In Sweden CMM is estimated to be the fifth and sixth most common cancer diagnosed in women and men, respectively, representing greater than 5% of all new cancer cases (Cancer incidence in Sweden 2011, www.socialstyrelsen.se). In 2011, more than 3000 new melanoma cases were diagnosed in Sweden with approximately 500 deaths. Worldwide, the highest incidence rates of CMM are those reported in Australia and New Zealand, whereas the lowest are in Asia (Liang *et al.*, 2010). In Australia and New Zealand, CMM represents the third and the fourth most common

cancer diagnosed in men and women, respectively, accounting for approximately 10% of all malignancies. Before age 40, the incidence of melanoma is significantly higher in women than in men, while after age 40, melanoma is more prevalent among men (Anderson *et al.*, 2009; Watson *et al.*, 2011). The incidence rates of melanoma are significantly higher in individuals with light complexion than those with dark skin or with Asian origin. The lifetime risk of melanoma in the US is about 1 in 50 for whites and only 1 in 1000 for blacks (Kabigting *et al.*, 2009).

3 RISK FACTORS

The likelihood of developing sporadic or familial melanoma depends on the interactions of genetic, phenotypic and environmental risk factors. There are several widely accepted genetic, host and environmental risk factors.

3.1 ENVIRONMENTAL FACTORS

The major well-established environmental risk factor for development of CMM and also for non-melanoma skin cancer (squamous cell and basal cell carcinomas), is UV radiation, from either sunlight or indoor tanning devices (Garibyan and Fisher, 2010; Lazovich *et al.*, 2010; Young, 2009). In general, UV radiation is believed to enhance tumor formation by damaging DNA and creating mutations in key regulatory genes such tumor suppressor genes (e.g. *CDKN2A*, *PTEN* and p53) and proto-oncogenes (e.g. *BRAF* and *NRAS*) (Besaratinia and Pfeifer, 2008; Garibyan and Fisher, 2010).

It has been shown that exposure to artificial UV radiation from tanning devices, which are widely available especially in Europe and US, increases significantly the risk of CMM. The results from a meta-analysis, where the association between artificial UV radiation and melanoma risk was investigated, showed that the individuals who use tanning devices before 35 years of age have a significantly higher risk of developing melanoma (based on 7 informative studies; summary relative risk, 1.75; 95% CI, 1.35–2.26) (International Agency for Research on Cancer Working Group on artificial ultraviolet light and skin, 2007). In addition, melanoma can be induced in some animals upon exposure to UV radiation (Besaratinia and Pfeifer, 2008; Noonan *et al.*, 2001; Wang *et al.*, 2009). Nowadays, in several countries the indoor tanning devices are illegal for those under the age of 18 and in some other countries the amount of UVB radiation from tanning devices is limited.

The spectrum of solar UV radiation reaching the earth's surface consists mainly (~95%) of UVA (320-400 nm) and a small fraction (~5%) of UVB (280-320 nm). Both UVA and UVB radiation are considered carcinogenic to humans (El Ghissassi *et al.*, 2009; Garibyan and Fisher, 2010). Although UVA is more abundant in the sunlight and can penetrate the skin deeper, it is the UVB that can cause direct DNA damage through the formation of cyclobutane pyrimidine dimers and pyrimidine 6-4 photoproducts

(Gilchrest *et al.*, 1999; Noonan *et al.*, 2012). In contrast, the effect of UVA radiation is mainly indirect, and causes DNA damage through formation of ROS; reactive oxygen species (Rizzo *et al.*, 2011). However, it has been suggested that also UVA radiation can result in DNA damage via the production of cyclobutane pyrimidine dimers (Mouret *et al.*, 2006). UVA rays contribute to skin aging, whereas UVB rays are mainly responsible for sunburns.

Moreover, the pattern of sun-light exposure might play an important role in melanomagenesis. People who are exposed to UV radiation on an intermittent and intense base are postulated to be at higher risk of melanoma development (Elwood and Jopson, 1997; Young, 2009). In contrast, chronic and regular exposure to sun-light might be protective against melanoma, but significantly increases the risk of non-melanoma skin cancers (Gandini *et al.*, 2011). In addition, the risk of melanoma is strongly linked to early-life sun exposure and severe sunburns, blistering, especially during childhood (Cust *et al.*, 2011).

Finally, it is also important to remember that the sunlight has some beneficial effects; for instance, sunlight is the best natural source for vitamin D production and has been used as a treatment option for some types of skin diseases, such as vitiligo and psoriasis.

3.2 HOST RISK FACTORS

3.2.1 History of melanoma

As with many other types of malignancies, CMM may cluster in families, and family history of CMM is a strong risk factor for the development of melanoma (Gandini *et al.*, 2005b). Melanoma patients with family history of the disease are characterized by younger age at diagnosis and higher density and number of nevi (Chiarugi *et al.*, 2012). Patients with family history of melanoma and very high number of total body melanocytic nevi (with some nevi displaying histologically features of atypical/dysplastic nevi) are classified to have the familial atypical multiple mole melanoma syndrome (FAMMM) (Hansson, 2008). The effect of family history is independent of age, nevus count, hair and eye color (Ford *et al.*, 1995). In general, the risk of melanoma increases by two-fold in a person with an affected first-degree

relative (Ford *et al.*, 1995). Moreover, patients with family or personal history of melanoma are at greater risk of developing multiple primary melanomas (Hansson, 2008; Psaty *et al.*, 2010). Individuals in families with three or more melanoma cases in blood relatives (strong family history) are considered to have even a higher risk than those with only 2 cases in non-first degree relatives (weak family history) (Thompson *et al.*, 2005). Even a personal history of non-melanoma skin cancer has been shown to increase the risk of a subsequent melanoma (Marghoob *et al.*, 1995). Thus, approximately 8% of patients with melanoma will suffer from a subsequent second melanoma (Ferrone *et al.*, 2005).

3.2.2 Skin, hair and eye color

The personal pigment-related characteristics of an individual such as fair complexion; fair hair (red, blond and light brown) and light eye colors (blue, green and hazel) increase the risk of developing melanoma as compared to those with dark complexion and dark hair/eye color. A meta-analysis found that the subjects with light eye color were at significantly higher risk of developing melanoma (RR= 1.62; 95% CI: 1.44; 1.81) compared to those with dark eyes (Gandini *et al.*, 2005b). The same study showed that the relative risk of melanoma in red-haired persons tended to be greater (RR=3.64; 95% CI: 2.56, 5.37) than in those with blond (RR= 1.96; 95% CI: 1.41, 2.74) and light brown-hair (RR= 1.62; 95% CI: 1.11, 2.34), compared to dark hair, suggesting that different hair colors confer different risk for melanoma development (Gandini *et al.*, 2005b).

3.2.3 Nevi

The nevi represent nests of benign melanocytic proliferation and typically most of the nevi appear early during life and reach high frequency in young adults (Bauer and Garbe, 2003). However, unlike melanoma most of the nevus cells express markers of senescence such as senescence associated β-galactosidase (SA-betagal) and therefore the majority of nevi remain dormant and regresses spontaneously or very seldom might further progress to develop melanoma. Furthermore, nevi very frequently harbor $BRAF^{V600E}$ mutation and nearly all, especially common nevi, express $p16^{INK4A}$ protein, which also believed to contribute to maintain nevi in a senescent state.

There are different types of nevi including congenital, common and atypical/dysplastic nevi. The total body number and the phenotype of nevi (common and dysplastic) are regarded as potent markers for increased risk of CMM (Chang *et al.*, 2009). Approximately 20-30% of CMMs are derived from pre-existing nevi, as it is evident by presence of remnants of nevi on the histological examination of CMMs (Chiarugi *et al.*, 2012).

Dysplastic nevi (also known as BK-mole, Clark's nevi and atypical nevi) are present in about 5-10% of the Caucasian populations and are generally larger (5 mm or more) and display more color variegation than common nevi (Gandini *et al.*, 2005a; Goldstein and Tucker, 2013). The acronym FAMMM is also known as dysplastic nevus syndrome and atypical mole syndrome (Hansson, 2008). This syndrome was first described in the late 1970s and is associated with increased risk of melanoma development, as well as other malignancies such as pancreatic cancer. Dysplastic nevi may derive from pre-existing common nevi or from *de novo*. Dysplastic nevi may arise anywhere on the skin including sun-protected areas, however, they are more commonly located on skin with intermediate sun-exposure such as the trunk and the back (Naeyaert and Brochez, 2003). Diagnosis of dysplastic nevi require certain histopathological and clinical features, misdiagnosis of dysplastic nevi as melanoma or vice versa is not uncommon (Brochez *et al.*, 2002).

Intermittent sun-exposure (holiday sun-exposure) is one of the major determinants of both nevus number and size in adults (Newton-Bishop *et al.*, 2010). In addition, several single nucleotide polymorphisms in genes on different chromosomes have been associated with both increased number and large size of nevi (Newton-Bishop *et al.*, 2010). The higher the number of nevi an individual has, the higher the risk of developing melanoma. The risk of melanoma in people with very high number of common nevi (more than 100) is approximately seven times greater than in those with less than 15 common nevi (Gandini *et al.*, 2005a). The association between melanoma development and dysplastic nevi is even stronger. Individuals with five dysplastic nevi are six times more likely to acquire melanoma compared to those with no dysplastic nevi (Gandini *et al.*, 2005a).

3.2.4 Pigmentation

It is the amount and type of melanin pigment produced by melanocytes rather than the number of melanocytes that differ among individuals. The rate of melanin synthesis is under the control of genetic and environmental factors. Cutaneous melanocytes produce two kinds of melanin pigments; eumelanin (brown/black) and pheomelanin (red/yellow). At the time of skin exposure to UV radiation, keratinocytes stimulate the melanocytes to produce the melanin pigment. Keratinocytes produce α-melanocyte stimulating hormone (α-MSH), which will then bind and activate the melanocortin-1 receptor (MC1R) on the surface of melanocytes to generate cyclic adenosine 3′, 5′-monophosphate (cAMP). Ultimately, there will be activation of certain genes (e.g. *microphthalmia-associated transcription factor; MITF*) and synthesis of melanin from tyrosine. The melanin pigments are then transported in melanosomes and distributed to the surrounding keratinocytes to protect their nuclei from radiation. Eumelanin is more protective than pheomelanin against the damaging effect of UV radiation.

The synthesis of the two different melanin pigments (eumelanin/pheomelanin) depends on the genotype of the *MC1R* gene, as well as on other genes such as *tyrosinase* (*TYR*), *tyrosinase-related protein (TYRP1)* and *dopachrome tautomerase* (*DCT*) (Busca and Ballotti, 2000). Binding of α-MSH to wild-type MC1R will trigger production of eumelanin, whereas stimulation of MC1R that harbor certain genetic variants results in production of pheomelanin. There are several different common variants of *MC1R* which impair the production of eumelanin and instead result in synthesis of pheomelanin. Interestingly, it has recently been observed that the pheomelanin production in mice carrying an inactivating mutation in the *MC1R* gene and melanocyte-specific $BRAF^{V600E}$ mutation contributes to melanoma formation independently of UV radiation (Mitra *et al.*, 2012).

3.3 GENETIC FACTORS

Familial melanoma has an autosomal dominant pattern of inheritance. Familial melanoma can simply be defined as families with two or more affected members and a more stringent definition is families with either two or more first-degree relatives (parents, siblings or children) or three members affected with melanoma irrespective of degree of relationship. Overall, approximately up to 10% of the patients diagnosed with melanoma have a first degree relative with melanoma.

Familial and sporadic melanomas are similar with regard to clinical and histopathological features (e.g. anatomical site and histological subtype) and also share the same prognostic factors such as Breslow thickness, ulceration and Clark level (Chiarugi *et al.*, 2012; Nagore *et al.*, 2008). Superficial spreading and nodular melanomas are the most frequent histological subtypes in patients with familial melanomas, while the acral lentiginous and lentigo maligna melanomas rarely run in families (Chiarugi *et al.*, 2012). The genetic susceptibility for induction of melanoma has a variable penetrance, spanning from high- to low-penetrance susceptibility genes. Analysis of multiple-case families has identified alterations in high penetrance genes that strongly predispose to melanoma formation.

3.3.1 High-penetrance genes

Inheritance of germline mutations in high-penetrance susceptibility genes is rare. So far two genes have been identified that confer high susceptibility to melanoma development; *CDKN2A* and *CDK4*. These genes are inherited in an autosomal dominant pattern.

3.3.1.1 Cyclin-dependent kinase inhibitor 2A

The tumor suppressor gene *CDKN2A* was the first identified major melanoma susceptibility gene. The risk of developing melanoma is significantly higher in individuals carrying a germline *CDKN2A* mutation. Overall, approximately 20% to 40% of familial melanomas are caused by germline *CDKN2A* mutations (Hayward, 2003). It is estimated that *CDKN2A* mutation carriers in families with multiple CMMs have a 30% risk of developing melanomas at 50 years of age and 67% at 80 years of age (Bishop *et al.*, 2002). In the general population, the frequency of *CDKN2A* mutations is very rare and the lifetime risk of melanoma in germline *CDKN2A* mutation carriers, regardless of family history, is estimated to be 14% at 50 years of age and 28% at 80 years of age (Begg *et al.*, 2005). Moreover, controversial results have been reported regarding association of few *CDKN2A* polymorphisms (e.g. c.442G > A, c. 29C >G and c.69C>T) with a possible risk of CMM development (Veinalde *et al.*, 2013).

3.3.1.2 Cyclin-dependent kinase 4

The other known high-penetrance susceptibility gene for familial CMM development is *CDK4*. In contrast to *CDKN2A*, germline *CDK4* mutations are rare (approximately 2%) and have been reported only in a limited number of melanoma prone families (Goldstein *et al.*, 2006; Puntervoll *et al.*, 2013). *CDK4* is located on chromosome 12q14, consisting of eight exons (the first exon is non-coding) and encoding for a serine/threonine protein kinase and play an important role in cell-cycle progression. The CDK4 protein with CDK6 form a heterodimeric complex with cyclin D which leads to phosphorylation and inactivation of retinoblastoma (Rb) protein and subsequently releases the E2F family of transcription factors, which in turn upregulate genes responsible for cell cycle progression through the G1-to-S phase (Sheppard and McArthur, 2013).

