MELANOMA SUSCEPTIBILITY GENES AND THEIR ROLE IN CUTANEOUS MALIGNANT MELANOMA DEVELOPMENT IN LATVIA

Dace Pjanova

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Author: Dace Pjanova

Promoters: Dr. biol. Olita Heisele
Dr. med. Ludmila Engele

RIGA 2007

University of Latvia
Promotion Council
Biology
Riga, Latvia

Chairman: Prof., Dr.habil.biol. Pauls Pumpens

Opponents: Prof., Dr.biol., MD Viesturs Baumanis
(University of Latvia, Riga, Latvia)

Dr.biol. Janis Klovins
(Latvian Biomedical Research and Study Centre, Riga, Latvia)

Asoc.prof., MD, PhD Johan Hansson
(Karolinska Institutet, Stockholm, Sweden)

Dissertation is accepted for the commencement of the degree of Dr. biol. (in Molecular Biology)

Commencement: June 8th, 2007

ABSTRACT

Cutaneous malignant melanoma (CMM) is a very aggressive tumour the incidence of which is rising rapidly in most fair-skinned populations worldwide and in Latvia as well. The increase in CMM incidence during the last 5 years in Latvia is raised from 5.4 cases in the year 2000 up to 7.5 cases in 2005. The disease is complex and heterogeneous with genetic, phenotypic, and environmental factors contributing to its development. With regard to genetic factors, approximately 5-12% of melanoma cases occur in a familial setting (Goldstein and Tucker 2001). To date, two high-penetrance melanoma susceptibility genes, cyclin-dependent kinase inhibitor 2A (*CDKN2A*) on chromosome *9p21* and cyclin dependent kinase 4 (*CDK4*) on *12q13*, have been identified. Germline inactivating mutations of the *CDKN2A* gene are the most common cause of inherited susceptibility to melanoma and only few families worldwide have been found to harbour *CDK4* mutations. However, predisposing alterations are still unknown in a large proportion of melanoma kindreds and other causal genes have yet to be identified. There have also been only few published estimations of the prevalence of such mutations among incident cases of melanoma and much ongoing research is being focused on the identification of low-penetrance melanoma susceptibility genes that confer a lower melanoma risk with more frequent variations.

The aim of the present study was to characterise the *CDKN2A* and *CDK4* gene status in CMM patients and their tumours, relate these changes to melanoma susceptibility and pathogenesis, and assess the criteria for identification of individuals at evaluated risk for CMM in Latvia.

One hundred and seventy-six melanoma patients with histologically confirmed CMM, for the first time in Latvia, were screened for germline mutations in *CDKN2A* and *CDK4* exon 2 using single stranded conformational polymorphisms analysis (SSCP) or high resolution melting curve analysis (MCA) and sequencing. In addition, *CDKN2A* mutational status in 27 primary melanoma tissues and promoter methylation in 19 tumours was analysed as well. In order to relate the allelic loses to mutations and promoter methylation four microsatellite markers (D9S974, D9S942, D9S1870, and D9S171) around *9p21* were assessed in 10 of tumours analysed.

No disease related *CDKN2A* germline mutations were identified in observed 176 melanoma patients and no tumour associated *CDKN2A* mutations were found in any of the 27 tumours analysed. Promoter methylation was detected in 2/19 tumours analysed. Previously described *CDK4* germline mutation, R24H, was found in two patients both with a strong family history of melanoma. The investigation of one of the corresponding *CDK4* mutation positive families has shown the segregation of the mutation with the disease on the unique haplotype (113-194-206-*CDK4*-174-201) background. In addition, *CDKN2A* polymorphisms were studied as putative low penetrance melanoma susceptibility as well as melanoma progression alleles. The proportion of cases with polymorphisms in this Latvian melanoma population was c.442G>A (A148T) (6%), c*29C>G (18%), and c*69C>T (17%), however, only the frequency of the A148T polymorphism was higher in melanoma patients compared to that in 203 control individuals (6% vs 1%, p=0.01). A148T has also been reported in association with melanoma in Polish series but not in an English series. Therefore, the examination of the A148T carrier's haplotype in 10 Latvian and 39 Polish samples was done. No significant difference was seen between these both populations and the predominant haplotype observed in English samples, giving no indication therefore that the discrepancy could be explained by population differences in linage disequilibrium. The association between *CDKN2A* polymorphisms and tumour invasiveness was not found, giving no support that polymorphisms could be related to melanoma progression. No significant association between *CDKN2A* polymorphisms and unselected cancer patients groups (breast, ovarian, gastric, colon, and rectum) was found, however, this analysis was underpowered. In melanoma tumours, LOH for at least one microsatellite marker was found in three out of 10

primary melanoma tissues (30%) and LOH at two markers, D9S942 and D9S974, located close to the *CDKN2A* gene, was found in one melanoma tissue, which was obtained from patient with the family history of the disease. However, the expected "double hit" for tumour suppressor gene inactivation was not observed. None of the cases with LOH had mutation or were methylated at the promoter region of the gene.

In summary, results show that germline mutations at the *CDKN2A* locus are rare in sporadic melanomas in Latvia. The study does, however, provide some additional evidence for a role for the *CDKN2A* polymorphism A148T as a low-penetrance susceptibility allele and LOH at *CDKN2A* locus in melanoma tissues suggests the possibility of the existence of an additional tumour suppressor locus on chromosome region *9p* yet to be discovered. At least, two new melanoma families with rare *CDK4* gene mutation were identified in the present work.

PUBLICATIONS

Results of this work have been published in the following papers:

I. **Pjanova D,** Engele L, Randerson-Moor JA, Harland M, Bishop DT, Newton Bishop JA, Taylor C, Debniak T, Lubinski J and Heisele O. CDKN2A and CDK4 variants in Latvian melanoma patients: analysis of a clinic-based population. Melanoma Res. (Accepted 23 November 2006).

II. **Pjanova D,** Heisele O, Engele L, Randerson-Moor JA, Kukalizch K, Bishop DT, Newton Bishop JA. Analysis of Latvian melanoma families for 9p21 germline deletions by multiplex ligation-dependent probe amplification approach. Acta Universitatis Latviensis. 2006; 710: 7-16.

III. **Pjanova D,** Eņģele L, Ignatoviča V and Heisele O. CDKN2A mutations and loss of heterozygosity in primary melanomas. Proc. Latvian Acad. Sci. Section B. 2006; 60(2/3): 98-100.

IV. **Pjanova D,** Heisele O, Engele L, Desjatnikova I. Tumour suppressor gene CDKN2A/p16 germline mutations in melanoma patients with additional cancer and cancer in their family history. Acta Universitatis Latviensis. 2003; 662: 25-32.

Results of his work have been presented in the following international conference:

1. **Pjanova D,** Heisele O, Engele L, Streinerte B, Ignatovica V, Kleina R. CDKN2A and CDK4 alterations in melanoma patients and primary melanomas from Latvia. Perspectives in Melanoma X and The Third Annual International Melanoma Research Congress. Noordwijk, 2006, September 14-16, Melanoma Research 2006, Vol. 16, Supplement 1, S28.

2. Heisele O, Engele L, **Pjanova D.** Mutation analysis of CDKN2A and CDK4 genes in sporadic and familial melanoma patients from Latvia. 4th Baltic Congress of Oncology, Tartu, 2006, May 25-27, p147.

3. Heisele O, **Pjanova D,** Desjatnikova I, Engele L. Single nucleotide polymorphisms in CDKN2A gene in different groups of malignant melanoma patients. 12th Meeting of the ESPCR, Paris, 2004, September 22-25, Pigment Cell Res. 2004, Vol. 17, N5, p.588.

4. **Pjanova D,** Heisele O, Engele L, Balkovska I, Desjatnikova I. CDKN2A and CDK4 gene variants and histopathological features of cutaneous malignant melanoma. European and UK Melanoma Study Group Conference, Birmingham, 2004, June 17-18, p.14.

5. **Pjanova D,** Heisele O, Engele L, Desjatnikova I. Analyses of CDKN2A and CDK4 genes in sporadic melanoma patients with additional non-melanoma cancer in their family history. 7th International Symposium on Predictive Oncology & Intervention Strategies, Nice, 2004, February 7-10, Cancer Detection and Prevention 2004, Symposium Volume, p.109.

6. **Pjanova D,** Heisele O, Engele L, Desjatnikova I. “Tumour suppressor gene CDKN2A variants in melanoma patients of Latvian population”. 11th Meeting of the ESPCR, Gent, 2003, September 17-20, Pigment Cell Res. Vol.16, Nr.5, p.599.

7. **Pjanova D,** Heisele O, Engele L, Desjatnikova I. “Sreening for tumour suppressor gene CDKN2A/p16 germline mutations in patients with melanoma and unrelated cancer in family history”. Third research meeting on melanoma, Milan, 2003, May 26-27, p.51.

8. Heisele O, **Pjanova D,** Engele L, Desjatnikova I. “The CDKN2A/p16 germline mutations in sporadic melanoma patients of Latvian population”. 1st Conference in Functional Genomics and Disease, Prague, 2003, May 14-17, p.110, abstr. PD3/145.

CONTENTS

ABBREVIATIONS

3'UTR – 3' untranslated region
ASR – age standardised rates
ARF – alternative reading frame
bp – base pairs
CCM – cutaneous malignant melanoma
CDK4 – cyclin dependent kinase 4
CDKN2A – cyclin-dependent kinase inhibitor 2A
FM – familial melanoma
LOH – loss of hetorozygosity
MC1R – melanocortin 1 receptor
MCA – melting curve analysis
MLPA – multiplex ligation-dependent probe amplification
MPM – multiple primary melanomas
MSP – methylation specific PCR
PCR – polymerase chain reaction
RFLP – restriction fragment length polymorphism
SNP – single nucleotide polymorphism
SPM – sporadic primary melanoma
SSCP – single-strand conformational polymorphism

INTRODUCTION

Melanoma develops from the malignant transformation of melanocytes, the pigment producing cells that residue in the basal epidermal layer in human skin. As in the case with most cancer types, both genetic and environmental factors are believed to contribute to melanoma genesis.

Hereditary predisposition to cutaneous malignant melanoma has been recognised since the 19th century, when William Norris, a general practitioner, reported a family with numerous moles and several family members with metastatic lesions (Norris 1820). The most direct evidence for a role of genetic susceptibility to melanoma comes from the clinical identification of relatively rare but clearly evident families with dense clustering of melanoma. Since the first human genetic map in 1987, linkage analysis of families with a high incidence of melanoma culminated in the identification of two melanoma susceptibility genes – *CDKN2A* and *CDK4* – the products of which are known to be components of potent tumour suppressor pathways (Kamb et al., 1994a; Zuo et al., 1996).

The most prevalent in families with melanoma is the *CDKN2A* gene on chromosome *9p21* and only few families worldwide have been found to carry germline mutations in the *CDK4* gene on *12q13*.

Much less progress has been made in the identification of low-penetrance melanoma susceptibility genes which are believed to account for a much larger percentage of melanoma susceptibility in the general population. Only one important common low-penetrance gene has been identified, *MC1R*, variants of which play a major role in determining hair colour (Duffy et al., 2004), freckles (Bastiaens et al., 2001a), susceptibility to sun, and melanoma (Box et al., 2001a). In terms of other low-penetrance melanoma susceptibility genes, there are conflicting data on their role in melanoma susceptibility, including the above mentioned *CDKN2A* gene polymorphisms.

To date, the only effective melanoma treatment is surgery which is effective for melanomas in stage I and II. It means that early detection is very critical. Often the result depends not only from appropriate diagnosis but also from the biological features of the cancer including its gene characteristics. Adding the information about genetic alterations in the classification of melanomas might help to choose the appropriate therapy.

Little is known about the genetic background of melanoma disease. There are not serious genetic investigations and follow-up of melanoma patients from Latvia at all. Furthermore, only few published data on patients from putatively ethnically similar populations and only a few estimations of the prevalence of *CDKN2A* and *CDK4* germline mutations in sporadic melanoma patients or population-based samples have been published (Aitken et al., 1999; Begg et al., 2005; Berwick et al., 2006). In the present study, there was characterised, for the first time in Latvia, the *CDKN2A* and *CDK4* status in consecutive consenting melanoma patients, *CDKN2A* common polymorphisms, A148T, c.*29C>G, and c.*69C>T, were analysed as putative low-penetrance susceptibility and melanoma progression alleles, and somatic alterations in primary melanoma tumours were characterised as well.

The present work was carried out at the Latvian Biomedical Research and Study Centre, in the laboratory of Melanoma genetic lead by Dr. Olita Heisele in collaboration with Latvian Oncological Center and Dr. Ludmila Engele and at the Genetic Epidemiology Division, Cancer Research UK Clinical Centre, St. James's University Hospital in Leeds, UK (Prof. Julia Newton Bishop and Prof. Timothy Bishop).

1. REVIEW OF THE LITERATURE

1.1. Cancer genetics

Cancer is a genetic disease caused by progressive accumulation of mutations in critical genes that control cell growth, differentiation, cell death, or genomic stability. Several types of genes are known to contribute to the development of cancer.

The discovery that the oncogenes of tumour-producing retroviruses are related to cellular genes (proto-oncogenes) has led to intensive research into the role of these genes in normal and tumour cell growth, proliferation, and differentiation. Many cellular genes may act as **oncogenes** when expressed inappropriately or mutated. These genes act in a dominant way at the cellular level to drive proliferation or prevent normal differentiation.

Genes that provide negative regulatory signals in the normal cell are also implicated in the development of cancer. If such a gene requires loss or inactivation to contribute to the transformation process, then it is likely that both copies of the gene must be altered and that such **tumour suppressor genes (TSG)** would be genetically recessive at the cellular level.

The first TSG identified, retinoblastoma (*RB*) gene, was found from studies on retinoblastoma, a rare inherited cancer syndrome occurring in children. As suggested by Knudson 1971, inactivation of the gene is a "two hit" phenomenon. One of the changes is inherited whereas the second occurs somatically during embryogenesis or early in the life (Figure 1.1). In such cases of inherited (familial) tumour predisposition, the first mutation is present in the germ cells (sperm or ovum) and is therefore inherited by every cell in the body. Only one further somatic mutation is required for complete gene inactivation in these cases. In the more common non-familial cancers, two somatic mutations in the gene are required and the chances of this happening in the same cell are much less (Figure 1.1). Usually, the first mutation in the "two-hits" is a point mutation or other small change confined to the TSG. The second mutation often involves the loss of all or part of the chromosome. The mechanisms underlying this loss are non-disjunction (leading to the loss of a whole chromosome), mitotic recombination (leading to the loss of the parts of chromosome), or an interstitial deletion. Genomic loss is the most common event that inactivates a TSG and is called **loss of heterozygosity** (LOH). The detection of recurrent LOH in a chromosomal region is considered to be evidence for the localisation of TSGs. The other possibility is that **epigenetic inactivation** (e.g. promoter methylation) of TSG is implicated in the tumorogenesis (Jones and Baylin 2002).

Many TSGs have now been identified (e.g. *RB, APC*) and many appear to confirm the Knudson's "two-hit" hypothesis (reviewed in Knudson 1996). However, there are exceptions to this rule and there are several examples where loss of function of one allele of a TSG is sufficient to generate an altered cell phenotype that can contribute to transformation. This is termed **haploinsufficiency** and the levels of protein required for adequate function may vary from gene to gene, leading to the prediction that some genes will be more strongly haploinsufficient than others. Quon and Berns 2001 propose that all TSGs that are frequently mutated in sporadic human cancers may show varying degrees of haploinsufficiency.

