GENETIC AND EPIGENETIC ALTERATIONS IN MELANOMA

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by Rainer Tuominen

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All that's in a name is a puff of sound, a lungful of wind, and yet it is an airy enclosure. How is it that the gist, the spirit, the complicated web of bone, hair, brain, gets stuffed into a syllable or two? How do you shrink the genie of human complexity? How the personality? Unless, that is, your mother gives you her name, Other Side of the Earth.

By Louise Erdrich in the Antelope Wife

This work is dedicated to my wife Pia and my children Felix and Mira without whom I wouldn't have insisted on continuing to decipher the endless unknowns of the clinical data...
ABSTRACT

Malignant melanoma is a disease that may arise in several different parts of the body, preferentially the skin, rarely in the mucosal membranes or the choroidal tissues of the eye. The incidence of cutaneous melanoma (CMM) is steadily increasing in the Caucasian populations, unlike uveal melanoma (UM) that shows a stable incidence. The increase is likely to be related to UV-irradiation leading to genetic aberrations that allow skin melanocytes to develop unlimited growth and immortality and ultimately lead to metastases.

Paper I presents a genomic and epigenomic screening of 77 metastatic cutaneous melanoma metastases for the protein expression of p16\textsuperscript{INK4A} in relation to 3 well-known causes of expression loss: truncating and non-synonymous mutations in CDKN2A, the gene for p16INK4A, transcriptional silencing of p16\textsuperscript{INK4A} gene promoter and previously studied deletions in the CDKN2A loci encompassing p16\textsuperscript{INK4A}. These aberrations were compared to p16\textsuperscript{INK4A} expression in tumours and presence of mutations in BRAF and NRAS genes. Unexpectedly, a significant association between tumours carrying NRAS mutations and transcriptional silencing of p16\textsuperscript{INK4A} promoter was observed.

Paper II was a case study of a family with multiple cases of uveal melanoma. Family members with were found to be negative for germ-line CDKN2A aberrations, why next generation sequencing was employed. The proband, the proband’s sister and both parents were analyzed. The final mapped and filtered variants were filtered against variants found in the DNA of the non-carrier mother. A germ-line, frame-shift, insertion in BAP1 (exon 3 c.75insG) was identified and validated by Sanger sequencing. The insertion leads to a truncation at codon 43 and was found to segregate with the disease.

Paper III is a retrospective study to evaluate the naturally occurring transcriptional silencing of DNA repair protein \textsuperscript{O\textsubscript{6}}-methylguanine DNA methyltransferase, MGMT. MGMT activity counteracts the efficacy of alkylating chemotherapy. Two cohorts of patients mainly derived from Sweden (n=74) and Belgium (n=79) were included, in total encompassing 191 tumours. The hypermethylation of MGMT gene promoter was found in 21.5% of tumours successfully analyzed (28 positive, 130 total) and to be associated with a significantly longer progression free survival (PFS) and to be an independent variable in a multivariate analysis for PFS.

Paper IV is an in vitro melanoma study for combination therapy efficacy. The BRAFV600E-melanoma cell line A375 and a mutant BRAF inhibitor (BRAFi)-resistant subline were subjected to sequential and simultaneous exposures for BRAFi PLX4720 and temozolomide (TMZ). Administration order was found to influence the treatment outcome: administration of BRAFi followed by TMZ displayed a poorer efficacy compared to exposure simultaneously or administration in the reverse order. This effect was related to BRAFi induction of MGMT mRNA and protein, but also induction of the DNA damage marker \textgammah2ax by BRAFi and TMZ.
LIST OF SCIENTIFIC PAPERS


IV. Alireza Azimi, Rainer Tuominen, Samaneh Ghashghaei, Hanif RassoolZadeh, Marianne Frostvik Stolt, Marianne Farnebo, Suzanne Egyházi Brage, Carolina Hertzman Johansson Induction of MGMT by BRAF inhibitors decreases the response to temozolomide in melanoma cells – DNA damage response central for the combination therapy outcome , manuscript.
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2 associated death promoter</td>
</tr>
<tr>
<td>BAD</td>
<td>BCL2-associated agonist of cell death</td>
</tr>
<tr>
<td>BAP1</td>
<td>Bap1, BRCA1 associated protein-1 (ubiquitin carboxy-terminal hydrolase)</td>
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<tr>
<td>BARD1</td>
<td>BRCA1 associated RING domain 1</td>
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<tr>
<td>BCL2</td>
<td>B-cell CLL/lymphoma 2</td>
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<tr>
<td>BRAF</td>
<td>Braf, B-Raf proto-oncogene, serine/threonine kinase</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1, early onset</td>
</tr>
<tr>
<td>CDK4/6</td>
<td>Cdk4, cyclin dependent kinase 4/ Cdk4, cyclin dependent kinase 6</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>p16INK4A , Inhibitor of CDK4/CDK6 and p14ARF ,Alternative Reading Frame</td>
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<tr>
<td>CMM</td>
<td>Cutaneous malignant melanoma (skin melanoma excluding melanoma in mucosal membranes but including acral melanoma)</td>
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<tr>
<td>CpG</td>
<td>Cytosine-phosphate-guanine</td>
</tr>
<tr>
<td>CREB1</td>
<td>cAMP responsive element binding protein 1</td>
</tr>
<tr>
<td>E2F1</td>
<td>E2F transcription factor 1</td>
</tr>
<tr>
<td>EGFR/ERBB1</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELK1</td>
<td>ELK1, member of ETS oncogene family</td>
</tr>
<tr>
<td>ERBB2/Her2</td>
<td>Erb-b2 receptor tyrosine kinase 2</td>
</tr>
<tr>
<td>ERBB3/4</td>
<td>Erb-b2 receptor tyrosine kinase 3f/ erb-b2 receptor tyrosine kinase 4</td>
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<tr>
<td>ERK1/2,MAPK3/1</td>
<td>Mitogen-activated protein kinase 3/ mitogen-activated protein kinase 1</td>
</tr>
<tr>
<td>Gene Symbol</td>
<td>Description</td>
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<tr>
<td>-------------</td>
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<tr>
<td>EZH2</td>
<td>Enhancer of zeste 2 polycomb repressive complex 2 subunit</td>
</tr>
<tr>
<td>FOS</td>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>GAB1/2</td>
<td>GRB2-associated binding protein 1/2</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine bis-phosphate</td>
</tr>
<tr>
<td>GNA11</td>
<td>Guanine nucleotide binding protein (G protein), alpha 11</td>
</tr>
<tr>
<td>GNAQ</td>
<td>Guanine nucleotide binding protein (G protein), q polypeptide</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<tr>
<td>GRB2</td>
<td>Growth factor receptor-bound protein 2</td>
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<tr>
<td>GRM3</td>
<td>Glutamate receptor, metabotropic 3</td>
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<tr>
<td>GSK3β/GSK3B</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine tris-phosphate</td>
</tr>
<tr>
<td>IGF1R</td>
<td>Insulin-like growth factor 1 receptor</td>
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<tr>
<td>JUN</td>
<td>Jun proto-oncogene</td>
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<tr>
<td>KIT/CD117</td>
<td>v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog</td>
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<tr>
<td>MEK1/MAP2K1</td>
<td>Mitogen-activated protein kinase kinase 1</td>
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<tr>
<td>MEK2/MAP2K2</td>
<td>Mitogen-activated protein kinase kinase 2</td>
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<tr>
<td>MGMT</td>
<td>Mgmt, O-6-methylguanine-DNA methyltransferase</td>
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<tr>
<td>MITF</td>
<td>Mitf-M, Microphthalmia-associated transcription factor</td>
</tr>
<tr>
<td>MTOR</td>
<td>mTOR, mechanistic target of rapamycin (serine/threonine kinase)</td>
</tr>
<tr>
<td>mTOR/MTOR</td>
<td>Mechanistic target of rapamycin (serine/threonine kinase)</td>
</tr>
<tr>
<td>NGFR/CD271</td>
<td>CD271, Nerve growth factor receptor</td>
</tr>
<tr>
<td>Non-RTK</td>
<td>Non-receptor tyrosine kinase</td>
</tr>
<tr>
<td>NRAS</td>
<td>Neuroblastoma RAS viral (v-ras) oncogene homolog</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>PIK3CA/PIK3K</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphoinositol-1,4,5-trisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PLCγ/PLCG1</td>
<td>Phospholipase C, gamma 1</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SETDB1</td>
<td>SET domain, bifurcated 1</td>
</tr>
<tr>
<td>SHC/SHC1</td>
<td>SHC (Src homology 2 domain containing) transforming protein 1</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of sevenless homolog 1 (Drosophila)</td>
</tr>
<tr>
<td>SRC</td>
<td>SRC proto-oncogene, non-receptor tyrosine kinase</td>
</tr>
<tr>
<td>STAT3/5A/B</td>
<td>Signal transducer and activator of transcription 3/ signal transducer and activator of transcription 5A and 5B</td>
</tr>
<tr>
<td>TP53</td>
<td>P53, tumor protein p53</td>
</tr>
<tr>
<td>UM</td>
<td>Uveal melanoma including anterior and posterior uveal melanoma, but excluding melanoma in the accessory parts of the eye (the wider term being ocular melanoma)</td>
</tr>
<tr>
<td>ZEB1/2</td>
<td>Zeb1, Zinc finger E-box binding homeobox 1/2</td>
</tr>
</tbody>
</table>
1 MELANOMA ETIOLOGY AND CHARACTERISTICS

