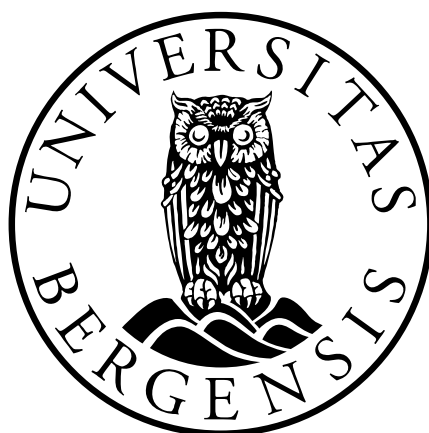


Genetic biomarkers as prognostic and predictive factors in metastatic malignant melanoma

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Dissertation for the degree philosophiae doctor (PhD)
at the University of Bergen

2010

Dissertation date: 4808202

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Acknowledgements

The work presented in this thesis started September 2005 at the facilities of Dept of Molecular Biology, and was done in collaboration between Section of Oncology, Institute of Medicine, Faculty of Medicine, the Department of Molecular Biology, University of Bergen and the Department of oncology, Haukeland University Hospital. I am grateful to the institutions for giving me the opportunity to perform my experiments in this academic environment. The work was funded by the Norwegian Cancer Society for which I am grateful.

The work was performed under the main supervisor, Professor Per Eystein Lønning, and the co-supervisors Professor Jürgen Geisler and Professor Johan Lillehaug. I appreciate the high medical expertise, hard work and excellent writing guidance provided by Per Eystein; Johan's scientific enthusiasm and ability to make a creative scientific atmosphere at his lab and for keeping his door open for discussing problems along the way; Jürgen, for inviting me into this project, for helping me to keep my moral when the progression was slow.

Also, I am grateful for the contribution from the staff at the Department of Surgery, Section of Plastic Surgery and the Dept of Oncology, Haukeland University Hospital in the biopsy and clinical data collection, in particular the oncologists, Stephanie Geisler and Svein Inge Helle and the plastic surgeons, Marit Nordskar and Åse Sivertsen. Of course, I want to recognise all the patients who volunteered to participate in this study.

Most of the laboratory methods used were established by the Breast Cancer Group and introduced to me by Beryl Leirvåg and Stian Knappskog. Also, I want to recognise Nhat Kim Doung and Elise de Faveri for technical assistance.

The collaboration with Professor Åke Borg's research group at Lund University, Sweden has been a fruitful experience, and I am grateful for their contribution in the array based experiments.

Lastly, I want to recognise my wife, Hilde Schøning, for her patience during these years and for giving birth to our three wonderful children, Carl Henrik, Maria, and Jesper. Your support has meant a great deal to me, and the joy you and our children have given me really helped me to overcome scientific problems that seemed impossible to solve.

Abstract

Malignant melanoma is one of the most chemoresistant malignancies in man. Although effort is put into developing new drugs to treat metastatic melanoma, still dacarbazine, an alkylating agent approved in 1975 for therapeutic use in the USA, is considered standard treatment, despite response rates as low as 10%.

Today, there is no biomarker predicting response to this drug. Therefore, we have aimed at identifying such biomarkers and also prognostic markers in metastatic melanomas undergoing dacarbazine therapy.

In the present papers we have revealed that metastatic melanoma patients can be classified into 4 different groups according to expression of immuneresponse, proliferative, pigmentation, and stromal genes. Most important, these classes have significant different overall survival.

Further, we have identified expression of *MGMT*, a gene coding for an enzyme responsible for repairing DNA damage caused by alkalyting agents like dacarbazine, to predict disease stabilisation on dacarbazine treatment and to independently predict survival.

In a gene known to be activated through mutation in melanomas, *BRAF*, we have identified alternative spliced forms where a part of the gene coding for the catalytic part of the enzyme is not included, and expression of these alternative spliced forms are correlated to response to dacarbazine treatment. In cell culture, knockdown of this gene recovered dacarbazine sensitivity in a melanoma cell line wild type (codon 600V) in *BRAF*, whereas a mutated cell line (codon 600E) did not respond to this knockdown.

Lastly, we have shown alterations in the p53 pathway to be associated with inferior survival. If combined with low *p16INK4a* expression, the correlation was even stronger.

List of publications

Paper I

Göran Jönsson, **Christian Busch**, Stian Knappskog, Jürgen Geisler, Hrvoje Miletic, Markus Ringnér, Johan R. Lillehaug, Åke Borg, and Per Eystein Lønning

Gene Expression Profiling-Based Identification of Molecular Subtypes in Stage IV Melanomas with Different Clinical Outcome. *Clinical Cancer Research*; 2010, in press, doi: 10.1158/1078-0432.CCR-09-2509

Paper II

Christian Busch, Jürgen Geisler, Johan R. Lillehaug, and Per Eystein Lønning

MGMT expression levels predict disease stabilisation, progression free and overall survival in patients with advanced melanomas treated with DTIC. *European Journal of Cancer* (2010), in press, doi:10.1016/j.ejca.2010.04.023

Paper III

Christian Busch, Stian Knappskog, Jürgen Geisler, Johan R. Lillehaug, and Per Eystein Lønning

Expression of *BRAF* alternative splices lacking the catalytic domain predicts DTIC response in advanced melanoma. *Manuscript in revision*

Paper IV

Christian Busch, Jürgen Geisler, Stian Knappskog, Johan Richard Lillehaug, and Per Eystein Lønning

Alterations in the p53 pathway and *p16INK4a* expression predict overall survival in metastatic melanoma patients treated with dacarbazine. *Journal of Investigative Dermatology*, 2010, in press, doi:10.1038

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Abbreviations

aCGH	Array comparative genomic hybridization
AP1	Activator protein 1
APC	Adenomatosis polyposis coli gene
ARF	Alternative reading frame
ASF	Alternative splicing factor
ATM	Ataxia teleangiectasia mutated
ATR	Ataxia telangiectasia Rad3-related
B2M	Beta 2 microglobulin
Bcl-2	B-cell CLL/lymphoma 2
bp	Base pair
BRAF	V-raf murine sarcoma viral oncogene homolog B1
BRCA1/2	Breast cancer 1/2
CDK	Cyclin dependent kinase
CDKN2A	Cyclin dependent kinase inhibitor 2 A
CHK2	Checkpoint kinase 2
C-MYC	V-myc myelocytomatosis viral oncogene homolog (avian)
DTIC	Dacarbazine
E2F	E2 transcription factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ERK	Extracellular signal-regulated kinase
FOXO	Forkhead box O3
Grb2	Growth factor receptor-bound protein 2
GTP	Guanine triphosphate
HIF	Hypoxia inducible factor
IHC	Immunohistochemistry
IL-2	Interleukin 2
INK4a	Inhibitor of Kinase 4a
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin 1 Receptor
MDM2	Mouse double minute 2 homolog
MEK	Mitogen-activated protein kinase-kinase or Erk Kinase
MGMT	O-methylguanine-DNA methyltransferase
MRI	Magnetic Resonance Imaging
MTIC	3-methyl-(triazene-1-yl)imidazole-4-carboxamide
p53	Tumour protein 53
p-AKT	Phosphorylated v-akt murine thymoma viral oncogene homolog
PET-CT	Positron Emission Tomography Computed Tomography
PIP	Phosphatidylinositol (4,5)-bisphosphate

PRC	Pre-replicative complex
qRT-PCR	Quantitative real time polymerase chain reaction
RAS	Rat Sarcoma
Rb	Retinoblastoma protein
SF2	Splicing factor 2
siRNA	Short interfering RNA
SOS	Sons of senvenless
Src	V-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian)
TMZ	Temozolomide
TNM	Classification of Malignant Tumours with mandatory parameters: Tumour, Node, and Metastasis
<i>TP53</i>	Gene encoding p53
β -Catenin	Beta-Catenin

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1. Introduction

1.1 General remarks

Malignant melanoma is a cancer type arising from melanocytes, a cell type with embryonal neural crest origin, whereas keratinocytes from the skin originates from non-neural ectoderm. 91.2% of melanoma diagnosed are skin melanomas, 5.2% ocular, 1.3% mucosal and 2.2% of unknown origin (Chang et al., 1998). In rare occasions, primary tumours are found in the mucosa from different body compartment like intestines (mainly small intestine), sinuses of the face and brain membrane.

Melanomas on the palms of the hands, under the feet or in the nail beds are classified as acral melanomas. Acral melanoma accounts for about 5% of skin melanoma in Caucasians, but is the predominant form of melanoma in dark skinned (African) populations (Thorn et al., 1994). Melanomas can otherwise be found throughout the body surface including uvea of the eye and genitals, but mostly on sun-exposed areas. Superficial spreading malignant melanomas in general develop from pre-existing nevi whereas nodular malignant melanomas develop *de novo*. Although other skin cancer forms like basaliomas and squamous cell carcinoma are more common than melanoma, they metastasize infrequent, and therefore melanoma is responsible for most skin cancer deaths (Sladden et al., 2009).

In general, melanocytes detect sun exposure and produce the skin pigment, melanin, which they distribute through their dendrites in plasma membrane coated packages called melanosomes to surrounding keratonocytes. The melanosomes containing melanin form a cap over the nucleus of the keratonocyte to protect its DNA against UV light (Figure 1).

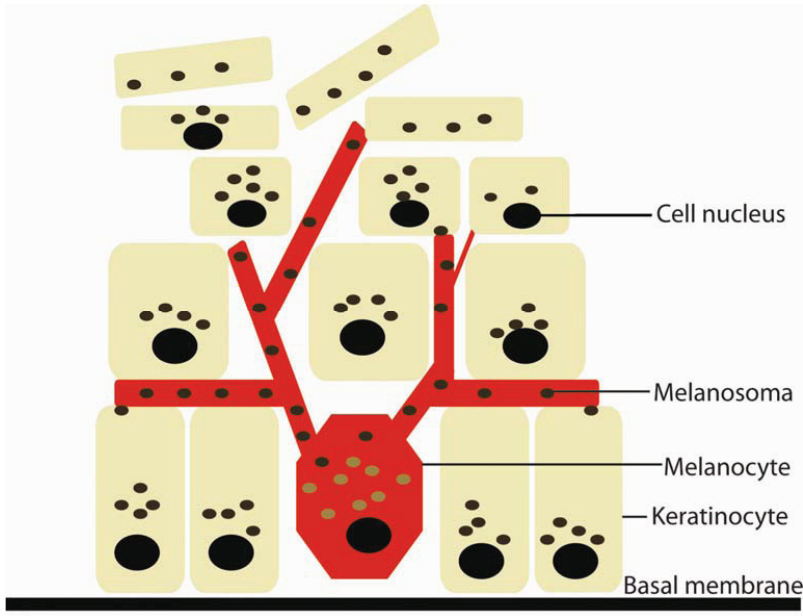


Figure 1: Melanosomas containing eumelanin and pheomelanin are produced in the soma (body) of the melanocyte. The melanin precursors are oxidated to give darker, mature melanin when exposed to UVA light. The melanosomas are transported out to the dendrites of the melanocytes and transferred to the keratonocytes where they cover the nucleus to protect it against UV light (Park et al., 2009).