In addition, TSGs can have different action modes which have led to the invention of the terms 'gatekeeper' and 'caretaker' to describe different suppressor roles (Kinzler and Vogelstein 1998). Gatekeeper genes are defined as rate-limiting for a step in the pathway of tumour development. Thus, the adenomatous polyposis coli gene *APC* is considered to be an initiation gatekeeper as its inactivation is required early in colorectal carcinogenesis. Caretaker genes include those which when functionally inactivated lead to defective DNA repair or other loss of function that leads to mutation, for example, some DNA repair genes, *MSH2 and MLH1.* In many cases, TSGs may be involved in both gatekeeping and in caretaking functions (e.g. *TP53, BRCA1*).

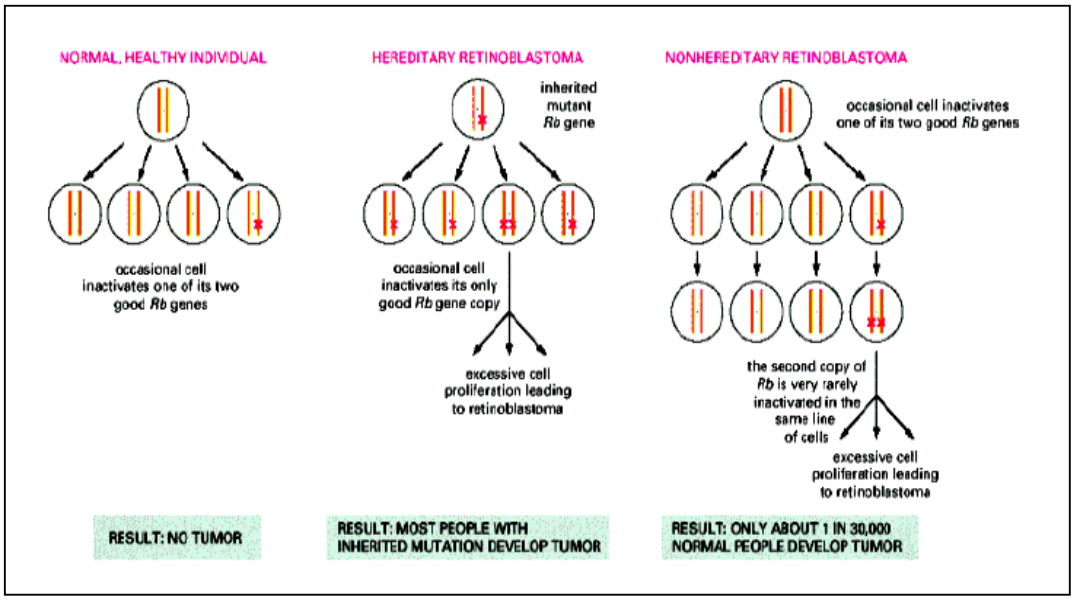


Figure 1.1. *Knudson's "two-hit" model. The genetic mechanisms in the RB tumour suppressor gene underlying retinoblastoma (redrawn from Alberts, Molecular Biology of the Cell, New York, 2002).*

Inherited susceptibility to cancer

Cancers can be classified as hereditary or sporadic based on the difference in the involvement of genetic factors. Hereditary cancers, which account for approximately 2% of all cancers, are caused by germline mutations of defined genes inherited in an autosomal dominant or recessive manner. More than 20 different hereditary cancer syndromes have now been defined and attributed to specific germline mutations in various inherited cancer genes (Fearon 1997). Genes associated with the most common cancer types and syndromes are shown in Table 1.1. A few of the genes are oncogenes or DNA repair genes, but most are TSGs. Some tumor suppressors regulate transcription, while others operate in signal transduction pathways that are involved in regulating processes of cell birth, differentiation, and death (Knudson 2002). All the main cancers in general population appear to have a familial component that ranges 1% - 15%, depending on the particular sites (Hemminki et al., 2004).

Cell cycle

The cell cycle can be separated in four distinct phases: initial growth (G1), DNA replication (S), a gap (G2), and mitosis (M). The transitions between all of these phases are tightly regulated and involve concerted action of cyclins and cyclin- dependent kinases (CDKs). The tight control of the cell cycle is important for cell division. A critical point in cell cycle control is the G1 to S transition. After passing this checkpoint, the cell is irreversibly committed to the next cell division. This has led to the proposal that alterations in this checkpoint are critical for the development of cancer.

The key regulators of the G1 to S checkpoint are the [cyclin d:cdk4/cdk6] and the [cyclin e:cdk2] complexes (Figure 1.10). These complexes phosphorylate the retinoblastoma gene product (Rb) relieving the repressive effects of Rb protein on transcription factors belonging to the E2F family and therefore activate genes involved in DNA synthesis.

Table 1.1. *Genes associated with predisposition to common cancers (summarised by Bishop 2005)*

Primary cancer	Syndrome	Genes	Normal function of the gene	Localisation
Breast	Breast and breast/ovarian	*BRCA1*	DNA repair	17q21
		BRCA2	DNA repair	13q12.3
	Li-Fraumeni	*TP53*	Cell cycle regulation, apoptosis, transcription factor	17q13.1
		CHEK	DNA repair	22q12.1
	Cowden syndrome	*PTEN*	Protein tyrosine phosphatase	10q23.31
Bowel	Familial adenomatous polyposis	*APC*	Cell proliferation, adhesion	5q21-q22
	Hereditary non-polyposis colorectal cancer	*hMSH2*	DNA mismatch repair	2p22-p21
		hMSH6	DNA mismatch repair	2p16
		hMLH1	DNA mismatch repair	3p21.3
		hPMS1	DNA mismatch repair	2q31-q33
		hPMS2	DNA mismatch repair	7p22
	Juvenile Polyposis Coli	*SMAD4*	Serine/treonine kinase	18q21.1
		BMPR1A	Serine/treonine kinase receptor	10q22.3
	Peutz-Jeghers	*STK11*	Serine/treonine kinase	19p13.3
Skin	Familial melanoma	*CDKN2A*	Cell cycle regulation	9p21
		CDK4	Cell cycle regulation	12q14
	Nevoid cell sarcoma	*PTCH*	Regulator of cell division	9q22.3

The kinase activities of the [cyclin d:cdk4/cdk6] and the [cyclin e:cdk2] complexes are negatively regulated thought two cyclin-dependent kinase inhibitor (CDKI) families:

i) the INK4 (inhibitor of CDK4/CDK6) proteins: p16/INK4A/MTS1/CDKN2A, p15/INK4B/MTS2/CDKN2B, p18/INK4C/CDKN2C, and p19/INK4D/CDKN2D;

ii) the KIP (kinase inhibitor protein) molecules: p21/CIP1/CDKN1A/WAF1, p27/KIP, and P57/KIP2.

The KIP family of CDKIs regulates the activities of kinases complexed with cyclins d and e whereas the INK4 family binds exclusively to cdk4 and cdk6. The INK4 family of CDKIs are induced during cellular senescence and upon growth-inhibitory signals. A well characterised KIP-family member p21CIP1 is activated by p53 upon DNA damage and other stresses.

1.2. The skin

The skin is the largest organ of the body composed of specialised epithelial, neural crest and connective tissue cells. As the major interface with the environment it has important protective functions providing a barrier against body fluid loss and entry of external factors such as pathogenic micro-organisms. Elaborate neural receptors and small nerve endings mediate touch, pressure, temperature, and pain. The skin also has an important role in thermoregulation, has an endocrine function, and produces vitamin D in response to sun

exposure. The upper layer of skin is subjected to the penetration of solar radiation and the pigment melanin which is produced in the skin acts to provide protection against the damaging effects of ultraviolet light (UV).

The skin consists of a thin outer layer referred to as *epidermis* (about 0.1 mm), an inner connective tissue matrix known as *dermis* (up to 3 mm and more), and subcutaneous tissue (Figure 1.2). The later is a layer of fat and connective tissue that houses larger blood and lymphatic vessels and nerves. The dermis is composed of mechanically tough connective tissue consisting mostly of collagen fibres and a semi-solid matrix of glycosaminoglycans (GAGs) produced by fibroblasts, the main cell of the dermis. Various immune cells such as mast cells, macrophages, and lymphocytes are also present in dermis. The epidermis is a stratified squamous epithelium composed mainly of keratinocytes (~ 95%) which differentiate to form five layers (Figure 1.3): the *stratum basale* (basal cell layer), *stratum spinosum* (spinous or prickle layer), *stratum granulosum* (granular cell layer), *stratum licidum* (transition layer) and *stratum corneum* (surface layer). There are three major cell types in human epidermis namely keratinocytes, melanocytes (Figure 1.3) and Langerhans cells. Although innervated the epidermis does not contain blood vessels and relies upon diffusion from capillaries present in the upper dermis.

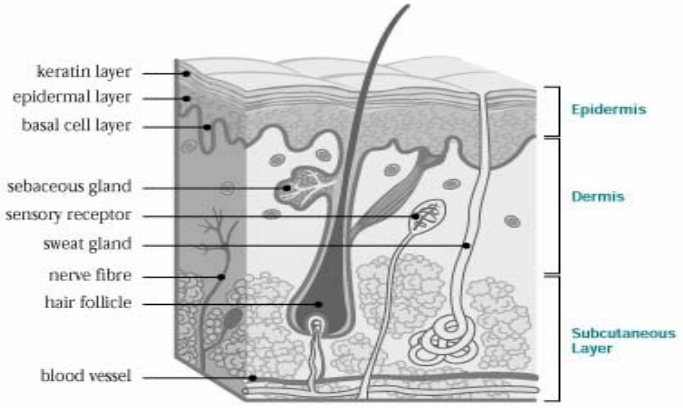


Figure 1.2. *Skin anatomy (www.myskintherapy.com/skinlayers.htm).*

Keratinocytes in the basal layer of epidermis are mitotically active epithelial cells. So called undifferentiated stem cells remain in the basal layer but others differentiate terminally from the basal (least keratin) to the cornfield cells (most keratin). There is a progressive synthesis of keratin proteins as cells migrate upwards through *stratum spinosum*. In *stratum granulosum* the nuclei and organelles of the keranocytes undergo degradation and the cells become flattened. The whole differentiation process takes approximately 28 days and finishes in *stratum corneum* with dead, flattened keratinocytes that have thickened cell envelopes encasing a matrix of keratin fibrils.

Melanocytes are dendritic cells located in *stratum basale* layer of epidermis and epidermal appendages, i.e. sebaceous glands and hair follicles (Figure 1.3). These cells are derived from the neural crest and migrate into epidermis early in embryonic life (week 10 in humans). Once in epidermis, melanocytes extend dendrites from the cell body and create contacts with the surrounding keratinocytes (Figure 1.3). This close anatomical relationship between these two cells is called the “epidermal melanin unit”. Melanocytes produce the pigment melanin and transfer it via their dendrites to keratinocytes. This is responsible for skin colour and has an important role in protection against UV radiation.

Langerhans cells are dendritic, bone-morrow-derived antigen presenting cells found mostly in *stratum spinosum*. These cells represent the outermost sentinels of the cellular immune system of the skin and can migrate into the dermis and local lymph nodes.

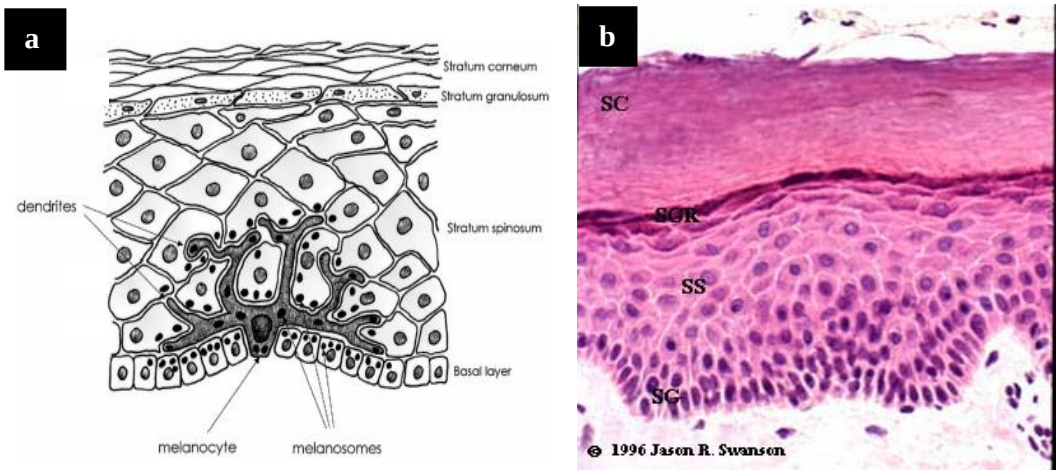
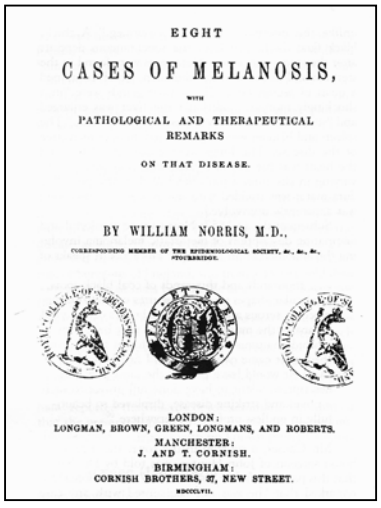


Figure 1.3. *Shematic diagram* ***(a)*** *and a thin section light microscopy picture* ***(b)*** *of the epidermis: Stratum corneum (SC), stratum granulosum (SGR), stratum spinosum (SS), stratum basale or basal layer (SG) with basal keratinocytes and melanocytes interspersed amongst basal keratinocytes, all situated on the basement membrane which separates epidermis from dermis (a, redrawn from Rees 2004; b, from http://www.meddean.luc.edu/lumen/MedEd/medicine/dermatology/skinlsn/epider.htm).*

1.3. Melanoma

The first report of melanoma is found in the writings of the legendary Greek physician Hippocrates in the fifth century BC, where he described "fatal black tumours with metastases". However, it was not until 1806, when René Laennec (1781-1826) described "la melanose" to the Faculté de Médecine in Paris, that the disease was characterised in detail and named (see Davis and McLeod 1992). The first English-language report (Figure 1.4) that describes the entity now known as cutaneous malignant melanoma (CMM) was, in fact, a familial occurrence of the disease described by general practitioner William Norris (1792-1877) who noticed a family with numerous moles and several family members with metastatic lesions (Norris 1820).


EIGHT

CASES OF MELANOSIS,

WITH

PATHOLOGICAL AND THERAPEUTICAL REMARKS

ON THAT DISEASE.

BY WILLIAM NORRIS, M.D.,

LONDON:
LONGMAN, BROWN, GREEN, LONGMANS, AND ROBERTS.
MANCHESTER:
J. AND T. CORNISH.
BIRMINGHAM:
CORNISH BROTHERS, 37, NEW STREET.

Figure 1.4. *The first comprehensive study of a series of melanoma patients suggesting hereditary predisposition to melanoma.*

These observations went unnoticed for many years, until Cawley made a similar observation in 1952 (Cawley 1952). Later in 1978, Lynch and Clark separately reported a novel nevus phenotype characterised by a large number of common and atypical nevi commonly found in families with a genetic susceptibility to melanoma (Lynch et al., 1978; Clark et l., 1978). This nevus phenotype was first labelled "the BK mole syndrome". Since then many groups around the world have studied the nevus phenotype in the context of familial melanoma which has been known as "dysplastic nevus syndrome" or, alternatively, "familial atypical mole and multiple melanoma syndrome" (FAMMM) (Greene et al., 1985; Bergman et al., 1992; Newton Bishop et al., 1994). It appeared then, that the presence of an abnormal nevus phenotype was a necessary part of familial melanoma. More recently, an abnormal nevus phenotype has been recognised as a characteristic of some, but not all, familial melanoma. Molecular insights over the last 20 years have confirmed the significant genetic contribution to the etiology of both familial and sporadic melanomas (summarised in chapters below).