1.1 Cutaneous melanoma incidence, trends and mortality

Cutaneous malignant melanoma (CMM) is a cancer disease that displays a growth of incidence in most populations of Caucasian origin, including Sweden. Among 61297 registered cancer tumours in Sweden year 2013, 3027 tumours (from 2831 patients) i.e. 4.9 % were diagnosed as cutaneous malignant melanoma. This made cutaneous malignant the 6th most common cancer disease among men and the 5th most common cancer diagnosis among women (National Board of Health and Welfare, 2013).

The age-standardized incidence of CMM in Sweden 2013 was 35.6 for males and 32.1 for females per 100 000 inhabitants (age-standardized according to the Swedish population). These, relatively equal, incidences have nearly doubled in Sweden since year 2000, the present yearly increase in incidence being 5.5 % for men and 5.2 % for women (National Board of Health and Welfare, 2013).

The cumulative risk for development of CMM before the age of 75 years of age 2013 was 2.1 %, same for both sexes, but also 0.2 % before the age of 35 years for women (0.1 % for men), i.e. at the same level as the risk of developing breast cancer for this age group (National Board of Health and Welfare, 2013).

As shown in Figure 1, the incidence of CMM in Nordic countries years 2000-2012 shows a near linear increase, but this increase is not followed by increase in mortality, indicating diagnostic and therapeutic progress. Despite the higher incidence for CMM for women in the Nordic countries, CMM mortality for females is lower than that of males (Figure 1). Reasons for the better survival for females may include lower median age at diagnosis, lower median Breslow thickness of primary tumors and earlier detection, but female gender is a positive, independent variable for survival even when tumor stage is accounted for (1), (2). This gender difference on overall survival has been related to differences between sexes regarding tumor localization and pathologic variables: proliferative index, histological subtype and tumor vascularization and ulceration in a study with relatively few patients (n_{females}=132, n_{male}=123 (3). The gender difference is also evident in a larger proportion of males experiencing emergency presentation, i.e. disease is diagnosed together with need of immense actions by the health care as analyzed using hospital records from 27 different countries. This difference was suggested to be related to lower level of body consciousness and ability to seek help for males compared to females (4).

The long-term trend in cutaneous melanoma with improved therapy outcome is evident as surviving fractions by 4-year intervals between 1964 and 2012 in the Nordic countries, but also shows a larger improvement among males compared to females (Figure 2) (NORDCAN). The mortality trends for both CMM and UM can be collectively described as cautiously optimistic, mortality possibly reaching a plateau.
Figure 1. Incidence and mortality trends for cutaneous malignant melanoma in the Nordic countries 2000-2012. Mortality data is not available for all countries for 2010-2012. Incidence and mortality as age standardized rates/100 000 inhabitants (NORDCAN).

Figure 2. Long-term improvement in surviving fraction (median for all Nordic countries) for CMM 1964-2012. Adapted from NORDCAN database numerical data.
Factors found to be associated with an increased risk for developing cutaneous malignant melanoma are Caucasian ancestry and phenotypic traits like poor tanning response (skin types I and II), red hair color, skin with appearance of freckles, dysplastic nevi or excessive number of normal nevi and documented familial history of CMM (5). In general, cumulative UV irradiation is not regarded to be related to CMM risk unlike the other skin diseases like squamous cell carcinoma and basal cell carcinoma. Whether an intermittent UV irradiation causing sunburns, possibly in particular during childhood, increases risk for CMM is debated and, due to the retrospective nature and length of the retrospective period, highly challenging to study (6). Still, the apparent increased CMM risk for individuals migrating from higher to lower latitude countries compared to non-migrating subjects and the higher CMM incidence found to be associated with higher socio-economic status (SES), suggestive of more recreational UV exposure, strongly implies intermittent UV irradiation to be involved in CMM etiology (7), (8). Possibly, vitamin D influx via sun exposure and diet and UVR-initiated formation of ROS in melanocytes are confounding factors here (9), (10).

Behind the phenotypic traits are differences associated with impaired function of the eumelanin production, the common phenotypic variation (without outright albinism) being most prominent for melanocortin-1 receptor (MC1R) protein by non-synonymous amino acid changes. MC1R is regarded as a low to median penetrance CMM susceptibility gene. Carriage of 2 or more variants for MC1R was found to increase the risk for CMM with an OR 2.51 (95% CI: 1.83–3.44) (OR corrected for study-specific factors). All variants associated with appearance of red or blond hair color were significantly associated with increase in CMM risk. In addition, carriage of MC1R variants without the typical red hair/blond hair and fair skin color was shown to be associated with increase in CMM risk. This implies that MC1R function may have CMM related effects beyond those related to the phenotype (11).

Heredity for CMM includes shared genetic variants associated with the phenotype, but also in ~5-10% of all families with increased number of CMM cases, germ-line aberrations are found. Around the world, these mutations are dominated by regionally distinct variants in CDKN2A. Also, rare germ-line mutations in CDK4 are found as well as aberrations in genes MITF, TERF2IP, ACD, POT1 and BAP1 albeit all at a low frequency (12). As the prevalence of heredity in CMM is low, the contribution of germ-line mutations for CMM pathology is limited, but several of the germ-line altered genes are found to be somatically altered as well. In addition, as some of these genes increase the risk for development of other neoplasms (pancreatic cancer for CDKN2A and CDK4; UM for BAP1), screening of the alterations is useful for identification of subject on heightened risk for cancer.
1.3 Uveal melanoma etiology and association to phenotype

The incidence of uveal melanoma (UM) is considerably lower than that of CMM, a total of 114 cancer cases in the eye were registered in Sweden during 2013, including retinoblastoma, uveal melanoma and some rare neoplasms, but excluding tumors in the accessory organs of the eye, like the eyelids. In adults, a majority of eye neoplasms are uveal melanoma with posterior localization being most common, anterior neoplasms in the ciliary body or iris are rare, but have a better prognosis compared to the posterior tumors (13). A difficulty in diagnosis of UM is distinguishing UM from benign uveal nevi, necessitating watchful waiting to avoid unnecessary therapeutic intervention.

The UM risk factors are fairly similar to the risk factors for CMM, increased risk is confirmed to be associated with light eye color (blue or grey, i.e. absence of eumelanin in the iris), Caucasian ancestry, light skin complexion and sun sensitive skin. A difference may however be that red/blond hair color appears not to be associated with higher risk of UM (13). Also, presence of dysplastic dermal nevi or freckles, presence of iris nevi or numerous normal dermal nevi increase the risk of UM development (13). For UV exposure, no obvious effect of latitude or occupational/recreational UV exposure on UM risk was found, with the exception of welding as an occupation (14). An obvious difficulty in studying risk factors for UM is the relative rarity of the disease, why virtually all studies are either low in power or ethnically limited, consequently well-controlled meta-analyses are better suited for the risk estimates offering enhanced statistical power. Overall, the recognized risk factors for CMM and UM are overlapping to a high degree.