1.1.1 Causes of melanoma

There is a solid body of evidence linking melanoma carcinogenesis to sun exposure (Menzies, 2008). However, there are some observations suggesting other etiologic agents (unknown) than the total amount of UV radiation. First, about 9% of melanomas are non-skin melanomas, which have not been sun-exposed. In addition, about 5% are acral melanomas, and here the skin is so thick that UV light will not reach the melanocytes of the skin as the melanocytes are located deep in epidermis near the basal membrane. Second, the anatomical distribution of malignant melanoma does not match the distribution of other sun-related cancers like squamous cell carcinoma and total UV dose, but relates more to intermittent sun exposure (Bulliard, 2000). Third, whereas the incidence of squamous cell cancer continues to rise

exponential with age, for melanoma there is not a clear relationship. In the face, the incidence rises with age (and sun exposure) as for squamous cell carcinoma, the incidence peak is earlier in adult life with respect to other body locations (Rees, 2008). The anatomical site of high and low sun exposure predicts the patterns of melanoma distribution. The incidence variations between different races tend to follow the risk to burn rather than to tan (Cormier et al., 2006). An exception is formation of acral melanomas where the aetiology is unknown and the incidence is equal across all ethnic groups (Jennifer et al., 2002). There has been a debate whether UV-B alone or in combination with UV-A is responsible for cancer initiation, contemporary evidence suggests both parts of the UV spectrum are mutagenic (Bennett, 2008).

1.1.2 Hereditary melanoma

The most studied locus in inherited melanoma, *CDKN2A*, is located on chromosome 9 and code for two distinct proteins, p16^{INK4A}, a tumour suppressor and p14^{ARF}, a protein which stabilises the important tumour suppressor, p53 (Greene, 1999). In melanoma families, most of the observed mutations in the *CDKN2A* locus target the p16^{INK4A} transcript only. In contrast, only a few families harbour disturbances in the p14^{ARF} transcript only (Goldstein et al., 2006). Similar to Retinoblastoma and Rb mutations, germline *CDKN2A* mutations cause melanoma in a younger age than melanoma without inherited disposition. However, the patients are not as young as in retinoblastoma, rather young adolescence (Knappskog et al., 2006, 2007). An international consortium named GenoMEL (<http://www.genomel.org/index.htm>) is continuously coordinating research in this field and administrates a database for registration of different *CDKN2A* mutations identified (<http://chromium.liacs.nl/LOVD2/home.php>). Although *CDKN2A* mutations is the most frequent cause of inherited melanoma, germline mutations are only found in 25-50% of melanoma prone families (Goldstein et al., 2007). Genetic testing of melanoma prone families is still controversial (Kefford and Mann, 2003).

CDK4 located on chromosome 12 is another melanoma subspecialty gene. Only a few families with inherited melanoma harbour this mutations worldwide, but *CDK4*

mutations have high penetrance like *CDKN2A* mutations (Fargnoli et al., 2006). Variation in *MC1R* on chromosome 8 is also related to familial melanoma, but with a low penetrance (Bataille, 2003; Höiom et al., 2009; Kennedy et al., 2001).

1.1.3 Clinical features

A malignant melanoma usually presents as a pigmented lesion. If the lesion is in line with the level of the skin, it is called superficial spreading malignant melanoma. A lesion that stands up from the skin is called nodular. Lesion in the palms of the hands or feet or nail bed it is classified as acral melanoma (Figure 2). The ABCD rule is often used to distinguish between a melanoma and a benign nevus by general practitioners. The following criteria are used to identify moles that should be further investigated:

- **A**symmetrical skin lesion. One half is different from the other half.
- **B**order of the lesion is irregular. The edges are notched, uneven or blurred.
- **C**olour. Melanomas usually have multiple colours, and shades of brown, tan and black are present whereas benign lesions tend to have more homogenous colour.
- **D**iameter. Pigmented lesions greater than 6 mm are more likely to be melanomas than smaller lesions.

Dermatoscopic is often used by dermatologists to better distinguish benign from malignant lesions. When in doubt, the lesion should be excised *in toto* to allow proper diagnosis.



Figure 2: Clinical presentation of primary malignant melanomas. Upper left: Superficial spreading melanoma, lower left: Nodular spreading melanoma, and right: Acral malignant melanoma.

1.1.4 Prognostic factors in melanoma

The most evident prognostic markers in melanoma are histopathologic and clinical aspects like Breslow's depths, Clark's level of invasion, mitotic rate, ulceration of the tumour, and metastatic tumour load. Many so-called biomarkers are identified in melanoma. But in routine clinical practice, only lactate dehydrogenase (LDH) serum levels and now also mitotic rate are included. In the current TNM classification system, LDH is used to differentiate between M1 b and c because of its prognostic value, whereas mitotic rate is used together with ulceration to differentiate between T1 a and b. (Balch et al., 2009; Balch et al., 2004; Balch et al., 2001b). The melanoma marker serum-S100B is also well documented as a prognostic marker (vonSchoultz et al., 1996). In the German and Swiss melanoma guidelines, serum-S100B is included in the follow of patients with melanoma over 1 mm (Dummer et al., 2005; Garbe et al.,

2007). A rise in serum value indicates metastatic spread. HMB-45 is an additional immune-histochemical marker for melanoma (Skelton et al., 1991), although it is not considered as specific as S-100 it is often used as a package together with S-100 in diagnosing melanoma (Cochran et al., 1993; Steven et al., 2008). In primary melanoma, several biomarkers are identified, but only few are found as independent prognostic markers, independent of tumour thickness (Bosserhoff, 2006). These include p-AKT, Bcl-2, β -Catenin, and HIF2alpha. In metastatic melanoma, Bcl-2 expression is associated with overall survival (DiVito et al., 2004).

1.2 Clinical practice

1.2.1 Epidemiology

The incidence of malignant melanoma has been increasing in western countries for the last 5-6 decades (MacKie et al., 2007; Tucker and Goldstein, 2003). The highest incidence has been registered in Australia (48/100 000) and New Zealand (31/100 000). The Norwegian population has the 4th highest incidence in the world (MacKie et al., 2009), female: 16.3/100 000 and men: 15.6 /100 000 per year in 2007, an increase from 2.1/100 000 (female) and 1.8/100 000 (male) during the period of 1953-1957 (Cancer Registry of Norway, 2008). The high incidence in Norway (also true for the rest of Scandinavia) is believed to be due to a combination of intermittent, high sun exposure and fair skin. Lack of sun in Scandinavia during winter time encourage people to visit sunny areas to sunbathe without having any pre-existing melanin protection in their skin, often leading to sun burns. A similar habit is common among Norwegians in the summer time, high sun exposure during a short season. Intermittent sun exposure is recognised as a stronger risk factor than chronic sun exposure in relation to melanoma development (Elwood and Gallagher, 1998). In the middle aged (25-49 years) group of the Norwegian population, melanoma is the second most common cancer form after testis cancer in men and breast cancer in women (Cancer Registry of Norway, 2008). The same rise in incidence the last 50 years is reported in

other cohorts like Scotland (MacKie et al., 2007) and in more dark skinned Caucasians in southern and eastern Europe (Vries et al., 2003).

1.2.2 Staging systems

The 7th TNM (Tumour, Node and Metastasis) classification was modified in 2009 (Balch et al., 2009), and includes different prognostic factors (Appendix, Table A1). T status is now based on Breslow thickness (see below) and whether there is ulceration or not in the tumour. Also, in this version, the mitotic rate is included in the sub-classification of T1 tumours. N-status is given by the number of nodal metastases and N1 is sub-classified whether the node is palpable or is found by histopathological examination after Sentinel node biopsy. M status is given after the anatomical localisation of the metastases or if serum lactate dehydrogenase level is elevated.

Clinical (and Pathologic) stage (Balch et al., 2001a) is an even easier system that combines different TNM classes into the same groups if the prognosis is similar (Appendix, Table A2).

Breslow's thickness of the primary tumour was proposed to be a prognostic factor by Alexander Breslow in 1970 (Breslow, 1970). This is measured using a micrometer on the microscope during histopathologic examination of the primary tumour. Breslow was able to show a correlation between tumour thickness and risk of relapse. Tumours thinner than 1mm relapsed in rare occasions only, whereas tumours with thickness greater than 3.0mm had a recurred in more than half the cases. The invasion level is given in mm.

Clark's level of invasion was devised by the pathologist Wallace Clark (Clark et al., 1969) and measures the depth of penetration of a melanoma into the skin according to the anatomic layer. The different levels in this system are related to the risk of developing metastases (Appendix, Table A3). The reproducibility of this measure is not as good as for Breslow's thickness, and it is no longer included in the current TNM classification of melanomas.

1.2.3 Established treatment modalities

Surgery

Generally, treatment of all solid malignant tumours has been surgical excision whenever possible. If the cancer has proceeded too far or has metastasised, the term inoperable has been used. Still, in some tumour forms, metastatic or locally advanced tumours are curable with radiation or chemotherapy (Klimm et al., 2005; Pectasides et al., 2009). The surgical treatment of the primary malignant melanoma has changed little over the last decades, although a higher awareness of the diagnosis has led to earlier surgical excision (decreased Breslow thickness) of the primary tumour, especially in women (MacKie et al., 2007). Partly because of that, and also after large, thorough prospective studies (Balch et al., 1993; Lens et al., 2007; Ringborg et al., 1996; Thomas et al., 2004; Veronesi et al., 1988), with changes in the National guidelines, the excision margins of the primary tumour have decreased causing less morbidity and infrequent need for reconstruction at the site of the primary tumour (Testori et al., 2009; van Aalst et al., 2003). This is also true for eye melanoma where enucleation no longer is the only treatment option for all patients, but local radiation can save the eye for patients with limited tumours (Bergman et al., 2005; Chang et al., 1998; Diener-West et al., 2001).

Regional lymph nodes are thought to play a role as a barrier for lymphatic cancer spread, but new insight and *in vitro* findings have questioned this hypothesis (David, 2005). Still, resection of lymph nodes harbouring metastatic spread (TLND=therapeutic lymph node dissection) will reduce tumour load, and these lymph nodes are available to surgical excision. If the resected metastatic lymph nodes are diagnosed with tumour free borders, 30-50 % of the patients will remain relapse-free (Allan et al., 2008; Mack, 2004; White et al., 2002). However, there has been no prospective, randomized, controlled trial comparing TLND and no treatment. Recurrence rate after therapeutic lymphadenectomi is depending mainly on numbers of lymph nodes involved but also tumour growth through the lymph node capsule and which region that harbours lymph node spread (Mack, 2004; Meyer et al., 2002).