Melanoma incidence and mortality

Melanoma incidence varies by latitude and altitude worldwide, with areas closer to the equator and higher in altitude generally having higher rates (Figure 1.5). This pattern varies however, by the pigmentation of the population and their sun exposure patterns.

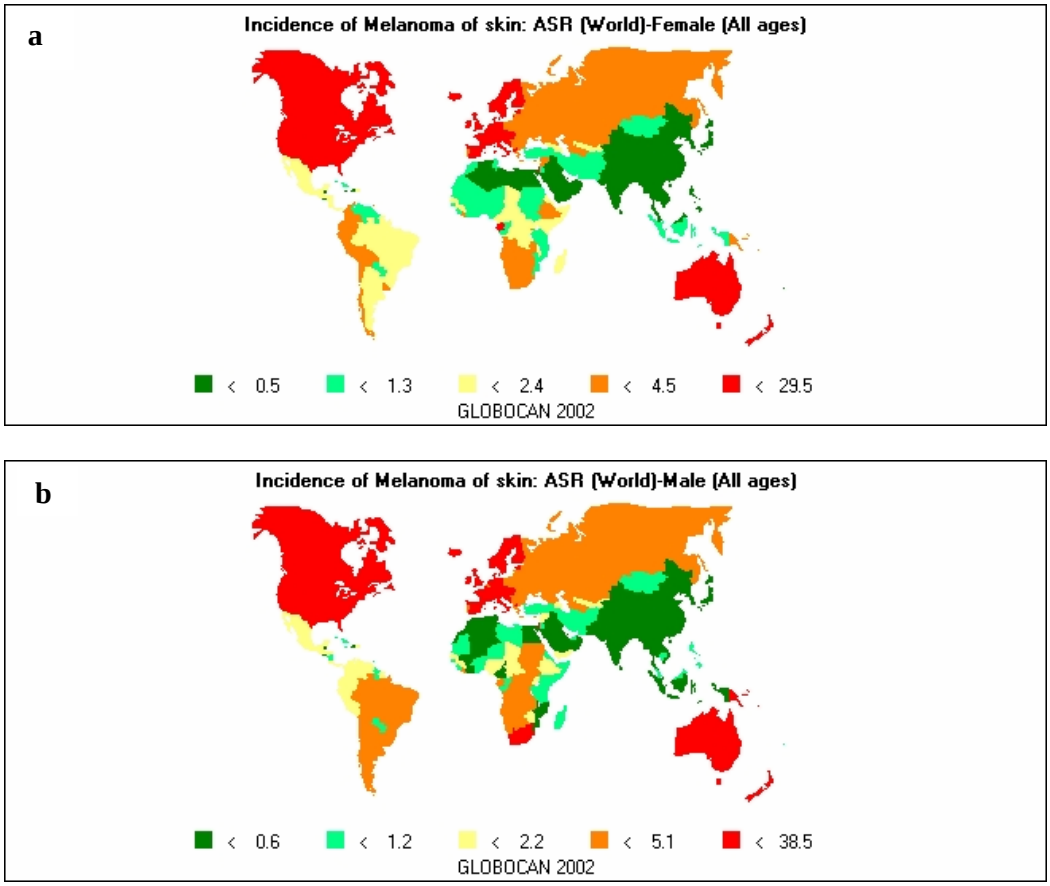


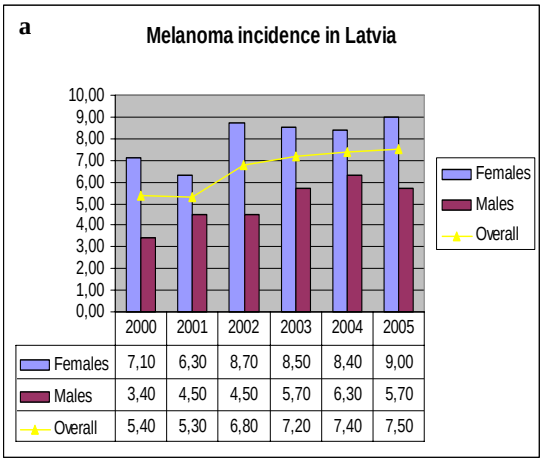
Figure 1.5. *Melanoma incidence worldwide, age standardised rates (ASR).* ***(a)****, females;* ***(b)****, males (GLOBOCAN 2002).*

Recognised as the most common fatal skin cancer, overall melanoma incidence in fair-skinned populations has risen throughout the world (Jemal et al., 2000; Bulliard and Cox 2000; Marrett et al., 2001; MacKie et al., 2002; Mansson-Brahme et al., 2002; de Vries et al., 2003; Lasithiotakis et al., 2006; Coory et al., 2006). In some countries namely in the USA, New Zeeland, and Australia the incidence rates among men are rising more rapidly than among women (Jemal et al., 2000; Bulliard and Cox 2000; Marrett et al., 2001; Coory et al., 2006). In the most countries, there is no evidence of a decline in incidence overall, but in Australia there is some evidence of a stabilisation to decrease in rates probably due to the educational programmes (Marrett et al., 2001). Rates increased most among thick melanomas (MacKie et al., 2002; Mansson-Brahme et al., 2002; Coory et al., 2006). The patterns of increase over an extended time period, and over the shorter period in thick lesions, suggest a true increase in incidence even the increases is due to more intensive screening. Over time, a higher percentage of earlier lesions with high cure rate have been diagnosed (Mansson-Brahme et al., 2002).

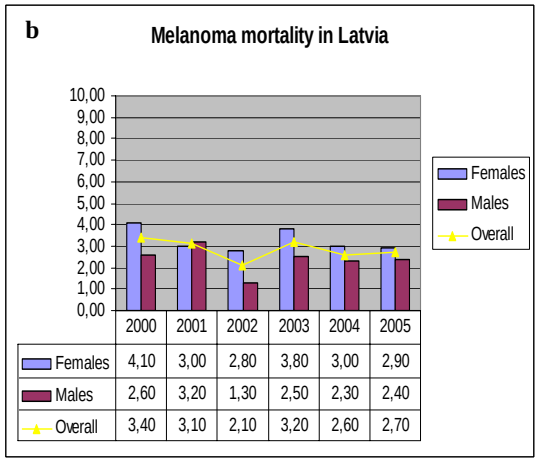
Melanoma mortality rates have overall risen more slowly than the incidence (Jemal et al., 2000), have been stable (Coory et al., 2006) or even have felt slightly (MacKie et al., 2002; Baade and Coory 2005).

In Europe melanoma incidence rates were by far highest in northern and western Europe, whereas mortality was higher in eastern and southern Europe. Melanoma rates have been rising steadily, albeit with substantial geographic variation. Mortality rates in western Europe have recently levelled off, whereas in eastern and southern Europe both incidence and mortality rates are still increasing (de Vries et al., 2003).

In Latvia approximately 170 new melanoma cases are diagnosed annually. The increase in incidence is observed overall from 5.4 in 2000 to 7.5 in 2005. For both genders, the increase rose by a similar amount (Figure 1.6a). The mortality, with little changes between years, is stabilising in women and still increasing in males (Figure 1.6b).



	2000	2001	2002	2003	2004	2005
Females	7,10	6,30	8,70	8,50	8,40	9,00
Males	3,40	4,50	4,50	5,70	6,30	5,70
Overall	5,40	5,30	6,80	7,20	7,40	7,50



	2000	2001	2002	2003	2004	2005
Females	4,10	3,00	2,80	3,80	3,00	2,90
Males	2,60	3,20	1,30	2,50	2,30	2,40
Overall	3,40	3,10	2,10	3,20	2,60	2,70

Figure 1.6. *Melanoma incidence* ***(a)*** *and mortality* ***(b)*** *in Latvia by sex and overall (data of Latvian Cancer Registry).*

Clinical features of cutaneous malignant melanoma

Melanoma develops from the malignant transformation of melanocytes and can be subdivided into several subtypes, primarily based on anatomic location and patterns of growth (Table 1.2).

Table 1.2. *Clinical classification of melanoma (as summarised in Chudnovsky et al, 2005)*

Subtype	Frequency	Common site	Key distinguishing features
Superficial spreading melanoma	70%	Trunk of men Legs of women	RGP, 1-5 years
Nodular melanoma	10-25%	Trunk of men Legs of women	RGP, 6-18 month
Acral lentiginous melanoma	5%	Palms, soles, nails	Not related to sun damage All races affected Accounts for 30-70% of melanoma in dark-skinned individuals
Lentigo maligna melanoma	<1%	Head and neck of elderly	Associated chronic sun exposure RGP, 3-15 years
Noncutaneous melanoma	5%	Ocular, mucosal	Not associated with sun exposure Prognostic factors and treatment differ from that of cutaneous subtypes

RGP – radial-growth phase

The majority of melanoma subtypes are observed to progress through distinct histologic steps (Figure 1.7). Five distinct steps have been proposed in the evolution of melanoma on the basis of such histological criteria: i) common acquired and congenital nevi without displastic changes (benign nevus), ii) dysplastic nevi with structural and architectural atypia, iii) radial-growth phase (RGP) melanoma, iiii) vertical-growth phase (VGP) melanoma, and iiiii) metastatic melanoma. As the presumed precursor to melanoma, both benign and dysplastic nevi are characterised by disruption of the epidermal melanin unit, leading to increased number of melanocytes in relation to keratinocytes. These precursor lesions progress to *in situ* melanoma, which growth laterally and remains largely confined to the epidermis, so this stage is defined as the radial-growth phase (RGP). RGP melanoma is generally cured by excisional surgery. Most melanoma subtypes demonstrate a slow RGP, followed by a potentially more rapid VGP (Clark et al., 1984). VGP melanoma invades both the upper layer of the epidermis and beyond, and penetrates into the underlying dermis and subcutaneous tissue through the basement membrane, forming expansile nodules of malignant cells. It is believed that the transition from radial- to vertical-growth phases is critical step in the evolution of melanoma that presages the acquisition of metastatic potential and poor clinical outcome (Meier et al., 1998; Rusciano 2000). Correlating with these histological pictures, RGP-melanoma cells remain dependent on exogenous growth factors supplied by surrounding keratinocytes, are incapable of independent growth, are not tumorogenic in immunodeficient mice and do not metastasise in patients. By contrast, VGP-melanoma cells have completely escaped from keratinocytes control and established communicative networks with fibroblasts, acquire growth factor independent growth, are tumorogenic in animals and are highly metastatic both in patients and in experimental animal models (Hsu et al., 2002).

Melanoma can be further classified into clinical stages according to significant prognostic factors, as specified by the American Joint Committee on Cancer (AJCC) (Table 1.3).

a

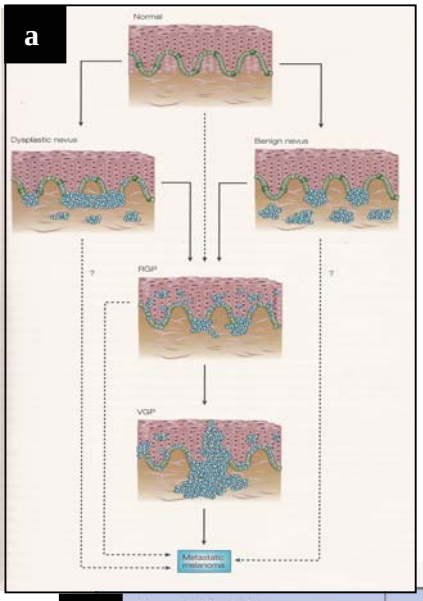


Figure 1.7. *The Clark model (Clark et al., 1984) describing the histologic changes that accompany the progression from normal melanocytes to malignant melanoma:* ***(a)****, pictorial representation (Chin 2003);* ***(b),*** *corresponding Hematoxylin and Eosin stained histologic sections with description (Miller and Mihm 2006).*

b

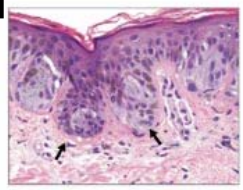
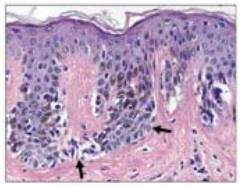
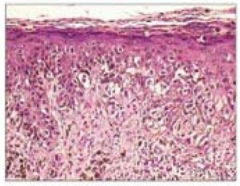
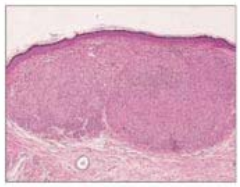
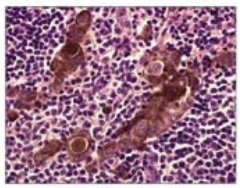
Histopathological Appearance	Description	Histologic Features
 Benign nevus	**Step 1** The first event is a proliferation of structurally normal melanocytes leading to the benign nevus. Clinically, these nevi present as flat or slightly raised lesions with either uniform coloration or a regular pattern of dot-like pigment in a tan or dark brown background. Histologically, such lesions have an increased number of nested melanocytes along the basal layer (arrows).	Proliferation of melanocytes Benign lesions
 Dysplastic nevi	**Step 2** The next step is the development of aberrant growth. This may occur within a preexisting benign nevus or in a new location. Clinically such lesions may be asymmetric, have irregular borders, contain multiple colors, or have increasing diameters. Histologically, such lesions have random and discontiguous cytologic atypia (arrows).	Dysplastic cells Random atypia
 Radial-growth phase	**Step 3** During the radial-growth phase, cells acquire the ability to proliferate intraepidermally. Clinically, they sometimes present as raised lesions. These lesions no longer display random atypia and instead show cytomorphologic cancer throughout the lesion. In addition to the intraepidermal cancer, the cells can penetrate the papillary dermis singly or in small nests but fail to form colonies in soft agar.	Intraepidermal growth Continuous atypia
 Vertical-growth phase	**Step 4** Lesions that progress to the vertical-growth phase acquire the ability to invade the dermis and form an expansile nodule, widening the papillary dermis. The cells can also extend into the reticular dermis and fat, are capable of growth in soft agar, and have the capacity to form tumor nodules when implanted in nude mice.	Dermal invasion
 Metastatic melanoma	**Step 5** The final step in the model is the successful spread of cells to other areas of the skin and other organs, where they can successfully proliferate and establish a metastatic focus. These cells can grow in soft agar and can form tumor nodules that may metastasize when implanted in nude mice.	Metastasis

Table 1.3. *Staging of cutaneous melanoma: a simplified overview*[a]

Stage 0	Melanoma involves the epidermis but has not reached the underlying dermis.
Stage I and II	Melanoma is characterised by tumour thickness and ulceration status. No evidences of regional lymph nodes or distant metastasis.
Stage III	Melanoma is characterised by lymph nodes metastasis. No evidences of distant metastasis.
Stage IV	Melanoma is characterised by location of distant metastasis and the level lactate dehydrogenase.