Hereditary accumulation of UM has been shown to be associated with germ-line aberrations in BAP1, in particular truncating BAP1 mutations, leading to an increased risk for UM, CMM, mesothelioma, clear cell renal cancer and paraganglioma. Other rare cancer diseases have also been suggested to be associated with BAP1 germ-line alterations in single family contexts. Whether specific alterations in BAP1 are differentially associated with the tumor types is not well understood, but this appears unlikely for UM and CMM, the two most commonly seen tumor types. As apparent, this wide spectrum of cancer diseases associated with BAP1 requires a multi-disciplinary approach to identify and manage the family members who are carriers of these germ-line variants (15).

1.4 Genetic aberrations and melanoma associated pathways

Genetic aberrations associated with neoplastic development in CMM and UM have long been regarded to be rather different, mainly due to obvious lack of evidence for inactivation of CDKN2A and nearly absent presence of oncogenic mutations in BRAF and NRAS in UM. Lately, however, some common genetic aberrations have been confirmed to exist, forming a more coherent picture of these diseases, both associated to Caucasian origin and fair complexion. This common phenotypic context makes it likely that phenotype-related gene variants are common in both patient groups and that the effect of phenotype is related to
mutational patterns typical of UV induced mutations in UM similar to CMM. Evidence for this is, however, only available for CMM presently.

The early expansion of tumor cells in CMM and UM is illustrated in cartoons (Figures 3 and 4).

Figure 3. Schematic illustration of early CMM spread though the basal cell membrane to the dermal space. A. Normal dermis-epidermis boundary B. Spread of melanoma cells to the dermis. The cells and dermal layers are not proportionally illustrated.

Figure 4. Schematic illustration of posterior uveal melanoma initiation and growth towards the retina.
1.4.1 Major pathways activated in melanoma

The major pathways activated in CMM tumors are MAPK pathway (RAS-RAF-MEK-ERK) and PI3K pathway (PIK3CA-AKT-mTOR). Both pathways are known to be directly activated by oncogenic signaling by some RTKs (EGFR, MET, KIT), whereas any of the two pathways are activated by many RTKs (VEGFR, IGF1R, EPHA2, FGFR). In addition, MAPK pathway is activated by oncogenic mutations in RAS (in CMM dominantly NRAS) or BRAF leading to a constitutively active MAPK downstream signaling and unregulated expression of Cyclin D1 protein as one of the high impact oncogenic events (Figure 5)(16).

Some G-protein coupled receptors (GPCRs) have been shown to carry mutations in the intercellular C-terminal part rendering them to signal constitutively downstream activating MAPK pathway. GRM3 mutations are best studied leading to increased ERK phosphorylation, although the exact downstream signaling details are not known (Figure 5)(17) In addition, phenotype-related GPCR MC1R with downstream signaling via the cyclic AMP mediated pathway may be involved in oncogenic signaling in CMM.

Similarly, in UM, constitutive MAPK pathway activation has been shown to take place, but the identified alterations associated with this activation are overexpression of RTKs (KIT, IGF1R and MET) and constitutive signaling from GPCRs GNAQ and GNA11 leading to activation of MAPK, but also signaling to PLCγ in the PI3K-AKT pathway (18).

Figure 5. Cartoon depicting some major oncogenic pathways in CMM and their upstream activation. Data compiled using (19), (20),(21),(22),(23),(24)
In malignant melanoma, a number of mechanisms leading to neoplasia have been described. Cutaneous malignant melanoma development has been known since 1994 to be associated with sporadic loss of p16\textsuperscript{INK4A} protein translated from one of two reading frames of the gene \textit{CDKN2A} . Similarly, association between germ-line mutations in \textit{CDKN2A} and aggregation of CMM in families has been recognized (25). \textit{CDKN2A} has two reading frames giving P16\textsuperscript{INK4A} protein which is a repressor of cyclin dependent kinases CDK4 and CDK6, preventing their association with type D cyclins, and most prominently cyclin D1. This results in negative regulation of the retinoblastoma protein due to hypophosphorylation preventing Rb-E2F1 association and E2F1 function as a transcription factor. Loss of this tumor suppressive mechanism leads to attenuated G1-S cell cycle blockage and inability to induce cellular senescence. The second reading frame gives p14\textsuperscript{ARF} protein which inhibits MDM2. Loss of this tumor suppressive protein inhibits the p53-dependent functions in apoptosis and prevents G2/M cell cycle checkpoint engagement (26).

CMM are loss of the tumor suppressor gene, \textit{CDKN2A} derived proteins and constitutive, oncogenic activation of the RAS-RAF-MEK-ERK (MAPK) pathway by oncogenic mutations, primarily in genes for BRAF and NRAS. When accounting only alterations (mutations and copy number changes) in any of these three genes, 67% of CMM possess such alterations with 32% in \textit{CDKN2A}, 39% in \textit{BRAF} and 21% in \textit{NRAS}. These alterations are convincingly related to melanoma development as \textit{BRAF} and \textit{NRAS} alterations are significantly mutually excluding both being within the MAPK pathway, and that virtually all alterations are deactivating for \textit{CDKN2A} (deletions and mutations), but activating for \textit{BRAF} and \textit{NRAS} (mutations and amplifications)(28)(TCGA database). In addition, \textit{CDKN2A} is deactivated transcriptionally by promoter methylation in \textasciitilde20 % of melanoma metastases (27).

Among relatively common alterations not accounted above are the enhanced mRNA expression or, rarely, mutation of GLI family zinc finger 1/2/3 (GLI1/GLI2/GLI3) in 8%, 11% and 16% of CMM. The two former are positive transcription factors of the sonic hedgehog (SHH) pathway, whereas GLI3 is regarded a negative regulator for the same pathway. The pattern for the aberrations does not support a simple association to CMM, in particular as the protein expression of both GLI1 and GLI3 appears to be elevated in CMM tumors (28)(TCGA database), (29)(Human Protein Atlas). In addition and important for CMM tumors without \textit{BRAF} or \textit{NRAS} mutations, cyclin D1, the commonly deregulated cyclin in many cancers including melanoma and a downstream target for transcriptional activation by MAPK pathway and a RB1-E2F1 transcriptional target, is increased by mRNA up-regulation in 10% and by gene amplification in 5 % of CMM tumors. The gene amplifications are mainly found in acral or lentiginous subtypes which are associated with chronic sun exposure. A similar downstream example of aberrant signaling in melanoma is the increased mRNA expression and gene amplification of the transcription factor v-myc avian myelocytomatosis viral oncogene homolog (MYC), present in 9 % of CMM (with additional, rare mutations) (28) (TCGA database). These two last aberrations are likely to represent downstream signaling activation and may have effects equal to upstream activating oncogenic events for these tumors.
1.4.3 RTK activation in CMM

In addition to these genetic features CMM tumors display a prominent activation of multiple receptor tyrosine kinases (RTKs), primarily by enhanced mRNA and protein expression rather than by mutation or gene amplification. The RTKs found to be commonly deregulated account for 14% alterations in CMM for the hepatocyte growth factor receptor MET proto-oncogene (MET), 13% for the insulin-like growth factor 1 receptor (IGF1R), 13% KDR (VEGFR and VEGFR2), 12% for KIT, 11% EGFR, 11% PDGFRA and 10% in EPHA2. The increased mRNA expression of RTKs may possibly be partially a result of downstream RTK signaling activating the melanocyte lineage specific transcription factor MITF via beta-catenin (CTNNB1) or via the leucine zipper transcription factor cAMP responsive element binding protein 1 (CREB1) downstream of MAPK pathway. CREB1 may also respond to cAMP signaling downstream of MC1R. Enhanced mRNA and protein expression in melanoma tumors is also indicated for MET, IGF1R, AXL, ERBB3, PDGFRβ and EPHA2 (29)(Human Protein Atlas),(20). Autocrine signaling has been described for RTKs AXL, PDGFRβ and FGFR3 (30), (20). KIT RTK, which is highly expressed in normal melanocytes, may have both oncogenic and tumor suppressive roles in CMM as it is found to be mutated and or amplified in 11% of CMM tumors (TCGA), but has also been reported to be transcriptionally inactivated by promoter methylation in ~30% of CMM metastases (31).