Attempts of improving the prognosis by performing a lymph node dissection as adjuvant treatment before a lymph node metastasis is clinical diagnosed, so called elective lymph node dissection (ELND) have not been able to prove beneficial (Cascinelli et al., 1998; Lens et al., 2002).

In addition to therapeutic lymphadenectomi, sentinel lymph node dissection (SLND) has proven to be a strong, independent prognostic indicator, and is now adopted in the newest TNM classification (1.2.2). SLND followed by therapeutic lymphadenectomi if positive is being evaluated for improving prognosis (Morton et al., 2006). In this method, a radioactive compound and blue dye are injected into the scar where the primary tumour was located, and the lymph node(s) that harbour radioactive signal and blue dye is dissected and tested for tumour metastases. If metastasis is present, a formal lymph node toilette is performed. So far, studies have not been able to prove survival benefits for patients receiving SLND compared to controls, but there is a significant benefit when looking at time to progression (Testori et al., 2009).

Patients showing long lasting survival after surgical excision of single metastases are few (Frenkel et al., 2009; Leo et al., 2000). Still, surgical excision of metastatic deposits may provide palliation for many patients (Essner et al., 2004; Ollila, 2006; Testori et al., 2009). New methods like MRI, PET scan and various serum markers for detecting distant metastases are being adopted, showing increased sensitivity to conventional methods (Holder et al., 1998; Laurent et al.). When these methods are used preoperatively for staging patients before surgical excision of what is considered to be a single metastasis (diagnosed by screening with conventional methods or symptoms), more metastases are often diagnosed, revealing the patient to be inoperable.

Chemotherapy

So far, different regimen of adjuvant chemotherapy has revealed no overall survival or relapse-free survival benefits (Eggermont and Gore, 2007) in stage II or stage III melanoma.

Although dacarbazine (DTIC) was introduced more than 30 years ago and was FDA approved in 1976, DTIC monotherapy is still considered standard treatment for metastatic melanoma. Further, the response rate is as low as 10-15 % (Eggermont and Kirkwood, 2004; Tawbi and Kirkwood, 2007). DTIC is a prodrug, and like the more recently approved Temozolomide (TMZ), it is activated to mitozolomide (MTIC). Whereas the TMZ is activated in a non-enzymatic degradation process in physiological pH, DTIC is activated through oxidative demethylation in cytochrome p-450 enzymes (Baker et al., 1999). In vivo, MTIC alkylates DNA in the O⁶ position of guanine. Methylated O⁶ of guanine is mismatched with thymidine. During replication, this mismatch is recognized by the mismatch repair system, leading to thymidine, and not the methyl group in O⁶ of guanine, to be removed. Then, the same O⁶metG and T mismatch is gathered, and the mismatch repair system is once more activated. This cycling causes secondary lesions and DNA double-strand breaks (Ochs and Kaina, 2000), and subsequent activation of apoptotic pathways, mainly p53 dependent, but also through the intrinsic apoptotic pathway in TP53 mutated tumours (Roos et al., 2006).

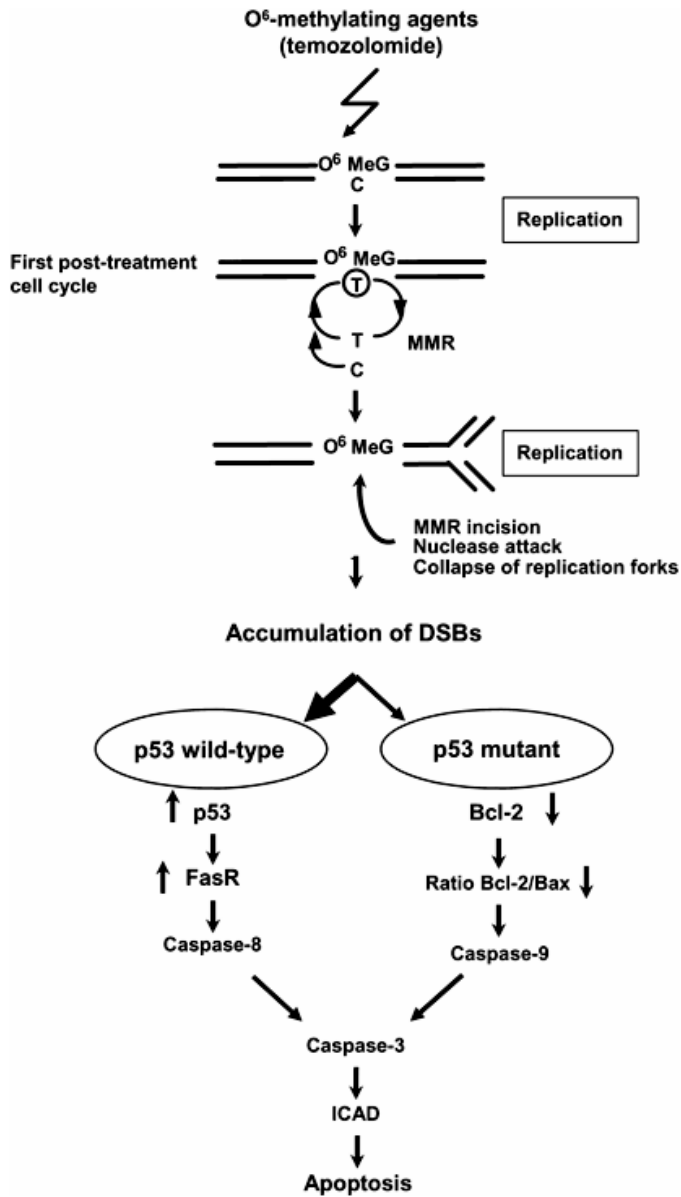


Figure 3: A model of O⁶MeG-triggered apoptosis in p53 wild-type and p53 mutated glioma cells (Roos et al., 2006).

DTIC requires activation in the liver (Tsang et al., 1991) whereas TMZ is metabolized to mitozolomide in peripheral blood at physiological pH (Villano et al., 2009). TMZ,

unlike DTIC crosses blood-brain barrier (Agarwala and Kirkwood, 1998). In addition to a more convenient 4-weekly oral application, this drug is also suitable for treatment of brain metastases (Villano et al., 2009).

Combination of drugs in addition to DTIC has not proven superiority to DTIC alone (Chapman et al., 1999; Keilholz et al., 2005; Lui et al., 2007). The “Darthmouth” regimen was regimen contained different chemotherapeutics (DTIC, carmustine, cisplatin and tamoxifen). Although promising results were observed in phase II trials, no significant benefit as compared to DTIC treatment was shown in a large phase III trial (Chapman et al., 1999). Notably, novel drugs, like taxanes, have not provided therapeutic benefits (Eggermont and Kirkwood, 2004; Hodi et al., 2002; Lui et al., 2007).

Table 5: Success rates, response odds ratios from studies with dacarbazine monotherapy as reference (Lui et al., 2007).

Comparators	Author	Year	Complete + Partial response				Odds (response)	95% Confidence limits (response)	
All treatments with or without dacarbazine			Comparator		DTIC		Ratio	Lower	Upper
			Successes	Failures	Successes	Failures			
Fotemustine	Avril et al	2004	17	95	8	109	2.44	1.01	5.90
Dacarbazine	Bajetta et al	1994	40	138	16	72	1.30	0.68	2.49
interferon									
Carmustine	Bellet et al	1976	5	20	7	18	0.64	0.17	2.39
vincristine									
Vincristine	Carter et al	1976	34	161	8	40	1.06	0.45	2.46
lomustine									
dacarbazine									
Dacarbazine	Chapman et al	1999	20	99	12	109	1.84	0.85	3.95
carmustine									
cisplatin									
tamoxifen									
Dacarbazine	Chauvergne et al	1982	8	14	4	22	3.14	0.79	12.43
detorubicin									
Dacarbazine	Chiarion Sileni et al	2001	10	31	1	18	5.81	0.69	49.16
carmustine									
cisplatin									
tamoxifen									
Dacarbazine	Clunie et al	1980	6	16	6	21	1.31	0.36	4.84
<i>C. parvum</i>									
dacarbazine									
Dacarbazine	Constanza et al	1972	12	65	9	56	1.15	0.45	2.93
carmustine									
Dacarbazine	Constanza et al	1977	36	238	19	122	0.97	0.53	1.76
semustine									
Dacarbazine	Falkson et al	1991	16	16	6	26	4.33	1.41	13.36
interferon-alpha									
Dacarbazine	Falkson	1995	17	20	7	29	3.52	1.23	10.05
interferon									
Dacarbazine	Falkson et al	1998	39	163	10	59	1.41	0.66	3.01
interferon									
tamoxifen									
Dacarbazine	Gough et al	1978	4	12	3	17	1.89	0.36	10.03
<i>C. parvum</i>									
dacarbazine									
Vincristine	Kongoniaa et al	1981	14	44	13	43	1.05	0.44	2.50
nitrosomethylurea									
dactinomycin									
Dacarbazine	Lopez et al	1984	4	16	2	20	2.50	0.40	15.43
epirubicin									
Vinblastine	Luikart et al	1984	2	19	4	20	0.53	0.09	3.22

Radiation

Melanoma has historically been considered to be a radio-resistant malignancy (Doss and Memula, 1982; Shuff et al., 2010). The introduction of higher fractions (greater than 4 Gy) (Overgaard et al., 1986) has gradually changed this view.

In the treatment of primary uveal melanoma, radiation alone is also preferable to enucleation if the tumour is limited (COMS, 2006).

In the treatment of primary skin melanoma, there are no randomised trials supporting adjuvant treatment radiation of primary melanoma (Testori et al., 2009). However, in some situations like desmoplastic primary tumours and mucosal tumours, adjuvant therapy is recommended (Temam et al., 2005; Vongtama et al., 2003).

In the stage III melanoma, many guidelines recommend radiation as adjuvant therapy after lymphadenectomy. The recommendations are based on phase II trials showing increased loco-regional control (Bonnen et al., 2004; Chang et al., 2006). However, the only randomised study published, revealed no benefit from radiation on overall survival or disease free survival (Creagan et al., 1978).

In palliation of ulcerated or painful metastases radiation have been proven beneficial (Kirova et al., 1999). Two large retrospective studies have identified radiation dose as an independent prognostic factor (Olivier et al., 2007; Seegenschmiedt et al., 1999). In the treatment of multiple or solitary brain metastases radiation can give good palliation (Grob et al., 1998). Multiple brain metastases are treated with whole brain radiation and give symptom relief in 50-70% of the patients (Ellerhorst et al., 2001; Kirova et al., 1999) whereas solitary or few (three or less) metastases are treated with stereotactic radiosurgery, and give transient local control in 85-90% of patients (Hara et al., 2009; Noel et al., 2002). Recent studies also suggest a modest survival benefit when stereotactic surgery is used in combination with or instead of whole brain radiation on patients with a few brain metastases (Brennum et al., 2002; Majer and Samlowski, 2007; Stone et al., 2004).