[a] - *a complete staging system is reviewed in Balch et al., 2001*

In the absence of known distant metastasis, the most important prognostic indicator is regional lymph node involvement. However, the majority of melanoma patients present with clinically normal lymph nodes. Thus, in clinically node-negative patients, the microscopic degree of invasion of melanoma is of importance in predicting outcome. There are two systems described for microscopic staging of primary cutaneous melanoma, Clark level and Breslow thickness. Clark levels classify melanoma according to anatomic landmarks in the epidermis, dermis, and fat (Clark et al., 1969). While this system correlates with prognosis, an inherent concern with Clark microscopic staging is that the thickness of the skin, and thus the location of the defined landmarks, varies in different parts of the body. Breslow thickness is a measure of the absolute thickness of the tumour from the granular layer (*stratum granulosum)* to the deepest contiguous tumour cell at the base of the lesion (Breslow 1979). Breslow thickness has strong prognostic value in those with nonmetastatic melanoma. Melanoma prognosis also worsens with the histologic findings of ulceration, high tumour cell mitotic rate, and vascular invasion. Increasing age, male sex, and tumour location on the trunk, head, or neck also worsen prognosis. Once metastasis to lymph nodes occurs, the 5-year survival ranges from 13% to 69%, depending on the number of lymph nodes affected and tumour burden (Balch et al., 2001).

Because there is no effective therapy for advanced melanoma, the classic clinical signs of melanoma are important to reduce mortality by detecting the disease in the early stages. These signs include change in colour, recent enlargement, nodularity, irregular borders, and bleeding. Cardinal signs of melanoma are also referred as ABCDE (asymmetry, border irregularity, colour, diameter, elevation) rule (Figure 1.8).

Regarding familial melanoma, studies have not demonstrated a less favourable outcome in patients with familial compared with sporadic melanoma (Burden et al., 1999; Puig et al., 2005; Florell et al., 2005). The melanoma mortality risk factors for both sporadic and familial melanoma are still considered to be ulcerated tumours increased tumour depth and male sex (Puig et al., 2005; Florell et al., 2005). In a population-based study recently done in Utah (Florell et al., 2005), authors have shown that although familial melanomas tend to arise at an earlier age than sporadic tumours, the other prognostic indicators and survival of these patients are the same. Consistent with theses findings, melanoma tumours from high-risk families exhibit phenotypic features similar to those sporadic melanoma tumours with regard to site, mean tumour thickness, prognostic and histopathologic subtypes, and pattern of metastasis (Masback et al., 2002; Puig et al., 2005). Melanoma occurring in a familial setting may be more likely to be of superficial spreading type and less invasive (Hemminki et al., 2003a), although this difference may be due to increased surveillance in high-risk families, who thus way spot suspicious moles earlier than individuals in the general public. These data suggest that, which the exception of age of onset and number of primary tumours (which would be expected in the context of an inherited predisposition), the biologic behaviour of

familial melanoma is similar to sporadic melanoma and represents an excellent model system for the investigation of the disease.

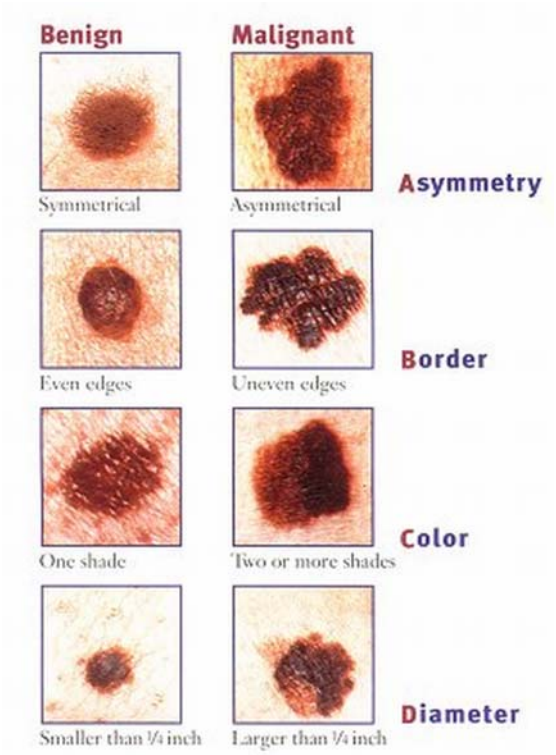


Figure 1.8. *The* ***ABCDE*** *rule for the diagnosis of malignant melanoma:* **A** *stands for asymmetry, the mole or lesion is asymmetric;* ***B*** *stands for irregular borders, the mole or lesion has irregular borders;* ***C*** *stands for irregular colors, the mole or lesion is irregularly colored ranging from light brown to dark brown, black, red, blue or white;* ***D*** *stands for diameter, a mole or lesion is suspicious if it is over 5 mm in diameter;* ***E*** *stands for elevation, the elevation of a mole or lesion may be a hint for the lesion being malignant (not shown). Picture from* www.cancerresearch.org/melanomabook.html

1.4. Melanoma risk factors

As in many cancers, both genetic predisposition and exposure to environmental agents are risk factors for melanoma development. It is true for sporadic melanoma and the same is likely to be true for familial melanoma.

The strongest risk factor for melanoma is a family history of melanoma (Kefford et al., 2002). Any family history of melanoma is associated with a doubling risk for close relatives. A study from the Utah population database estimates relative risk to first-degree relatives of melanoma patients to be 2.1 (95% CI: 1.4-2.9) (Goldgar 2002). A similar study from the Swedish Cancer Registry estimated the incidence for melanoma to be 2.40 (95% CI: 2.10-2.72) for offspring if one parent had a melanoma, 2.98 (95% CI: 2.54-3.47) for an affected sibling, and 8.92 (95% CI: 4.25-15.31) if a parent and sibling were both affected. The highest ratio was 61.78 (95% CI: 5.82-227.19) for offspring when a parent had multiple melanomas (Hemminki et al., 2003b). Some familial clustering is attributable to known cancer predisposition genes, as is discussed later.

Strong risk factors for melanoma development are also previous melanoma, increased numbers of both common and dysplastic moles, and tendency to freckle as well (reviewed in Tucker and Goldstein 2003). Population-based studies estimate that 15-20% of healthy individuals have at least one atypical mole, whereas 40-50% of melanoma patients have at least one atypical mole (Bataille et al, 1996). More, many melanoma families exhibit atypical nevus/mole syndrome (AMS) characterised by atypical nevi and increased number of banal nevi as well (Lynch et al., 1978; Slade et al., 1995). A high relative risk of 46.1 (95% CI: 21.0-87.5) of melanoma development in patients with sporadic atypical nevi have also been reported (de Snoo et al., 2007). Although this study was conducted in an area with a founder mutation in melanoma susceptibility genes in many of its melanoma families and, therefore

results may not be applicable to other populations, this study shows a significant association between atypical nevi and melanoma development.

Immunosuppression, sun sensitivity, and exposure to ultraviolet radiation are additional risk factors. Melanoma primarily affects fair-haired and fair-skinned individuals and those who burn easily or have a history of severe sunburn than their darkly pigmented matched controls (Landi et al., 2002; Veierod et al., 2003; Cho et al., 2005), which supports the role of pigmetation in melanoma susceptibility. The predominant sites of melanoma, however, are those exposed to intermittent, intense sunlight, such as the back or legs, and not those receiving more constant exposure, such as a face and dorsal hands and forearms (an exception is lentigo maligna melanoma) (Kennedy et al., 2003; Gillgren et al., 2003). Similarly, several epidemiologic studies have linked brief intense sun exposures, such as severe sunburns, especially during the childhood or in relation to sun vacations, to increased melanoma risk (Oliveria et al., 2006). Collective studies on intermittent versus chronic (occupational) ultraviolet exposure (Berwick et al., 2005) suggest that chronic exposure may be protective, possible owing to increased skin thickness and melanin production that result from chronic ultraviolet exposure. This is in contrast to the nonmelanoma skin cancers, basal cell carcinoma and squamous cell carcinoma, which arise from epidermal keratinocytes and are more strongly associated with cumulative sun exposure (Kennedy et al., 2003; Wang et al., 2005). The exact mechanism and wavelengths of UV light that are the most critical remain controversial, but both UV-A (wavelength 320–400 nm) and UV-B (290–320 nm) have been implicated (Jhappan et al., 2003). The relation of melanoma incidence to latitude of residence, with the highest incidence found in Australia, also strongly supports the role of UV-induced damage in melanoma pathogenesis (Marks 2000). Finally, patients with Xeroderma pigmentosum, an autosomal recessive trait of impaired DNA repair to ultraviolet light injury, have a high risk of melanoma (Kraemer et al., 1994).

Regarding familial melanoma, only a few studies, thus far, directly correlate the combination of familial risk and ultraviolet exposure to melanoma pathogenesis, and these results are conflicting. A study of high risk melanoma families (Hemminki et al., 2003a) found increased melanoma risk on sun-protected compared with sun-exposed body sites. Siskind et al., 2002 found no differences in cumulative solar exposure during childhood or adolescence among low-risk, intermediate-risk, or high-risk melanoma families. The authors suggest that genetic determinants are more predictive of melanoma risk in higher risk families, and exposure to ultraviolet radiation is an important environmental factor. A recent study (Chaudru et al., 2004) of *CDKN2A* mutation carriers shows that melanoma risk increases with sun exposure and sunburns. In contrast, the above mentioned population-based study of Berwick et al., 2005 inversely correlated melanoma survival with sun exposure (including intermittent levels and sunburns). The authors have suggested that solar damage might act to increase melanisation or DNA repair of damage due to ultraviolet radiation. The reason for these discrepancies could be that melanoma patients who report a family history of melanoma in population studies are less likely to have hereditary melanoma as compared with those from well characterised pedigrees or those with an associated, known germline mutation. Given such different findings, it is not currently possible to unequivocally determine the effect of sun exposure on familial melanoma.

A systematic meta-analysis of observational studies of melanoma and sun exposure, the number of nevi, family history, actinic damage, and phenotypic factors was recently conducted by Gandini et al, 2005a, 2005b, 2005c as part of a comprehensive meta-analysis of all major risk factors for melanoma. The analysis showed that intermittent sun exposure and sunburn history were shown to play considerable roles as risk factors for melanoma, whereas a high occupational sun exposure seemed to be inversely associated to melanoma (Table 1.4). The number of common nevi was confirmed as an important risk factor (Table 1.5) and pooled estimates were obtained for family history, skin type and colour, freckles, eye and hair colour, pre-malignant and ski n cancer lesions, and actinic damage indicators (Table 1.6).

Table 1.4. *A meta-analysis of sun exposure as a risk factor for melanoma[a]; summarised from Gandini et al, 2005a*

Risk factor	RR (95% CI)	Heterogeneity
Sunburn in chilhood	2.24 (1.73-2.89)	Chi squared 72.75, p<0.001
Sunburn in adult life	1.92 (1.55-2.37)	Chi squared 35.81, p=0.003
Intermittent dun exposure	1.46 (1.19-1.79)	
Chronic sun exposure	1.09 (0.86-1.37)	

[a] *- together 57 studies. Some latitudinal effects so that studies conducted at higher latitudes were associated with a stronger association with sunburns. RR, relative risk; 95% CI, confidence interval.*

Table 1.5. *Pooled estimates for risk of melanoma for increasing number common nevi (Gandini et al, 2005b)*

No. nevi	RR	Lower 95% CI	Upper 95% CI
Whole body			
0-15	1.00		
16-40	1.47	1.36	1.59
41-60	2.24	1.90	2.64
61-80	3.26	2.55	4.15
81-100	4.74	3.44	6.53
101-120	6.89	4.63	10.25
Arms			
0	1.00		
1-5	1.44	1.29	1.60
5-10	2.48	1.90	3.23
11-15	4.82	3.05	7.62

For whole body, No. of studies = 26, Heterogeneity χ^2 *= 181.970, p<0.001. For arms, No. of studies = 17, Heterogeneity* χ^2 *= 111.738, p<0.001.*
No., number; RR, relative risk; 95% CI, confidence interval.

Table 1.6. *Pooled estimates for family history, skin type and colour, freckles, eye and hair colour, pre-malignant and skin cancer lesions, actinic damage indicators[a] (Gandini et al, 2005c)*

Risk factors	Categories	RR and 95% CI	Heterogeneity Chi-square p-value
Family history	Yes vs. No	1.74 (1.41, 2.14)	0.368
Actinic damage indicators	Pre-malignant and skin cancers lesions vs. No	4.28 (2.80, 6.55)	<0.001
	Other indicators vs. No	2.02 (1.24, 3.29)	<0.001
Density of freckles	High vs. Low	2.10 (1.80, 2.45)	<0.001
Phototype	I vs. IV	2.09 (1.67, 2.58)	0.002
	II vs. IV	1.84 (1.43, 2.36)	<0.001
	III vs. IV	1.77 (1.23, 2.56)	<0.001
Eye colour	Blue vs. Dark	1.47 (1.28, 1.69)	<0.001
	Green vs. Dark	1.61 (1.06, 2.45)	<0.001
	Hazel vs. Dark	1.52 (1.26, 1.83)	0.499
Hair colour	Red vs.Dark	3.64 (2.56, 5.37)	<0.001
	Blond vs. Dark	1.96 (1.41, 2.74)	<0.001
	Light brown vs. Dark	1.62 (1.11, 2.34)	<0.001
Skin colour	Light vs.Dark	2.06 (1.68, 2.52)	<0.001

[a] *-results extracted from 60 studies; RR, relative risk; 95% CI, confidence interval.*

1.5. Melanoma and cancer syndromes

In family studies, melanoma is reported as a relatively infrequent component of family cancer syndromes such as familial retinoblastoma (Kaye et al., 2004), in male carriers of *BRCA2* mutations (Liede et al., 2004), and Li-Fraumeni syndrome (Potzsch et al., 2002). Much more commonly families are encountered with a predominance of melanoma and therefore referred as familial melanoma. Often the presence of an abnormal nevus phenotype is also observed in familial melanoma and the co-existence of melanoma and atypical nevi within families has been named as familial atypical multiple-mole melanoma (FAMMM) (Lynch et al., 1978). One of examples of a family with melanoma and atypical multiple-mole is presented in Figure 1.9. However, an abnormal nevus phenotype has been recognised as a characteristic of some, but not all, familial melanoma.

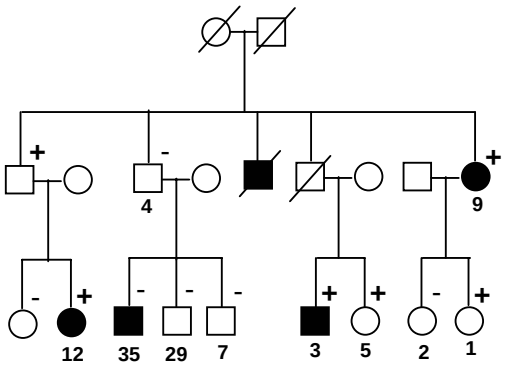


Figure 1.9. *Pedigree of a FAMMM family: o - female; □ – male; • or ■ – melanoma; + - CDKN2A mutation positive; - - CDKN2A mutation negative; number below symbol – number of atypical nevi (redrawn from de Snoo et al., 2003).*

1.6. High penetrance melanoma susceptibility genes

1.6.1. *CDKN2A* [OMIM 600160]

The *CDKN2A* tumour suppressor gene, located at chromosomal region *9p21*, is the major high-risk melanoma susceptibility gene identified to date.

Involvement of a *9p* locus in melanoma development was first indicated by cytogenetically detectable loss or translocation of this region in melanomas (Cowan et al., 1988). Loss of heterozygosity (LOH) studies in melanoma cell lines and metastatic melanoma tumours (Fountain et al., 1992) have implicated chromosome *9p21* as the region that contains a tumour suppressor gene. These findings were supported by germline loss of this region in a person with multiple primary melanomas (Petty et al., 1993) and linkage studies in large familial melanoma pedigrees (Cannon-Albright et al., 1992), which were further conformed by several other studies (Nancarrow et al., 1993; Gruis et al., 1993; Goldstein et al., 1994). This led to the cloning and identification of the first melanoma susceptibility gene, from the *9p21* region (Kamb et al., 1994a). This melanoma predisposing gene has been known by several different names: *MTS1, CDK4I*, *INK4A*, *CDKN2*, but the currently accepted gene nomenclature is *CDKN2A*, which stands for cyclin-dependent kinase inhibitor 2A.