These RTK alterations are accompanied by loss of PTEN in 16% of CMM leading to loss of negative regulation of PI3K-AKT pathway and its downstream effector mTOR. Also, in 17% of CMM, alterations in neurofibromin 1 (NF1) are found, allowing an enhanced RAS activation due to loss of NF1 induction of GTPases (i.e. loss of GTP depletion for RAS proteins). NF1 is often co-altered with NRAS mutation in CMM, but also, transcriptionally, both decreased and increased indicating diverse signalling. PTEN loss is primarily due to gene deletion or transcriptional silencing and shows a significant co-occurrence with BRAF mutation (28) (TCGA database). In addition mutations, and to a lesser degree mRNA up-regulation, of GRIN2A and GRIN2B, two ion-gates for mono- and divalent cations and glutamate receptors, are commonly found in CMM (19% and 15%, respectively)(28) (TCGA database). These receptors are important in mammalian neuronal cells as excitatory neurotransmitter receptors, but may have functions of similar importance in melanocytes and melanoma cells belonging to the neuro-endocrine lineage of cells.

1.4.4 Downstream signaling from RTKs in CMM

The classic RTKs found to signal downstream in unregulated way are HGF receptor cMET and EGF receptor EGFR. Both receptors have intercellular domains capable of signaling via GRB2 and GAB1 to activate both MAPK and PI3K-AKT pathways (Figure 5). The signaling may be constitutively active as a result of RTK mutation leading to persistent tyrosine kinase activity by the RTK kinase domain or by mutational inactivation of RAS locking the RAS protein in a constitutively active, GTP-bound state. This constitutive activation leads to a deregulated signaling via MAPK and PI3K-AKT pathways resulting in increased proliferation, inhibition of apoptosis, loss of contact inhibition, altered cellular metabolism, increased migration and enhanced metastatic capacity of melanoma cells.
Among the most important downstream targets of MAPK are the AP-1 transcription complex (Jun/Fos) and transcription factors CREB1, ELK1 and STAT3. The most prominent downstream effector for the PI3K-AKT pathway is regarded to be mTOR (Figure 5).

ERBB-family RTKs like EGFR, ERBB2 and ERBB3 may have a different signaling via formation of heterodimers between EGFR or ERBB2 and ERBB3, followed by trans-phosphorylation of the auto-phosphorylation-negative ERBB3 kinase domain (Figure 5). These heterodimers are not inhibited by common small molecule inhibitors of EGFR or targeted antibodies for ERBB2 resulting in a constitutive activation of the PI3K-AKT pathway.

The non-RTK dependent effects include overexpression or aberrant phosphorylation of Src family kinases leading to downstream activation of STAT3 and STAT5, two transcription factors with pro-survival effects via inactivation of apoptosis (23) JAK activation of STATs may take place in a subgroup of melanomas, but mutations in JAK1 and JAK2 are rare (28) (TCGA database).

1.4.5. Genetic aberrations in uveal melanoma

Unlike CMM, in uveal no BRAF or NRAS mutations are demonstrated in UM tumors (32). Instead, oncogenic mutations in two G-protein coupled receptors, guanine nucleotide binding protein (G protein), q polypeptide (GNAQ) and guanine nucleotide binding protein (G protein), alpha 11 (Gaq class) (GNA11), two α-type G proteins that are frequently found to be mutated in UM in glutamine 209 (33% and 39% of UM, respectively) rendering the proteins constitutively active in the GTP-bound state (Figure 6). CMM carries GNAQ and GNA11 mutations also, but at a much lower frequency ~1.5% of CMM. Few inactivated tumor suppressor proteins have been demonstrated in UM, BAP1 being the sole commonly found tumor suppressor gene found to be altered (18) but recently epigenetic inactivation of p16INK4A has been reported in UM (33) indicating a role for p16INK4A in UM development. The metastatic potential of UM has been reported to be associated with a decrease in ERBB3 mRNA and an increase in KIT mRNA (34)

On the other hand, loss of heterozygosity in 3p arm (including locus for BAP1) and amplification of 8q are associated with increased propensity of the UM to metastasize and therefore a worse prognosis (34).

Aberrant RTK protein expression has also been associated with UM tumors for RTKs KIT, IGF1R and MET, although no frequent mutations in these RTKs have been demonstrated (Figure 6)(35), (36),
Figure 6. Cartoon for known activated oncogenic pathways in UM. The data was compiled from (18), (35), (36).
1.5 Melanoma epigenetics

In addition to gene deletion and mutational alteration of protein activity, epigenetic alterations in DNA and histones have recently become a part of melanoma genetic aberrations. Epigenetic alterations are regarded to be related to transcriptional deregulation leading to loss of tumor suppressor gene expression (transcriptional silencing) and/or up-regulation of genes for proteins with enhanced expression in malignant cells compared to normal cells (37) Also, epigenetic alterations of transcription with effect on transcription factor binding to DNA have obvious potential to lead to cellular adaptation towards increased survival (38).

1.5.1 Transcriptional silencing and histone modifications

Epigenetic regulation of gene expression has been associated with silencing of tumor suppressor genes in melanoma, more prominently genes like CDKN2A, RASSF1A and PTEN (39),(40), (41),(42). The transcriptional silencing by aberrant promoter methylation is associated with di-methylated lysine 9 in histone 3 (me2H3K9), resulting from activity of H3K9 histone methyltransferases like SETDB1 and EHMT2 and counteracted by H3K9 histone demethylase LSD1 (43). The transcriptional repression is believed to require binding of methyl CpG binding protein 2 (MECP2) and methyl CpG domain binding protein 1 and 2 (MBD1 and MBD2) activity. MBD1 is also a binding partner to SETDB1 (38).

Another epigenetic complex that regulates gene transcription is the polycomb repressive complex 2 (PRC2) that interacts with DNA methyltransferases and is associated with tri-methylated H3K27. EZH2 is the catalytic subunit of PRC2 with H3 methyltransferase activity. Ectopic overexpression of miR-124a, that regulates EZH2 negatively, has been shown to decrease tumor growth in vivo in CMM and to be related with tumor aggressivity. Similar effects have been observed by miR-124a in UM verifying that EZH2 down-regulation is important in melanoma (44, 45) (46).

Other histone lysine modifications that are suggested to be related with epigenetic modification of transcription and accessibility of gene promoters are histone (lysine) acetyltransferases (HATs) like PCAF that is associated with p300 and CREB transcriptional activation and histone deacetyltransferases (HDACs) that remove acetyl groups from histone lysines (37).

Similar to CMM transcriptional silencing by promoter methylation for p16INK4A in UM has been reported in 50% of tumors analyzed, but only six respective 22 tumors were included in these screenings (33), (47).

1.5.2 miRNA related regulation

Additional epigenetic regulation of protein expression derives from miRNA that may have effects on gene expression or protein stability that affects the oncogenic process. One example of this is miR-125b having been shown to be a negative regulator of the MAPK
downstream target c-Jun by altering translation or protein stability. Forced expression of miR-125b was found to suppress cellular proliferation and migration in line with MAPK pathway down-regulation (48).

1.5.3 Alterations in epigenetic regulators

Loss of gene regulation in melanoma may be association to epigenetic regulation may be a result of mutations in genes coding for epigenetic regulators of gene expression. Alterations in EZH2 or SETDB1 are found in 37% of all CMM tumors accounting increased mRNA transcription, gene amplification and mutation (28)(TCGA database). This high frequency together with appearance of EZH2 mutations in the catalytic domain suggest a role these and possibly other epigenetic regulators to be associated with the oncogenic potential of melanoma.

1.6 Malignant melanoma therapy

1.6.1 CMM therapy options and outcomes

Although early detection and surgical removal of melanoma does, in ~90% of diagnosed cases offer cure for the disease, when surgery is not sufficient to remove the melanoma cells, risk for metastatic spread of the disease is high. For cutaneous melanoma primary tumor thickness (Breslow thickness) and melanoma propensity to metastasize to multiple tissues (lymphatic tissues, soft tissues, lung, CNS) (49) For uveal melanoma, the metastatic spread is often limited to the liver and may allow therapy using liver perfusion and high local dose of chemotherapy (50).