1.2.4 Experimental therapy of metastatic melanomas

Isolated limb perfusion/isolated liver perfusion

To save one affected limb with massive loco-regional melanoma relapse from amputation a special treatment option exists where the affected limb are given an intra-arterial high toxic dose of a chemotherapeutic compound (Melfalan), tumour necrosis factor and hyperthermia (Kroon and Thompson, 2009). The limb is coupled to a heart/lung machine for oxygenation so that the toxic compounds do not reach systemic circulation. The treatment has high efficacy on local tumour control, and may be long lasting, although the majority of patients eventually die of other metastases (Hayes et al., 2007).

A similar therapy exists for liver metastases (van Etten et al., 2009) where the liver is perfused and oxygenated isolated from the body. This treatment can also be performed transcutaneously (Jones and Alexander, 2008).

Immunogenicity and immunotherapy

There have been some trials with interferon as adjuvant treatment in melanoma (Kirkwood et al., 2001; Kirkwood et al., 1996). Although this adjuvant treatment became approved in USA, follow up studies have been unable to repeat superiority to observation alone (Atkins et al., 2008).

Malignant melanomas as well as benign nevi are highly immunogenic. Benign nevi disappearing after a period of inflammation around the lesion are well known in the literature (Zeff et al., 1997). There are also a few case reports of single metastases disappearing completely (High et al., 2005), probably because of involvement of the immune system. Based on that, there have been several attempts of stimulating the immune system by various approaches, like immunisation with vaccines, interferon and interleukin (IL-2 and T-Cell activator) treatment. There have also been attempts to use IL-2 as monotherapy or in combination with chemotherapeutic drugs (biochemotherapy), but these regimes have not demonstrated increased survival compared to dacarbazine treatment. Instead, the patients have experienced increased

toxicity (Dummer et al., 2006; Ives et al., 2007; Schadendorf et al., 2009). Still, a small subgroup of patients can achieve long lasting responses on this therapy (Dummer et al., 1995). These treatments are expensive, resource demanding, and patients suffer from side effects. Nevertheless, huge efforts are put into development and trials with these regimes. One compound, a T-cell activator, Ipilimumab, has recently shown prolonged survival in stage III and IV melanomas compared to a more general immune activator gp 100 (Hodi et al., 2010; O'Day et al., 2010).

Targeted therapy

The lack of efficient chemotherapy for disseminated disease opens for early testing of new drugs or drug combinations, i.e. there is no standard second line therapy, the threshold for testing new drugs are low after failure to standard therapy (DTIC). In targeted therapy a group of patients having a genetic alteration resulting in an enzymatic defect or activation in a specific signal pathway are given a specific treatment recovering or inhibiting that enzyme. In tumour forms like chronic myelogenous leukemia and metastatic renal cancer, targeted therapy has proven successful (Druker et al., 2006; Facchini et al., 2009).

As BRAF and the MAP-kinase pathway is activated in melanoma (Davies et al., 2002), much of the focus has been on developing anti-BRAF compounds and other MAP kinase inhibitors. One of the first anti BRAF compounds, Sorafenib has so far not proven effectiveness alone (Eisen et al., 2006) or in combination with other drugs (Hauschild et al., 2009). However, new compounds are emerging and promising in Phase I and II trials (Kefford et al., 2010; Patel et al., 2010; Smalley, 2010). Nevertheless, no targeted therapy has been proven beneficial in Phase III trials (Hersey et al., 2009; McDermott et al., 2008).

Table 4: Examples of trials in melanoma with drugs targeting survival and apoptosis (Eberle et al., 2007; Straume et al., 2009; Tawbi and Nimmagadda, 2009)

Drugs	Targets	Phase	# Pat	Response	Reference
OSI-774 (Erlotinib)	EGFR	II	14	1 SD ^a	Sosman and Puzanov, 2006
Imatinib mesylate	BCR-ABL, c-Kit and PDGF-R	II	26	0 SD	Wyman et al. 2006
R115777 (Tipifarnib)	Farnesyl transferase	II	14	0 CR	Gajewski et al. 2006
Sorafenib	B-Raf, Raf-1, c-Kit VEGFR and PDGFR	II	37	6 SD	Eisen et al. 2006
Sorafenib + carboplatin/paclitaxel	B-Raf, etc.	I/II	35	11 PR, 19 SD	Flaherty et al. 2004
Sorafenib + temozolomide	B-Raf, etc.	II	78	15 PR, 37 SD	Amaravadi et al. 2007
Sorafenib + DTIC vs. DTIC	B-Raf, etc.	II	101	PFS: 21/12 weeks, ORR: 24%/12%	McDermott et al. 2007
Sorafenib + carboplatin/paclitaxel vs. carboplatin/paclitaxel	B-Raf, etc.	III, 2. line	270	PFS: 18/17 weeks; ORR: 11%/12%	Agarwala et al. 2007
Sorafenib + carboplatin/paclitaxel vs. carboplatin/paclitaxel	B-Raf, etc.	III, 1. line		Underway (E2603)	Flaherty 2007
Bevacizumab	VEGF	II	27	5 PR, 5 SD	Straume et al. 2008
PD0325901	MEK	I/II	27	1 PR, 4 SD	Lorusso et al. 2005
Perefosine	PKB/Akt	II	18	3 SD	Ernst et al 2005
CCI-779 (Temozolimus)	mTOR	II	33	1 PR	Margolin et al. 2005
RAD-001 (Everolimus)	mTOR	II	20	7 SD	Rao et al. 2006
Oblimersen + DTIC	Bcl-2	I/II	14	1 CR, 2 PR, 3 MR	Jansen et al. 2000
Oblimersen + DTIC (1) vs. DTIC (2)	Bcl-2	III	386, 385	52 OR (1), 29 OR (2)	Bedikian et al. 2006
Bortezomib (PS-341)	Proteasome, NF-κB	II	27	6 SD	Markovic et al. 2005
Bortezomib (PS-341) + Temozolomide	Proteasome, NF-κB	I	19	3 MR, 1 PR	Sosman and Puzanov 2006
Bevacizumab+/-INFα2b	VEGF	II	16/16	5/3 SD	Varker et al. 2007
Bevacizumab, carboplatin and paclitaxel	VEGF	II	53	9 PR, 30 SD	Perez DG et al. 2009
Thalidomide + DTIC	VEGF and other	II	13	1 PR, 3 SD	Ott PA et al. 2009

^a Abbreviations: OR, objective response; ORR, objective response rate (median); PR, partial response; CR, complete remission; MR, moderate regression; CR, clinical response; SD, stable disease; PFS, progression-free survival (median).

1.3 Molecular Biology of malignant melanoma

1.3.1 Genetic alterations leading to cancer development

“The two hit theory” was described by Alfred Knudson (Knudson, 1971) when he published a statistical analysis of retinoblastoma in children. In this disease inherited cases got the disease at young age as compared to spontaneous case. Also, affected children frequently got the tumour in both eyes. The findings were consistent with “two hit” events, and later the retinoblastoma gene (Rb) was found as a tumour suppressor gene (Hong et al., 1989). In inherited cases of retinoblastoma, patients receive one mutated allele of the Rb locus and later acquire another hit (mutation or loss of heterozygosity) on the other allele, thus no Rb tumour suppressor with biological function is left. The Rb protein is central in the “Rb pathway”, one of the most important pathways regulating cell cycle (Massague, 2004). The “two hit” theory is applicable to all tumour suppressors. If one allele is mutated, still, there is a protein with biologic function transcribed and translated from the other allele. Therefore, the second allele has to be altered in some way so that biological function is reduced before carcinogenesis can be initiated.

In 1969 Frederick Li and Joseph Fraumeni identified early onset of different soft tissue cancers in siblings and cousins (Li and Fraumeni, 1969). This syndrome was later named Li-Fraumeni cancer syndrome, and years later, in 1990, germline mutations in the already known tumour suppressor p53 was identified in all family members suffering from this syndrome (Malkin et al., 1990).

For p53, unlike classic tumour suppressors, a mutant type of the protein translated from one allele can inhibit function of the protein from the normal allele by interference with the hetero-tetramer of the protein, probably driving the wild type

proteins into a mutant type of conformation in this structure (Dridi et al., 2006; Ishimaru et al., 2003; Willis et al., 2004). Tumour suppressors normally have a negative control over cell cycle or promote apoptosis.

Oncogenes, on the other hand, are genes that, when mutated, code for proteins which gain another or stronger biological function and promote carcinogenesis. Mutation in one allele is sufficient. As for tumour suppressors, in addition to activation of an oncogene, an additional step is often required for cancer to occur. This step can be mutation in other genes, environmental factors or viral infections. The first oncogene to be discovered was the v-src (viral sarcoma), a viral gene which causes sarcoma in chicken (Tal et al., 1977). A similar gene in human cell (c-src) was later found (Oppermann et al., 1979), and this gene is activated by a mutation. Proto-oncogenes are genes that do not need mutation to be activated, but through overexpression or duplication their normal function is enhanced (Pall, 1981). Oncogenes often code for proteins involved signal transduction pathways important for cell growth, examples are RAS and ERK (Fang and Richardson, 2005; Sivaraman et al., 1997).

Gene alterations can occur at several levels in the transcription - translation process to mature protein, and a brief overview of the normal function of a gene is given. A gene locus has coding (exons) and non coding (introns) areas (Sharp, 1985). When a gene is transcribed, a pre-mRNA is first produced containing both exon and introns, secondly introns are removed by a process called splicing (Berget et al., 1977). Then, the mRNA is translated to a protein (Söll et al., 1965). There are possibilities for introduction of aberrations on all levels listed here, and in several control levels in between. At the chromosomal level, gross deletions, duplications, translocations, or inversions are well known, giving hereditary syndromes like Down's syndrome and cri du chat (Lejeune et al., 1959; Lejeune et al., 1964). Although one would expect all expressed genes on chromosome 21 to be over-expressed, expression studies have revealed that only 20% of the genes are in fact over-expressed, the later 80% are compensated for through regulatory mechanisms (Ait Yahya-Graison et al., 2007).