The gene is composed of four exons: exons 1α, 1β, 2, and 3 and encodes two distinct tumour suppressor proteins involved in cell cycle regulation by using different first exons, 1α

or 1β spliced in different reading frames to common exons 2 and 3. Both proteins are tumour suppressors involved in cell cycle regulation (Figure 1.10).

The α-transcript, comprising exons 1α, 2, and 3, encodes $p16^{INK4a}$ protein of 156 amino acids, a negatively regulator of the cell cycle which bids to cdk4 and cdk6 and inhibits its ability to phosphorylate the retinoblastoma protein (Rb), thus preventing the release of the E2F transcriptional activating proteins, thereby blocking progression through the G1 phase of the cell cycle (Serrano et al., 1993). Therefore, mutations in the *CDKN2A* gene allow cell to escape cell cycle arrest in G1 phase, leading to uncontrolled cell proliferation and growth (Figure 1.10b).

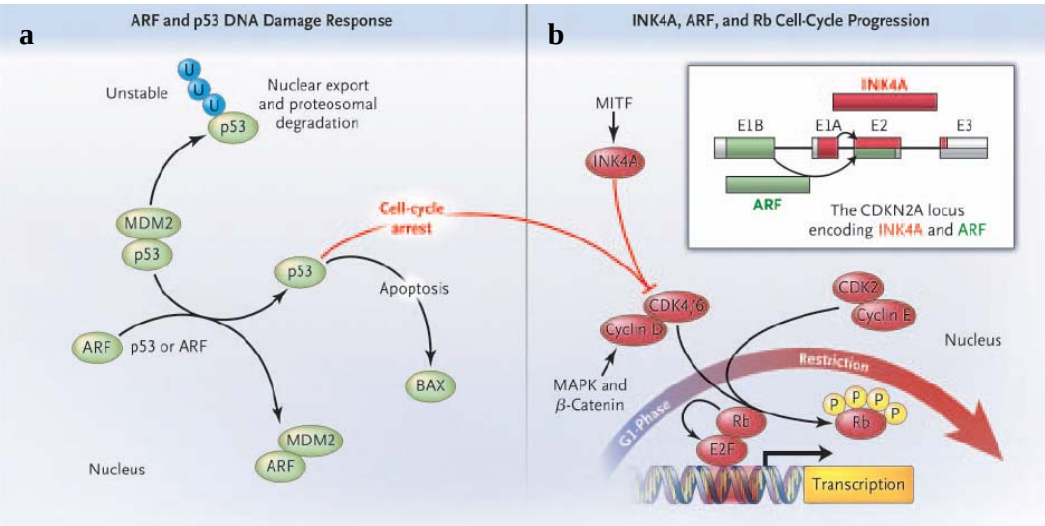


Figure 1.10. *CDKN2A tumour suppressor locus and cell cycle regulation. CDKN2A encodes two distinct tumor-suppressor genes; separate first exons that are spliced into alternate reading frames of the second and third exons permit the expression of two different proteins from the same genetic locus. The gene has 4 exons. Transcription of messenger RNA (mRNA) can be initiated at either exon 1β (E1B) or exon 1α (E1A), and the initiation site determines which gene the locus will express. RNA that is transcribed from either exon is spliced with the remaining two exons, E2 and E3, to produce mRNA for either INK4A or ARF. However, ARF uses a different reading frame of the exon 2 and 3 codons (box). In the cell-cycle progression involving INK4A, ARF, and retinoblastoma protein (Rb), a family of cyclins and cyclin-dependent kinases (CDKs) regulate progression through the cell cycle, and a family of CDK inhibitors opposes this action. In particular, the two phases of the G1–S checkpoint are governed primarily by cyclin D associated with cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) at its early phase and cyclin A or E associated with CDK2 at the later restriction phase.* ***(a)****, in response to DNA damage, mouse double minute 2 (MDM2) protein binds to the transcriptional activation domain of protein 53 (p53), blocking p53-mediated gene regulation while simultaneously leading to p53 ubiquination, nuclear export, and proteosomal degradation. ARF opposes this action by sequestering MDM2. This disruption of the MDM2–p53 interaction stabilizes p53 and increases p53 activity. Depending on other events, p53 either activates DNA repair and cell-cycle arrest or causes apoptosis and the formation of BCL-2–associated X protein (BAX). In the absence of ARF, p53 levels are decreased and the response to DNA damage is blunted.* ***(b)****, INK4A encodes a cyclin-dependent kinase inhibitor that inhibits CDK4 and CDK6. Ordinarily, these two kinases associate with D-type cyclins and drive the cell cycle by phosphorylating Rb, releasing it from its inhibitory interaction with the E2F transcription factor, thereby allowing the expression of E2F-related genes and progression from G1 to S. The absence of INK4A leads to unopposed CDK4 or 6 activities and increased cell-cycle activity. (Adapted from Miller and Mihm 2006).*

The smaller β-transcript is encoded by exon 1β, located approximately 20 kb centromeric to exon 1α, and exons 2 and 3, and specifies the alternative product $p14^{ARF}$ (alternative reading frame). Protein $p14^{ARF}$ is involved in cell cycle regulation by binding to HDM2 (human double minute 2) protein (MDM2 in mouse) (Pomerantz et al., 1998; Zhang et al., 1998), which in turn inhibits HDM2-induced p53 degradation, resulting in stabilisation and accumulation of the p53 protein (Figure 1.10a) as well its downstream target p21CIP1 (not shown in the figure), an inhibitor not only of cdk4 and cdk6, but also other cdks (reviewed in Ortega et al., 2002). This dual role of HDM2 indicates that specific targeting of $p14^{ARF}$ will affect the p53 and Rb cell cycle regulation pathways simultaneously. This is in contrast to $p16^{INK4a}$, for which a role in cancer control seems to be restricted to the pRb pathway only (Figure 1.10). Thus, inactivation of a single *CDKN2A* locus affects two of the major pathways in cancer biology: the Rb and the p53 pathways.

In addition to the role of $p16^{INK4a}$ and $p14^{ARF}$ as negative regulators of the cell cycle, loss of the *CDKN2A* locus has been reported to lead to a reduced repair of UV-induced DNA damage and an increased mutation rate, thus further contributing to melanoma development (Sarkar-Agrawal et al., 2004).

Since *CDKN2A* has identified as the major melanoma susceptibility gene, groups around the world have identified melanoma-prone families and reported the prevalence rate of *CDKN2A* mutations among them. Previous reports of *CDKN2A* mutation rates in familial melanoma are summarised in Table 1.7. Among these studies, the standard for what constitutes "familial" predisposition to melanoma has varied significantly, with inclusion criteria ranging from any person with multiple primaries to at least three affected first-degree relatives over two generations. These differences in inclusion parameters can be well understood in the context of the differing incidences of sporadic melanoma among the respective populations (e.g. England has about 10 cases per 100,000 per annum, while Australia about 50 cases per 100,000 per annum). These differences in defining "familial melanoma" make the comparison of these data difficult.

Most germline *CDKN2A* mutations found in melanoma families are of the missence type and have been found in exons 1α and 2, some of which are recurrent (Figure 1.11). Wherever recurrent mutations have been analysed, they have been shown to be a founder mutations (Table 1.8 and references therein). To date, only one mutation seems to appear repeatedly at a hotspot, a 24bp duplication at the N-terminus (33ins24) that has presumable arisen many times because of DNA polymerase slippage (Pollock et al., 1998). An online database of locus-specific variants named eMeanoBase (www.wmi.usyd.edu.au:8080/melanoma.html) is maintained online by the Sydney group of the Melanoma Genetic Consortium "GenoMel" (Fung et al., 2003).

Although novel *CDKN2A* mutations have been reported all the time (Avbelj et al., 2003; Huber and Ramos 2006; Ghiorzo et al., 2006; Hocevar et al., 2006; Knappskog et al., 2006; Erlandson et al., 2007), the percentage of families with identified *CDKN2A* mutations still is moderate (Table 1.7). Mutation analysis has been therefore extended to the non-coding regions of *CDKN2A* and has led to the finding of some such a mutations. A single-base change in the 5' UTR (c.-34G>T) that creates an aberrant transcription start codon was identified in Canadian melanoma families (Liu et al., 1999) and has now been reported in families from other continents (Harland et al., 2000; Eliason et al., 2006). There is a little evidence of other causal promoter mutations (Harland et al., 2000; Pollock et al., 2001a), but recently a deep intronic mutation (IVS2-105A>G) that creates an alternative splice site was found to explain susceptibility in a significant proportion of English melanoma families (Harland et al., 2001). This mutation has been also found by other groups, but by lower frequency (Loo et al., 2003; Majore et al., 2004). Additional rare splice mutations, IVS1-1G/C and IVS2+1G/T; c.457G/T; c.149A/C (Q50P), have also been reported by Petronzelli et al., 2001 and Loo et al., 2003 respectively.

Table 1.7. *CDKN2A mutation rate in melanoma kindreds worldwide*

Geographic origin	No. of families	*CDKN2A* mutation rate	Definition of familial melnoma	Reference
USA	36[a]	2/36 (6%)	At least two cases of melanoma per family or one melanoma case and at least two cases of DNS	Kamb et al., 1994a
USA	18	13/18 (72%)	At least two cases of melanoma in first-degree relatives	Hussassian et al., 1994
USA	28	5/28 (18%)	Two or more cases of melanoma in first- or second-degree relatives	Fitzgerald et al., 1996a
Australia	48	10/48 (21%)	Three cases of melanoma in two consecutive generations	Flores et al., 1997
England	27	6/27 (22%)	At least two cases of melanoma or one MPM patient	Harland et al., 1997
Sweden	64	5/64 (8%)	Population based study; at least two cases of melanoma in first-degree relatives	Platz et al., 1997
Italy	10	4/10 (40%)	Two or more cases of melanoma in first- or second-degree relatives	Fargnoli et al., 1998
France	48	21/48 (44%)	At least three affected family members, or two affected members if one diagnosed before the age 50 years	Soufir et al., 1998
Scotland	16	6/16 (38%)	Two melanoma cases per family	MacKie et al., 1998
Australia	131	11/131 (8%)	At least two cases of melanoma in the family	Holland et al., 1999
Australia	87	9/87 (10%)	Population based study; high-risk families defined on the basis of number of melanoma cases in the family, age at diagnosis, sex, and birth cohorts	Aitken et al., 1999
Spain	34	6/34 (17%)	At least two cases of melanoma in first- or second-degree relatives or one melanoma patient and one patient with DNS in the family	Ruiz et al., 1999
Italy	14	7/14 (50%)	Two melanoma cases diagnosed in first-degree relatives	Ghiorzo et al., 1999
England	20	3/20 (15%)	At least two melanoma cases per family	Newton Bishop et al., 1999
Israel	30	2/30 (7%)	Two or more cases of melanoma in first- or second-degree relatives in the family	Yakobson et al., 2000
Italy	15	5/15 (33%)	Two and more melanoma cases in the family or one MPM patient	Della Torre et al., 2001
Italy	62	21/62 (34%)	At least three affected family members (maximum third-degree relative) or two affected members, one younger than 50 years at diagnosis, the other complying with one of the following: being a first-degree relative, an additional relative with pancreatic cancer or having MPM	Mantelli et al., 2002
Poland	16	0/16 (0%)	Two or three cases of melanoma per family	Lamperska et al., 2002
France	23	3/23 (13%)	At least two melanoma cases in first- or second-degree relatives	Soufir et al., 2004

The table is continued on the next page.

Table 1.7.*(continuation) CDKN2A mutation rate in melanoma kindreds worldwide*

Geographic origin	No. of families	*CDKN2A* mutation rate	Definition of familial melnoma	Reference
Poland	16	1/16 (6%)	Two or more first-degree relatives affected by melanoma	Debniak at al., 2004a
Italy	55	4/55 (7%)	At least two relatives with melanoma in the family	Landi et al., 2004
Scotland	48	13/48 (27%)	At least two cases of melanoma in first-degree relatives	Lang et al., 2005
USA	116	13/116 (11%)	One or more first-degree relatives with melanoma or at least two affected relatives with melanoma on one side of the family	Niendorf et al., 2006
USA	60	5/60 (8%)	Population-based study; at least two affected first-degree relatives	Eliason et al., 2006
France	36	0/36 (0%)	At least two cases of melanoma per family or one MPM patient	Laud et al., 2006
Slovenia	11	5/11 (45%)	At least two family members with melanoma in the family	Hocevar et al., 2006

DNS – dysplastic nevus syndrome; MPM – multiple primary melanomas;
[a] – only 8 melanoma kindreds studied by Kamb et al., 1994a showed linage to 9p21, other kindreds had not been tested for 9p linage. Both mutations were found in 9p21 linked families.

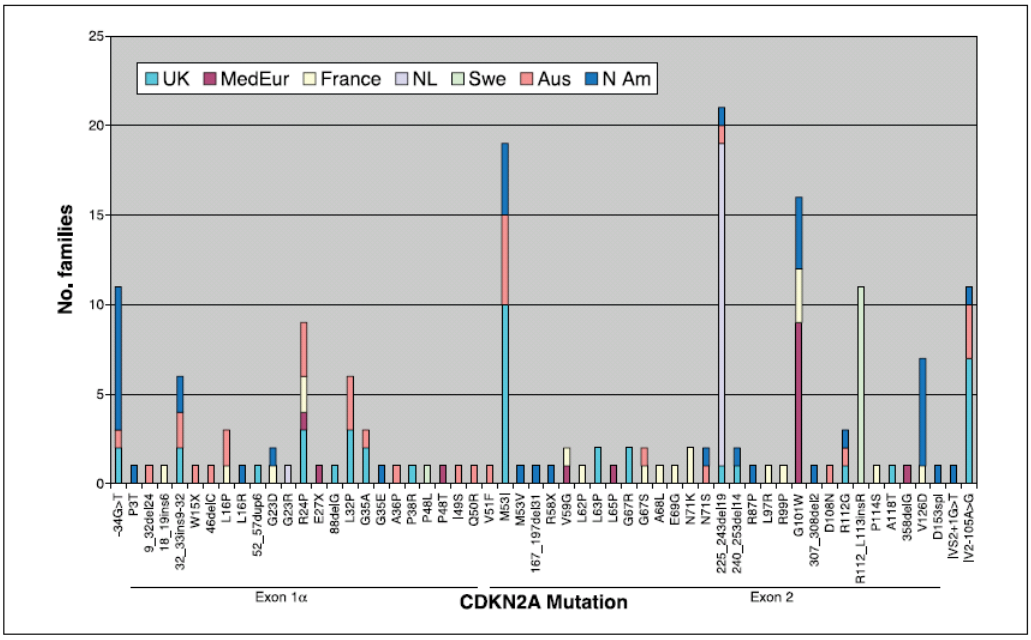


Figure 1.11. *Number of families with CDKN2A mutations according to family's geographic locale. Seven locales are defined as follows: UK, United Kingdom; MedEur, Mediterranean Europe (Spain, Italy); France; NL, the Netherlands; Swe, Sweden; Aus, Australia; N Am, North America (Goldstein et al., 2006).*

More recently the screening of English and Australian melanoma pedigrees for *CDKN2A* intronic mutations identified also two rare intronic variants (IVS1+1104C/A; IVS1-C/G) that predispose to melanoma (Harland et al., 2005a).