The classic systemic therapy for disseminated cutaneous melanoma has been alkylating agents like DTIC (dacarbazine), TMZ (temozolomide) as monotherapies or together with platinum compounds (cisplatin or carboplatin), other alkylating drugs (fotemustine, melphalan) or immunostimulatory agents (interleukin-2 (IL-2), interferon (IFN)). Neither the single agent therapies nor combinations of different types of agents have been particularly successful in therapy, displaying response rates (RR) of 5-12% and median overall survival (OS) <1 month to 8 months (51). However, a large phase III trial NCT00091572 for stage IV patients, 429 patients receiving TMZ (150mg/m² orally 7 consecutive days every 14 days) and 430 patients receiving DTIC (1000mg/ m² intravenously day 1+/- 3days every 3 weeks) did show median OS of 9.1 months and 9.4 months, respectively, with corresponding objective response rates of 10% and 14% for TMZ and DTIC (52)(www.clinicaltrials.gov). In a meta-analysis data, and allowing pooling of data for DTIC and TMZ clinical trial monotherapies, a median OS length of 7.9 months has been established for chemotherapeutic drugs (51). As few patients respond to therapy and the sustained therapeutic efficacy is poor, alternatives to classic chemotherapy have been to treat patients systemically with high,
intermediate or low dose IFN in the adjuvant setting. Meta-analysis of these clinical trials (3593 treated patients vs 2539 control patients (observation)) showed an overall decreased risk of death with IFN administration (OR 0.88 [CI 0.79-0.99], p=0.03) displaying a significant, but rather weak enhancement of therapeutic efficacy (51).

1.6.2. Uveal melanoma therapy options and outcomes

For uveal melanoma, surgical removal of the eye, enucleation, is often required. Overall about 50% of all uveal melanoma patients develop metastatic disease, nearly 90% of these metastases being hepatic (50). As ocular melanoma has, similar to cutaneous melanoma, high propensity to metastasize, systemic therapy used historically composed of combination of dacarbazine and cisplatin together or combined with other chemotherapeutic agents or immunostimulatory drugs IL-2 or IFN (53). Local therapies to avoid enucleation like brachytherapy (54), transpupillary thermotherapy (TTT), stereotactic radiotherapy showing 73% (95% CI; 61-86%) progression free survival at 3 years after single dose stereotactic radiotherapy in 60 patients, but these therapy forms are often limited to use for eligible patients only (anterior involvement for brachytherapy and TTT, stereotactic radiotherapy for posterior tumors) and is associated with partial loss of eyesight due to damage to normal tissues(50, 55).

MEKi selumetinib was compared to chemotherapy with TMZ or DTIC as a monotherapy in an open-label phase II trial with 101 patients. Therapy with selumetinib was shown to prolong median PFS (15.9 weeks 95% CI [8.4-21.1] compared to 7.0 weeks 95% CI [4.3-8.4] for TMZ/DTIC) and a non-significant increase for median OS (11.8 months 95% CI [9.8-15.7 months] compared to 9.1 months 95% CI [6.1-11.1 months])(56). In this trial, the randomization for the therapy groups resulted in the chemotherapy group to receive a higher proportion of patients with measured levels of elevated serum lactate dehydrogenase (59% vs 50%) possibly leading to a bias towards shorter than expected PFS and OS for patients in the chemotherapy group. Also, 37% receiving MEKi selumetinib had dose reductions compared to 2% for the chemotherapy group plausibly causing opposing bias in the study. Ipilimumab (anti-CTLA-4 Ab) was also tested in a phase I trial for 13 patients, but no objective responses were observed (57).

Regarding therapy efficacy in UM, loss of BAP1 expression in UM has been suggested to be related to induction of de-differentiation leading to a neuro-endocrinal stem cell like phenotype in UM cells associated with a pronounced membranous β-catenin staining. De-differentiation may have pronounced effect on the therapy efficacy as stemness is generally associated with low proliferative capacity but high survival capacity (58).
1.6.3 Targeted and immunotherapies in cutaneous melanoma

The introduction of targeted therapies for therapy of cutaneous melanoma has led to improvements of patient care (59). A phase III study comparing use of inhibitor against codon 600 mutated BRAF (Vemurafenib/Zelboraf, NCT01006980) versus chemotherapy with DTIC) in 336 treatment naive patients showed significantly prolonged OS for patients treated with targeted therapy with OS of 84% of patients at 6 months (95% CI; 78-89%) compared to 336 patients receiving DTIC 64% (95% CI;56-73%). This study allowed crossing-over why final PFS and OS measurements were not reached (60),(52, 61)(www.clinicaltrials.gov).

In line with this, addition of inhibitor of non-mutated MEK (trametinib) did increase the efficacy of another BRAFi, dabrafenib, further compared to using dabrafenib alone (NCT0158464) combination median PFS 9.3 [CI 7.7-11.1] RR 67% vs. monotherapy median PFS 8.8 months [CI 5.9-10.8] with RR of 51%. This can be compared efficacy measures of monotherapy with DTIC with median PFS 2.3 [CI 2.2-2.4] ORR 14% and monotherapy with TMZ median PFS 2.2 [CI 2.1-2.3] ORR 10% (61)(www.clinicaltrials.gov)

These improvements in cutaneous melanoma therapy have been highly necessary, but require sustained drug administration and do not appear to give stable remissions. Median OS for BRAFi dabrafenib treated patients was 18.2 months [CI 16.6-not reached] at update of the study at Dec 2012 (Journal of Clinical Oncology, 2013 ASCO Annual Meeting Abstracts. Vol 31, No 15_suppl (May 20 Supplement), 2013: 9013), showing that half of the patients in dabrafenib monotherapy had succumbed at 1.5 years after therapy start.

Cutaneous melanoma is considered to be responsive to immune therapies due to observations of rare, spontaneous regressions of primary tumours and more common appearance of tumor-infiltrating lymphocytes (TILs) in melanoma tumours (62) immunotherapy against disseminated cutaneous melanoma has been developed in parallel with targeted therapy.

The first immunotherapeutic to show potency for therapy was the cytotoxic T lymphocyte-associated antigen-4 blocking antibody (anti-CTLA-4 antibody). CTLA-4 is a receptor found on TILs and regulatory T cells and does have higher affinity with the T cell co-activating receptor B7 (necessary for antigen-presenting derived immune response) than its endogenous ligand CD28. The latter is clearly a disadvantage in tumour cell vaccination with tumour cell derived antigens. A prolonged inactivating signaling for TILs leads to anergy of the cells. Blockage of CTLA-4 with the CTLA-4 specific antibody ipilimumab/Yervoy has led to a slow-onset therapeutic effect and short PFS but, in a minority of patients, a long-lasting remission. This was originally shown in a phase II, three-arm, clinical trial involving 676 stage III and IV patients comparing glycoprotein 100 alone, ipilimumab/Yervoy alone or a combination. Inclusion of ipilimumab/Yervoy was statistically beneficial for the patients, with ipilimumab/Yervoy alone (137 patients) showing a median PFS of 2.86 months (95% CI 2.76 to 3.02), but a median OS of 10.1 months (95% CI 8.0 to 13.8). In this clinical trial, 7 (of total 14) deaths were due to severe immune response related adverse events (NCT00094653,
Given the rather serious side effects of ipilimumab/Yervoy, in particular for GI tract, response markers for use of this drug would be very helpful.

A clinical phase I, two-arm trial, NCT01295827 (64), with the anti-programmed-death-receptor-1 (anti-PD-1) antibody pembrolizumab in 173 patients with ipilimumab-refractory advanced melanoma showed an ORR at 26% at both doses used (2mg/kg or 10mg/kg). Only 3% of patients experienced grade 3-4 adverse effects.

As a part of another clinical dose-escalation study for anti-PD1 antibody, nivolumab, 107 systemically pre-treated melanoma patients were studied (mixed cutaneous and uveal melanoma patients, NCT00730639) (65). Median PFS was 3.7 months, but at 2 years 27% of patients had not progressed. 2-year OS was 43% and the median OS was 16.8 months showing a reasonably rapid onset of therapeutic efficacy and improved OS compared to chemotherapy. This clinical trial is difficult to evaluate due to multi-dosing and mixed patient inclusion, but shows that previously treated patients are not refractory to this therapy.