In familial melanoma all mutations detected in *CDK4* are characterized by substitution of arginine by histidine ($CDK4^{R24H}$) or cysteine ($CDK4^{R24C}$) in codon 24 in exon 2 (Hill *et al.*, 2013). These mutations prevent interaction between CDK4 and $p16^{INK4A}$, thus causing CDK4 resistance to inhibition by $p16^{INK4A}$.

3.3.2 Intermediate-penetrance genes

3.3.2.1 Microphthalmia-associated transcription factor (MITF)

The *MITF* oncogene, located on chromosome 3p14, plays an important role in melanocyte differentiation, survival and proliferation and induces transcription of genes associated with melanin synthesis such as *TYR* and *TYRP1* (Hou and Pavan, 2008).

A rare germline missense *MITF* mutation (substitution of glutamic acid by lysine in codon 318; $MITF^{E318K}$) has been identified to increase the susceptibility to melanoma development by more than five-fold in carriers from melanoma families and from the general population (Bertolotto *et al.*, 2011; Ghiorzo *et al.*, 2013; Yokoyama *et al.*, 2011). This novel *MITF* variant is associated with family history of melanoma, multiple primary melanomas, nevus count and non-blue eye color (Yokoyama *et al.*, 2011).

3.3.2.2 BRCA-1 associated protein 1 (BAP1)

BAP1 is a tumor suppressor gene, located on chromosome 3p21. Recently, germline mutations in *BAP1* have been found to predispose patients to development of uveal and CMMs, as well as other malignancies such as mesothelioma (Abdel-Rahman *et al.*, 2011; Carbone *et al.*, 2013; Hoiom *et al.*, 2013). In addition, a very high frequency (84%) of somatic *BAP1* mutations have been described in metastatic uveal melanomas (Harbour *et al.*, 2010). In a population-based study, analysis of 66 patients with uveal melanomas, unselected for family history, revealed that only 2 out of the 66 (3%) cases had a *BAP1* mutation (Aoude *et al.*, 2013).

3.3.3 Low-penetrance genes

3.3.3.1 Melanocortin-1 receptor

MC1R is located on chromosome 16q24 and one of the key genes determining skin, hair and eye color. This single exon gene encodes for a seven-transmembrane G-protein coupled receptor, which is expressed on the surface of melanocytes and keratinocytes. The *MC1R* gene is highly polymorphic; many variants in this gene increase the susceptibility to CMM formation (Bloethner *et al.*, 2009; Lin *et al.*, 2009). There are few *MC1R* variants known as red hair color (RHC) variants (e.g., D84E, R142H, R151C, R160W and D294H) due to their association with red hair and fair skin phenotype. These variants can significantly increase susceptibility to melanoma development (Hayward, 2003). The other *MC1R* polymorphisms are designated as non-red hair color (NRHC) variants (e.g., V60L, V92M and R163Q) and have much weaker association with melanoma risk (Raimondi *et al.*, 2008).

Carriers of *MC1R* variants are more sensitive to the effect of UV radiation and have a poor tanning ability and as a results are at higher risk of developing melanoma. The risk of melanoma development increases with the increase in the number of *MC1R* variants (Demenais *et al.*, 2010). The risk of melanoma increases from 1.5 fold among individuals with one *MC1R* variant to 2-7 folds in those with multiple *MC1R* variants (Hill *et al.*, 2013). Besides, presence of an *MC1R* variant in *CDKN2A* mutation carriers significantly increases the penetrance of *CDKN2A* mutation and decreases the age of onset by 20 years as compared to individuals with a *CDKN2A* mutation alone (Box *et al.*, 2001).

Furthermore, few reports with controversial results have studied the association of *MC1R* variants and *BRAF* mutations in sporadic melanomas. Some researchers have found that patients with germline *MC1R* variants had a higher risk of developing *BRAF* mutated melanomas (Fargnoli *et al.*, 2008; Landi *et al.*, 2006); while others have found no association between germline *MC1R* variants and *BRAF* mutation (Hacker *et al.*, 2010; Thomas *et al.*, 2010).

3.3.3.2 Other low-penetrance genes

Genome-wide association studies (GWAS) have discovered many common loci that are associated with a low increase in melanoma risk. Example of chromosomal regions and genes associated with low-penetrance susceptibility include *TYR, TYRP1, ATM, MX2, MTAP/CDKN2A, OCA2/HERC2, SLC45A2, IRF4, PLA2G6, ASIP, TERT, PARP1, CASP8* and the *SETDB1* region (Hill *et al.*, 2013).

4 MELANOMA CLASSIFICATION

More than forty years ago, CMM was classified into different subtypes, which differ clinically, histopathologically and also have different genetic alterations. Based on anatomic location and the growth patterns of the melanoma cells, CMM is generally divided into several subtypes including superficial spreading melanoma (SSM), nodular melanoma (NM), acral lentiginous melanoma (ALM) and lentigo maligna melanoma (LMM) (Clark *et al.*, 1969). However, the impact of this classification on the prognosis and treatment of melanoma patients is very limited (Romano *et al.*, 2011). Mucosal and ocular melanomas represent non-cutaneous melanoma subtypes.

4.1 CUTANEOUS MALIGNANT MELANOMA

4.1.1 Superficial spreading melanoma

This is the most common subtype, accounting for about 60-70% of all primary melanomas diagnosed in the Caucasian populations (Greenwald *et al.*, 2012). SSM is characterized by lateral spreading of single or nests of melanoma cells within the epidermis, known as a pagetoid pattern (Smoller, 2006). This subtype of melanoma is most commonly located in areas with intermittent sun-exposure, such as the trunk in males and the extremities in females, and associates with presence of pre-existing nevi (Elwood *et al.*, 1987). Moreover, SSMs are also characterized by a high frequency of *BRAF* mutations (Lee *et al.*, 2011).

4.1.2 Nodular melanoma

NM is the most aggressive and the second most common subtype of CMM. NM accounts for about 15% of all melanomas, however, it accounts for a much larger proportion of melanoma with ≥2 mm thickness (Bergenmar *et al.*, 1998; Demierre *et al.*, 2005). Unlike in SSM, the malignant cells in NM are characterized by a rapid growth and direct invasion of the dermis forming a well-circumscribed vertical growth phase (VGP) which usually lacks the radial growth phase (RGP). The median thickness of NM has not changed significantly over time, whereas the thickness of SSM at diagnosis has decreased significantly (Greenwald *et al.*, 2012). Furthermore, NM is usually diagnosed in older patients as compared with SSM patients. Several studies have shown that the frequency of *BRAF* mutations is significantly lower in NM than in

SSM, while *NRAS* mutations are more frequently present in NM (Greenwald *et al.*, 2012; Lee *et al.*, 2011).

4.1.3 Lentigo maligna melanoma

LMM represents about 10% of all melanomas. This subtype of CMM is commonly diagnosed on chronically sun-damaged (CSD) skin, such as in the head and neck region, especially in elderly people, and is very rarely diagnosed in patients younger than 50 years (McGuire *et al.*, 2012). Histologically, LMM shows marked solar elastosis and predominant proliferations of single cells along the dermal-epidermal junctions, which then grow very slowly and invades the dermis (Reed and Shea, 2011).

4.1.4 Acral lentiginous melanoma

In 1976, ALM was reported as the fourth distinct histological subtype of melanoma (Reed and Martin, 1997). Although ALM is a rare subtype of CMM in the Caucasian populations, accounting for 3-13%, it represents the most frequent subtype of melanoma diagnosed in Asian, Hispanic and dark-skinned individuals (Stalkup *et al.*, 2002). It can arise anywhere on the body but predominantly occurs in distal relatively sun-protected parts of the body, particularly soles, palms and nailbeds (subungual areas), hence the name (Kuchelmeister *et al.*, 2000). The feet constitute the most commonly involved site. Approximately 60% of melanomas diagnosed in the acral parts of the body are histologically ALM (Stalkup *et al.*, 2002). The diagnosis of ALM depends upon the presence of a lentiginous pattern of intraepidermal growth of melanocytes.

Although trauma has been suggested to be a possible risk, the classical risk factors for CMM such as fair complexion, sun exposure and family history, seem to be of less importance in ALM development. Patients with ALM are typically old and have a poor prognosis compared to those with other subtypes; partly due to delayed diagnosis (O'Leary *et al.*, 2000). ALM is also characterized by high frequency of focal amplification of certain genes including cyclin D1 (*CCND1*), GRB2-associated binding protein 2 (*GAB2*) and *CDK4* (Chernoff *et al.*, 2009; Curtin *et al.*, 2005). Cyclin D1 amplification is an early genetic event in ALM development. *KIT* mutations and/or amplification are also commonly detected in ALM, whereas *BRAF* and *NRAS* mutations are relatively infrequent (Curtin *et al.*, 2006).

A recent study has shown that platelet-derived growth factor receptor alpha (*PDGFRA*), a RTK, is mutated in a small proportion (7%) of ALM (Dai *et al.*, 2013). Interestingly, these mutations were mutually exclusive with *KIT* but coexisted with *BRAF* and *NRAS* mutations. They also observed that almost all mutations detected in the *PDGFRA* were sensitive to tyrosine kinase inhibitors such as imatinib (Dai *et al.*, 2013).

4.2 NON-CUTANEOUS MELANOMA

Primary non-cutaneous melanoma represents a small percentage (approximately 5%) of all melanomas. This group of melanomas affects older people and the prognosis is worse compared with CMMs. Ocular and mucosal melanomas constitute the majority of non-cutaneous melanomas. Similar to the CMM, the frequency of non-cutaneous melanoma is significantly higher among whites than among blacks (McLaughlin *et al.*, 2005). However, the pathogenesis and risk factors predisposing to non-cutaneous melanoma are to a large extent not known. For example, UV radiation is not likely to be a causative agent.

4.2.1 Ocular melanoma

The majority of primary ocular melanomas arise from the uveal tract (choroid, ciliary body and iris) and very rarely from the conjunctiva. Ocular melanoma is the most common primary malignancy of the eye in adults, with an annual incidence of 2-6 new cases per million in Europe.

The MAPK pathway is commonly activated in ocular melanoma, although very rarely through *BRAF*, *NRAS* or *KIT* mutations (Beadling *et al.*, 2008; Zuidervaart *et al.*, 2005). More than 80% of ocular melanomas harbour activating somatic mutations that result in substitution of glutamine by leucine at position 209 (Q209L) in either *GNAQ* or the *GNA11* oncogenes, which can lead to constitutive activation of the MAPK pathway (Van Raamsdonk *et al.*, 2009; Van Raamsdonk *et al.*, 2010). Mutations in these oncogenes are mutually exclusive. Uveal melanoma also characterized by containing a high frequency of somatic *BAP1* mutations, that might coexist with *GNAQ* mutation (Harbour *et al.*, 2010). In addition, rare germline *BAP1* mutations predispose to uveal melanomas and also other malignancies (Abdel-Rahman *et al.*, 2011).

4.2.2 Mucosal melanoma

Primary mucosal malignant melanoma (MMM) is a distinct very rare subtype, representing less than 2% of all melanomas (Clifton *et al.*, 2011; McLaughlin *et al.*, 2005). MMM melanoma originates from malignant transformation of melanocytes located in any mucosal surface, but mostly arise in the mucosal membrane lining the nasal cavity, paranasal sinuses, oral cavity, vulva, vagina and anorectum (Patrick *et al.*, 2007). The diagnosis of MMMs usually occur at advanced stages and patients are older (median age at diagnosis of 70 years) and have a worse prognosis (5-year survival is 25%) as compared with CMMs (Patrick *et al.*, 2007). Unlike CMM, there are no known carcinogens implicated in the pathogenesis of mucosal melanoma. The environmental and genetic risk factors such as UV radiation, family history of melanoma and nevus count have not been associated with mucosal melanoma development. In addition, Breslow thickness and Clark level are not applicable as prognostic factors in MMM. The incidence of MMM is believed to be remained stable. However, in Sweden the incidence of MMM in the nasal cavity and sinuses has slightly increased from 1960 through 2000 (Jangard *et al.*, 2013).

More than half of the MMM tumors are located in the head and neck region (Clifton *et al.*, 2011). Head and neck MMMs are commonly located in the nasal cavity and paranasal sinuses, known as sinonasal mucosal melanoma (SNMM) (Patrick *et al.*, 2007). The vast majority of the SNMM tumors occur in the nasal cavity, and approximately 80% of patients present with a localized disease. The tumors in the paranasal sinuses are most common in maxillary and ethmoid sinuses (Gore and Zanation, 2012). Classification of SNMM is different from that of CMM; SNMM are usually staged according to the Ballantyne classification which is based on anatomical, clinical and computed tomography (CT) data. Stage I represents tumor confined to the site of origin, stage II; tumor with regional lymph node metastasis and stage III represents tumors with systemic metastasis. Recently, the seventh edition Union for International Cancer Control (UICC) established the TNM staging for melanoma in the upper aerodigestive tract, which is believed to be more effective in predicting patient outcome (Sobin and Compton, 2010). According to the 7th UICC staging, patient's tumors are classified into four stages (the first and second stages are omitted): stage III (T3); for melanomas localized to the mucosa, stage IVa (T4a); for tumors invading deeper tissues without lymph node involvement, and stage IVb (T3-T4a or T4b); for

tumors spread to lymph nodes, and IVc (any T, any N); for melanomas with distant metastasis.

In addition, MMM differs from CMM by harboring a distinct genetic background. Comparative genomic hybridization has shown that MMM have a significantly higher degree of chromosomal aberrations including copy-number changes and amplification of different genomic region, as compared with other subtype of CMMs (Curtin *et al.*, 2005). MMM is also characterized by high frequency of *KIT* amplifications and/or mutations and a low frequency of *BRAF* mutations (Beadling *et al.*, 2008; Curtin *et al.*, 2006). Recently, we have found that the *KIT* mutation frequency varies among different anatomical sites of mucosal melanoma, with significantly higher frequency detected in vulvar melanoma (Omholt *et al.*, 2011).