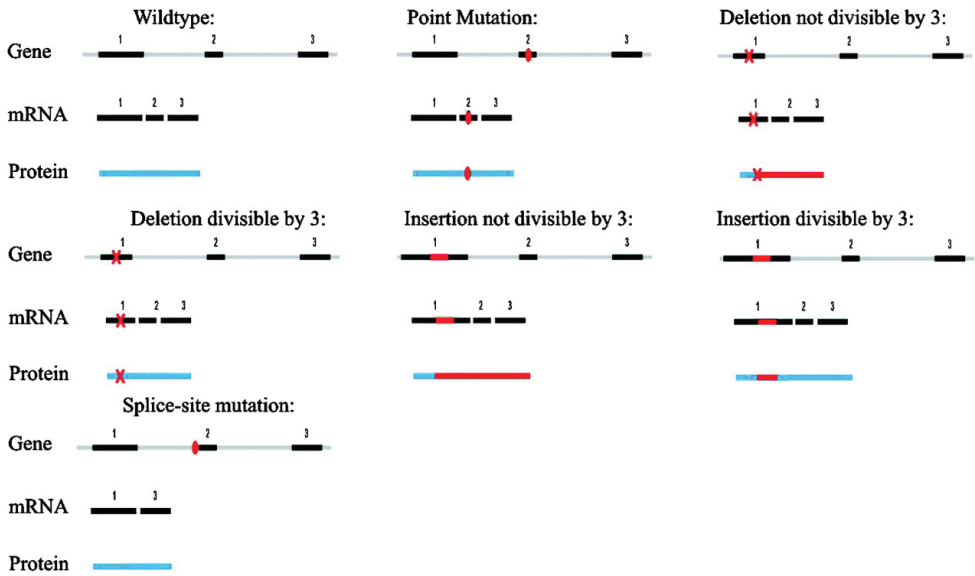
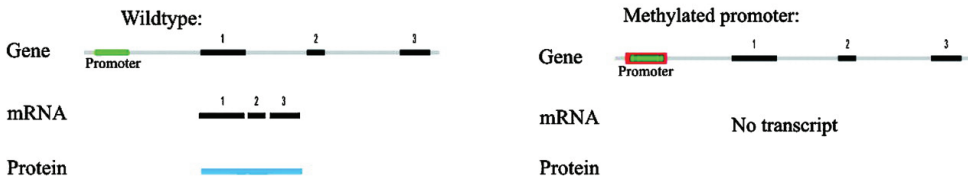
I) Mutations:**II) Promoter methylation:**

Figure 4: Different types of gene mutations and mechanism of epigenetic silencing (Lonning, 2007).

On the DNA level the same errors can occur. Again, deletions, mutations, and insertions are possible (Fig 4). The transcription and translation process are regulated on many levels, and dys-regulation results in high (over-expression) or low (silencing) mRNA or protein levels. Lastly, proteins are modified after translation by addition for functional groups like acetate, phosphate, lipids and carbohydrates, or structural changes by introduction of disulfide bridges in the protein (Burnett and Kennedy, 1954; Fleischer, 1983; Plevoda and Sherman, 2003; Sevier and Kaiser, 2002), Figure 5. Amino acids can also be removed inside the protein or at the end. When insulin is synthesised, pro-insulin is first produced in the cell. Then a polypeptide is removed after the introduction of disulfide bonds giving mature insulin (Massague et al., 1981).

Phosphorylation is a normal mechanism for controlling enzyme function, turning the enzyme on and off (Hurley et al., 1990). The transcription process is also regulated by different mechanisms including interaction of trans and cis acting factors. A trans acting transcription factors is a DNA sequence or a gene that code for a protein or siRNA that regulate transcription of the target gene. A cis acting factor is a DNA sequence usually located at the 5' end of the coding region, but can also in a intron or 3' end of the coding region that bind trans acting factors. Silencing of genes through promoter methylation (Kass et al., 1997), interaction of transcription factors at the promoter (Mitchell and Tjian, 1989), and histone acetylation (DNA packing) (Elgin, 1996) have been explored, whereas mechanisms responsible for silencing of the extra X chromosome in women include all these mechanisms and are incomplete understood (Minks and Brown, 2009). mRNA splicing is regulated by spliceosome assembly and splicing factors (Chen and Manley, 2009) and translation is inhibited by siRNA and other decay mechanisms of mRNA (Matzke et al., 2001). Mechanisms for controlling protein processing are less studied than gene transcription and translation, and proteomics is a relative new, but promising cancer research field.

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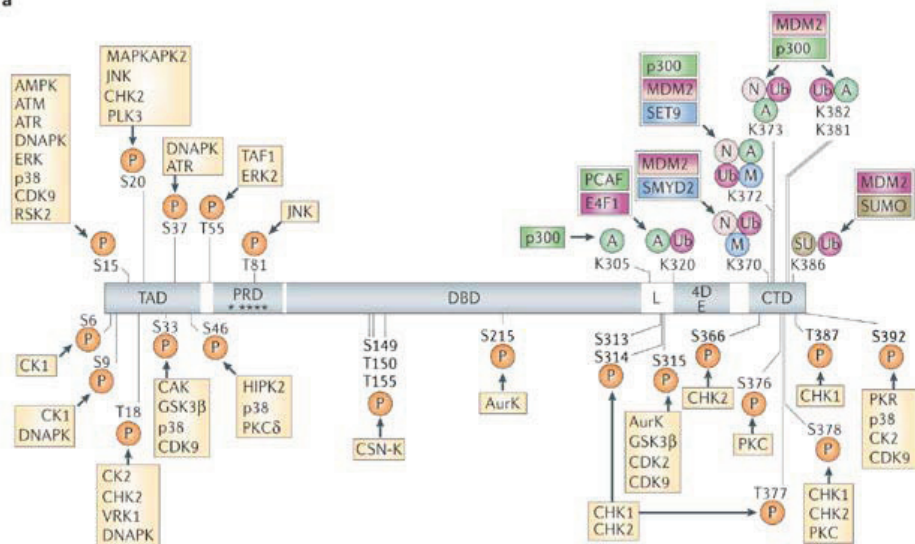


Figure 5. Post-translational modifications of human p53. Specific residues are modified as shown, with phosphorylation (P) in orange, acetylation (A) in green, ubiquitylation (Ub) in purple, neddylation (N) in pink, methylation (M) in blue and

sumoylation (SU) in brown. Proteins responsible for these modifications are shown in matching colours (Toledo and Wahl, 2006).

1.3.2 Epigenetic changes

In addition to genetic changes affecting cancer development, there is an emerging knowledge of DNA information outside the human 30 000 genes. The term epigenetics refers to study of heritable changes that can not be explained by changes in DNA sequence (Bird, 2007). The most studied epigenetic changes are histone modification and promoter methylation (Cheung and Lau, 2005).

DNA is wrapped around proteins which are called histones. Histones are responsible for further condensation of DNA. In its highest condensed form, DNA is not accessible for gene transcription, and modification of these histones like methylation, acetylation and phosphorylation regulates the tightness of this wrapping and thereby transcription (Strahl and Allis, 2000). During mitosis, these protein modifications are thought to be inherited into its daughter cells and several models exists for explaining this inheritance in the replication fork, however in some situations a faithful replication is not always desired, and therefore there is a window for change during mitosis (Probst et al., 2009). Also, modification of histones is a continuous process by different enzymes which active regulate gene transcription (Shilatifard, 2006).

The second epigenetic regulation mechanism is methylation at CpG sites. Here, cytosines are converted into 5-methylcytosine. 5-methylcytosine is not recognised by the transcription apparatus, and when this occurs in the DNA promoter, the gene is turned off (Siegfried et al., 1999), Figure 4. DNA methylation is regulated by several enzymes, and methylation and demethylation is believed to be a continuous process (Kangaspeska et al., 2008). DNA methylation is the most studied of the two epigenetic regulation mechanisms. Promoter methylation is closely related to cancer by silencing of tumour suppressors in cancer development (Esteller, 2008; Kanai, 2008), and assays identifying promoter methylation has been designed for several tumour suppressor genes.

1.3.3 Alternative splicing

Alternative mRNA splicing is found in more than half of multi exon genes (Johnson et al., 2003), and alternative splicing is responsible for increasing the functional diversity of the human genome in making isoforms of the protein (Stamm et al., 2005). Although the function of alternative splicing driving cells into a cancer phenotype is not completely understood (Keren et al., 2010), alternative splicing is regulated through expression of enhancers or silencers with important functions in the spliceosome assembly or by activation of splice specific transcription factors (House and Lynch, 2008). The splicing factor 2/alternative splicing factor (SF2/ASF) has been identified as a proto-oncogene and is upregulated in various tumours stimulating alternative splicing by antagonising the inhibition of heterogeneous nuclear ribonucleoprotein (hnRNP) family on spliceosome assembly (Karni et al., 2007). Alternative splicing has been identified in tumour suppressors like BRCA1/2 (Gutierrez-Enriquez et al., 2009), APC (De Rosa et al., 2007), CHK2 (Staalesen et al., 2004), TP53 and TP73 (Hofstetter et al., 2010) with inactivation of the genes as a result and susceptibility to cancer, both inherited and sporadic, has been reported (Venables, 2004). Alternative splicing has been explored both on high throughput platforms including all known genes and on a single gene level, and this will hopefully provide new diagnostic and therapeutic tools in cancer treatment in the future (Pajares et al., 2007).

1.3.4 Cancer stem cells

The models for cancer stem cells were first introduced in hematopoietic cancers in 1997 (Bonnet and Dick, 1997), but the concept has later been applied to the field of solid tumours. The hypothesis is that a malignancy is created from pluripotent cells like ordinary stem cells, which have the ability to differentiate into all cell types. Cancer treatment with traditional chemotherapy will only kill differentiated tumour cells, but the cancer stem cell will survive and cause relapses later on (Woodward et al., 2005). Therefore, according to this model, a successful cancer treatment will have to include a specific cancer stem cell therapy. They postulate that after specific cancer

stem cell treatment, the tumour will regress just by lack of growth stimuli from the cancer stem cell.

Investigators have been able to isolate melanoma cells with some stem cell characteristics, but they have not been able to conclude that these cells are in fact cancer stem cells (Dou et al., 2007; Na et al., 2009). Also, whether melanoma stem cells are derived from melanocyte stem cells, melanocyte progenitors or de-differentiated melanocytes remains unclear (Zabierowski and Herlyn, 2008).

1.3.5 Important signal transduction pathways with disturbances in melanoma

Cell cycle

Because of p16's importance both in inherited melanoma and as a prognostic factor in primary melanoma, a brief overview of cell cycle is presented. The cell cycle consists of four phases, M (mitosis), G1, G2 and S (synthesis) phase. The two G (gap) phases allow repair of DNA. G1, S and G2 are called interphases. A cell passage through the cell cycle is thoroughly regulated by different proteins called cyclins. These proteins have different associated proteins called cyclin dependent kinases (CDKs). The concentration of the different cyclins and their associated kinases rises and falls during cell cycle, and these complexes drive the cell through the cell cycle (Massague, 2004). In G1, G2 and M, there are cell cycle checkpoints which control cell cycle. Different biochemical signalling pathways converge at these checkpoints and stop cell cycle if the genome is damaged. Stimuli to the first checkpoint (G1) like response to DNA damage leads to arrest of cell cycle. The next checkpoint, the G2 checkpoint, also has an effect on cycle in response to DNA damage or unreplicated DNA (Nyberg et al., 2002). This ensures that damaged DNA is not replicated during S-phase. Finally, the M-checkpoint reacts on misalignment on the mitotic spindle and arrest chromosomal segregation (Malmanche et al., 2006). Disruption of these checkpoints allows new mutations to be integrated in the genome, mutations that for instance may induce carcinogenesis.