Collectively, the clinical trials in cutaneous melanoma show a successive improvement in PFS and OS for melanoma patients with introduction of targeted and immunotherapies, but also a shift in therapy resistance from intrinsic to acquired resistance. The acquired resistance to targeted therapy is a very large field for research in CMM today, but the clinically most relevant question is likely not why resistance appears but how to avoid it from appearing in the first place.

1.6.4 Targeted and immunotherapies in uveal melanoma

A retrospective study has indicated therapies with ipilimumab, MEKi or anti-VEGF Ab bevacizumab to provide an inferior therapeutic outcome compared to local therapies (66).

1.6.5 Future perspectives for melanoma therapy

In general, the high mutational rate observed in (cutaneous) malignant melanoma cells is a potential source of therapy resistance. It increases the likelihood for the melanoma cells to become heterogenic as clones of melanoma cells develop aberrations in same pathways but in different components of the pathways (28)(TCGA database) (67)(the COSMIC database).

The heterogeneity inducers other than diversity in genetic aberrations may include hypoxia, tumor microenvironment, selection by therapeutic agents and may be executed by reversible alterations of epigenetic changes in miRNA profiles or histone modifications and be seen as adaptations. Malignant cutaneous melanoma exhibits showing relatively small number of tumors activated by mutated RTKs and non-RTKs whereas overexpression of the RTKs, often several in same tumors (including IGF1R, MET, SRC, EPHA2, KIT, ERBB3, EGRF), is a relatively common feature (28)(TCGA database).
Two additional and closely related concepts are the phenotypic plasticity and stemness observed in melanoma cells. Reasons behind the phenotypic plasticity are largely unknown, although involvement of the melanosomal, lineage-specific transcription factor, MITF, has been suggested. In this model, low expression of the melanotic lineage related transcription factor MITF is associated with invasive and stem cell markers and ZEB2 whereas high MITF expression is associated with differentiation markers, high proliferative capacity and high ZEB1 expression (68).

If phenotypic plasticity does take place reversing the malignant cell phenotype depending by extracellular stimuli, the targeting of tumor cells may become much more difficult. For traditional chemotherapy with DTIC, therapy resistance has been shown to largely depend on loss of p16\(^{INK4A}\) and alterations in p53 pathway (27). If phenotypic plasticity is associated with genetic aberrations being replaced with reversible transcriptome-level alterations, then future therapies need to address the therapy-induced phenotypic variation. The transition from general cytotoxic therapies to targeted therapies has led to a change in therapy-associated resistance: from intrinsic resistance to acquired resistance. Whether the recent advances in immunotherapy can help to avoid the acquired therapy resistance is to be seen.
2 AIMS OF THE THESIS

The research presented in the thesis aims at understanding the mechanisms behind the aggressive nature of melanoma, here cutaneous and uveal melanoma. The ultimate purpose of the studies is to find prognostic markers, therapy efficacy markers and combination treatments that allow a more efficient, personalized cancer therapy to be used, the basis of which, I believe, is the genetic alterations and epigenetic modifications that are found in the tumors.

The specific aims for the presented papers are:

Paper I: to account for all known genetic and epigenetic causes of p16<sup>INK4A</sup> tumor suppressor protein inactivation within a group of CMM tumors and to relate these with p16<sup>INK4A</sup> protein expression. Secondarily, relate the genetic and epigenetic alterations in p16<sup>INK4A</sup> to pathological parameters and oncogenic mutational status.

Paper II: to screen for germ-line aberrations within a family with multiple cases of UM and a low-age of onset proband. The primary aim was to study the hereditary causes of UM, but also appearance of cutaneous melanomas within the family was investigated. The study was aimed at germ-line aberrations that segregate with UM and are present in the proband tumor.

Paper III: to re-evaluate the previously published lack of association between MGMT inactivation and efficacy therapy with alkylating agents. Comparison between single agent therapy with dacarbcine (DTIC) or temozolomide (TMZ) and combination therapy including DTIC/TMZ in relation to MGMT promoter methylation, MGMT mRNA expression, therapy related toxicity and therapy outcome were studied.

Paper IV: to investigate an in vitro observation regarding administration sequence dependency for the outcome for co-administration of mutant BRAF inhibitor and temozolomide. The roles of DNA damage response in relation to therapy efficacy and MGMT induction by mutant BRAFi were specifically focused on.
3 RESULTS AND DISCUSSION

3.1 Results and Discussion, Paper I

Results. Screening of transcriptional silencing of p16$^{\text{INK4A}}$ via promoter methylation in CDKN2A and presence of mutations in p16$^{\text{INK4A}}$ coding sequence revealed presence of silencing in 25% of tumors analyzed (15 tumors positive for methylation out of 59 eligible for analysis). Table 1 presents the collective results of the study with p16$^{\text{INK4A}}$ deletion data derived from a previous publication (69).

Twenty-one of the 77 analyzed metastases had bi-allelic deletions, in practice excluding them from mutational and methylation analyses (3 randomly chosen bi-allelic tumors were tested in the analyses to ascertain this). Unexpectedly, p16$^{\text{INK4A}}$ protein expression was found to be absent in all of the 15 tumors confirmed to be positive for promoter methylation. Presence of p16$^{\text{INK4A}}$ coding sequence alterations were confirmed in 9 out of 56 tumors successfully analyzed (16 % of total), with 4 tumors exhibiting typical UV exposure associated variants. Within NRAS mutated tumors only 9 % (n=2) were found to carry a p16$^{\text{INK4A}}$ mutation despite that NRAS mutated tumors made up 41 % of all tumors eligible for analysis (Table 1). On the other hand, transcriptional silencing was significantly more common in NRAS mutant metastases compared to non-NRAS mutant metastases (p=0.0004, Table 2).

Overall p16$^{\text{INK4A}}$ nuclear and cytoplasmic protein expression was found to be lost in 55 tumors (82%, N$_{\text{analyzed}}$= 67). In 26 out of total 56 tumors (46%) no gene deletion nor mutation neither transcriptional silencing could explain the lack of nuclear p16$^{\text{INK4A}}$ protein expression, leaving of the observed loss of nuclear p16$^{\text{INK4A}}$ protein expression loss unexplained by the mechanisms accounted for in the study.

Fourteen of the studied metastases were accompanied by analysis of p16$^{\text{INK4A}}$ protein expression in the corresponding primary tumor (Table 1). Six primary-metastasis pairs exhibited loss of p16$^{\text{INK4A}}$ in the metastatic process, six pairs had identical status and two had reversed status with negative primary tumor and positive paired metastases.

Discussion. The high proportion of tumors with negative p16$^{\text{INK4A}}$ nuclear staining suggests that, in CMM, a large part of loss of p16$^{\text{INK4A}}$ expression remains to be explained by additional mechanisms. This paper contributes to the basic understanding of p16$^{\text{INK4A}}$ inactivation in CMM and points out the knowledge gap regarding alternative mechanisms by which p16$^{\text{INK4A}}$ protein expression can be lost.

There are other suggestions to add to mechanisms leading to loss of p16$^{\text{INK4A}}$ expression: ID1 protein may function as a transcriptional repressor of CDKN2A (70). This has, however, later been disputed (39). In addition, high expression of enhancer of zeste homolog 2 (EZH2) protein, a member of polycomb protein group, has been shown to be associated with low p16$^{\text{INK4A}}$ protein expression (46). In addition, transcriptional suppression by β-catenin protein...
has been shown to be able to transcriptionally suppress p16<sup>INK4A</sup> gene expression (71).

Finally, and relatively recently, a transcriptional co-repressor, CtBP1, has been suggested to transcriptionally inactivate p16<sup>INK4A</sup> (72). Several of these inactivating mechanisms have, however, not been demonstrated to be differentially expressed in melanoma tumors compared to normal melanocytes or normal skin.