4.3 OTHER UNCOMMON MELANOMA SUBTYPES

Desmoplastic melanoma is one of the very rare subtypes of CMMs. This subtype of melanoma usually presents as histologically non-pigmented lesion and predominantly occurs in the head and neck area in elderly people (Wood, 2013). Other rare types of cutaneous melanoma include amelanotic melanoma, nevoid, verrucous and Spitzoid melanomas.

4.4 MOLECULAR CLASSIFICATION OF MELANOMA

As the traditional classification of melanoma has not been sufficiently precise in term of predicting patient outcome and also because of the advances in melanoma genetics and with the identification of high mutation frequencies in certain genes, there have been attempts to molecularly classify melanoma with the aim of having a more biologically relevant classification.

Accordingly, based on sun-exposure and anatomical site, melanoma can be subdivided into four groups which are characterized by distinct molecular alterations; chromosomal aberrations, *BRAF*, *NRAS* and *KIT* mutations (Bastian *et al.*, 2003; Curtin *et al.*, 2005). This classification includes melanomas on CSD skin, melanoma on non-CSD skin; acral melanoma and mucosal melanomas. Melanoma on the non-CSD skin possesses a very high frequency of *BRAF* and *NRAS* mutations and, conversely, melanoma on the CSD skin, acral and mucosal melanomas are characterized by a low

frequency of *BRAF* and *NRAS* mutations and a high frequency of *KIT* aberrations (mutation and/or copy number increase) and an increase in the copy number of *CDK4* and *CCND1* (Curtin *et al.*, 2006; Curtin *et al.*, 2005). In addition, acral and mucosal melanomas also have a significantly higher number of chromosomal aberrations, as compared with melanoma on the skin with and without CSD (Curtin *et al.*, 2005). Uveal melanoma might be added to this classification as it has a different genetic profile that characterized by a high frequency of *GNAQ* and *GNA11* mutations.

Others have classified melanoma into several subtypes according to the status of the key genes/pathways that are altered in melanomas. These subtypes are characterized by alterations in different pathways, through mutations in various oncogenes and tumor suppressor genes. These include *BRAF*, *BRAF/PTEN*, *BRAF/Akt*, *BRAF/CDK4*, *KIT*, *GNAQ*, *GNA11*, *NRAS*, *MITF*, *PTEN*, *Akt*, *PI3K*, *CDKN2A*, *CDK4*, *CCND1*, *Bcl*-2 and *p53* (Vidwans *et al.*, 2011). Basically, both diagnostic technologies and potential targeted therapies are also available for some of these molecular subtypes (Vidwans *et al.*, 2011).

5 MOLECULAR ALTERATIONS AND SIGNALING PATHWAYS IN MELANOMA

Next generation sequencing analyses have shown that melanoma contains a higher number of somatic mutations as compared with most other types of malignancies (Hill *et al.*, 2013). However, only a small fraction of these mutations are thought to be implicated in melanoma development. There are several molecular pathways that have a key role in the initiation and progression of melanoma. For instance, the RAS-RAF-MEK-ERK pathway, also known as the MAPK pathway, represents a critical early step in melanocyte proliferation, and it is implicated in about 90% of the common melanoma subtypes, particularly through activating mutations in the *BRAF, NRAS* and *KIT* proto-oncogenes (van den Hurk *et al.*, 2012). Other key signaling pathways involved in melanoma development include the phosphatidylinositol 3-kinase (PI3K)-Akt, $p16^{INK4A}$-Rb and the $p14^{ARF}$-p53 pathways.

5.1 MITOGEN-ACTIVATED PROTEIN KINASE PATHWAY

MAPK is a signal transduction pathway which becomes activated in response to growth factors, cytokines and hormones. This pathway is involved in the regulation of a variety of cellular processes such as survival, proliferation, senescence and differentiation (Fecher *et al.*, 2008). The MAPK cascades consist of multiple components; these include three RAS (HRAS, KRAS and NRAS), three RAF (ARAF, BRAF and CRAF), two MEK (MEK1 and MEK2) and two ERK (ERK1 and ERK2) proteins. A simplified description of the MAPK pathway is represented in Figure 1.

5.1.1 *RAS*

The first *RAS* gene was discovered in 1960s with the observation of the ability of murine viruses (Harvey and Kirsten viruses) to initiate sarcoma in other new born rodents; this oncogene was termed *RAS* (for *Ra*t *s*arcoma virus). These genes in the Harvey and Kirsten sarcoma viruses were closely related but not totally identical and were termed after Harvey (*HRAS*) and Kirsten (*KRAS*). Later on, the cellular homologues of the *HRAS* and *KRAS* genes were identified in the human genome. In the early 1980s, the third *RAS* family member was discovered in a neuroblastoma cell line and named *NRAS* (Karnoub and Weinberg, 2008).

The RAS superfamily consists of five distinct subfamilies; RAS, RHO/RAC, RAB, ARF, and RAN (Castellano and Santos, 2011). A multitude of proteins belong to the RAS subfamily. However, in human cancers three isoforms of the *RAS* gene are commonly mutated namely *HRAS*, *KRAS* and *NRAS*. The products of these three oncogenes are highly homologous, sharing about 80% of the amino acid sequence (Castellano and Santos, 2011).

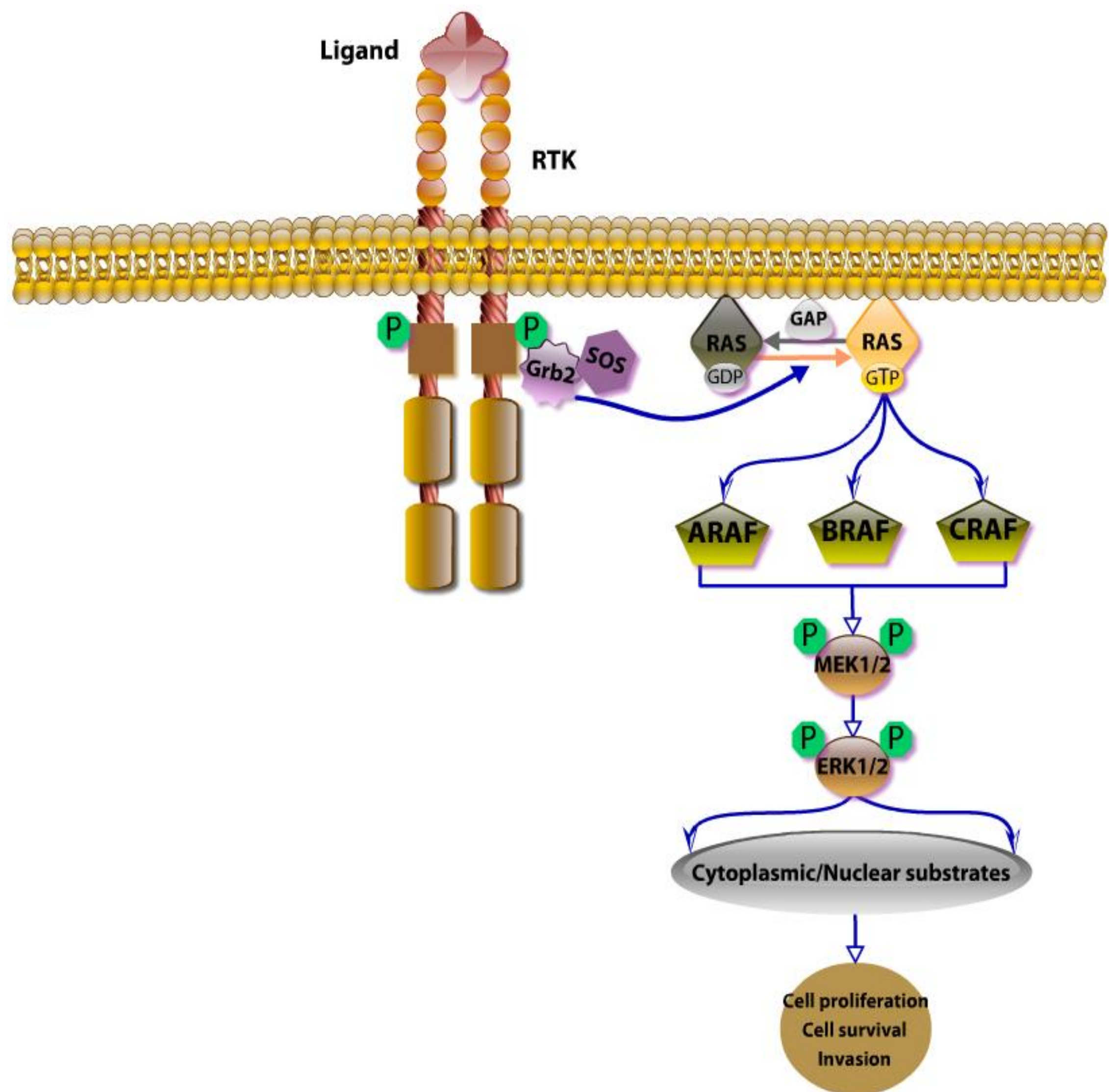

Figure 1. Schematic representation of the RAS-RAF-MEK-ERK (MAPK) pathway.

Binding of a ligand (e.g. growth factors) to the surface of RTK (e.g. KIT, PDGFR, EGFR, FGFR and c-MET) stimulates the tyrosine kinase activity and autophosphorylate the receptor, which then initiate intracellular signal transduction through different pathways. RAS proteins transduce mitogenic signals from the plasma membrane to the inside of the cells. Autophosphorylation of the cytoplasmic domain of the RTK is accompanied by recruitment of adaptor proteins and activation of RAS protein through switching their binding state from the guanosine diphosphate (GDP)- to the guanosine triphosphate (GTP)-binding (Figure 1). Transition of RAS proteins between the inactive form (RAS-GDP) and active form (RAS-GTP) is tightly controlled by a group of regulator proteins. For example, GTPase-activating proteins (GAPs) cause inactivation of RAS proteins by stimulating the intrinsic GTPase hydrolysis activity of RAS, whereas guanine nucleotide exchange factors (e.g. son of sevenless; SOS) and the adaptor protein growth factor receptor-bound protein 2 (Grb2) facilitate activation by switching of RAS-GDP to RAS-GTP form (Rajalingam *et al.*, 2007). In the GTP-bound state the RAS proteins stimulate a wide range of downstream effector proteins belonging to multiple signaling cascades, such as MAPK and the PI3K pathways.

Oncogenic mutations in the RAS isoforms are commonly detected in a wide variety of human malignancies. However , the types and frequencies of the RAS mutations differ among different types of tumors, and mutations are predominantly detected in exons 1 and 2 (Prior *et al.*, 2012). Missense mutations in the *HRAS* and *NRAS* oncogenes are commonly reported in exon 2 codon 61 and less frequently in exon 1 codon 12 (Prior *et al.*, 2012).In contrast, the majority of *KRAS* mutations are present in exon 1 codon 12 (Bello *et al.*, 2013). Mutations in exon 1 codon 13 are generally less frequent. Most of the mutations in the codon 61 are the result of substitution of glutamine by arginine, leucine or lysine ($NRAS^{Q61R}$, $NRAS^{Q61L}$ and $NRAS^{Q61K}$), whereas the common alterations in codon 12 include substitution of glycine by aspartic acid, valine or serine ($NRASG^{G12D}$, $NRAS^{G12V}$and $NRAS^{G12S}$) (Prior *et al.*, 2012). Substitution of these amino acids leads to inhibition or impairment of intrinsic RAS GTPase activity and thereby constitutive activation of RAS proteins independently of RTK stimulation. Activated RAS proteins will eventually stimulate cell proliferation and inhibit apoptosis by activating both the MAPK and PI3K pathways.

In melanomas the *RAS* mutations are predominantly found in the *NRAS* oncogene, whereas *HRAS* and *KRAS* are very rarely mutated (Ball *et al.*, 1994). *NRAS* consists of seven exons (4 coding exons) and is mapped on chromosome 1q13. In CMM the majority of the *NRAS* mutations occur in codon 61, however, this might not be the same in MMM where codons 12 and 13 are also frequently mutated (Omholt *et al.*, 2011). Activating *NRAS* mutations were first identified in melanoma cell lines in 1984 (Platz *et al.*, 2008). The frequency of *NRAS* mutations in primary CMM ranges from 17% to 29% and are also present in the RGP of melanoma (Edlundh-Rose *et al.*, 2006; Omholt *et al.*, 2002). *NRAS* mutations have also been reported at high frequency in nevi, especially in large congenital nevi (Bauer *et al.*, 2007). Expression of an activating *NRAS* mutation alone results in hyperproliferative melanocytes, and ultimately in development of senescence. Thus, it seems that *NRAS* mutations alone are insufficient for malignant transformation of melanocytes and additional genetic alterations are required for melanoma development. For instance, in combination with *p53* or *CDKN2A* loss-of-function mutations, *NRAS* can contribute to establishment of CMM (Ackermann *et al.*, 2005; Dovey *et al.*, 2009). *NRAS* mutations in melanoma are associated with high Breslow thickness, nodular subtype and older age at diagnosis (Ball *et al.*, 1994; Edlundh-Rose *et al.*, 2006; Ellerhorst *et al.*, 2011; Lee *et al.*, 2011).

5.1.2 *RAF*

A few decades ago the *RAF* gene was discovered in the murine sarcoma virus; 3611-MSV. It was observed that this virus increases the development of fibrosarcoma in newborn mice; hence the *RAF* abbreviation was derived for *r*apidly *a*ccelerated *f*ibrosarcoma. Shortly after the discovery of *v-RAF,* the human homologue was cloned and designated as *c-RAF*, also known as *RAF-1* (Wellbrock *et al.*, 2004). Soon thereafter, the second member of the *RAF* gene family was described and designated as *ARAF*. In 1988, the third isoform was discovered, termed *BRAF* (Ikawa *et al.*, 1988). The three *RAF* genes are located on separate chromosomes and encode three serine/threonine kinases (ARAF, BRAF and CRAF).