The G1 checkpoint is the best characterised of these checkpoints. Many signalling pathways intervene here on the basis on metabolic status, stress and gene status, and a decision is made regarding self renewal, differentiation and death. In order to continue with cell cycle, different Cyclin/Cyclin dependent kinase (CDK) - complex has to be activated. For G1, the CDK includes CDK2 which combines with Cyclin E and activate a pre-replicative complex (PRC). Activated PRC recruits DNA helicases, primases and polymerases causing unwinding of DNA helix and DNA replication. Newly synthesised DNA cannot reassemble new PRC until CDK activity drops at the end of the mitosis ensuring that DNA is only replicated once (Prasanth et al., 2004). The level of Cyclin E is constant high in early embryos allowing CDK2 to initiate S-phase as soon as the M-phase is over (Murray, 2004). In most other cells, however, the levels of Cyclin E are low until mitotic signal intervene. Cyclin E expression is under control of E2F transcription factor. E2F is bound to the retinoblastoma protein (Rb), and Rb binding turns the transcriptor factor into a repressor. Mitotic stimuli increases the levels of Cyclin D which combine with its CDK (CDK 4 and 6), and this dimer phosphorylates Rb so that E2F dissociate from Rb. As a consequence, E2F becomes active and can support transcription of Cyclin E. Cyclin E-CDK2 complex also phosphorylates Rb causing a positive feedback loop ensuring S-phase entry once CDK2 has been activated. Research done on mice deficient of Cyclin D show that some tissues are strictly Cyclin D dependent like hematopoietic tissue whereas other tissues are Cyclin D independent (Kozar et al., 2004).

CDKs are also regulated by direct binding by inhibitory proteins which combine with the Cyclin/CDK complex and disrupt its catalytic centre. Many of these proteins, p15, p16^{INK4a} and p57 are mediators of cytostatic signals. Another inhibitory protein, p21 is a downstream protein of p53 in the p53 cascade (Gil and Peters, 2006). Another inhibitor that binds to the Cyclin-CDK complex is p27. Mitogenic signals like CDK4/6 neutralise p27 together with its effect on Rb so that CDK2 is activated both through transcription and removal of the inhibitory protein p27. When the cell balance tips towards CDK2 activation, CDK2 bites back at p27 by phosphorylating the protein and marks it for polyubiquitination and destruction (Montagnoli et al., 1999).

As mentioned above, $p16^{INK4a}$ is another inhibitor of Cyclin-CDK complex (Sherr, 1996). $p16^{INK4a}$ expression is induced by transcription factors in response to excess RAS-MEK-ERK activity, thereby protecting cells against hyperactive RAS pathway inducing senescence by blocking CDK4/6 activity (Serrano et al., 1997).

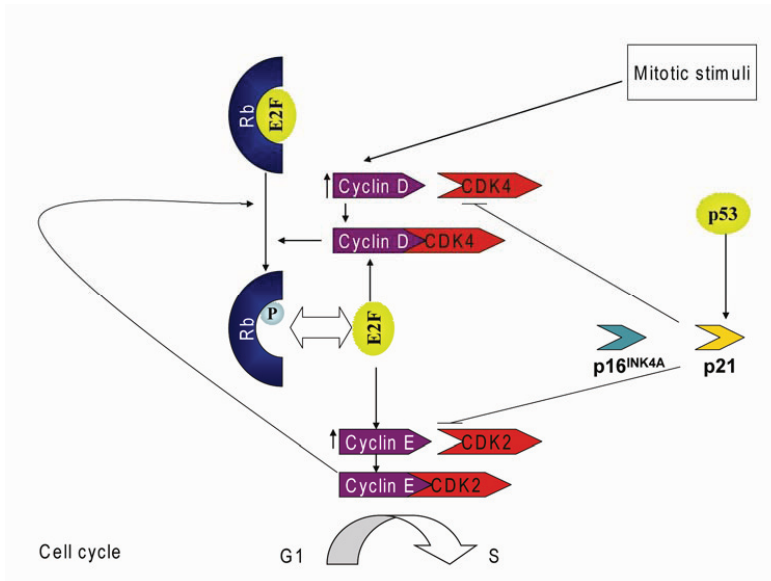


Figure 6: The Rb pathway

RAS-RAF-MEK-ERK (MAPK) pathway

Activation of the growth kinase pathway, MAPK, has been proven in several tumour forms, but is predominant in melanoma with activated BRAF V600E in 50-70% of melanoma cell lines, and additional 10-20% of NRAS in codon 61 (Davies et al., 2002), and great effort are put into identifying and testing molecules targeting this pathway specific by the pharmaceutical industry. This signal transduction pathway couples binding of growth factors at the cell membrane to intracellular response (Figure 7). Upon EGF receptor activation by EGF, two molecules of EGFR are dimerised, and their intracellular parts are autophosphorylated. Grb2 and SOS (sons of sevenless) are recruited to EGFR's intracellular domain, and RAS is translocated from

cytosol to the cell membrane. RAS is activated by GTP binding, and RAF is activated by phosphorylation. RAF phosphorylates MEK which again phosphorylates ERK (Schlessinger, 2000). ERK enters the nucleus and activates different transcription factors like C-MYC and AP1 by phosphorylation (Karin, 1995; Marampon et al., 2006). AP1 binds to the Cyclin D gene and facilitates its transcription whereas C-Myc targets p53. (Hennigan and Stambrook, 2001; Reisman et al., 1993). Thereby, activation of this pathway by EGF results in Cyclin D expression and facilitates cell cycle progression.

PIP₂-AKT pathway is another downstream pathway of RAS. Activated RAS can recruit membrane bound Phosphatidylinositol 3-kinase (PI3K), and phosphatidylinositol-4,5-bisphosphate (PIP₂) is phosphorylated to PIP₃. PIP₃ recruits two serine/threonine kinases to the membrane, PDK-1 and AKT. Here PDK-1 phosphorylates AKT, and AKT translocates to the nucleus where it phosphorylates different transcription factors and also proteins that are involved in apoptotic and survival pathways (Franke et al., 1997). In relation to cell cycle AKT inhibits glycogen synthase kinase 3-β, preventing this kinase from phosphorylating and destabilising Cyclin D (Sears and Nevins, 2002). AKT also inhibits FOXO transcription factors by barring them from the nucleus and thereby prevent transcription of its gene targets like p27 and p21 (Arden, 2004; Brunet et al., 1999; Medema et al., 2000).

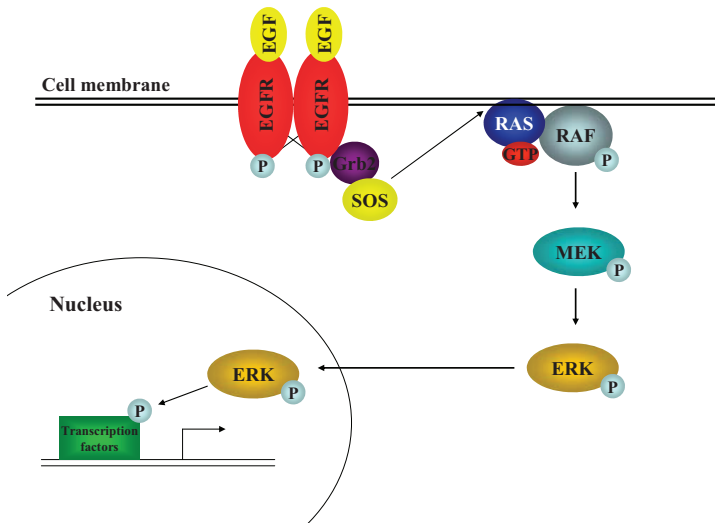


Figure 7: The MAPK pathway

1.4 Chemoresistance in melanoma

1.4.1 The p53 pathway

Unlike other cancer forms (Toledo and Wahl, 2006), melanoma has a low frequency of TP53 mutations (Akslen et al., 1998; Florenes et al., 1994). This conclusion was supported by studies where p53 was analysed by immunohistochemistry and sequencing of known hot spot exons. The open reading frame of TP53 was not sequenced. Still, biallelic deletions and inactivation of the *p14^{ARF}* transcript of *CDKN2a* gene, coding for a protein binding and inactivating MDM2 and thereby stabilising p53, are frequent in melanoma (Grafstrom et al., 2005). One of the aims of this study was to see if such mechanism could be involved in chemoresistance in this cohort. Radiation and some cytotoxic drugs, anthracyclins like Doxorubicin and Epirubicin, cause double stranded DNA breaks and exert their effect through the p53 pathway (Figure 8): When a DNA double-stranded DNA break is sensed by ataxia teleangiectasia mutated (ATM), ATM phosphorylates and activates the transducer

checkpoint kinase, CHK2, which again signals through p53. Both ATM and CHK2 phosphorylate MDM2 (and p53) so that MDM2's inhibitory effect of p53 is disrupted. p53 is a transcriptional factor for many different downstream effectors, amongst them, p21. Upon DNA damage, p53 is activated and p53 induces p21 transcription. p21 inhibits cell cycle by binding to the Cyclin-CDK complex (see 1.3.5).

Resistance to alkylating agents like DTIC has been linked to expression of the DNA repair enzyme MGMT (see 1.4.2). No alternative pathway has been identified as responsible for the primary resistance to anti neoplastic drugs in melanoma.

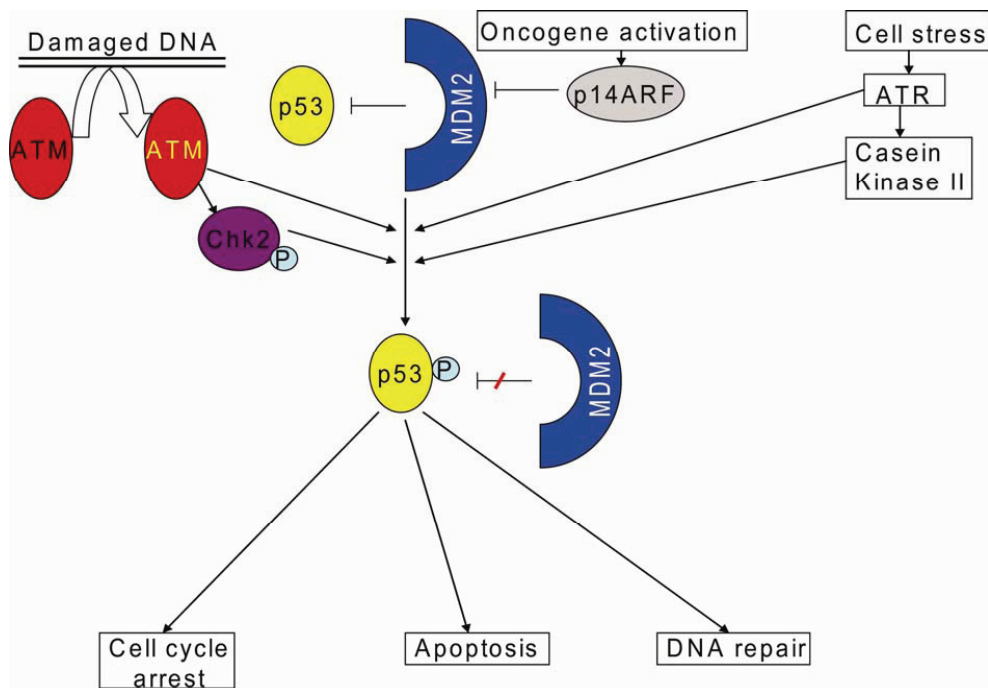


Figure 8: The p53 pathway

1.4.2 O-methylguanine-DNA methyltransferase

MTIC, the active compound of DTIC and temozolomide adds methyl groups to several localisations in DNA, amongst them N^7 position of guanine and O^6 position of guanine (Denny et al., 1994; Middleton and Margison, 2003). Although methylation of the O^6 position of guanine is the less common event of these, it is believed to be of most importance (Friedman et al., 2000). The DNA repair enzyme O-methylguanine-