The antibody clone JC8 for detection of p16<sup>INK4A</sup> used in Paper I has been evaluated and found to give cytoplasmic and nuclear staining for p16<sup>INK4A</sup> protein with staining intensity being affected by siRNA silencing of p16<sup>INK4A</sup> gene and Western blot giving a band of correct molecular weight (73). This is, however, not evidence for proper identification of p16<sup>INK4A</sup> as a stringent evaluation would require knock-out and knock-in tissue for p16<sup>INK4A</sup> gene for the human protein.

This paper has until the end of February 2015 been cited 12 times, but no citing publications present data to verify or to question the published significant association between transcriptional silencing and presence of a NRAS mutation. One publication commented on the lack of mRNA expression data in studies in general, including our study. This is simply due to that we were not confident about being able to measure mRNA expression for p16<sup>INK4A</sup> reliably using the archival materials available. Developments in the field of RNA extraction from FFPE material have profoundly diminished this uncertainty.

3.2 Results and Discussion, Paper II

Results. The clinical manifestations of the proband UM are displayed in Figure 1. A large, highly vascular, intra-uveal melanoma of posterior origin was removed by enucleation. The tumor was positive for HMB-45 suggestive of presence of melanosomes of low differentiation grade and appeared to have a mixture of epithelioid and spindle-shaped cells. The metastatic tumor spread was evident in a CT scan of abdomen and scintigrafi of the skeleton showing numerous hepatic and skeletal metastases.

Next-generation exome sequencing was performed using peripheral blood derived DNA from the proband, her sister and both parents (of which father was the obligate carrier). After mapping, and filtering of the variants likely to be artefactual, the remaining sequence variants were filtered to remove common SNP and variants present in the healthy non-carrier (mother). The remaining variants are listed in Supplementary Table 2. As a frame-shift, truncating insertion in the BAP1 gene was observed in the proband DNA and the BAP1 germ-line alterations had been linked to UM risk, DNA extracted from the proband-derived tumor was examined using Sanger sequencing. The preservation of the observed frame-shift insertion chr3:52,443,617 c.75insC in the tumor DNA was verified and a microsatellite-based LOH analysis was performed to confirm corresponding loss of the wild-type BAP1 locus allele at 3p21. This frame-shift insertion results in a premature termination of the BAP1 protein in amino acid 43.
After analysis of DNA from additional family members by Sanger sequencing, the observed frame-shift insertion was found to segregate with UM within the family. In Figure 2, the family pedigree with the observed BAP1 mutational status and cancer diagnoses are given together with a schematic picture of BAP1 with the insertion in exon 4 and representative fragment analyses results for the proband tumor microsatellite analysis (two lower panels) showing nearly complete loss of the D3S1578 157 bp repeat fragment and the D3S3026 211 bp repeat fragment in tumor DNA compared to blood DNA. The inheritance for the microsatellite fragments is given by comparisons of the microsatellite analysis for the parents blood derived (upper panels) with the proband’s corresponding blood DNA pattern (left lower panel). In Figure 2D electropherograms for Sanger sequencing for mutation positive and mutation negative BAP1 screening analyses are presented for the two out of three brothers in first generation (I-1 and I-2), subject I-1 being analyzed for an archival UM tumor and subject I-2 for an archival prostate cancer tumor, probably showing a hemizygous BAP1 c.75insC in the former (poor DNA quality) and a heterozygous BAP1 c.75insC in the latter. A confirmation of the next generation analysis of proband’s parents in shown, father displaying a heterozygous germ-line BAP1 c.75insC, mother wt sequence (panels II-1 and II-2). The two bottom panels show the Sanger sequencing results for the proband blood DNA (heterozygous c.75insC) and UM tumor (hemizygous BAP1 c.75insC). Note that the hemizygous BAP1 c.75insC was in both cases associated with UM tumors (I-1 and III-1) whereas the LOH for BAP1 locus was absent in the prostate cancer tumor (I-2) and that the proband’s father carried a germ-line BAP1 c.75insC, but had no cancer diagnosis.

Discussion. This publication was early in showing that BAP1 mutations occur as hereditary mutations in UM and that carriage of these mutations is associated with increased risk for CMM development. Both of these aspects have been since shown in a number of publications (summarized in (74), Table 2). It should be pointed out that this publication reflects the urgency felt by the involved researchers: the clinical condition of the young proband was rapidly deteriorating and her biological relatives could have high risk for developing similar malignancies. All this together with the apparent association between the truncating BAP1 frame-shift insertion c.75insC led to a narrow focus on this aberration within the paper.

In hindsight, the other findings from the study (Supplementary Table 2) should have been more emphasized as the extended analyses for the family showed the penetrance of the germ-line BAP1 mutation for UM and CMM to be limited to ~50% (4 family members UM or CMM out of 8 BAP1 c.75insC carriers) when age was not regarded. This suggests that for the strongly affected family members, BAP1 c.75insC may not have been the only susceptibility gene aberration emphasizing the usefulness of full genome sequencing data.

There are seven external citations for this publication, two of which are review articles. This publication is not challenged or commented in any of the citing articles, but general comments concerning positive association between UM and CMM susceptibility and carriage of truncating BAP1 mutations are done.
3.3 Results and Discussion, Paper III

Results. This paper presents a retrospective study on two CMM patient cohorts for therapy outcome comparison between patients with or without methylated promoter for MGMT. This spontaneous methylation is, similar to methylation of p16\textsuperscript{INK4A} promoter in Paper I, transcriptionally inactivating and leads to low or absent expression of the DNA repair protein MGMT. MGMT counteracts the effect of alkylating chemotherapy by removing the methyl groups from O6-methylguanine, thereby eliminating the most cytotoxic DNA lesions formed by dacarbazine (DTIC) or temozolomide (TMZ). The cohorts consisted of DTIC or TMZ single agent therapy patients (n=74, cohort S, mainly Swedish patients) and combination therapy patients (n=79, cohort C, mainly Belgian patients). The patients cohorts are described in Table 1 showing significant differences between the cohorts regarding median age at the start of therapy (higher in S cohort) and proportion of patients with normal or elevated level of pre-therapy serum lactate dehydrogenase (LDH) in blood (cohort S having higher proportion patients with elevated level).

In Figure 1, images from the analyses of MGMT promoter methylation are shown, panel A and B including results of same four FFPE samples, using methylation-specific PCR and HRMA, respectively. Panel C shows a comparison of the pyrogram for the negative and positive methylation control DNAs derived from two melanoma cell lines. Table 2 shows a comparison of patients found to carry MGMT methylation positive (n=28) or negative (n=102) tumors. This stratification showed significant differences between the patient groups regarding follow-up, progression-free survival (PFS) and overall survival (OS) in a univariate analysis. Importantly, the pre-therapy serum LDH did not differ between these groups indicating no difference in tumor burden prior to therapy. When response to therapy within the cohorts was compared to the MGMT methylation status (Table 2) a significant overrepresentation of therapy responders among patients found to carry methylated tumors was observed in cohort S, whereas corresponding association was not found in cohort C. Response here was defined as either complete or partial response, whereas stable disease was or progression was defined as no response. A logistic survival analyses on the pooled cohorts (Figure 2) further displayed a significant benefit for having positivity for MGMT methylation for longer PFS. To investigate whether the effect of MGMT methylation was an independent variable associated with PFS length, a multivariate Cox regression analysis was performed including dichotomized variables for gender, age at therapy start, tumor stage, pre-therapy serum LDH level and MGMT methylation, controlling for cohort association. Gender, pre-therapy serum LDH and MGMT methylation were found to be independent variables for PFS length in these patients.