Overall, *CDKN2A* mutations have been found in 20-40% of families with 3 or more affected members and in 10% of families with 2 melanoma cases (de Snoo and Hayward 2005). In populations with the prevalent founder mutation, the likelihood of detecting a *CDKN2A* mutation might be also higher in families with fewer members affected than in populations without a founder mutation. These are also some suggestions that the proportion of identifiable mutations is greater in areas of the world with lower melanoma incidence e.g. Europe compared with Australia. This finding would also be expected for a high-penetrance susceptibility gene, as clustering of cases by chance is predicted to be more likely in sunny areas of the world with higher baseline incidence rates.

For most cancers, inherited susceptibility is identified by positive family history, multiple primaries and an early age of onset. Hence, a number of studies have been sought for germline *CDKN2A* mutations in patients with multiple primary melanomas and the mutation rates between studies are different. However, in the majority of studies the mutation rate ranges from 8-26% and once again many of the mutation-positive cases had documented family history of melanoma (Table 1.9 and references therein).

Early age at diagnosis in the absence of a family history does not appear to be a strong indicator of *CDKN2A* germline in melanoma patients, since several studies demonstrated a low frequency of *CDKN2A* mutations in patients with early-onset melanoma. In a study of children aged less then 15 years with melanoma only 1/31 individual tested carried germline *CDKN2A* mutation and this person had a family history of melanoma (Whiteman et al., 1997). Screening of 147 adolescents aged 15-19 years with melanoma found germline *CDKN2A* mutations in only two persons (Youl et al., 2002). Similarly, analysis of 60 persons with melanoma before the age 20 years yielded one mutation carrier who belongs to melanoma family (Berg et al., 2004). In a clinic-based series of melanoma cases younger than 40 years of age, 1/49 patients was found to carry a mutation and this individual had again a family history of the disease (Tsao et al., 2000a). Another studies investigating early-onset melanoma, similarly, identified low prevalence of *CDKN2A* mutations in the studied cohorts and mainly mutations have been found in persons with family history of melanoma (Soufir et al., 2004; Nagore et al., 2005; Stratigos et al., 2006b; Hocevar et al., 2006). However, early-age of onset might be useful to predict the CDKN2A mutations in populations with a predominant founder mutation (Mantelli et al., 2004).

The incidence of germline *CDKN2A* mutations in the general population is low. In the first population based study of melanoma cases from Queensland, Australia, it has been estimated that just 0.2% of all melanoma cases are caused by *CDKN2A* mutations (Aitken et al., 1999). Given that the lifetime risk of developing melanoma in Queensland is approximately 1:15 – 1:20 it has been extrapolated that approximately 1:10 000 of the general population of Queensland are germline *CDKN2A* mutation carriers (Hayward 2003).

The penetrance of *CDKN2A* mutations has been analysed in several studies with differences in risk based on study design and analytical approach. Most of the studies have used data ascertained from hereditary melanoma families. Cannon-Albright et al., 1994 estimated that *CDKN2A* mutation carriers have a 53% risk of developing melanomas by age 80 years, based on three families with multiple cases of melanoma. Newton Bishop et al., 2000 reported a penetrance estimate of 64% at age 85 years, based on three families. Box et al., 2001a, using 15 Australian *CDKN2A* mutation-carrying pedigrees that were also assessed for variants in the melanocortin receptor gene (*MC1R*), obtained relative frequencies of melanoma of 50% in *CDKN2A* mutation carriers without *MC1R* variants and 84% in carriers with *MC1R* variants (see also section 1.8.1).

Table 1.8. *CDKN2A founder mutations*

Mutation	Main population	Ancestry	Reference
"p16-Leiden" (c.225-243del19)[a]	The Netherlands	Dutch	Gruis et al., 1995b
insR113 (c.337-336insGTC)	Sweden	Swedish	Borg et al., 1996 Hashemi et al., 2001
M53I (c.159G>C)	Scotland	Scottish	Pollock et al., 1998 Lang et al., 2007
c.-34G/T[b]	Canada	British	Liu et al., 1999
G101W (c.301G>T)	France/ Italy	Celtic	Ciotti et al., 2000
V126D (c.377T>A)	North America	German/English	Goldstein et al., 2001
V59E	Israel	Jewish	Yakobson et al., 2003

[a] – a 19 basepair deletion in CDKN2A exon 2, which leads to a chimeric protein consisting of the N-terminal region of p16 and the C-terminal region of p14ARF; [b] – nucleotide substitution upstream of the p16 start codon, which results in a novel start site that generates a protein in a different reading frame.

Table 1.9. *Frequency of germline CDKN2A mutations in patients with multiple primary melanomas*

Reference	Population	Number with mutation (%)	Number with family history of melanoma
Monzon et al., 1998	Canada	5/33 (15%)	2
MacKie et al., 1998	Scotland	2/17 (12%)	0
Ruiz et al., 1999	Spain	0/9 (0%)	0
Burden et al., 1999	Scotland	6/23 (26%)	4
Hashemi et al., 2000	Sweden	9/80 (11%)	7
Auroy et al., 2001	France	9/100 (9%)	0
Blackwood et al., 2002	USA	8/96 (8%)	1
Peris et al., 2004	Italy	3/14 (21%)	0
Soufir et al., 2004	France	1/18 (6%)	0
Puig et al., 2005	Spain	17/104 (16%)	6
Stratigos et al., 2006b	Greece	2/2 (100%)	0

The largest study carried out to date by the Melanoma Genetics Consortium (GenoMel) has been based on 80 families from Europe, North America, and Australia (Bishop et al., 2002) and estimated the penetrance of 30% (95% CI: 12% to 62%) by age 50 years and 67% (95% CI: 31% to 96%) by age 80 years. This same study also showed the penetrance figures in hereditary melanoma to be different depending on geographical location. These differences between geographic locations were proportionate to the differences seen in sporadic melanoma, with the penetrance in Australia, USA and Sweden being 3.74 times higher than in Western Europe (Bishop et al., 2002). These estimates were considered upper bounds of penetrance because of the selection of families. To obtain risk estimates more representative of carrier risk in the general population, Begg et al., 2005 evaluated the lifetime risk of melanoma among relatives of *CDKN2A* mutation carriers, who were ascertained using a population-based study design. Probands were incident CMM patients with either first (SPM) or subsequent melanoma (MPM). The analysis estimated that the risk of melanoma in

mutation carriers was 14% (95% CI: 8% to 22%) by age 50 years, 24% (95% CI: 15% to 34%) by age 70 years, and 28% (95% CI: 18% to 40%) by age 80 years. The risk varied depending on whether the proband had one or more melanomas with risk 19% (95% CI: 7% to 37%) by age 80 years for SPM and 35% (95% CI: 22% to 51%) for MPM. These results expand the spectrum of melanoma risks by extending findings to CMM patients without extensive familial aggregation but do not provide a lower bound because mutation carriers were identified by their melanoma status. Differences between the both largest estimations of melanoma risks, along with the population beseline rates corresponding to the mixed geographic populations in the GEM (Genes Environment and Melanoma study) samples are shown in Figure 1.12 adapted from Begg et al., 2005. Similar low *CDKN2A* mutation rate in individuals with melanoma using population-based study design was recently also noted by Orlow et al., 2007.

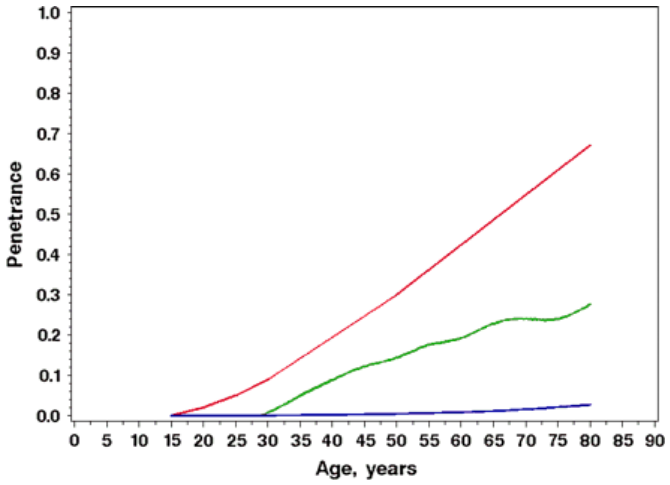


Figure 1.12. *Comparison of age-specific penetrance estimates of melanoma for CDKN2A mutation carriers. Estimates from the Genes Environment and Melanoma study (**green line**), benchmark population cumulative incidence rates for melanoma (Population Incidence) (**blue line**), and the results published using 80 multiple-case melanoma families (Melanoma Genetics Consortium) (**red line**) are shown. Adapted from Begg et al., 2005.*

Melanoma risk derived from population-based or other non-family-based sources would be anticipated to be lower than the risks observed in multiple-cases families. Similar phenomenon has been observed in the much larger body of literature on the risk of breast cancer in carriers of *BRCA1/BRCA2* mutations. Studies of high-risk, multiple cases families or studies involving ascertainment through genetic counselling clinics have led consistently to high estimates of penetrance (Ford et al., 1998; Antoniou et al., 2006), while studies based on carrier families ascertained without regard to family history of breast cancer or in a population-based manner have generally led to considerably lower estimates of risk (Satagopan et al., 2001; 2002; Antoniou et al., 2003; Chen et al., 2006). Clearly, a carrier identified in the population is likely to have a much lower risk profile than do carriers identified in multiple-cases families. This difference is probable due to the existence of genetic variants that affect risk (Antoniou et al., 2001; 2002; Pharoah et al., 2002), some of which have recently been identified (Bhattacharyya et al., 2000; Tutt et al., 2002) and are possible due to differences in risk conferred by different mutations within the same gene (Antoniou et al., 2003). For melanoma it has been shown in several family-based studies that genetic (e.g. *MC1R*), host (e.g. dysplastic and typical nevi), and environmental factors (e.g. sun exposure) increase the risk for melanoma in *CDKN2A* mutation carriers and may influence the penetrance of melanoma (Chaudru et al., 2004). Given the importance of these other modifying factors for penetrance, it is expected that families with many cases of melanoma may share the *CDKN2A* mutation as well as the other factors (Bishop et al., 2002; Begg et al., 2005).

Currently, it is not possible correctly predict who might be carrying a *CDKN2A* mutation. More, different mutations on the *CDKN2A* gene may confer substantially different risk of melanoma (Berwick et al., 2006). However, two factors previously consistently shown to be associated with an increased frequency of *CDKN2A* mutations have been observed in the population based study of Begg et al.,
2005 – the number of melanoma patients in the family and MPM. Overall, 3% of MPM patients compared to 1.3% of SPM patients have been shown to carry *CDKN2A* mutations and the frequency of mutations significantly increases as the number of reported relatives with melanoma increased (Begg et al., 2005). Given that the estimated mutation frequencies are representative for the U.S. population, Goldstein and Tucker 2005 extrapolated to impute that 1.3% of all newly diagnosed melanoma patients in 2005 would carry the *CDKN2A* mutation and of these 36% would have neither a family nor personal history of melanoma. A recent publication by Casula et al., 2007 looking for clinical predictors for germline mutations in the *CDKN2A* gene in large clinic-based population of melanoma patients confirmed previous findings. Relative risk of carrying a *CDKN2A* mutation for CMM patients was demonstrated to significantly increase with the presence of familial recurrence of melanoma (RR 6.31; p=0.0009), multiple primary melanomas (RR 3.43; p=0.0014) and early onset age as well (RR 4.56; p=0.0026). These findings also suggest that mutations rates of candidate cancer genes may deeply vary among CMM patients from different geographical areas (Casula et al., 2007).

Cancer risk in *CDKN2A* mutation positive families

Given that somatic mutations in *CDKN2A* have been documented in a wide variety of different tumour type (Foulkes et al., 1997, Ruas and Peters 1998) attention has focused on the possible contribution of germline *CDKN2A* mutations to risk of non-melanoma cancers in individuals from mutation-positive families. Although, the majority of families appear to be predisposed to melanoma alone, in some studies an increased risk of pancreatic cancer has also been observed. The first evidences of a broader tumour spectrum in melanoma prone kindreds was the report of an increased risk of pancreatic cancer in Dutch melanoma families even before *CDKN2A* was identified (Bergman et al., 1990). After the *CDKN2A* identification Goldstein et al., 1995 identified an increased risk of pancreatic cancer in families with *CDKN2A* germline mutations. There occurred seven cases of pancreatic cancer in 4/10 families carrying a mutation, versus 0.32 cases expected, which equates to a 21.8-fold increased risk of this tumour type in individuals with a germline *CDKN2A* mutations. At the same time, Whelan et al., 1995 reported a *CDKN2A* mutation in kindred with three cases of pancreatic cancer and two cases of melanoma, and suggested that *CDKN2A* may underlay susceptibility to the familial cancer syndrome comprising these tumour types. Subsequently, several other groups have reported similar occurrence of pancreatic cancer in mutation-positive melanoma families (Soufir et al., 1998; Ghiorzo et al., 1999; Liu et al., 1999; Borg et al., 2000; Goldstein et al., 2000b; Rulyak et al., 2003). Since these two malignancies are often observed together, this association has been proposed as a new hereditary cancer syndrome (Lynch et al., 2002). Penetrance estimation for pancreatic carcinoma in patients carrying a *CDKN2A* mutation has not yet been well established, although a Dutch study estimated a 17% (95% CI: 3% to 30%) penetrance by age 75 in familial atypical multiple-mole melanoma syndrome families (FAMMM) with the $p16^{INK4a}$-Leiden 19-bp deletion (Vasen et al., 2000). A recent analysis examining the relationship between familial melanoma, pancreatic cancer and germline *CDKN2A* mutations using published data of 42 families with pancreatic cancer among 189 melanoma-prone families revealed that, at present, there is no genotype or phenotype predictive of increased risk of pancreatic cancer in these families (Goldstein 2004). Even more, it is not currently understood why some families appear to be susceptible to pancreatic cancer and others not. Bartsch et al., 2002 screened families with clustering of

pancreatic cancer but found *CDKN2A* mutations only in families with melanoma as well. The link of the relationship between pancreatic cancer and *CDKN2A* is indicated, however, by Ghiorzo et al., 2004, who found G101W mutations in sporadic pancreatic cancer patients, particularly if there was clustering of this cancer in the family. Significantly, Lal et al., 2000 found germline *CDKN2A* mutations in 2/14 patients with both pancreatic cancer and melanoma as primary tumours.

Besides pancreatic carcinoma, in several families the co-occurrence of eye and skin melanoma is reported. This raises the question whether eye melanoma is also a part of the FAMMM syndrome. Although *CDKN2A* gene inactivation by methylation plays a role in uveal melanoma (van der Velden et al., 2001b), lack of germline mutations in *CDKN2A* and *CDK4* genes (Singh et al, 1996; Soufir et al., 2000; Vajdic et al., 2003) indicates that hereditary eye melanoma seems to be a separate entity with separate causal genetic defects. However, more recently a melanoma-prone family with *CDKN2A* germline mutation, where one carrier was affected by uveal melanoma and the other by a cutaneous melanoma, have been reported (Kannengiesser et al., 2003).