All three RAF isoforms share three conserved regions (CR1, CR2 and CR3), with a high degree of homology (Figure 2). The CR1 contains the RAS-binding and cysteine-rich domains, which are essential for interaction with RAS-GTP proteins. The CR2 is rich in serine and threonine residues, while the CR3 is the catalytic kinase and most

homologous domain of the RAF proteins (Wellbrock *et al.*, 2004). The RAF proteins also contain a non-conserved negative-charge regulatory phosphorylation site (N-region). Unlike ARAF and CRAF, BRAF become fully active by T599 and S602 phosphorylation and no additional phosphorylation of activating residues are required, whereas ARAF and CRAF require phosphorylation of a motif in the N-region besides the phosphorylation of two amino acids in the kinase domain for a maximal activation (Garnett and Marais, 2004; Maurer *et al.*, 2011). In addition, the basal kinase activity of the BRAF is higher, and BRAF is subjected to alternative splicing, producing different isoform of BRAF proteins ranging from 75 to 100 kDa. The wild-type BRAF protein is expressed in most of tissues with relatively higher level of expression in neuronal tissues and melanocytes (Wellbrock *et al.*, 2004).

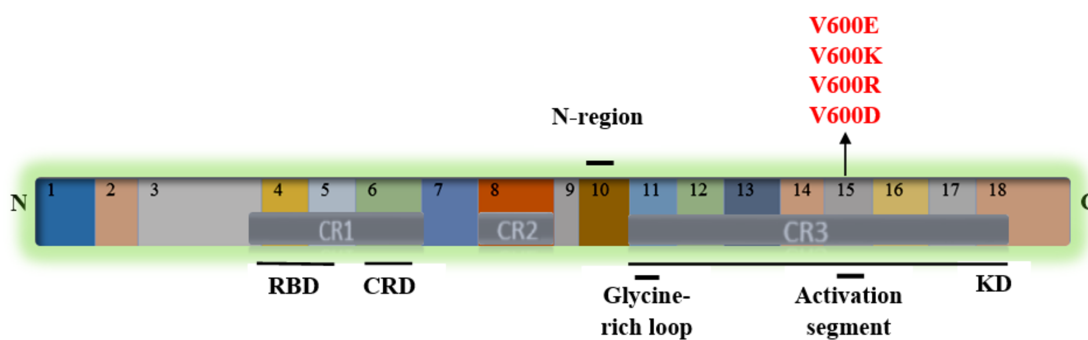

Figure 2. Structure of the BRAF protein. The conserved regions (CR1, CR2 and CR3) are shown inside the BRAF protein structure. The numbers represent the BRAF exons. The bars represent the BRAF protein domains; RBD, Ras binding domain; CRD, cysteine-rich domain and KD, kinase domain. The common $BRAF^{V600}$ mutations are shown in exon 15 which is located in the activation segment of the kinase domain.

Phosphorylation of the RTK results in activation of the RAS proteins (HRAS, KRAS and NRAS), which then activate the MAPK pathway through stimulation of the serine/threonine RAF protein kinases. RAF protein activation will, in turn result in activation and phosphorylation of MEK; MAP kinase extracellular signal regulated kinase (the only known substrate for BRAF protein), with BRAF having the strongest activity for MEK activation. Finally MEK phosphorylates and activates its only known downstream effector ERK; extracellular-signal regulated kinase (Garnett and Marais,

2004). ERK regulates a very large number of targets in the nucleus, as well as in the cytoplasm. In the nucleus ERK affect gene expression by stimulating several transcription factors and eventually results in stimulation of cellular proliferation, differentiation, and survival (Fecher *et al.*, 2008).

Dysregulation of the MAPK pathway is a very frequent event in melanoma. Mutations in the *ARAF* and *CRAF* isoforms are significantly less common than in the *BRAF* isoform. *BRAF* is a proto-oncogene located on chromosome 7q34 and comprises 18 exons. In 2002, the importance of *BRAF* mutations in human cancers was discovered (Davies *et al.*, 2002). *BRAF* is commonly mutated in a variety of cancers, with an overall frequency of about 7% (Davies *et al.*, 2002; Fecher *et al.*, 2008). A subset of tumors including CMM, papillary thyroid cancer, colorectal cancer and ovarian cancer harbor a high frequency of *BRAF* mutations. In melanoma, *BRAF* is the most commonly mutated oncogene so far identified. The most common *BRAF* mutations are located in exon 15, in the activating segment of the kinase domain. Substitution of valine by glutamic acid at codon 600, a single nucleotide mutation (c.1799T>A; $BRAF^{V600E}$), accounts for approximately 70-80% of the *BRAF* mutations. $BRAF^{V600E}$ possesses a high basal kinase activity that induces transformation of NIH3T3 cells (Davies *et al.*, 2002; Fecher *et al.*, 2008). Substitution of valine by lysine (c.1798_1799GT>AA; $BRAF^{V600K}$) accounts for nearly 20% of the *BRAF* mutations (Bucheit *et al.*, 2013; Menzies *et al.*, 2012). Other relatively common *BRAF* changes include substitution of valine by arginine (c.1798_1799GT>AG; $BRAF^{V600R}$) or aspartic acid (c.1799_1800TG>AT; $BRAF^{V600D}$) (Menzies and Long, 2013; Rubinstein *et al.*, 2010). Outside exon 15, mutations in the glycine-rich loop in exon 11 have also been described, albeit less frequently. In melanoma cells, presence of an activating *BRAF* mutation constitutively stimulates signaling through pERK which is necessary for their proliferation, and depletion of *BRAF* mutation significantly induces marker of apoptosis, indicating that BRAF is essential for growth and survival of melanoma cells (Karasarides *et al.*, 2004).

The reported frequencies of *BRAF* mutations vary among different studies, but overall, nearly 50% of CMMs harbor *BRAF* mutations (Davies *et al.*, 2002; Edlundh-Rose *et al.*, 2006; Lee *et al.*, 2011; Omholt *et al.*, 2003; Shinozaki *et al.*, 2004). *BRAF* mutations are most frequently found in thin melanomas and younger patients and are

also associated significantly with melanomas arising on intermittently sun-exposed body sites, and with the SSM subtype (Devitt *et al.*, 2011; Edlundh-Rose *et al.*, 2006; Ellerhorst *et al.*, 2011; Liu *et al.*, 2007; Long *et al.*, 2011). However, interestingly, the *BRAF*^{V600K} mutation has been associated with older age and head/neck tumor location and LMM subtype, as compared with *BRAF*^{V600E} (Bucheit *et al.*, 2013; Menzies *et al.*, 2012; Stadelmeyer *et al.*, 2013). In CMMs, several studies have shown no significant association between *BRAF* mutations and clinical outcome (Devitt *et al.*, 2011; Edlundh-Rose *et al.*, 2006; Ellerhorst *et al.*, 2011; Shinozaki *et al.*, 2004), while other have demonstrated an association with worse survival (Mann *et al.*, 2013).

Importantly, *BRAF* mutations are also very frequently detected in benign and dysplastic nevi (Kumar *et al.*, 2004; Omholt *et al.*, 2003; Pollock *et al.*, 2003). The presence of *BRAF* mutations in nevi, and in the RGP of melanoma indicates that *BRAF* activation represents an early somatic event in melanoma development (Omholt *et al.*, 2003; Pollock *et al.*, 2003). As with *NRAS*, there are data showing that presence of *BRAF*^{V600E} alone is not sufficient for malignant transformation of melanocytes and that additional genetic alterations such as inactivation of *P16*^{INK4A}, *p53* or *PTEN* might be required for the establishment of melanoma (Ko *et al.*, 2010).

5.2 PHOSPHATIDYLINOSITOL 3-KINASE-AKT PATHWAY

The PI3K-Akt pathway is also commonly activated in melanomas, affecting multiple cellular processes such as cell growth and survival. A simple diagrammatic scheme is represented in Figure 3.

5.2.1 *KIT*

The *KIT* gene (also known as *c-KIT* or *CD117*), discovered in 1987, is the cellular homologue of the viral oncogene (*v-KIT*) which was derived from Hardy-Zuckerman-4 feline sarcoma virus (Yarden *et al.*, 1987). The *KIT* proto-oncogene is located on chromosome 4q12 and consists of 21 exons. *KIT* encodes for a transmembrane RTK, which is expressed on the surface of different cell types including melanocytes. The structure of the KIT receptor protein consists of an extracellular ligand binding domain (encoded by exons 1-9), a transmembrane region (encoded by exon 10) and intracellular domains (Figure 3). The intracellular domains comprised a juxtamembrane domain (encoded by exon 11) and two tyrosine kinase domains (encoded by exons 12-21). Binding of ligand, the stem cell factor (SCF, also known as kit ligand, steel factor or mast cell growth factor), to the extracellular domain will induce dimerization and autophosphorylation of the intracellular tyrosine kinase domains. The intracellular juxtamembrane domain has a negative regulator function that inhibits activation of the KIT receptor in the absence of ligand binding.

Activation of the KIT receptor will trigger stimulation of many downstream effectors including the MAPK and PI3K-Akt pathways (Fecher *et al.*, 2008). Signaling through the KIT receptor and subsequent phosphorylation of Akt is essential for melanocyte development, proliferation, migration and survival and is likely to be important in melanoma tumorigenesis (Woodman and Davies, 2010). Earlier studies have shown that KIT is expressed in normal melanocytes, benign and dysplastic nevi, whereas the expression is lost in melanoma cells (Hocker *et al.*, 2008). KIT expression is also observed in melanoma *in situ* and junctional component of invasive lesions, however, the expression is absent in their invasive component. This pattern of expression indicates loss of KIT expression during melanoma progression (Willmore-Payne *et al.*, 2005). Approximately 40% of melanomas display positive KIT expression by immunohistochemistry (IHC) (Kong *et al.*, 2011; Omholt *et al.*, 2011). A positive correlation has been described between immunohistochemical expression of KIT

protein and *KIT* mutational status in melanoma tumors (Omholt *et al.*, 2011; Torres-Cabala *et al.*, 2009).

Initially, it was believed that *KIT* mutations are very rare (2%) in melanoma (Willmore-Payne *et al.*, 2005). Subsequently it was found that the low frequency of *KIT* mutation in melanomas was the result of analyses of unselected cases. Curtin and colleagues later on showed that activating *KIT* mutations and/or copy number increases are frequently detected in mucosal melanoma (39%), acral melanoma (36%) and melanoma on CSD skin (28%) and are mutually exclusive with *BRAF* and *NRAS* mutations (Curtin *et al.*, 2006).

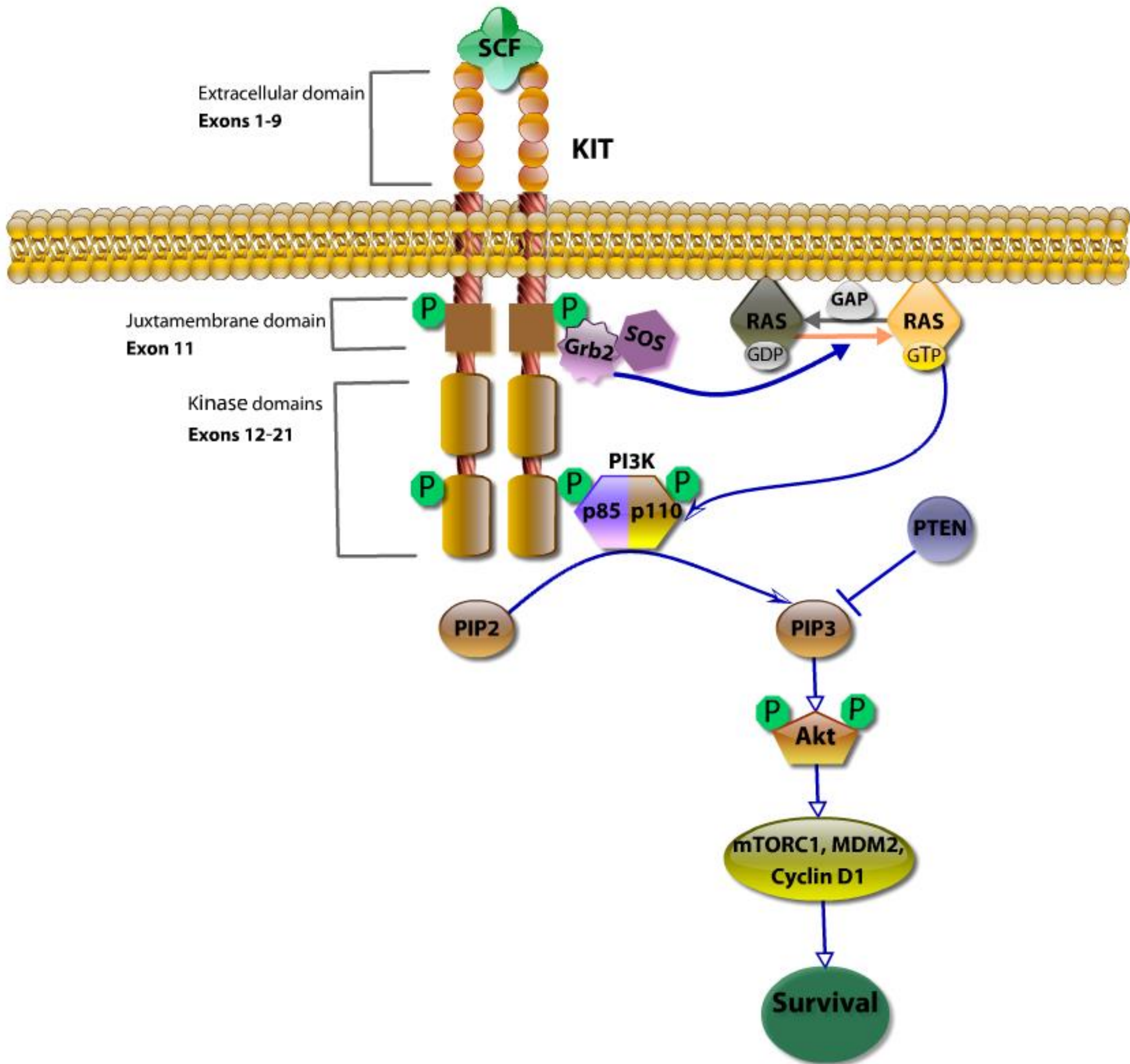

Figure 3. The structure of KIT receptor and a schematic representation of the PI3K-Akt pathway.