DNA methyltransferase (MGMT) reverses this reaction and thereby inhibits the killing of cancer cells by removing drug induced methyl group of guanine (Graves et al., 1989; Souliotis et al., 1991). Hence, the DNA damage will not be recognised by the cell, and no apoptosis will occur. Tumour expression of this enzyme is recognised as a major contributor to resistance to alkylating agents *in vitro* (Scudiero et al., 1984), but the correlation has not been so evident in clinical trials. Hypermethylation analysis of *MGMT* has been shown to correlate with positive clinical response to temozolomide in gliomas (Esteller et al., 2000; Hegi et al., 2004; Paz et al., 2004). Also, a report in 2002 showed a trend correlating MGMT immunostaining to DTIC response in melanoma, although not significant (Ma et al., 2002). Recently, a paper found a correlation between expression of *MGMT* and temozolomide response in melanoma cell culture (Augustine et al., 2009). Interestingly, the authors were unable to find a correlation to methylation of the *MGMT* promoter, suggesting other regulating mechanism than methylation of the promoter to be of importance for *MGMT* expression. Clinical trials using other substrates for MGMT (i.e. inhibiting the enzyme) in combination with alkylating agents have been performed. They have shown efficacy in reducing MGMT activity, but so far not on clinical outcome (Quinn et al., 2009; Watson et al., 2009).

1.4.3 Predictive factors in melanoma

Regarding predictors of therapy, there are no such for standard treatment. A combination of BRAF mutational status and *CDKN2A* expression has been identified as predictors in isolated limb perfusion with high dose melphalan and actinomycin D (Gallagher et al., 2008). Also *MGMT* expression has been linked to temozolomide cytotoxicity in melanoma cell lines (Augustine et al., 2009) but so far not *in vivo*.

2. Aim of study

The primary aim of this project was to identify predictive factors in relation to dacarbazine treatment in metastatic melanoma. Secondly, we wanted to identify molecular factors predicting survival in patients suffering from metastatic melanomas.

3. Material and methods

3.1 Patients

From year 1999 and onwards, 85 consecutive patients referred for metastatic malignant melanomas to the Department of Oncology, Haukeland University Hospital were included in the study. The last follow up was May 2009. All patients were given information about the study and signed an informed consent form.

For paper I, 57 patients of the total material were included in the main study. Initially, we excluded 28 patients having tumour samples obtained by Tru-cut biopsy from this study due to a limited amount of tissue available. Subsequent, tissue from 20 out of these 28 were analysed in the second part of the microarray as a validation set.

3.2 Therapeutic protocol

The protocol for this study was designed by the authors JG and PEL, and the protocol was approved by the regional ethic committee. The patients were preferably treated with dacarbazine (DTIC 800-1000mg/m² every 21 days), but also a related drug, Temozolomide. This drug is converted to the same active substance as DTIC, MTIC. Temozolomide was given to a few patients with advanced disease complicated by brain metastases.

Clinical responses to be used for the present thesis were evaluated retrospective after the UICC-criteria every 6 weeks by CB in collaboration with the medical oncologist JG.

3.3 Tissue sampling

Surgical biopsies from accessible lymph nodes and subcutaneous metastases were obtained the day before implementing the first chemotherapy cycle. For patients with metastases to internal organs biopsies were obtained under ultrasound guidance with a Tru-cut biopsy needle. For most of the patients, Tru-cut biopsies were obtained on the day after treatment and whenever therapy was changed due to disease progression. This was done to look at molecular changes caused by the therapy and disease progression, but these samples have not been analysed as a part of this thesis. A part of the biopsy was examined histopathological to make sure that the sample was representative, and indeed some of the samples had to be rejected as expected for the study (6 patients). The rest of the sample was snap frozen in liquid nitrogen until examination to conserve RNA.

3.4 Nucleic acid purification

RNA was extracted with Trizol[®] reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Some of the samples were contaminated with melanin after the RNA extraction, and further steps had to be added to separate RNA from melanin (Carninci and Hayashizaki, 1999). Finally, RNA was dissolved in H₂O and kept in -80°C.

DNA was extracted from the biopsies using QIAamp DNA Mini Kit[®] (Quiagen, Venlo, The Netherlands). Also for the DNA analyses, some of the samples had to undergo further steps to remove melanin (Lagonigro et al., 2004; Nozawa et al., 1999). Isolated DNA was dissolved in H₂O and kept in -20°C.

Complementary DNA (cDNA) was synthesised using reverse transcriptase (Roche diagnostics, Basel, Switzerland) following the manufacturer's instructions, and oligo dT was used as transcriptase primer. For the lightcycler assays, the cDNA was made from the same RNA concentration, and random hexamers was added to the solution

to standardise the quantity of the cDNA in the whole open reading frame. Three parallel cDNA preparations were made for combined analyses.

3.5 PCR

For mutation screening of the tumour samples, we used cDNA as a template and performed PCR's up to 500bp of the open reading frame. For some genes we had to use nested PCR to amplify the product, in others, we had to use DNA as template and amplify all exons. The amplification was performed with the DyNazyme EXT polymerase system (FINNZYMES, Espoo, Finland). Another polymerase (AmpliTaq Gold™ DNA polymerase, Roche Diagnostics, Basel, Switzerland) was used for selected genes like *BRAF*.

3.5.1 Methylation specific PCR (MSP)

To determine the methylation status of the promoters of interest, we used primer and annealing temperatures as published previously by others. We used Ez DNA-methylation gold™ Kit (Zymo Research, Orange, CA) for the bisulfide conversion of tumour DNA. PCRs were performed with specific methylated or unmethylated primers for the promoter, and in the methylation specific PCRs we also used AmpliTaq Gold™ DNA polymerase for the amplification. Promoter status was determined after gel electrophoresis of the PCR products on a 3% agarose gel stained with ethidiumbromide. A CpGenome™ Universal Methylated DNA (Cemicon International, Temacula CA) was used as a positive standard and leukocyte DNA from healthy volunteers as negative control.

3.5.2 Quantitative Real Time PCR (qRT PCR)

A duplex qRT-PCR system (LighCycler®480, Roche Diagnostics, Basel, Switzerland) was used in the following concentrations: 1x LightCycler® 480 Probes Master, 0.5µM primers, and 0.125µM probes. In addition, 2µl template was added to the mixture in a final volume of 20µl. The cDNA was made from total RNA, in a

50ng/ μ l concentration. A housekeeping gene, β -2-microglobulin (*B2M*) served as a standard and was used as expression reference in each sample. Expression levels were presented as percentage of expression in the cell line SK-Mel 28. All qRT PCR assays were performed in three parallels.

3.5.3 Radioactive Quantitative PCR

For the analyses of the alternative BRAF splices we were not able to design a specific qRT-PCR because of resemblance in sequence at the breaking points. Therefore, we were forced to use a method with radioactive α -³²P-dCTP in the PCR and quantify the amount of the different splices based on radioactive count in different bands after gel electrophoresis and transfer of the products to a membrane.

3.6 Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA is a multiplex PCR method used to detect abnormal copy number of multiple genes or parts of genes down to 50-70 base-pairs. Primers and probes are designed to numerous sites on the locus of interest and also to other places on the same and different chromosomes for normalisation. Contrasting other PCR's it is not the primers that hybridise to the locus, but two adjacent MLPA probes that again are ligated. If the sequence is missing, no ligation will occur, with no subsequent amplification. The primers are marked with fluorescence and more amplification will give a higher fluorescence. For each target sequence, the distance between the two primers are unique and therefore the different targets can be discriminated by electrophoresis, and fluorescence intensity for each target is compared that of a standard.

3.7 Cell culturing

In the *in vitro* assay described in paper III two different melanoma cell lines were cultured, SK-mel 28 as a standard cell line mutated in BRAF, codon 600 and SbCl2 one of the few available melanoma cell lines wt for BRAF codon 600 and NRAS codon 61. These cell lines were cultured in RPMI 1640 (Carlsbad, CA, USA) added 10% foetal bovine serum (FBS) and L-glutamine. Incubation conditions were 37⁰C, 5% CO₂ and 100% humidity.

3.8 Western blot

Western blots were performed as previously described (Lyustikman et al., 2008). Briefly, the cells were lysed, and the cytosolic fraction loaded on a SDS-page gel for electrophoresis. The protein was then transferred to a nitrocellulose membrane and probed with monoclonal or polyclonal antibody specific to the target protein. A secondary antibody tagged with horseradish peroxidase was then added, and following incubation, a chemiluminescent substrate was added and the light detected on a photographic film.

3.9 Small interfering RNA (siRNA) knockdown

The cells were cultured on a dish over night and transfected with siRNA against the target gene according to the manufacturer's instruction using transfection reagent from Dharmacon (Lafayette, CO, USA). After incubating the cells for 48 hours, siRNA knockdown was considered most effective, and the cells were incubated with dacarbazine or control.

3.10 aCGH

Sample DNA and pooled reference DNA (Promega, Madison, WI, USA) was labelled and washed as described (Jonsson et al., 2007) and hybridised to 32k tiling

resolution BAC clone microarrays produced at the SCIBLU Genomics Centre, Lund University (www.lth.se/sciblu) as previously described (Jonsson et al., 2007). Median background-corrected intensities were filtered to remove spots that had been flagged in the image analysis or had a signal-to-noise ratio <5 in either of the intensity channels. Normalisation was performed as described (Staafl et al., 2007). Using estimated noise in each array, a moving average was applied to define adaptive thresholds for each sample (Staafl et al., 2007), subsequently used to call gains and losses by the CGH-Plotter segmentation algorithm (Autio et al., 2003). Fraction of the genome altered (FGA) was calculated as described (Chin et al., 2006).

3.11 Micro array (mRNA expression)

Gene expression analyses were performed using Illumina Beadarrays (HumanWG-6 v2 Expression Beadchip) (Illumina, San Diego, CA, USA) and the Illumina system (www.illumina.com) according to manufacturer's instructions at the SCIBLU Genomics Centre, Lund University (www.lth.se/sciblu). Illumina gene expression data was loaded into the Beadstudio v3 software (Illumina). Here, data was normalised using the cubic spine method. Normalised gene expression data was then exported, and only features with a detection p-value of <0.01 in at least 80% of the samples were used in further analyses. Next, data was loaded into MeV v4 (Saeed et al., 2003) where it was \log_2 transformed and mean centred across assays.