In addition, an increased susceptibility to breast cancer has been suggested in Swedish melanoma-prone families with the founder mutation (Borg et al., 2000). Swedish founder mutation has been also shown to be associated with meningiomas and neurinomas in patients with multiple primary tumours, of which at least one was cutaneous malignant melanoma (Nielsen et al., 2004). Although the degree of risk and whether it is restricted to a subset of mutations-positive families remains to be answered. There have also been suggestions of an increased risk of colon and lung cancers, but these are the most common of all cancers and therefore their occurrence in melanoma families may be because of chance. Recent study carried out by GenoMEL (Melanoma Genetic Consortium), comprising major familial melanoma research groups from Europe, North America, Australia, and Asia, provided the most extensive characterisation of mutations in high-risk melanoma susceptibility genes in families with three or more melanoma patients yet available and evaluated mutation relationship with pancreatic cancer, neural system tumours, and uveal melanoma (UM). This study included 466 families (2137 melanoma patients) from 17 GenoMEL centres. Overall, 41% (n=190) of families had mutations, most involved $p16^{INK4a}$ (n=178). There were striking differences in mutations across geographic locales (Figure 11). The proportion of families with the most frequent founder mutation of each locale differed significantly across the seven regions (p=0.0009). Single *CDKN2A* founder mutations were predominant in Sweden (p.R112_L113insR, 92% of family's mutations) and the Netherlands (c.225_243del19, 90% of family's mutations). France, Spain and Italy had the same most frequent mutation (p.G101W). Similarly, Australia and United Kingdom had the same most common mutations (p.M53I, c.IVS2-105A>G, p.R24P, and p.L32P). As reported also previously, there was a strong association between pancreatic cancer and *CDKN2A* mutations (p<0.0001). This relationship differed by mutation. In contrast, there was little evidence for an association between *CDKN2A* mutations and neural system tumours (p=0.52) and uveal melanoma (p=0.25). There was a marginally significant association between neural system tumours and *ARF* (p=0.05). However, this particular evolution had low power and requires confirmation (see also bellow) (Goldstein et al., 2006).

Another common phenotypic phenomenon in familial melanoma is the occurrence of a number of atypical nevi in the family – variously referred as a dysplastic nevus syndrome or atypical mole syndrome, and when there is the co-exsistence of melanoma and atypical nevi the syndrome has therefore been named familial atypical multiple mole melanoma (FAMMM). The first family to which the name FAMMM syndrome was assigned showed clear co-segregation of melanoma and multiple atypical moles, leading the authors to the assumption that a single dominant factor might be responsible for both phenotypes (Lynch et al., 1978). After the cloning of the first melanoma susceptibility gene, genotype/phenotype correlation studies in *CDKN2A* mutation positive families soon revealed the complexity of the

FAMMM syndrome. In the one of the first report of *CDKN2A* mutation positive families alone, only 30% of family members with atypical nevi were mutation carriers (Hussussian et al., 1994). Since several groups have studied the relationship between *CDKN2A* mutation status and the nevus phenotype. Even though *CDKN2A* seems to be nevogenic, the nevus phenotype cannot be used to distinguish mutation carriers from non-carriers (Gruis et al., 1995b; Wachsmuth et al., 1998; Newton-Bishop et al., 2000). The difficulty in defining the atypical mole phenotype (differs between studies), along with its inconsistent segregation with melanoma, make it as a ambiguous risk marker for melanoma and a very unreliable indicator of *CDKN2A* mutations carrier status. On the other hand, nevi *per se* represent the greatest risk factor for melanoma susceptibility and are often precursor lesions from which melanoma develop (Bogdan et al., 2003). Furthermore, a 15-years longitudinal assessment of nevus phenotype in a large melanoma family (Florell et al., 2004) have showed that p16/ARF mutation carriers continue to develop nevi over time whereas non-carriers and spouse controls in the same family show nevus regression. Recent genome-wide scan for naevus counts with a total of 736 markers for 424 families with 1024 twins and siblings, plus 690 parents, supported the linkage to the *CDKN2A* gene region (*9p21*) and discovered linkage to several novel regions that may also influence risk of developing malignant melanoma. So - for raised mole count, there was suggestive evidence of linkage to chromosome 16, and for atypical mole count on chromosomes 1, 6, and X (Zhu et al., 2007).

Additionally, the MPM phenotype is frequently found in high-risk families and studies (Burden et al., 1999; Puig et al., 2005) have found that 27% - 48% of individuals with MPM have a relevant family history of melanoma with a predisposing germline mutation, whereas only 8% - 26% patients with sporadic with MPM harbour a p16/ARF mutation (see Table 1.9). Additionally, p16/ARF mutations are more likely to be identified in individuals with MPM than in those with single melanomas, and these individuals also have other high-risk factors, including increased number of nevi, early age of onset, and fair skin (Monzon et al., 1998; Berwick et al., 2004; Puig et al., 2005). A recent study carried out by Ferrone et al, 2005 reviewed risk factors for MPM phenotype and confirmed previous findings. Twenty one percent of individuals with MPM had a family history of melanoma and 38% and had a presence of atypical nevi compared with 18% of individuals with single primary melanoma. Recently, it has been also found that patients with three and more MPMs have an increased probability of carrying $p16^{INK4a}$ mutation (Eliason et al., 2006).

A recent pooled data analysis carried out by GenoMEL examined the major risk factor reported to be associated with an increased frequency of *CDKN2A* mutations (number of melanoma patients in the family, early age at melanoma diagnosis, family member with MPMs or pancreatic cancer) confirmed previous findings (Goldstein et al., 2006).

Effects of the *CDKN2A* specific mutations

Although segregation of germline *CDKN2A* mutations with the disease in melanoma-prone families is a strong indicator that they are disease-related, demonstration of functional defects in the $p16^{INK4a}$ protein remains important in distinguishing disease-associated mutations from polymorphisms. As summarised by Castellano and Parmiani 1999, most (70%) familial $p16^{INK4a}$ mutations have not been formally tested for loss of function. About 25% of these are frameshift or nonsence mutations which produce a prematurely truncated protein and C-terminus truncations of exon 2 are known to cause loss of *CDK4* binding activity (Parry and Peters 1996), and it is therefore inferred that all familial truncations produce non-functional $p16^{INK4a}$ variants. The remaining 45% of non-tested familial mutations are missence alterations or inframe deletions and insertions, and they are considered to be non-functional because alter the amino acid residues in the ankyrin domains. This type of domain is involved in protein-protein interaction and $p16^{INK4a}$ is composed of four such tandemly repeated sequences (Serrano et al., 1993). Functional assessments of the

remaining mutant $p16^{INK4a}$ proteins using different assays have confirmed the functional impairment for most mutations but not all. These assays include cell cycle inhibitory actions or CDK4/6-binding and –inhibitory activity. Functional studies have compared ectopically expressed wild-type $p16I^{NK4a}$ with mutant $p16^{INK4a}$ in various cell lines (Walker et al., 1999; Becker et al. 2001). CDK kinase binding and inhibition by $p16^{INK4a}$ variants was also tested using *in vitro* expressed proteins (Ranade et al., 1995; Harland et al, 1997; Parry and Peters 1996), yeast 2-hybrid binding assays (Reymond and Brent 1995; Yang et al., 1995; Monzon et al., 1998), or mammalian 2-hybrid binding assays (Rizos et al., 2001). Generally, these assays confirmed that wild-type $p16^{INK4a}$ binds and efficiently inhibits CDK4 and CDK6, has the ability to arrest cells in G1 phase of the cell cycle and can inhibit colony formation. From these assays, $p16^{INK4a}$ variants identified in melanoma-prone kindreds can be separated into $p16^{INK4a}$ variants such as A148T, which are believed to be polymorphisms due to their frequent occurrence in the general population, poor segregation with familial melanoma and apparent lack of functional deficit, and melanoma associated mutations, which segregate closely with the disease, encodes significant amino acid changes and, when tested, have proven to be non-functional in various assays. Functional loss includes the inability to inhibit cell cycle progression and colony formation, changes in subcellular localisation and protein stability and diminished binding and inhibitory activity to *CDK4* and *CDK6*. Interestingly, some $p16^{INK4a}$ variants still remain puzzling. So, the N-terminal 24 base pair duplication, which results in a duplication of the first 8 amino acids of $p16^{INK4a}$, was identified in several melanoma families around the world (Walker et al., 1995; Flores et al., 1997; Pollock et al., 1998; Monzon et al., 1998). This mutation clearly segregates with melanoma and shows strong disease penetrance, but behaves as wild-type protein in almost all functional assays except one, which has shown that this mutation has diminished cell-cycle inhibitory activity (Becker et al., 2005).

1.6.2. *ARF* [OMIM 600160]

Since melanoma appears to segregate with chromosome *9p* markers in a greater proportion of families than have been shown to carry mutations of *CDKN2A*, it has been speculated that this high degree of linkage to *9p* may indicate the presence of another melanoma susceptibility gene in this region. One candidate gene is $p14^{ARF}$, the product of an alternative transcript arising from the same *CDKN2A* locus (Figure 1.10). *CDKN2A* mutations in exon 1α affect only the $p16^{INK4a}$ transcript, whereas some of these occurring in exon 2 can affect both $p16^{INK4a}$ and $p14^{ARF}$ confounding each gene specific role in melanogenesis. However, gene defects affecting only $p14^{ARF}$ have been described in some melanoma families (Randerson-Moor et al., 2001; Rizos et al., 2001; Hewitt et al., 2002) thus indicating the *ARF* locus as a melanoma susceptibility gene in its own right.

Mouse models have been helpful in addressing the specific effects of each product of the *CDKN2A* locus and have provided convincing evidence for both $p14^{ARF}$ and $p16^{INK4a}$ being tumour suppressors. Mouse strains lacking only $p16^{INK4a}$ were prone to the development of various tumours, including spontaneous development of melanoma (Krimpenfort et al., 2001; Sharpless et al., 2001). Although $p19^{ARF}$ knockout mice ($p19^{ARF}$ is the murine equivalent of human $p14^{ARF}$) show no development of melanomas, the tumour spectrum is similar to that seen in mice lacking both *CDKN2A* products. After introduction of a *Ras* transgene, $p19^{ARF}$ knockout mice do develop melanoma, which is also seen in $p19^{ARF}/p16^{INK4a}$ dual knockouts (Walker and Hayward 2002). Furthermore, when the $p16^{INK4a}$ null mice were crossed with $p19^{ARF}$ hemyzygotes, 50% of animals developed melanomas after treatment with the carcinogen 7,12-dimethylbenz(a)antracene, compared to 8% of mice lacking just $p16^{INK4a}$ (Krimpenfort et al., 2001). Melanoma development seen in $p16^{INK4a}$ knockout mice crossed with $p19^{ARF}$ hemyzygous suggests cooperation between the $p16^{INK4a}$ and $p19^{ARF}$ pathways in

melanoma development. All above mentioned findings in different mouse models make strong evidence for $p19^{ARF}$ as well as $p16^{INK4a}$, being *bona fide* tumour suppressors involved in melanoma development. Between mouse strains there were seen differences in the spectrum of tumours, rate of melanoma development, and melanoma invasiveness, suggesting phenotypic differences when either one or both genes are mutated. Although the data for such a conclusion in humans are not so clear, the first effort of the Melanoma Genetic Consortium (GenoMel) to address this question has resulted in the observation of a 1.8-fold more penetrant phenotype in mutation carriers with both genes being affected (Bishop et al., 2002). Large-scale studies are needed to determine whether this finding is significant.

There are evidences to accumulate with indications that $p14^{ARF}$ mutations independently predispose to melanoma, as well as nervous system tumours (NSTs). Interestingly, this combination of tumours has been proposed as a discrete syndrome (OMIM 155755), first identified by Kaufmann et al., 1993. The first documented individual to carry a germline deletion of part of chromosome *9p*, because of an unbalanced chromosome translocation, developed multiple melanomas and the neurofibroma (Petty et al., 1993). It was estimated that the deletion in this patient rendered the *CDKN2A, ARF*, and *CDKN2B* loci hemyzygous. Several years later, Bahau et al., 1998 reported two families with melanoma and a range of NSTs (including astrocytoma, meningioma, schwanomma and neurofibroma) that also carried hemyzygous deletions of a part of chromosome *9p*. Additional support for $p14^{ARF}$ inactivation being responsible, at least in part, for predisposition to NSTs and melanoma comes from Petronzelli et al., 2001 who reported a family in which there were nine melanoma cases, four of whom also developed neurofibromas. This family carries a germline spicing mutation that results in a lack of exon 2 sequencies, thus both proteins – $p16^{INK4a}$ and $p14^{ARF}$ – are defective.

So far, mutations affecting $p14^{ARF}$ exclusively have been reported in only few families. The first unequivocal report of a germline deletion of $p14^{ARF}$ in the absence of a loss of either *CDKN2A* or *CDKN2B* was found in a family segregating melanomas and NSTs (Randerson-Moor et al., 2001). This family included four melanoma cases, one of whom also developed a neurilemmona in the chest wall. Another deletion specifically of $p14^{ARF}$ has been reported by Hewitt et al., 2002 who describe a family in which a mother and daughter with melanoma carry a mutation of the terminal nucleotide of exo 1β. It was found that this mutation affects the splicing of the $p14^{ARF}$ mRNA and leads to complete absence of the mutant transcript and hence the haploinsufficiency for $p14^{ARF}$. In this family, there were three other individuals with breast cancer, at least one of whom carries the mutation but no documented NSTs. Germline intragenic mutation specifically affecting $p14^{ARF}$ has been documented in patients with multiple melanomas but without family history of melanomas or NSTs (Rizos et al., 2001; Puig et al., 2005). In these individuals, an insertion of 16 bp within exon1β (60ins16) gives rise to the frameshift in $p14^{ARF}$ (but leaves $p16^{INK4a}$ intact) and leads to the inactivation of the cell-cycle inhibitory function of this protein (Rizos et al., 2001). These mutations give further support to the premise that *ARF* is an independent melanoma predisposition gene that maps to chromosome *9p*. To date 5 more melanoma families with germline mutations at the $p14^{ARF}$ exon 1β splice donor site have been identified (Harland et al., 2005b). Recently the first $p14^{ARF}$ exon 1β missence germline mutation (G16D) was also identified in a melanoma-neural system tumour syndrome (CMM+NST) family (Laud et al., 2006). In the same study a germline deletion of 8474bp ranging from 196bp upstream of $p14^{ARF}$ exon 1β initiation codon to 11233bp upstream of exon 1α of $p16^{INK4a}$ was identifies in a family with five melanoma cases (Laud et al., 2006). Interestingly, most of the $p14^{ARF}$-only mutations (except one) found to date are either splice site mutations, frameshifts or deletions, which result in hemizygosity, thus indicating that dosage of $p14^{ARF}$ is critical in melanoma development.