However, the frequency of *KIT* point mutations alone (excluding copy number increase) seem to be less frequent in these melanoma subtypes (Handolias *et al.*, 2010). Approximately, 15% of ALMs harbor activating *KIT* mutations (Beadling *et al.*, 2008; Schoenewolf *et al.*, 2012). In contrast, such aberrations are absent or extremely rare in choroidal and conjunctival melanomas and melanoma on skin without CSD (Beadling *et al.*, 2008; Curtin *et al.*, 2006).

In MMM, the frequency of *KIT* mutations appears to differ significantly according to anatomical sites (Omholt *et al.*, 2011; Woodman and Davies, 2010). In vulvar mucosal melanoma the prevalence of *KIT* mutations is significantly higher (35%) compared with SNMM (4%) (Omholt *et al.*, 2011; Schoenewolf *et al.*, 2012). In melanoma, *KIT* alterations are most commonly detected in exon 11 and rarely in exons 9, 13, 17 and 18, and the majority of these alterations are point mutations (Woodman and Davies, 2010). Substitution of leucine by proline in codon 576 (KIT^{L576P}) in exon 11, is considered a hotspot *KIT* mutation in melanoma (Garrido and Bastian, 2010). Other prevalent *KIT* mutations in melanoma are KIT^{K642E} in exon 13 and KIT^{D816H} in exon 18. Inconsistent data have been published regarding the association of *KIT* alterations with overall survival. We have previously found no significant correlation between *KIT* mutation status and the overall survival in patients with MMM, whereas others have observed that overall survival in patients with *KIT* mutations is significantly shorter that in those without *KIT* mutated melanoma (Jin *et al.*, 2013; Kong *et al.*, 2011; Omholt *et al.*, 2011).

5.2.2 *PI3K*

The PI3K protein has a heterodimer structure comprised a catalytic (p110α) and regulatory (p85) units and also contain RBD domain that interact with RAS-GTP protein. The catalytic unit of the PI3K phosphorylates and changes phosphatidylinositol 4, 5-bisphosphate (PIP_2) to phosphatidylinositol 3, 4, 5-trisphosphate (PIP_3). PIP3 recruit phosphatidylinositol-dependent kinase 1/2 (PDK1/2) to the plasma membrane, which in turn enables phosphorylation and activation of Akt (Davies, 2012). Akt is a serine/threonine kinase (also known as protein kinase B) and consist of three highly homologous isoforms (Akt1, Akt2 and Akt3), which require phosphorylation of a threonine (Thr308) and a serine (Ser473) for its maximal activation. Akt has a wide range of substrates (e.g. mTORC1, BAD, p21, p27, cyclin D1 and MDM2) and thus

affects many important cellular processes such as proliferation and survival (Davies, 2012). Activation of the PI3K-Akt pathway in human cancer usually occurs through amplifications and/or mutations of RTKs (e.g. *KIT* and *ERBB4*), activation of upstream effectors (e.g. NRAS), inactivation of negative regulators (e.g. PTEN) or alterations in the *Akt* and *PIK3CA* genes (Davies, 2012).

All of the three Akt isoforms are expressed in melanoma, whereas only genetic alterations in the Akt1 and Akt3 isoforms have been reported in melanoma and are very infrequent (1-2%) (Kwong and Davies, 2013). The reported frequencies of pAkt expression in primary CMMs range between 46% and 86% (Dai *et al.*, 2005; Jovanovic *et al.*, 2008; Omholt *et al.*, 2006). Moreover, it has been shown that the level of pAkt expression is correlated with poor prognosis in melanoma patients (Dai *et al.*, 2005). Mutations in the *PIK3CA* gene, which encodes the catalytic unit p110α and enable PI3K to constitutively activate Akt, are frequently detected in many solid tumors, whereas such mutations are very infrequent (2-6%) in melanomas (Kwong and Davies, 2013; Omholt *et al.*, 2006).

5.2.3 *PTEN*

*P*hosphatase and *ten*sin homolog (*PTEN*) is a tumor suppressor gene located on chromosome 10q23. The PTEN protein has a lipid phosphatase activity and also acts as a dual protein phosphatase; dephosphorylating phosphorylated-tyrosine and phosphorylated-serine/threonine residues on proteins. With the lipid phosphatase activity, PTEN negatively regulates the PI3K-Akt pathway by dephosphorylating PIP_3. Thus, loss of *PTEN* will increase the PIP_3 level, which subsequently leads to activation of Akt signaling. In melanoma, loss of *PTEN* function is a common mechanism of Akt activation and often occurs through mutations, deletions and promoter methylation (Conde-Perez and Larue, 2012). The frequency of *PTEN* alterations in primary and metastatic melanomas is about 7% and 15%, respectively, while up to 30% of melanoma cell lines harbor *PTEN* aberrations (Aguissa-Touré and Li, 2012). Deletion and mutations in *PTEN* are often found together with *BRAF* mutations, but very rarely occur with *NRAS* mutations (Kwong and Davies, 2013). Recently, loss of PTEN was suggested to have a role in the abrogation of $BRAF^{V600E}$ induced senescence in melanocytes, and reduced expression of PTEN by IHC was seen in the progression from nevi to primary tumors in *BRAF* mutated melanomas (Vredeveld *et al.*, 2012).

5.3 CYCLIN-DEPENDENT KINASE INHIBITOR 2A-Rb-P53 PATHWAY

The *CDKN2A* locus on chromosome 9p21 comprises four exons (1α, 1β, 2 and 3) and encodes for two different proteins (through alternative splicing of the first and second exons) that have a tumor suppressing function. The product of exons 1α, 2 and 3 splicing is known as $p16^{INK4A}$, whereas splicing of exons 1β and 2 produce $p14^{ARF}$ (Hansson, 2008). A simple schematic illustration is represented in Figure 4.

The $P16^{INK4A}$ protein is a CDK inhibitor; that binds to CDK4/6 and inhibits formation of CDK4/6-cyclin D complexes. Inhibition of these complexes will maintain the Rb tumor suppressor protein in its active hypophosphorylated state and thereby preventing release of the E2F transcription factors, and eventually preventing cell cycle progression through G1-to-S phase. Interestingly, a progressive loss of expression of $p16^{INK4A}$ protein has been shown from benign nevi to metastatic melanoma, indicating that $p16^{INK4A}$ is involved in oncogene-induced senescence in nevi and that loss of $p16^{INK4A}$ is important for malignant transformation and establishment of melanoma (Sanki *et al.*, 2007).

On the other hand, $p14^{ARF}$ is involved in the p53 pathway regulation through binding to the human double minute 2 (HMD2) protein and thus promoting stabilization and inhibiting degradation of p53. The transcription factor p53 function as a guardian of the genome stability. P53 is capable of activating and repressing promoters of many genes, as well as interacting with many proteins resulting in cell-cycle arrest and apoptosis. In general, about half of all human malignancies harbor p53 alterations (Hussein *et al.*, 2003). However, a relatively less proportion (approximately 20%) of melanomas harbor p53 mutation (Hodis *et al.*, 2012)

Mutations in exon 1α exclusively affect $p16^{INK4A}$; mutations in exon 1β affect only $p14^{ARF}$, while mutations in exon 2 may impinge on both proteins. Germline *CDKN2A* mutations in melanoma are most frequently observed in exon 1α and exon 2, whereas mutations affecting exon 1β alone are relatively rare (Goldstein *et al.*, 2006; Orlow *et al.*, 2007). As a result, genetic lesions of the *CDKN2A* locus can affect both the Rb and p53 pathways. *CDKN2A* also likely play an important role in preventing malignant transformation of nevi by maintaining nevi in a senescence state (Hocker *et al.*, 2008).

In melanoma, somatic inactivations of the *CDKN2A* gene are frequent and usually occur through deletion, point mutation, insertion, and duplication, as well as via promoter methylation (Sheppard and McArthur, 2013). In addition, approximately 20% to 40% of melanoma prone-families harbor germline *CDKN2A* mutations (Hansson, 2008; Hill *et al.*, 2013). In population-based studies the prevalence of *CDKN2A* mutations is much lower, ranging from 0.2 to 3% (Hill *et al.*, 2013).

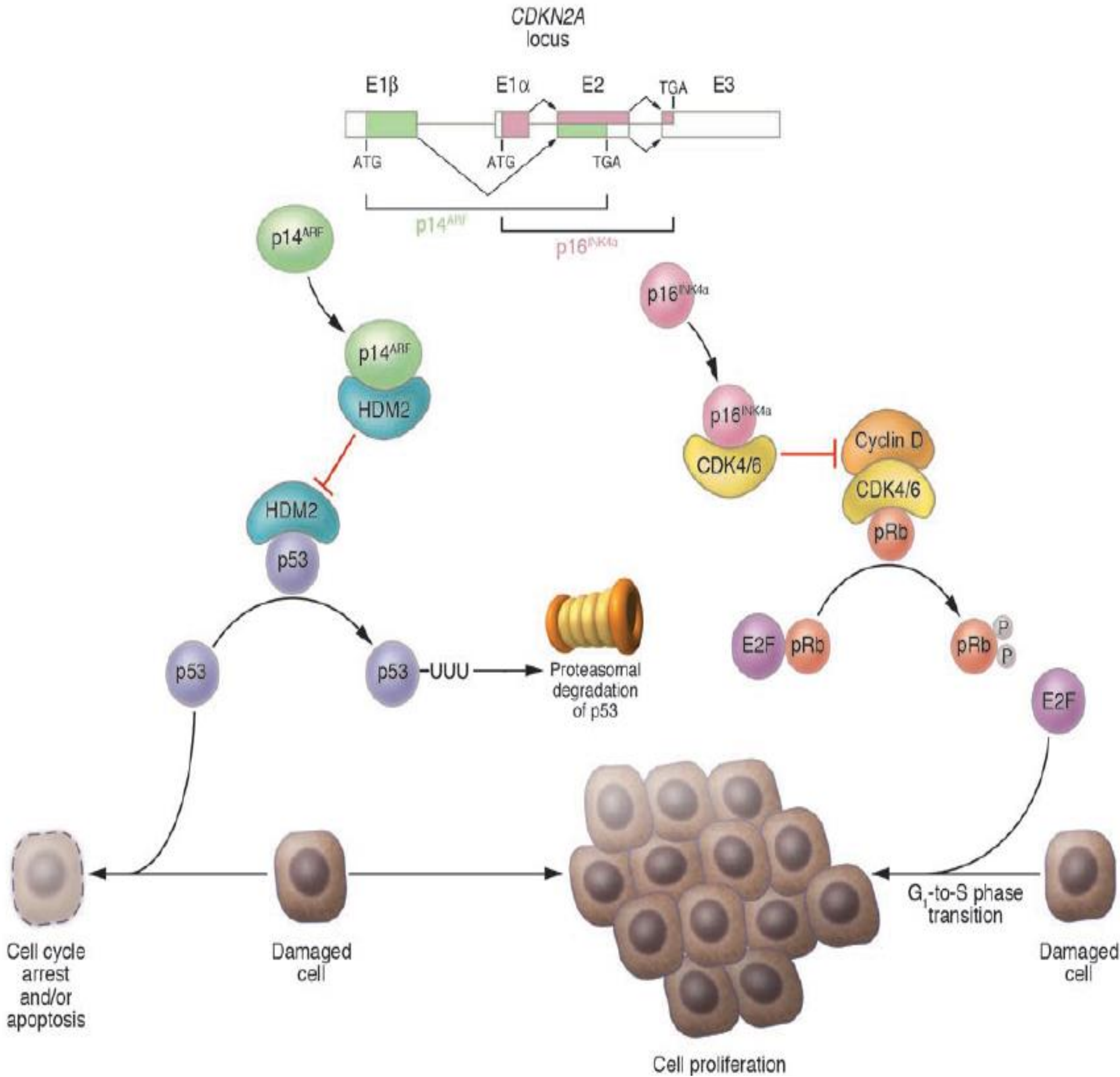

Figure 4. Schematic of *CDKN2A* locus and p16^{ink4A}-Rb and p14^{ARF}-p53 pathways. Adopted from (Chudnovsky *et al.*, 2005).

The likelihood of detecting germline *CDKN2A* mutations significantly increases with the number of melanoma cases in a family, early age at diagnosis of CMM and occurrence of multiple primary CMMs in the same individual, and also in the presence of family member with pancreatic cancer (Bishop *et al.*, 2000; Goldstein *et al.*, 2007). However, the frequency of *CDKN2A* mutation varies by geographic region. In a comparative study, conducted by GenoMEL consortium (www.genomel.org), analysis of a large number of families with three or more CMM cases showed that the frequency of germline *CDKN2A* mutations varies significantly between different populations, ranging from 20% (32 of 162) in Australia, 45% (29/65) in North America, to 57% (89 of 157) in Europe (Goldstein *et al.*, 2007).

In another GenoMEL study, analysis of families with three or more patients with melanoma revealed significant differences in the frequencies and distributions of *CDKN2A* mutation types across different countries (Goldstein *et al.*, 2006). For instance, in Sweden a single founder *CDKN2A* mutation, p.R112_L113insR mutation (also known as p.112dupArg) accounts for 92% of known familial mutations and in the Netherlands c.225-243del19, also called the p16-Leiden mutation, accounts for 90% of familial mutations. Italy, Spain and France share the same most common mutation (p.G101W), while p.M53I, c.IVS2-105A>G, p.R24P, and p.L32P are the most frequent *CDKN2A* mutations in Australia and the UK (Goldstein *et al.*, 2006). The penetrance of *CDKN2A* mutations with respect to CMM development also varies among different countries (Bishop *et al.*, 2002).