Discussion. The significant differences between the cohorts regarding age at therapy start and pre-therapy serum LDH levels were likely to be influenced by the site, the cohorts being dominated by patients from one site each (Karolinska University Hospital, Stockholm and Leuven University Hospital, Belgium). These differences reflect the younger diagnosis age among the Belgian patients, a selection bias could possibly have induced this difference as combination therapy is preferentially given to younger patients due to more adverse effects compared to single agent therapy. The significant difference in pre-therapy LDH is influenced by the respective site cut-off for elevated LDH levels, but that cut-off is defined by
the site-specific distribution and range for the absolute LDH levels. Possibly, the cut-off
being changed during the collection period at Karolinska University Hospital as a result of a
general decrease in LDH values at diagnosis by time influenced the distribution of patients
for this cohort. None of these factors were likely to have a profound effect on the MGMT
methylation as being an independent variable defining the PFS length, as the multivariate
model did control for both of these variables. On the other hand, gender was also found to be
an additional independent variable associated with PFS length. This association is not
obvious, as no significant skewness in the gender distribution for patients found to have
MGMT methylated and unmethylated tumors was observed (Table 2) in the univariate
analysis. Importantly though, the patient material from Leuven University Hospital
(constituting a great majority of cohort C patients) was found to have a gender difference
regarding the tumor stages and, consequently, males showed a borderline significance for
having lower tumor stage and median LDH value. This gender difference did, however, not
apply for the cohort S patients, why the initial advantage of males in cohort C patients must
be assumed to have caused the male gender to be independently associated with longer PFS.
Corresponding multivariate analysis for OS did also show the same independent variables to
be significant for OS length as for PFS, with gender being an exception (Supplementary
Table 2S). This difference was studied in univariate analyses showing that despite males in
cohort C had lower tumor stage and pre-therapy LDH levels, comparison of OS length for
males and females did not result in a significant difference. This can probably only be
interpreted as the beneficial effect by the female gender regarding survival as the effect by
MGMT methylation positivity in this cohort did not show significant association to OS length
either in a Kaplan-Meier survival analysis or in a univariate comparison of genders.

In relation to this, the OS length was significantly associated with MGMT methylation
positivity in cohort S, but not in cohort C why the multivariate analysis for OS did show
significant interaction between cohort and MGMT methylation (Supplementary Table 2S).
The inclusion of mRNA measurements for tumors with MGMT methylation status (n=42 and
n=44 for cohort S and C, respectively) did show a highly significant correlation to
methylation (chapter "MGMT promoter methylation in relation to MGMT mRNA
expression") strengthening the validity of the methylation analyses. As presented in the
Supplementary section, the association between the MGMT mRNA expression and MGMT
methylation was strong in both cohorts (Supplementary Figure 5S) while the associations
between HRMA Tm values and response status on one hand, and MGMT mRNA expression
and response status on the other hand, were stronger in cohort S. This is likely to reflect the
enrichment of cohort S patients with complete responders and the clearer association between
therapy outcome with alkylating agents alone compared to outcome with co-administration of
an alkylating drug with an additional agent.

3.4 Results and Discussion, Paper IV

Results. The therapeutic efficacy after co-administration of TMZ and mutant BRAF inhibitor
PLX4720 was found to be dependent on the administration order. This administration order
effect was only found to be associated with increased therapy efficacy when TMZ was
administered before PLX4720 both in the A375 parental cell line and the A375-PLX4720R
resistant subline (Figure 1 panels A-C). No corresponding effect by administration order was, however, observed in the SK-Mel-24 cell line that harbors a transcriptionally silenced MGMT (panels 1D and 1E). To investigate the possible role of MGMT in the combination therapy, both parental and PLX4720 resistant A375 cells were treated with PLX4720 and MEK inhibitor trametinib to show induction of MGMT mRNA by both drugs (Figure 2A). The constitutive MGMT expression of A375-PLX4720R was found to be lower (approximately 50% of corresponding level for parental A375) (Figure 2B). In addition, treatment of A375-PLX4720R with the clinically approved, specific inhibitor lomeguatrib, significantly reduced the MGMT protein level in administration with PLX4720 before TMZ (induction of MGMT was abolished) (Figure 2C). Utilizing Annexin V-FLUOS and PI staining the cytotoxicity of the single agent and combination therapy administrations were investigated. Administration of TMZ for 72h with addition of PLX4720 at 48 h showed similar early apoptotic/necrotic and apoptotic response in both parental A375 and A375-PLX4720R with only PLX4720 or TMZ exposure associated with approximately doubling of both types of cell death. Combination of these drugs enhanced cell death further, and addition of MGMTi lomeguatrib roughly doubled cell death when added to PLX4720 and TMZ (Figures 3A and 3B). To investigate the mechanistic background of these effects, immunoblotting was performed for cell lysates from both parental and resistant cells. Lomeguatrib (patrin) was found to efficiently down-regulate MGMT in both cell lines, PLX4720 was found to upregulate MGMT expression and this up-regulation was abolished by lomeguatrib (Figures 4A and 4B). Tumor suppressor protein p53 stabilization was found to be associated with TMZ administration, but TMZ alone did not induce γH2AX formation at 72h. Instead, combination of PLX4720 and TMZ induced γH2AX formation profoundly but the same was true for combination of MGMTi and PLX4720 for both cell lines (at 72h). The combination of MGMTi with PLX4720 and TMZ showed the strongest induction of γH2AX formation in both cell lines. The time-line for induction of γH2AX in parental A375 (Figure 5) did show separate peaks for induction of γH2AX form by PLX4720 (peaking at 48h) and TMZ (peaking starting at 96h) as expected by the results in Figure 4A and 4B.

Discussion. The studied effect of the administration order for combination TMZ and BRAFi PLX4720 is suggests that when combinations of drugs are employed, the administration order of the drugs may play an important role for therapeutic efficacy. These effects can only be studied pre-clinically given the numerous different drugs for therapy and the possible administration combinations. The data presented in Paper IV advocates a role for MGMT in the combination therapy with TMZ and BRAFi. The enhancing cytotoxic effect by inhibiting MGMT in the combination therapy setting is not surprising given that MGMTi can enhance the effect of TMZ in the combination. On the other side, the induction of γH2AX by addition of MGMTi to PLX4720 compared to PLX4720 or MGMTi (lomeguatrib, Patrin) alone is puzzling (Figure 4A and 4B). In addition to this effect being early compared to the enhanced induction of γH2AX by combining TMZ and MGMTi, the mere suggestion that BRAFi PLX4720 administration is associated with dsDNA break marker despite PLX4720 not being an alkylator is unexpected. Also, γH2AX expression was enhanced by inhibition of MGMT
together with BRAFi compared to BRAFi alone despite that MGMT has only been associated with efficacy of alkylating agents. These effects are not explained by the data presented in this manuscript, but offer surely a basis for further experiments.
4 THESIS CONCLUSIONS

Paper I: A large part of loss of protein expression for p16\textsuperscript{INK4A} in cutaneous melanoma metastases cannot be associated with conventionally studied inactivating mechanisms like gene deletion, gene mutation or p16\textsuperscript{INK4A} mRNA transcriptional inactivation by promoter methylation. A significant larger proportion of NRAS mutated metastatic CMM tumors appear to have simultaneous transcriptional inactivation compared to non-NRAS mutated tumors.

Paper II: Unlike CMM, deletion or mutation in p16\textsuperscript{INK4A} gene disruption appear to be rare in UM. Instead, BAP1 tumor suppressor gene is found to be mutated in the germ-line of hereditary UM families and loss of BAP1 wt allele in locus 3p21 is associated with UM development. The penetrance of the truncating BAP1 germ-line mutation was, however, found to be limited, but an association to an increased risk for CMM and formation of dysplastic nevi was observed.

Paper III: Opposite to earlier publication (75) presenting results for MGMT inactivation in CMM tumors for not being beneficial for therapy efficacy when using alkylating agents DTIC or TMZ, our published results suggest positivity for MGMT methylation being associated with longer PFS. The effect of MGMT silencing, confirmed by MGMT mRNA down-regulation, was more evident in DTIC/TMZ single agent therapy patients as would be expected by combination therapy resulting in responses unrelated to DTIC or TMZ.

Paper IV: The observed effect of administration order on efficacy of combination therapy with TMZ and mutant BRAF\textsuperscript{i} PLX4720 with enhanced efficacy with TMZ administered first was found to be related to PLX4720 induction of MGMT. As a consequence, enhanced MGMT in PLX4720 pre-incubation lowered the efficacy of following TMZ administration. Early time-point effects by PLX4720 on dsDNA strand break marker $\gamma$H2AX formation were observed with enhanced induction of $\gamma$H2AX by combining PLX4720 with MGMT\textsuperscript{i} lomeguatrib despite that MGMT is not known to be associated with targeted therapy.
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