4. Summary of results

Our main results may be summarized as follows:

In paper I we showed that metastatic melanoma patients could be classified into four separate groups based on hierarchical analyses of gene expression; The four subtypes were named (1) high-immune response, (2) proliferative, (3) pigmentation and (4) normal-like, as reflected by the set of genes characteristic of each group. Importantly, this classification gives prognostic information, thus the patients with expression of proliferative genes displayed an inferior survival to the others, and tumours from these patients also harboured homozygote *CDKN2A* deletions. Low expression of a predefined gene set associated with B and T-cell activity was significantly associated to poor outcome.

In paper II, for the first time, to our knowledge we were able to identify a predictor for the standard treatment of metastatic melanoma. *MGMT* expression was associated with disease stabilisation on DTIC treatment, and this marker also proved as a significant biomarker regarding overall survival as well as time to progression. Although *MGMT* methylation was associated with *MGMT* expression, *MGMT* methylation was not associated with clinical parameters, suggesting other regulation mechanisms of *MGMT* expression than methylation. In multivariate analyses, both *MGMT* expression and the old prognostic marker *p16^{INK4a}* proved to be independent factors in relation to overall survival, also when Breslow thickness and serum lactate dehydrogenase (LDH) were included. The observed correlation between Breslow thickness and *MGMT* expression further suggests a biological link to this histopathological measure other than tumour load.

In paper III, we were able to identify novel alternative splicing of *BRAF*, and expression of these splices corresponded to objective response to dacarbazine treatment. These splices were mostly seen in tumours wt in *BRAF*. Our findings were supported by *in vitro* studies of melanoma cell lines where knockdown of BRAF wild type for codon 600 displayed apoptosis in 30% of melanoma cells after DTIC

incubation whereas knockdown in mutated BRAF V600E did not respond to DTIC incubation.

Lastly, in paper IV, we identified aberrations in the p53 pathway, including p53, MDM2 and p14^{ARF} to predict reduced overall survival in our cohort when comparison was made between tumours with no or minor alterations in this pathway and tumours with major alterations. There was no correlation between mutational status and outcome when looking at the single genes in the pathway. In addition, when combining expression levels of *p16INK4a* to the analysis, a distinct group with no major alterations in the p53 pathway and high *p16INK4a* levels showed a significant better prognosis than the other patients.

5. Discussion

The main strength of this thesis is that the patient inclusion, tissue sampling and follow up were done in a prospective, consecutive manner in a single institution. In that way, bias in patient selection is excluded. Although tissue sampling and most of the laboratory analyses were performed by the undersigned who also participated in the collection of the clinical data, response and survival data were not matched with the laboratory findings until the follow up was closed (May 09). In that way, the investigator was blinded to the clinical data during the laboratory analyses.

Results from nucleic acids, DNA and RNA analyses have been presented in this thesis. The genes we have analysed are all genes coding for enzymes in cellular signal transduction pathways. One might advocate that immunohistochemistry or enzyme activity testing would be more appropriate than gene analysing. However, enzyme activity testing is difficult to perform in patient tissue due to low sample volume (Tru cut biopsies), and immunohistochemistry/Western Blots are semi-quantitative measurements. A positive immunohistochemistry (IHC) does not mean that protein function is normal. For instance, a single nucleotide mutation that does not interfere with protein folding or binding to the specific antibody used is not detected, although it could cause an impairment or loss in enzyme function. Yet, in paper I, we have included IHC analyses, and in paper III we have performed Western Blots from cell cultures to confirm our findings in RNA analyses.

We have not performed micro-dissection of tumour tissue to ensure that the tissue analysed really is tumour cells and not supporting, non-tumour cells like fibroblasts, endothelium cells and white blood cells. Indeed, in paper I the main finding is that expression of immune response genes in the tumour is caused by infiltration of CD3 (T-cells) and CD20 (B-cells) positive white blood cells and not expression of these genes in by the actual tumour cells. Regarding molecular findings in paper II-IV, at least in relation to mutation screening, infiltration of non-tumour cells conceals single nucleotide mutations rather than over-estimates them when sequencing is performed.

Therefore, the presence of non-tumour cells in the tissue analysed results in less sensitivity but does not reduce specificity in mutation screening.

Using array based mRNA expression, we were able to differentiate our patient population into four different groups with different prognosis Paper I. This differentiation was validated in an external set of metastatic melanoma patients (Bogunovic et al., 2009) with the same difference in prognosis between the groups. Also, it was confirmed in an internal dataset of 20 patients our own protocol initially excluded for this analysis due to small amount of tissue available for the aCGH analyses. However, for the microarray analyses the tissue was sufficient, but the number of patients was too low to validate the prognostic difference.

One of the problems we encountered in this study was the unfortunate low clinical response rate to DTIC therapy. In our cohort, only 5% responded objectively to DTIC monotherapy treatment. Although response rates up to 20% on DTIC has been reported (Lui et al., 2007), response rates differ greatly, and a response rate of 10% to DTIC is not uncommon (Eggermont and Kirkwood, 2004). This low response rate made it statistically difficult to identify biomarkers predicting response. Therefore, regarding the predictive factors, *MGMT* and *BRAF* (Paper II and III) splicing one might argue that these factors are of little value, given the fact that the objective response rate to dacarbazine in general is so low. Still, dacarbazine (and temozolomide) is the best treatment available today, even though not much superior to observation alone. In the *MGMT* paper (Paper II), we also included stable disease as good response which is quite common when evaluating response in melanoma treatment.

The novel splice variants of BRAF detected in paper III are hypothesised to reduce BRAF function in a dominant negative fashion. Although expression of splice variants of tumour suppressor genes and oncogenes are found in tumours, and they have proven carcinogenic in experimental systems, the biologic relevance of splice variants in tumours is still controversial (Korner and Miller, 2009). In our paper, we were able to identify these splice variants, and there seems to be a correlation to BRAF V600E mutation status and objective response to DTIC treatment. Whether or

not the isoforms without the kinase domain have a dominant, negative effect on normal BRAF is not shown by our experiments. Still, we were able to prove BRAF link to DTIC by synthesizing melanoma cell line wt for BRAF codon 600 to DTIC with BRAF knockdown whereas a mutated BRAF V600E could not be synthesized to this treatment.

Our identification of alterations in the p53 pathway (Paper IV) to correlate to overall survival in melanoma, contrasting former studies, emphasises this pathway's importance in melanoma regarding biological aggressiveness. Whether the survival benefit of not having disturbances in this pathway was influenced by DTIC treatment or not is a difficult question. Although alterations did not relate to response to treatment significantly in our cohort, there might be some effect, and therefore a treatment contribution to survival benefits is possible. Still, we believe that the identified correlation between the p53 pathway and survival reflects tumour biology and not treatment. Of course, a similar analysis in an untreated patient cohort would answer this question, but to include patients to this study would be difficult. Although the treatment results are poor, most patients will try available treatment.

6. Future perspectives

The identification of four distinct tumour-classes based on microarrays from biopsies of metastatic melanoma opens up for selectively offering novel, experimental drugs, like immune stimulating agents to subgroups of patients based on different predictive factors in future studies. So far, these new regimes have not been able to prove their superiority to standard treatment, at least in Phase III trials, and by offering this treatment to the subclasses of melanoma patients identified in our work in clinical studies, these regimes might show improved response rates. Performing microarray analysis has not become standard in medical laboratories and still remains a research tool. Likewise handling of the biopsies to preserve RNA for such analysis will require a change in clinical protocols. An ideal substitute for microarray analysis would therefore be an immunohistochemical (IHC) analysis that correlates thoroughly to our classification. We were able to correlate the microarray findings to IHC staining but there was not complete overlap, and further search for such ideal IHC analysis are warranted. Further, as RT-PCR techniques become more sophisticated one might hope that methods extracting RNA from formalin fixated biopsies might be sufficient for analyses using only fragments of RNA as substrates in predefined immune genes.

Recently, ipilimumab, T-cell activator proved survival benefit in advanced melanoma (Hodi et al., 2010; O'Day et al., 2010). This is in concert with our findings that T-cell activation is an important anti-tumour mechanism and target in metastatic melanoma. Therefore, a trial that tested responses in different groups after our classification of metastatic melanoma would be of importance. Whether this drug could further potent anti-tumour activity in patients already harbouring T-cell activation in their metastatic deposits or if patients without this activation have a better potential for ipilimumab treatment is not clear. If all the responders are in the immune activated group, this treatment can be offered to these patients selectively with even better responses than found by Hodi et al.

The identification of *MGMT* expression as a positive predictor for stabilisation of the disease on DTIC treatment is one of the findings that add to the development of a tailored medication of this disease on the basis of biomarker status. After validation of our findings, DTIC treatment can then be offered selectively to patients expressing low *MGMT* levels in metastatic deposits, thereby avoiding ineffective treatment to patients with high *MGMT* expression. Again, to use the analyse tools provided in this paper, RNA has to be harvested from metastatic tumours for analyses, and of course, IHC substitute would be easier to be incorporated in clinical practice.

The discovery of novel alternative *BRAF* splices variants further emphasises BRAF's importance in melanoma and also in prediction of chemosensitivity. Further clinical follow up studies are warranted, and the functional role of each isoform needs to be tested *in vitro* to prove their inhibitory effect on the wild type isoforms. The introduction of new inhibitors in this pathway, with improved response rates in early studies elucidates our findings, and it will be interesting to test out this treatment in relation to *BRAF* mutational/splicing status. One of the most exciting compounds in this aspect is the new drug, PLX 4032, which has shown promising results in phase I study with high objective response rate of the BRAF 600 mutated patients receiving the drug (Flaherty et al., 2009). Another compound which selectively inhibits BRAF 600E has shown promising response rates in a Phase I/II trial (Kefford et al., 2010).

Our discovery of the p53 pathway to be of importance in melanoma at least in respect to overall survival further adds to the prognostic implication of doing molecular testing in cancer. Although former studies have shown little importance of p53 in melanoma, analyses of other signal molecules in the p53 cascade question this theory. Still, we were not able to associate this finding to chemoresistance in this study, but in another cohort with better response rates such correlation may become significant.

The work presented in this thesis provides better knowledge about the molecular diversity in metastatic melanoma patients, both regarding response to treatment and survival. This is a field that has been poorly explored; work in the past has been focused on primary melanoma and cell lines from primary and metastatic spread.

There are few other cohorts on metastatic melanoma analysing molecular biomarkers' correlation to response to treatment and survival, and our findings could be adapted into clinical treatment after validation and some adjustments. Therefore, the results are crucial for tailoring existing treatment and will also serve as a guide in offering new, targeted therapy and immune-stimulating therapy to the right patient groups.

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