6 SYSTEMIC MELANOMA THERAPY

The majority of melanomas are locally confined at diagnosis, about 10% spread regionally and approximately 5% show signs of distant metastasis. Histopathological classification of melanoma according to TNM/anatomic stage grouping, based on American Joint Committee on Cancer (AJCC) staging, is essential for the selection of the appropriate treatment option and also to predict patient survival. The 5-year survival is more than 90% and 50% for patients with AJCC stage I and II, respectively (Balch *et al.*, 2009). However, the 5-year survival rate decreases dramatically for those with lymph node (stage III; 40%) and distance metastases (stage IV; 15%). While wide surgical resection might cure patients with localized primary melanomas, the standard systemic therapies provide a median survival of less than one year for patients with metastatic melanomas. Therefore, early diagnosis is critical for the cure and survival of patients.

6.1 CHEMOTHERAPY

Until very recently, patients who develop disseminated melanoma have had a limited and insufficiently effective systemic therapy options (cytotoxic chemotherapy, immunotherapy or a combination) and as a result had a very poor prognosis. For decades dacarbazine (known also as DTIC), the only US Food and Drug Administration (FDA) approved chemotherapeutic agent, remained the standard of care for patients with stage IV melanomas. Dacarbazine was approved in 1975, it has a low (average of 15%) and short response rate, with a median survival of 8 to 10 months (Lui *et al.*, 2007). The other drugs approved by the FDA for treatment of metastatic melanomas are high dose interleukin-2, approved in 1998, and hydroxyurea with response rates comparable to that of decarbazine (Bhatia *et al.*, 2009). Other chemotherapeutic agents that have a similar survival and response rate and are used in treatment of metastatic melanomas include temozolomide, fotemustine, paclitaxel, cisplatin, and carboplatin (Bhatia *et al.*, 2009; Guida *et al.*, 2012).

Combination chemotherapy has not shown significant survival or high response rate benefit compared with monchemotherapy in patients with metastatic melanomas (Bhatia *et al.*, 2009; Lui *et al.*, 2007). Trials using biochemotherapy (combination of interleukin-2 or interferon-alpha with cytotoxic chemotherapy) have shown a slight

improvement in response rate, but not in median overall survival, as compared with chemotherapy regimens alone (Bhatia *et al.*, 2009). In addition to low and short response rates, the standard chemotherapy is associated with severe toxicity including myelosuppression and liver toxicity, and a higher toxicity is associated with biochemotherapy and combination therapy (Bhatia *et al.*, 2009). Interferon alpha and radiotherapy can be used in the adjuvant setting following, surgical resection of a high-risk melanoma.

6.2 TARGETED THERAPY

6.2.1 Targeting RAS

Even before the discovery of high frequency of *BRAF* mutations, there were continuous attempts to develop molecules to inhibit signaling through the MAPK pathway in melanoma. Designing small molecules to specifically inhibit mutated NRAS protein, which is the second (after *BRAF*) most commonly mutated oncogene in melanoma, has not been successful. The first generation of inhibitors were designed to target RAS, either targeting RAS interaction with other adaptor proteins or inhibiting posttranslational modification (farnesylation) by for example farnesyltransferase inhibitors (such as tipifarnib and lonafarnib). It was soon realized that targeting RAS is not specific and sufficient inhibition is not achievable. Finally, there are data showing that targeting downstream effector of NRAS might be effective in patients with *NRAS* mutated melanomas, such as targeting MAPK and PI3K pathways simultaneously. Recently, a phase II clinical trial showed that a subset of patients with *NRAS* mutated melanoma might benefit from a MEK inhibitor (Ascierto *et al.*, 2013).

6.2.2 Targeting RAF

Among the targeted agents that were available prior to the identification of *BRAF* mutations and development of selective BRAF inhibitors was Sorafenib (Nexavar®). Sorafenib is a multi-kinase inhibitor targeting CRAF, mutated and wild-type BRAF, as well as RTKs such as vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR) and KIT (Nikolaou *et al.*, 2012). Several studies showed that sorafenib as a single agent or in combination with conventional chemotherapy had no significant effect in treatment of melanoma patients and also the response was not associated with the *BRAF* mutation status (Dhomen and Marais,

2009). The FDA eventually approved sorafenib for treatment of advanced renal cell and unresectable hepatocellular carcinoma.

The identification of high frequency *BRAF*^{V600E} mutations in melanoma in 2002 was the starting point for development of selective novel targeted therapeutic agents. In less than 10 years from the identification of *BRAF* mutations in melanoma, the results of phase III clinical trial of a selective *BRAF*^{V600E} inhibitor, named vemurafenib, were published (Chapman *et al.*, 2011). In this trial the researchers were able to show the superior effect of vemurafenib in patients with *BRAF* mutated melanomas compared with the standard dacarbazine treatment, with a response rate of 48% for vemurafenib and 5% for dacarbazine (Chapman *et al.*, 2011). These findings resulted in approval of vemurafenib in August 2011, by FDA in the US and later on by European Medicines Agency (EMA) in Europe, and other countries for treatment of patients with unresectable or metastatic melanoma with the *BRAF*^{V600E} mutation. Later on, it was shown that vemurafenib is also effective in melanomas with *BRAF*^{V600K} mutations (Sosman *et al.*, 2012). It is noteworthy that vemurafenib can enhance tumor progression through paradoxical activation and phosphorylation of ERK, if used in treatment of patients with *BRAF* wild-type or *NRAS* mutated melanoma (Bello *et al.*, 2013). This effect is believed to be driven formation of CRAF homodimeric or CRAF and wild-type BRAF heterodimeric complexes. Vemurafenib (Zelboraf®) is administered orally, 960 mg twice daily.

Despite the rapid and high clinical response rate (improvement of both overall and progression-free survival) with vemurafenib, eventually, unfortunately, in most patients the disease will progress and become resistant to treatment within the first year. The progression is mainly the result of acquired resistance and rarely (15%) due to primary resistance (Chapman *et al.*, 2011; Sosman *et al.*, 2012). So far, several different mechanisms of acquired resistance that usually results in reactivation of the MAPK pathway have been described. These include, *BRAF* amplification and expression of truncated BRAF through alternative splicing, CRAF upregulation, activating secondary *NRAS* and *MEK* mutations, COT activation and overexpression of RTKs such as PDGFRβ (Johnson and Sosman, 2013). In addition to the problem with resistance, vemurafenib has a range of adverse effects including arthralgia, rashes, fatigue and photosensitivity (Chapman *et al.*, 2011). The most intriguing adverse effect is the rather

rapid appearance of secondary skin tumors in approximately 20% of the cases, in form of squamous cell carcinoma or keratoacanthoma. Furthermore, some patients might even develop a second primary melanoma (Chapman *et al.*, 2011). Analysis of these tumors in patients treated with vemurafenib revealed a high frequency of *RAS* mutations, mostly found in *HRAS* (Su *et al.*, 2012).

The second potent selective BRAF inhibitor is dabrafenib (Tafinlar®), approved by FDA in May 2013 and later on by EMA, for the treatment of patients with unresectable or metastatic melanoma with $BRAF^{V600E}$ mutation. Dabrafenib is also administered orally, 150 mg twice daily. Dabrafenib, similar to vemurafenib, is associated with a high response rate, more than 50%, and significantly improves the median progression-free survival in patients with $BRAF^{V600E}$ mutated melanoma (5.1 months) compared with those receiving dacarbazine (2.7 months) (Hauschild *et al.*, 2012). Response to dabrafenib is also reported in patients with $BRAF^{V600K}$ or $BRAF^{V600R}$ mutated melanomas and in those with brain metastases, as well as in patients with non-melanoma tumors with $BRAF^{V600E}$ mutations such as papillary thyroid cancer (Menzies and Long, 2013). Except for photosensitivity, the adverse effects associated with vemurafenib are also seen in patients treated with dabrafenib, albeit with different incidence. The incidence of squamous cell carcinoma and keratoacanthoma are lower, whereas skin hyperkeratosis is higher in patients receiving dabrafenib (Hauschild *et al.*, 2012).

6.2.3 Targeting MEK

Targeting MEK is a very active area of research with many ongoing clinical trials testing different inhibitors. Trametinib (Mekinist®) is a selective MEK1 and MEK2 inhibitor, approved by the FDA simultaneously with dabrafenib, for the treatment of patients with unresectable or metastatic melanoma with $BRAF^{V600E}$ or $BRAF^{V600K}$ mutation. In a phase III open-label trial, trametinib demonstrated a median progression-free survival improvement (4.8 months) among patients who had $BRAF^{V600E}$ or $BRAF^{V600K}$ metastatic melanoma, as compared with patients received chemotherapy (dacarbazine or paclitaxel; 1.5 months) (Flaherty *et al.*, 2012b). With the aim of improving the response rates and decreasing development of resistance, the combination of trametinib and dabrafenib has been investigated. The phase II study of this combination showed significant improvement in both response rate and

progression-free survival, as compared with dabrafenib monotherapy (Flaherty *et al.*, 2012a). The other advantage of using MEK inhibitor, beside the efficacy in combination, is the activity of MEK inhibitor in *BRAF* wild-type and *NRAS* mutated melanomas.

6.2.4 Targeting KIT

Imatinib (Gleevec® or Glivec®) was first approved by the FDA in 2001 for the treatment of chronic myelogenous leukemia (CML) and later on for a number of other malignancies including gastrointestinal stromal tumors. Imatinib is a small-molecule that targets RTKs including KIT. The early trials of imatinib failed to show any clinical improvement in patients with metastatic melanomas, unselected for *KIT* mutations (Wyman *et al.*, 2006). After successful treatment of *KIT* mutated gastrointestinal stromal tumors and identification of *KIT* mutations in a subset of melanomas, several clinical trials with imatinib have been initiated in melanoma. Recently, the results of several phase II studies of imatinib in metastatic mucosal, acral or melanoma on CSD skin with a *KIT* mutation and/or amplification were published, with a response rate ranging between 16% to 25% (Carvajal *et al.*, 2011; Guo *et al.*, 2011; Hodi *et al.*, 2013). Promising results have also been observed with other small-molecule inhibitors of the KIT receptor such as sunitinib (Minor *et al.*, 2012). The response is believed to be better in melanoma with mutations in the juxtamembrane and proximal kinase domains of the KIT receptor (Carvajal, 2013).

6.2.5 Targeted immunotherapy

The other major advance in treatment of metastatic melanoma is the development of a human monoclonal antibody against cytotoxic T-lymphocyte antigen 4 (CTLA4), called ipilimumab. CTLA4 is a transmembrane protein expressed by T-lymphocytes and monocytes that negatively regulates activation of T-cells. Unlike targeted small molecules, ipilimumab can induce long-term responses, but only in a limited subset of patients. In 2011, ipilimumab (Yervoy®) was approved for treatment of patients with metastatic melanoma. Ipilimumab is administered intravenously, 3 mg/kg. Ipilimumab has shown a significant overall survival improvement in patients with unresected metastatic melanoma received ipilimumab plus dacarbazine or the gp100 peptide vaccine, as compared with those received dacarbazine plus placebo or the gp100 alone

(Hodi *et al.*, 2010; Robert *et al.*, 2011). The adverse effects are mainly associated with the immune system and can be very severe and even fatal.

Other promising immune targeted drugs that prevent inactivation of T-cells are Anti-PD-1 (e.g. nivolumab and lambrolizumab) and anti-PD-L1 antibodies. These monoclonal antibodies result in inhibition of the interaction between the inhibitory receptor PD-1 on the surface of tumor infiltrating T-lymphocytes and its ligand PD-L1, expressed selectively on tumor cells, and thus promoting antitumor response of T-cells (Menzies and Long, 2013). Phase I trials of nivolumab and lambrolizumab have shown a high overall response rate with a mild adverse effect profile (Menzies and Long, 2013). A phase I trial of combined ipilimumab and nivolumab treatment in patients with advanced melanoma has demonstrated a higher objective response rate than treatment with ipilimumab or nivolumab alone (Wolchok *et al.*, 2013).

7 AIMS OF THIS THESIS

The general aim was to better characterize the frequency of the most commonly mutated oncogenes (*BRAF*, *NRAS* and *KIT*) in the less common subtypes of melanoma such as familial, acral and mucosal melanomas. In addition, the aim was to assess the impact of the mutation status of these oncogenes on various clinical and histopathological features.

- **STUDY I**

  The purpose of this study was to evaluate the pattern of somatic *BRAF* and *NRAS* mutations in familial CMMs from individuals characterized for germline *CDKN2A* status. The aim was also to assess the activation of the MAPK and PI3K-Akt pathways, by immunohistochemical analysis of the tumors for the expression of pERK and pAkt, and to correlate the expression with *CDKN2A* mutation status. Finally, the impact of somatic *BRAF* and *NRAS* mutations on clinical and histopathological characteristics of familial CMM was investigated.

- **STUDY II**

  In this study the primary aim was to better define the rate of mutations in the *KIT*, *NRAS* and *BRAF* oncogenes in a large number of primary SNMMs. The second aim was to investigate if there is any association between mutations in these oncogenes and clinical and histopathological characteristics.

- **STUDY III**

  Here we aimed to analyze primary and metastatic ALMs for mutations in the *KIT*, *NRAS* and *BRAF* oncogenes and also a subset of primary ALM for mutations in the *PTEN* tumor suppressor gene. The effect of the mutation status on tumor and patients characteristics was also studied.

- **STUDY IV**

  The aim was to investigate the expression pattern of mutated BRAF protein ($BRAF^{V600E}$) in different subtypes of CMMs, which had been previously characterized for BRAF mutation status, and also to correlate the expression of $BRAF^{V600E}$ between matched primaries and metastases.

8 MATRIALS AND METHODS

8.1 TUMOR SAMPLES

In the first study, formalin-fixed paraffin embedded (FFPE) blocks of primary familial melanoma tumors were collected from eight GenoMEL centers in Europe (Barcelona, Genoa, Leeds, Leiden, Lund, Riga and Stockholm) and Australia (Brisbane). In all centers, familial melanomas (*i.e.* families with either two first-degree relatives with melanoma or three melanoma patients irrespective of degree of relationship) with or without germline *CDKN2A* mutations were identified and all tumor blocks that were traceable were obtained and examined for sampling. Overall, 223 familial melanomas with defined *CDKN2A* status (136 *CDKN2A* mutated and 87 *CDKN2A* wild-type) diagnosed between 1971 and 2007 were collected. The *CDKN2A* wild-type tumors were from families without known germline *CDKN2A* mutations. All tumors were histologically re-evaluated by an experienced pathologist to verify the diagnosis and to identify areas with a large proportion of tumor cells. Samples that contained too few tumor cells (65 cases) or where the extracted DNA was of insufficient quality (36 cases), were excluded. Thus, in total 135 familial CMMs from 128 patients were successfully analyzed. Six patients had multiple primary tumors (five patients had two tumors and one patient had three tumors). Of the 135 familial CMMs, 89 were from patients with germline *CDKN2A* mutations and 46 were from patients without germline *CDKN2A* mutations. Of the different *CDKN2A* mutations represented, 67 were located in exon 2, 13 in exon 1α, 5 in exon 1β and 4 in introns 1 and 2. For comparison purposes we included a control group consisting of 50 primary sporadic CMMs.

In the second study, the tumor samples were collected from pathology departments from different parts of Sweden. The tumors were from patients diagnosed with SNMM between 1986 and 2011. Sixty-one FFPE samples were collected, of which five cases were excluded because the sections did not contain enough tumor cells. Therefore, a total of 56 primary SNMMs were successfully analyzed.

In the third study, 115 primary tumors on acral body sites from patients diagnosed between 1990 and 2011 were collected from the pathology-department, Karolinska University Hospital, Solna in Stockholm. After re-evaluation of all samples by

pathologists, 27 were excluded because they contained too few tumor cells, were highly pigmented, infiltrated with lymphocytes or were not classified as ALMs. Overall, 88 primary ALMs (including 54 from feet, 28 subunguals, 5 from hands and 1 with unknown location) were analyzed. From 16 patients the corresponding metastases (15 were from lymph nodes and one from the skin) were also analyzed.

In the fourth study, we collected CMM samples that had already been analyzed for *BRAF* and *NRAS* mutations in the third project or a previously published study (Omholt *et al.*, 2003). Overall, 200 primary and metastatic CMMs were selected and successfully stained with VE1 antibody using IHC.

8.2 LASER CAPTURE MICRODISSECTION (LCM) AND DNA EXTRACTION

Five millimeter thick sections were prepared from paraffin blocks and fixed on plain glass slides. The sections were deparaffinized in two washes of xylene, rehydrated in increasing concentrations of ethanol, rinsed with deionized water, shortly stained with hematoxylin, rinsed with deionized water and dehydrated in decreased concentrations of ethanol and two washes of xylene. Tumor cells were microdissected from the sections using the Arcturus PixCell® LCM System (Arcturus, Mountain View, CA). Genomic DNA was extracted using a PicoPure DNA Extraction Kit (Arcturus) according to the manufacturer's recommendations. In the first project, samples from Lund (n=32) were not subjected to LCM; instead tumor cells were manually dissected from 10 µm thick paraffin sections and DNA extracted by using a Qiagen's QIAamp DNA FFPE Tissue Kit. For 15 of the Lund samples DNA amplification was performed using a BioScore Screening and Amplification Kit from Enzo Life Sciences Inc. (Farmingdale, NY, US).

8.3 MUTATION ANALYSIS

Genomic DNA was screened for mutations in *KIT* (exons 9, 11, 13, 17 and 18), *NRAS* (exons 1 and 2), *BRAF* (exons 11 and 15) and *PTEN* (exons 1, 3-6 and 10-12). DNA was amplified by PCR in a 10 µl mixture reaction containing 2.5 mM deoxynucleotide triphosphate, 5 U/µl platinumTaq DNA polymerase, 50 pmol/µl of each primer, 10 x PCR buffer, 50 mM $MgCl_2$ and 10 µg/µL bovine serum albumin (BSA). The PCR conditions were as follows: An initial denaturation at 95°C for 3 min, followed by 35

cycles of denaturation at 94°C for 30 sec; annealing at 54-63°C (depending on the exons examined) for 30 s; elongation at 72°C for 30 sec; and a final extension at 72°C for 10 min. Two microliter of the first PCR product was used as DNA template for amplification in a second PCR. The conditions for the second PCR were similar to that of the first PCR except that the numbers of cycles were reduced to 20. PCR products were electrophoresed in 1.6% agarose gel and visualized with GelRed (Biotium) under UV light. The DNA was retrieved from the gels by using QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA). After purification of the PCR amplicons, sequencing reactions were performed in a final volume of 20 µl using BigDye Terminator V1.1 Cycle Sequencing kit (Applied Biosystems). The cycle sequencing conditions were as follows: 10 min at 95°C, 25 cycles of 30 s at 96°C, 5 s at 50°C and 4 min at 60°C. The sequencing products were purified by ethanol precipitation, and automated DNA sequencing was performed by ABI PRISM3130xl Genetic Analyzer (Applied Biosystems). Sequencing was performed in both forward and reverse directions and all mutations were confirmed by a second independent PCR and sequencing reaction.

8.4 IMMUNOHISTOCHEMISTRY

8.4.1 pERK and pAkt immunohistochemistry

IHC for pERK and pAkt was performed on FFPE sections of 4 µm thickness. Briefly, sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Antigen unmasking was performed by heating sections in 10 mmol/l sodium citrate buffer at pH 6.0. Endogenous peroxidase activity was blocked by incubating sections in 3% hydrogen peroxide (H_2O_2) for 10 min in a pressure cooker. To prevent non-specific binding, sections were blocked with 5% normal goat serum (Vectastain Elite ABC Kit, Vector Laboratories) for 45 min. Sections were then incubated with rabbit monoclonal anti-phospho-p44/42 MAP kinase (ERK1/2) (Thr202/Tyr204, dilution 1:100, Cell Signaling Technology) or anti-phospho-Akt (Ser473, dilution 1:100, Cell Signaling Technology) antibody in a humidified chamber, at 4°C overnight. On the second day, a secondary antibody was added. For the pERK samples, the sections were incubated with goat anti-rabbit IgG biotinylated secondary antibody, followed by incubation with VECTASTAIN® Elite ABC reagents at room temperature for 30 minutes each. For the pAkt samples, the sections were covered with 1-3 drops of goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated polymer (SignalStain®

Boost IHC Detection Reagent, Cell Signaling Technology) at room temperature for 30 minutes. The reactions were visualized by using peroxidase substrate DAB kit (DAB, vector laboratories, Burlingame, CA). Sections were counterstained with hematoxylin, dehydrated in ethanol and mounted. The negative controls were incubated with tris-buffered saline (TBS) instead of primary antibodies. A metastatic melanoma sample with known positive pERK or pAkt was used as a positive control. The results were interpreted by three observers blinded to the clinical and mutational data. The scoring system was based on the percentage of stained cells and the intensity of the staining. The staining intensity was classified as follows: 0, negative; 1, weak; 2, moderate and 3, strong. Percentages were scored as: 0 (0-5%), 1 (6-25%), 2 (26-75%) and 3 (>75%). The final score was a summation of staining intensity and percentage of stained cells. Samples with a final score of 0-3 were considered negative, while those with >4 were considered having positive staining.

8.4.2 BRAF^{V600E} immunohistochemistry

Staining for BRAF^{V600E} and BRAF wild-type was performed on 4 µm FFPE sections using the mouse monoclonal BRAF^{V600E} specific antibody, VE1 (provided by Professor von Deimling, University of Heidelberg, Germany) and a BRAF wild-type (Raf-B, 1:500 dilution, Santa Cruz Biotechnology Inc.) antibody, respectively. Staining was performed on Ventana BenchMark XT immunostainer (Ventana Medical Systems, Tucson, AZ, US) according to a protocol described previously (Capper *et al.* 2011). Briefly, each slide was first labeled with a barcode referring to the protocol information and then loaded onto the BenchMark machine. Following pre-treatment (deparaffinization, antigen retrieval and endogenous peroxidase blockage), the slides were manually incubated with undiluted VE1 hybridoma supernatant or BRAF wild-type antibodies at 37°C for 60 minutes. This was followed by signal amplification using a Ventana amplifier kit, washing with DAB chromogen and counterstaining with hematoxylin and bluing reagent. After the autostaining was completed, the slides were washed with a drop of dish-washing detergent, dehydrated in ethanol, cleared in xylene and mounted. The staining was separately evaluated by three observers who had no information about the genotype of the samples. The evaluation was based on the intensity of the staining and was scored as negative, weak, moderate or strong.

9 RESULTS

9.1 STUDY I

The median age at diagnosis was 45 years in patients with germline *CDKN2A* mutations and 50 years in those without germline *CDKN2A* mutations and also in those with sporadic melanomas. The median tumor thickness was 0.7 mm in *CDKN2A* mutated, 0.9 mm in *CDKN2A* wild-type familial melanomas and 1.2 mm in the control sporadic group.

We found no significant difference in *BRAF* or *NRAS* mutation frequency between tumors from germline *CDKN2A* mutation carriers and tumors from non-carriers (*BRAF*: 45% *vs* 39% and *NRAS*: 9% *vs* 15%). There was also no difference in the frequency of *BRAF* mutations in tumors with *CDKN2A* mutations disrupting both $p16^{INK4A}$ and $p14^{ARF}$ compared to tumors with *CDKN2A* mutations affecting $p16^{INK4A}$ only. No association was found between specific *CDKN2A* founder mutations (p.M53I, p16-leiden, p.G101W and p.112dupR) and the *BRAF/NRAS* mutation status. Median tumor thickness in familial melanoma differed significantly among *BRAF* mutated (1.0 mm), *NRAS* mutated (1.4 mm) and *BRAF*/*NRAS* wild-type tumors (0.6 mm; p=0.001). No other examined features (including gender, anatomical site, histological subtype, Clark's level and ulceration) were associated with the *BRAF* or *NRAS* mutation status.

Positive pERK and pAkt staining was observed in 65% and 46% of the familial melanomas, respectively. In general, the expression of pERK and pAkt was mainly nuclear and showed a heterogeneous distribution pattern within the tumors. There was no difference in the level of pERK or pAkt expression between tumors from patients with germline *CDKN2A* mutations and tumors from patients without *CDKN2A* mutations. There was also no difference in pERK or pAkt expression in respect to the *BRAF* and *NRAS* mutation status of the tumors. The staining of pERK and pAkt in familial melanomas did not differ from that in sporadic melanomas, where positive pERK and pAkt staining was observed in 66% and 35% of cases, respectively.

To compare the rate of *BRAF* and *NRAS* mutations in different growth phases and to better define the timing of these mutations in familial melanomas, we selected a subset

of 29 tumors that had both RGP and VGP. Tumor cells from the two phases were dissected separately by LCM and subjected to mutation analysis. *BRAF* mutations were detected in 18 of the 29 tumors, 11 of which contained the same *BRAF* mutation in both the RGP and VGP. In the remaining seven tumors, *BRAF* mutations were present in the VGP but not in the corresponding RGP. Overall, *BRAF* mutation frequency was higher in the VGP (18 of 29, 62%) than in the RGP (11 of 29, 38%). Three of the 29 tumors contained *NRAS* mutations. In two of these tumors, *NRAS* mutations were detectable in both the RGP and VGP, whereas in one tumor, mutation was found in the VGP only (Table 1). Thus, in several, but not all of the cases tested, mutations in *BRAF* and *NRAS* seemed to be relatively early genetic events, (unpublished data).

9.2 STUDY II

The patient material included tumors from 35 females and 21 males with a median age at diagnosis of 76 years. Sixty-one per cent of the tumors were located in the nasal cavity and 39% in the paranasal sinuses. Ulceration and pigmentation were present in a significant proportion of the SNMM tumors.

The results showed that 21% (12 out of 56) of the tumors harboured *KIT*, *NRAS* or *BRAF* mutations. *KIT* mutations were observed in 4% of the SNMMs (2 out of 56). Both KIT mutations identified were the L576P in exon 11, whereas no mutations were observed in exons 13 and 17. We have previously reported a high rate of *KIT* mutations (35%) in vulvar melanomas (Omholt *et al.*, 2011). The finding of *KIT* mutations in only 4% of SNMMs indicates that the frequency of *KIT* mutations in MMM varies significantly with anatomical sites. *NRAS* mutations were identified in 14% of the SNMMs (8 out of 56). Four mutations were in exon 2 and four in exon 1. *BRAF* mutations were detected in 4% of the SNMMs (2 out of 56). One mutation was V600E and one was V600K.

We grouped the tumors according to the mutation status; *i.e.*, tumors with or without *KIT*, *NRAS* and *BRAF* mutations. The comparison showed that tumors with mutations were more likely to be located in the paranasal sinuses, whereas the wild-type group were more often found in the nasal cavity. This difference was statistically significant (p=0.045). No association was found between age at diagnosis, gender, ulceration and pigmentation and the mutation status of the tumors. In a multivariate analysis, the

overall survival was better for patients with melanomas in the nasal cavity than those with tumours in the paranasal sinuses (p=0.027).

9.3 STUDY III

There were 50 females and 38 males with a median age at diagnosis of 72 years. The most common location was the feet (61%), followed by subungual sites (32%) and hands (6%). The majority of the tumors were ulcerated, with a median tumor thickness of 3.5 mm.

Overall, mutations in *KIT*, *NRAS* and *BRAF* were detected in 15%, 15% and 17% of the ALMs, respectively. The majority of *KIT* mutations were observed in exon 11 and consisted of single amino acid changes. Five of the identified *KIT* alterations (V559del, P577del, D572G and Y823C) have not been described previously in melanoma. In no case were *KIT*, *NRAS* and *BRAF* mutations detected in the same tumor. Twenty-five primary tumors were also evaluated for mutations in the *PTEN* gene, and one tumor was found to carry a nonsense mutation (W111X). The 16 paired metastatic tumors showed an identical mutation status to the corresponding primaries; five were *KIT* mutated, three *NRAS* mutated, five *BRAF* mutated and three were wild-type for *KIT/NRAS/BRAF*. The *BRAF* mutations associated significantly with younger age at diagnosis (p=0.028), female gender (p=0.011), and were more commonly observed in tumors located on the feet (p=0.039). Other clinicopathological features such as age at diagnosis, thickness, ulceration, histological subtype and Clark's level showed no significant correlation with the mutation status. In a multivariate analysis, the anatomical site was an independent prognostic factor for overall survival; patients with ALMs on the hands or fingernails had a better overall survival than those with tumors on the feet or toenails (p=0.025).

9.4 STUDY IV

A total of 200 (124 primaries and 76 metastases) FFPE CMM samples (including 53 ALM, 45 SSM, 21 NM, 1 LMM, 3 unclassified and 1 with unknown location) were successfully stained using IHC with the VE1 antibody. Seventy-three of the 76 metastases were matched and were from 63 patients. The mean tumor thickness was 3.9 mm (range, 0.4-30 mm). The mutation status of the tumor samples was determined by either single-strand conformation polymorphism (SSCP) or direct DNA sequencing.

The mutation analyses results were as follow: *BRAF*^{V600E} mutations in 91 tumors, *BRAF*^{V600K} in 4 cases, *BRAF* mutations outside codon 600 in 2 tumors, *NRAS* mutations in 45 tumors and 58 tumors were wild-type for *BRAF* and *NRAS* mutations. The IHC results with the VE1 antibody were scored by three investigators. The scoring system was based on the intensity of the staining and classified as follows: negative, weak, moderate or strong.

Overall, positive VE1 antibody staining with was observed in 55% (110 out of 200) of the CMMs. In general, the staining was homogenous. Out of the 91 *BRAF*^{V600E} mutated tumors, 88 tumors displayed positive VE1 staining, and 3 cases were regarded as negative. Five out of the 45 *NRAS* mutated and 20 out of the 58 *BRAF/NRAS* wild-type tumors showed positive VE1 staining, with the majority being weakly stained. None of the melanomas with *BRAF*^{V600K} and *BRAF* mutations outside codon 600 showed positive VE1 staining. Thus, in 28 melanomas (24 primaries and 4 metastases) we observed a discrepancy between the *BRAF* mutation status and VE1 antibody staining results. Therefore, DNA re-analysis using pyrosequencing was performed for 25 cases (for the remaining 3 tumors, there was lack of material) to attest the *BRAF* mutation status of these tumors. The pyrosequencing confirmed the IHC results (positive staining) in three cases, meaning that the original mutation analysis results (*BRAF* wild-type) were inaccurate. In the remaining 22 cases (17 wild-type for *BRAF/NRAS* and 5 with *NRAS* mutations), which were positively stained with VE1 antibody, the pyrosequencing results were identical to that of the initial mutation analyses, meaning that no further *BRAF*^{V600E} mutation were present. The staining status was consistent between primary and their corresponding metastases; however, the intensity varied. A subset of tumors contained both RGP and VGP, and the VE1 staining status and intensity showed no variation between the two phases. The overall estimated sensitivity and specificity of VE1 antibody was 97% (88/91) and 80% (87/109), respectively.

Table 1. *BRAF* and *NRAS* mutations in RGP and VGP in familial melanoma

		BRAF mutation status		*NRAS* mutation status	
Tumor	Germline *CDKN2A* status	RGP	VGP	RGP	VGP
1	p.P38R	V600E	V600E	wt	wt
2	p.M53I	V600E	V600E	wt	wt
3	p.M53I	V600E	V600E	wt	wt
4	c.225_243del19	V600E	V600E	wt	wt
5	c.225_243del19	V600E	V600E	wt	wt
6	p.R112_L113insR	V600E	V600E	wt	wt
7	p.R112_L113insR	V600E	V600E	wt	wt
8	p.A118V	V600E	V600E	wt	wt
9	p14del	V600E	V600E	wt	wt
10	Wild-type	V600E	V600E	wt	wt
11	Wild-type	V600K	V600K	wt[a]	wt[a]
12	p.R24P	wt	V600E	wt	wt
13	p.Q50R	wt	V600E	wt	wt
14	c.225_243del19	wt	V600E	wt	wt
15	c.225_243del19	wt	V600E	wt	wt
16	p.G101W	wt	V600E	wt	wt
17	p.R112_L113insR	wt	V600E	wt	wt
18	Wild-type	wt	V600E	wt	wt
19	c.225_243del19	wt	wt	Q61K	Q61K
20	Wild-type	wt	wt	Q61R	Q61R
21	p.V51F	wt	wt	wt	Q61H
22	p.M53I	wt	wt	wt	wt
23	c.225_243del19	wt	wt	wt	wt
24	c.225_243del19	wt	wt	wt	wt
25	c.225_243del19	wt	wt	wt	wt
26	Wild-type	wt	wt	wt	wt
27	Wild-type	wt	wt	wt	wt
28	Wild-type	wt	wt	wt	wt
29	Wild-type	wt	wt	wt[a]	wt[a]

Abbreviations: RGP, radial growth phase; VGP, vertical growth phase; wt, wild-type.
[a]RGP and VGP were dissected and analyzed together.

10 CONCLUSIONS

- **Study I**
  - The frequency of *BRAF* and *NRAS* mutation in familial CMM (43% and 11%, respectively) is similar to that reported in sporadic CMM.
  - The germline *CDKN2A* mutation status has no significant effect on the frequency of somatic *BRAF* and *NRAS* mutations in familial melanoma.
  - The *BRAF* mutation incidence showed no significant difference between familial CMM harboring *CDKN2A* mutations affecting both $p16^{INK4A}$ and $p14^{ARF}$ and those affecting $p16^{INK4A}$ alone.
  - Evaluation of *BRAF* and *NRAS* mutation status in relation to the most frequent *CDKN2A* mutations (p.M53I, p16-leiden, p.G101W and p.112dupR) did not show any significant correlations.
  - In familial melanoma, *BRAF* and *NRAS* mutation status is associated with tumor thickness.
  - There was a high level of pERK and pAkt expression in familial melanomas, 65% and 46%, respectively. However, similar frequencies of pERK and pAkt (66% and 35%, respectively) expression were identified in the sporadic cases.
  - Expression of pERK or pAkt in CMM did not correlate with germline *CDKN2A* mutations or *BRAF*/*NRAS* mutation status.

- **Study II**
  - *KIT* and *BRAF* mutations are very infrequent in primary SNMM (4% each), whereas *NRAS* mutations are more common (14%).
  - *NRAS* mutations in exon 1 are as common as mutations in exon 2.
  - *KIT* mutation frequencies in MMMs seem to differ between various anatomical sites, with highest frequency in vulvar melanomas.
  - Mutations in *KIT*, *NRAS* and *BRAF* are more likely to be detected in MMM located in paranasal sinuses, as compared with tumors in the nasal cavity.
  - Overall prognosis is better for patients with melanomas in the nasal cavity than in those with tumors in the paranasal sinuses.
  - *KIT*, *NRAS* and *BRAF* mutations occur in a mutually exclusive manner.

- **Study III**
  - Our results show that *KIT, NRAS* and *BRAF* mutations occur at similar frequencies of about 15% in ALMs.
  - *PTEN* mutations are uncommon in primary ALMs (<5%).
  - The majority of the *KIT* mutations are present in exon 11.
  - *KIT*, *NRAS* and *BRAF* mutations occur before the melanomas metastasize.
  - In primary ALM, *BRAF* mutations are associated with younger age at diagnosis, female gender and feet location.
  - The anatomical site of primary ALM significantly associated with patient outcome.

- **Study IV**
  - The $BRAF^{V600E}$ expression in primary and metastatic CMMs is homogeneous, suggesting absence of *BRAF* clonal heterogeneity.
  - The VE1 antibody is highly sensitive and specific, especially when the staining is moderate to strong.
  - The specificity of the VE1 antibody is higher in metastatic than in primary CMMs.
  - Staining with VE1 antibody can be used as a rapid method for detection of $BRAF^{V600E}$ mutation, and positive strong result alone might be sufficient to precede and treat patient with a BRAF inhibitor.

11 FUTURE PERSPECTIVES

In our second paper, where we analyzed a relatively large number of primary SNMMs for the most commonly altered oncogenes (*KIT*, *NRAS* and *BRAF*) in cutaneous melanoma, the results showed a very low frequency of mutations in these oncogenes especially in *KIT* and *BRAF*. This indicates that SNMMs harbor mutations in other genes that have not been discovered yet, which may be of importance in the development and progression of this subtype of melanoma. This also indicates that the majority of patients with SNMMs will not benefit from the recent success in the molecular targeted therapy with small molecules such as vemurafenib and imatinib.

In the third project, where we screened a large number of ALMs for *BRAF*, *NRAS* and *KIT* mutations and a subset of tumors were also analyzed for *PTEN* mutations. The conclusion was that mutations in these genes are not common and majority of ALMs contain mutations in driver genes yet to be identified.

It would be interesting to perform a comprehensive mutation analysis using next generation sequencing platform to accomplish whole-genome or whole-exome sequencing to identify other driving genetic mechanisms involved in the development of SNMM and ALMs.

Recently, it has been shown that a large proportion of cutaneous melanomas contain recurrent somatic mutations at two position in the promoter region of the *telomerase reverse transcriptase* (*TERT*) gene. These mutations create a motif for E-twenty-six transcription factors, thus, resulting in increased transcriptional activity of the TERT promoter. However, ALM and MMM were not included in the analyzed tumors. Furthermore, such mutations have not been observed in ocular melanoma, indicating that such mutations might not be prevalent in all melanoma subtypes. Therefore, it will be interesting to investigate whether *TERT* promoter mutations are present in SNMM and ALM and also correlate the mutation status with clinicopathological characteristics.

12 SVENSK SAMMANFATTNING

I Sverige är malignt melanom (MM) den femte och sjätte vanligaste cancerformen hos kvinnor respektive män, och utgör mer än 5 procent av alla registerade tumörer. Basalcellscancer och skivepitelcancer är de vanligaste hudtumörerna, medan MM är den allvarligaste formen av hudcancer. Den vanligaste typen av melanom är hudmelanom (kutana melanom), medan andra subtyper som slemhinnemelanom (mukosala) och ögonmelanom (uveala) är sällsynta. Genomsnittåldern vid diagnos av kutana-melanom är omkring 65 år. I Sverige diagnosticerades under år 2011 mer än 3000 nya fall av MM och cirka 500 dödsfall orsakades av melanom. Riskfaktorer för MM är bland annat exponering av UV-strålning, ljus hy, blåa ellergröna ögon och röd eller blond hårfärg. Förekomst av banala och dysplastiska nevi och ärftliga faktorer, nedärva förändringar i gener som t.ex. *CDKN2A*, *CDK4* och *MC1R,* ökar också risken att insjukna. Aktiverande mutationer i protoonkogenerna *BRAF*, *NRAS* och *KIT* är vanligt förekommande i melanomentumörer. Målsökande behandling med BRAF-hämmare (vemurafenib och dabrafenib), MEK-hämmare (tramatenib) och KIT-hämmare (imatinib) har visat sig ge mycket bra initiala resultat hos patienter med avancerat melanom som bär på mutioner i dessa gener. Huvudsyftet med denna avhandlingen var att analysera frekvensen av *BRAF*, *NRAS* och *KIT* mutationer i olika typer av melanom och analysera vilka kliniska parametrar som är förknippad med de olika mutationer.

I den första studien samlades primära familjära och sporadiska kutana melanom in från åtta centra i Europa och Australien. *BRAF* och *NRAS* mutationer detekterades i 43% respektive 11% i de familjära melanomen. Frekvensen av *BRAF* och *NRAS* mutationer i sporadiska melanom skilde sig inte signifikant från den som identifierades i de familjära melanomen.

I det andra projektet analyserades 56 primära sinonasala mukosala melanom (SNMM) för mutationer i *BRAF*, *NRAS* och *KIT*. Resultaten visade att 12 SNMM innehöll en muterad onkogen: 2 tumörer hade *KIT* mutationer, ytterligare 2 tumörer bar på en *BRAF* mutationer och 8 tumörer innehöll *NRAS* mutationer.

I det tredje projektet utvärderades primära och metastaserande akrala lentiginösa melanom (ALM; melanom som är lokaliserade till fötter, fotsulor, händer, handflator och under naglar) för mutationer i *BRAF*, *NRAS*, *KIT* och *PTEN*. Resultaten visade en liknande mutationsfrekvens i både *KIT* och *NRAS* (15%), medan *BRAF* mutationer hittades i 17% av tumörerna. Av de 25 tumörer som utreddes för *PTEN* mutationer, påträffades endast en muterade tumör. *BRAF*, *NRAS* och *KIT* mutationsstatus i 16 analyserade metastaser överensstämde den som observrades i de matchande primärtumörerna. Jämfört med *BRAF*-vildtyp tumörer, var *BRAF*-muterade tumörer oftare diagnostiserades hos unga individer och det fanns även association med tumörer lokaliserade på fötterna och kvinnligt kön.

I det fjärde projektetd utvärderade vi 200 primärtumörer och metastaser från kutana melanom för $BRAF^{V600E}$ uttryck med immunhistokemi. Samtliga tumörer hade analyserats avseende BRAF mutationsstatus med DNA-sekvensering. 110 (55%) tumörer visade en positiv färgning. Infärgningen stämde överens mellan matchade primärtumörer och metastaser. I 28 tumörer observerades en diskrepans mellan VE1 färgning och resultat från DNA-sekvensering. Sensitivitet och specificitet av VE1 antikroppen var 97% respektive 80%. Diskrepanta resultat förelåg främst hos primära melanom medan överensstämmelsen mellan immunhistokemi och DNA-sekvensering var god bland metastaser.

Sammanfattningsvis, *BRAF*, *NRAS* och *KIT* mutationer är ovanliga i ALM jämfört med andra typer av hudmelanom. Dock är screening av ALM för mutationer i dessa onkogener fortfarande att rekommendera eftersom det finns möjlighet till behandling med målsökande mediciner. Då mutationer i *BRAF* och *KIT* är väldigt sällsynta i SNMM, är det mer tveksamt om man ska screena för mutationer i dessa gener. Immunohistokemi färgning med VE1 antikropp kan användas som en snabb metod för utvärdering av $BRAF^{V600E}$ mutation i melanom. Stark positiv färgning skulle kunna vara tillräcklig för att starta behandling med BRAF inhibitor.

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