1.6.3. *CDK4* [OMIM 123829]

A candidate gene search based on key cell cycle regulators led to the finding of another melanoma susceptibility gene, *CDK4* on chromosome *12q14* (Zuo et al.,1996). *CDK4* encodes cyclin-dependent kinase 4, which similar to the two *CDKN2A* gene products, is a key regulator of the cell cycle. In fact, $p16^{INK4a}$ prevents cdk4 from forming a complex with cyclin D, thereby bloking phosphorylation of RB1 (Figure 10) (reviewed in Ortega et al., 2002). When mutated *CDK4* becomes an oncogene not any more appropriately regulated by $p16^{INK4a}$, resulting in a similar molecular pathway defect to that occurring with a $p16^{INK4a}$ mutation (Zuo et al., 1996, Soufir et al., 1998). *CDK4* mutations are rare and previously have been reported in only 6 melanoma families worldwide – two American (Zuo et al., 1996), one French (Soufir et al., 1998), Norwegian, Australian, and English (Molven et al., 2005), despite many screens for *CDK4* alterations (FitzGerald et al., 1996a; Platz et al., 1998; Newton Bishop et al., 1999; Della Torre et al., 2001; Mantelli et al., 2002; Goldstein et al., 2002; Landi et al., 2004; Soufir et al., 2004). All *CDK4* mutations found to date are located in exon 2 and affect codon 24, with two families carrying an R24C change (Zuo et al., 1996) and other having an R24H substitution (Soufir et al., 1998; Molven et al., 2005). The arginine normally at this position is critical in allowing $p16^{INK4A}$ to bind to the kinase, and when the arginine is substituted, $p16^{INK4A}$ can no longer bind and inactivate the cdk4 protein (Wölfel et al., 1995; Coleman et al., 1997). Haplotype analysis, using microsatellite markers flanking the *CDK4* gene and single nucleotide polymorphisms (SNPs) within the *CDK4* gene, did not support the observation that the R24H mutation had a common origin in these families but indicate that there were at least two independent mutational events. The cytosine-guanine dinucleotide of codon 24, through its methylation, probably represents a mutational hotspot in the *CDK4* gene (Molven et al., 2005). Although the *CDK4* mutation rate and overall role in familial melanoma is low, mutations of this gene have a similar impact to those in *CDKN2A* (Goldstein et al., 2000b). Phenotypic characteristics of the families carrying *CDK4* germline mutations do not differ from *CDKN2A* affected families, and similarly show a high penetrance for melanoma in mutation-positive family members and no strong correlation between carrier status and nevus phenotype (Goldstein et al., 2000b). Because of the *CDK4* mutation rarity, it has been difficult to ascertain penetrance, but one study carried out by Goldstein et al., 2002 estimated the penetrance of *CDK4* mutation from two families to be 63% (95% CI: 42% to 85%). Thus far, there is no evidence for an increased risk of cancers other than melanoma in heterozygous carriers of *CDK4* codon 24 mutations (Zuo et al., 1996; Soufir et al., 1998; Molven et al., 2005).

In the reviewed literature sequencing of the whole *CDK4* has only been performed in the first *CDK4* germline mutation report (Zuo et al., 1996) and in those reported by Goldstein et al., 2002. However, since most investigations exclusively screened exon 2 of the *CDK4* gene, sequencing of the entire *CDK4* gene might increase the significance of this gene in melanoma predisposition in non-*9p*-linked melanoma families beyond the families that has been identified worldwide to date.

1.7. Additional melanoma susceptibility loci

The *CDKN2A* and *CDK4* genes account for predisposition in less than half of all multicase melanoma families. Homozygous deletions of *9p21* excluding *CDKN2A* in melanoma (Puig et al., 1995) was one of several lines of evidence leading to speculation of the *9p21* region harbouring an additional melanoma gene. Due to the *CDKN2B* close proximity (Figure 1.13), as well as its high sequence and functional homology to *CDKN2A*, *CDKN2B* (encoding $p15^{INK4b}$) has often been suggested as a third melanoma susceptibility gene on *9p21*. *CDKN2B* is one of the few genes to be extensively studied by several groups as

a candidate germline predisposition gene in familial melanoma. However, with the large number of families screened for *CDKN2B* germline mutations to date (Stone et al., 1995; Liu et al., 1997; Platz et al., 1997; Flores et al., 1997; Casula et al., 2003; Laud et al., 2006) and no mutations found so far, involvement of *CDKN2B* in melanoma development seems to be restricted to somatic defects.

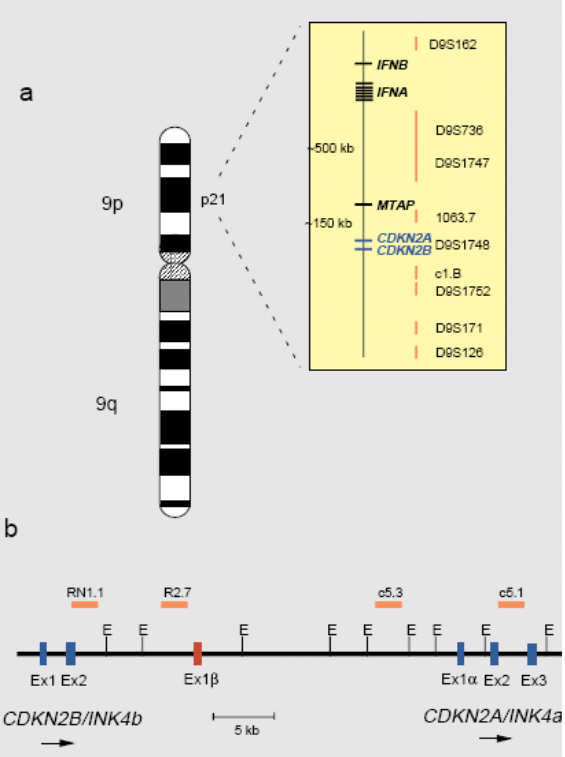


Figure 1.13. *Chromosome 9p21 and the genomic organisation of CDKN2A/INK4a and CDKN2B/INK4b.* ***(a),*** *the ideogram of human chromosome 9 identifies band and the linear map on the right shows the relative location of several known genes, including CDKN2A/INK4a and CDKN2B/INK4b genes;* ***(b)****, the expanded map of the CDKN2A and CDKN2B loci (Ruas and Peters 1998).*

Similarly, other members of the CDKI family, *CDKN2C* (encoding $p18^{INK4c}$) and *CDKN2D* (encoding $p19^{INK4d}$), have been tested for germline mutations and none was found (Platz et al., 1998; Newton Bishop et al., 1999).

Compared to other common familial cancers, such as breast cancer, it is surprising how few genome-wide linkage analyses have been performed in melanoma to localise high penetrance susceptibility genes. Only 4 such studies have been reported (Greene et al., 1983; Nancarrow et al., 1993; Gruis et al., 1993; Gillanders et al., 2003), the first three of which were underpowered (analysing between 3 and 14 families) had poor genomic coverage (using 23-172 markers spread across 12-22 autosomes) and failed to provide statistically significant support for the location of a melanoma predisposition locus. The most comprehensive and only high-density genome-wide scan carried out to date (Gillanders et al., 2003) used 414 highly polymorphic microsatellite markers, spread across all autosomes, in 49 Australian melanoma families in which linage to *9p21-9p22* region or mutation of *CDKN2A* or *CDK4* had been excluded. A novel melanoma susceptibility locus was localised to chromosomal region *1p22,* with the strongest linkage in families in families with earlier age at diagnosis. Analysis of 33 additional pedigrees provided further support for a melanoma predisposition locus mapping to this region of *1p22*. LOH studies in melanoma samples demonstrated that this susceptibility gene most probably encodes a tumour suppressor; however, sequence analysis of several candidate genes in this region has not detected any germline mutations in *1p22*-linked melanoma kindreds (Walker et al., 2004). Considerable effort continues to be put into searching for this novel melanoma susceptibility gene.

Additionally, linkage to a *1p36* locus (Bale et al., 1989; Goldstein et al., 1993) and the HLA region of chromosome band *6p21* (Hawkins et al., 1981; Demenais et al., 1984) were documented but not confirmed in other cohorts (Cannon-Albright et al., 1990; Gruis et al., 1990; Kefford et al., 1991; Walker et al., 1994).

Recently, Jonsson et al., 2005 reported mapping of a novel melanoma susceptibility locus to chromosome *9q21.32* between markers *D9S922* and *D9S152* in three Danish families with multiple cases of cutaneous and ocular melanoma and no germline mutations in *CDKN2A, CDK4, BRCA1* and *BRCA2*. Mutational analysis for germline mutations in the currently known coding regions of five genes located within this region has not resulted in any segregating sequence variants (Jonsson et al., 2005). Analysis of more families will be critical to confirm the presence of a melanoma susceptibility gene on *9q21.32*.

Another potential reservoir for the identification of novel melanoma susceptibility genes is the Ashkenazim. No Ashkenazi family has been reported to have a mutation in either *CDKN2A* or *CDK4* (Yakobson et al., 1998; Yakobson et al., 2000; Loo et al., 2005). Considering the common founder mutations identified in this population in other cancer predisposing genes (e.g. *BRCA1* and *BRCA2*) (Friedman et al., 1994; Neuhausen et al., 1996), it is quite likely that a recurrent mutation in an as yet unidentified gene is responsible for familial clustering of melanoma in Ashkenazim.

1.8. Low – penetrance melanoma susceptibility genes

1.8.1. *MC1R* [OMIM155555]

The melanocortin-1 receptor (*MC1R*) gene plays a critical role in the genetics of constitutive human pigmentation (Rees 2003). It maps to chromosome 16q24.3 (Gantz et al., 1994, Magenis et al., 1994) and encodes a seven-pass transmembrane G-protein-couplet receptor (Figure 1.14) with a high affinity for α-melanocyte-stimulating hormone (α-MSH).

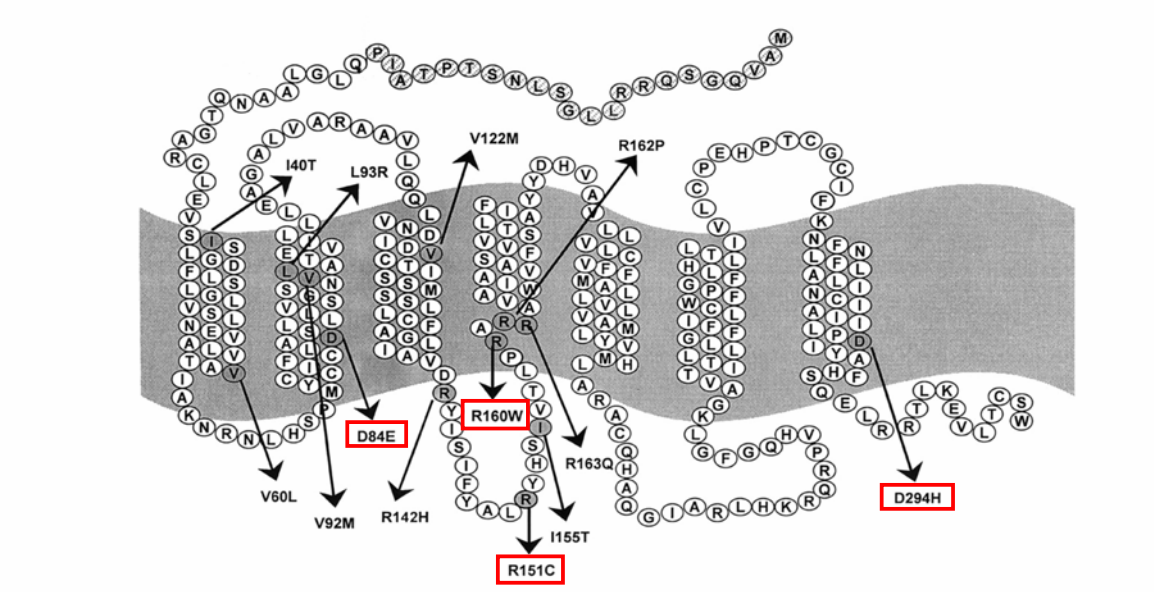


Figure 1.14. *Structure of MC1R. Localisation of natural loss-of-function mutations described in the literature (reviewed in Sturm et al., 2001) are highlighted by arrows and so called red hair variants "R" are marked by red boxes (adapted from Sanchez Mas et al., 2002).*

MC1R is expressed in a variety of cells, including skin melanocytes, endothelial cells, and keratinocytes (Chhajlani and Wikberg 1992; Chakraborty et al., 1999), and binding of α-MSH to *MC1R* leads to the activation of adenylate cylase and increased cyclic adenosine monophosphate (cAMP) levels, resulting in eumelanin synthesis and a concomitant increase in the eumelanin/phaeomelanin ratio in the skin (Schaffer and Bolognia 2001). Variants of *MC1R* gene appear to be compromised in their capability of inducing this switch in melanin production. Melanin is a very effective sunblock and it has been suggested that 2- or 3-fold differences in the black melanin pigment (eumelanin) can account for an up to 100-fold variation in sun sensitivity (Rees 2003). A second type of melanin (phaeomelanin), which is yellow/red in colour, is produced particularly in people with red hair and freckles. This type of melanin is functionally different from eumelanin in that it is less photoprotective and potentially mutagenic.

In contrast to the high-penetrance melanoma susceptibility genes, *MC1R* is highly polymorphic in whites and more common in the population. However, the prevalence is difficult to assess as it depends on the population and ability to distinguish "true" variants that cause disease versus polymorphisms. Considerable variation in the distribution of variant alleles has been demonstrated in European and, to a lesser degree, in Asian populations, but very little variation (only silent amino-acid changes) in African populations (Table 1.10), indicating the enormous evolutionary pressure in Africa against deviation from eumelanin production (Harding et al., 2000).

It is currently identified more than 77 human alleles of the *MC1R* with non-synonymous changes (Wong and Rees 2005). And there are many studies examining the association of *MC1R* variants with individual's pigmentation. In a group of Caucasians, Valverde et al., 1995 reported that 53% of individuals with red hair were found to carry one variant allele and 29% had two variant *MC1R* alleles. In contrast, no individual with any other hair colour was found to carry two variants and overall the frequency of heterozygous carriers of a variant allele in those with bond, brown and black hair did not exceed 33%. Similarly, the majority of individuals (76.5%) with skin type I (very fair skin) carried a variant *MC1R* allele, whereas no individuals with skin type IV (dark skin) possessed a variant. The frequencies were intermediate for individuals with sin types II (46.5) and III (5%). Box et al., 1997 confirmed and extended this work and showed that three particular variants of *MC1R* – R151C, R160W, D294H – were most significantly associated with red hair and fair skin (referred as "R" variants). Similar association of these variants with red hair and fair skin was also found by Flanagan et al., 2000. Other alleles, such as V60L, 86insA, D84E, R142H, R151C, I155T, and H260P, have been considered as partial "red hair colour" ("R") causing variants (Sturm 2002). Furthermore, there are evidences that the *MC1R* gene is associated with freckling (Palmer et al., 2000; Bastiaens et al., 2001a).

Given the associations between red hair, fair skin, freckling and melanoma risk is not surprising, certain variants of *MC1R* gene have been found to increase risk of cutaneous malignant melanoma. In the first study to report such an association Valverde et al., 1996 found that *MC1R* variants were significantly more common in melanoma patients than controls ($p=0.0094$) and conferred a 3.9-fold risk to carriers (95% CI:1.48-10.35). The bulk of this increased risk in the melanoma group was associated with the D84E variant. In the subsequent much larger study, the three major "R" variants (R151C, R160W, D294H) were found to each give a 2.2-fold increased risk of melanoma (95% CI: 1.6-3.0). Moreover, this effect was additive since individuals with two variants had a 4.1-fold relative risk of melanoma (95% CI: 2.1-7.9).

Table 1.10. *Allele frequencies (%) of the nine non-synonymous MC1R variants in different populations*

Population	**Non-synonymous *MC1R* variants**[a]									
	p.V60L (c.178G>T)	**p.D84E (c.252C>A)**	p.V92M (c.274G>A)	p.R142H (c.425G>A)	**p.R151C (c.451C>T)**	p.I155T (c.464T>C)	**p.R160W (c.478C>T)**	p.R163G (c.488G>A)	**p.D294H (c.880G>C)**	Reference
Greece	20.0	-	7.9	1.3	3.3	0.0	2.0	1.3	-	Stratigos et al., 2006a
Italy[b]	28.6	-	10.0	1.2	9.4	-	5.3	2.3	3.5	Landi et al., 2005
Italy	25.5	0.5	3.0	3.0	2.5	1.0	3.0	2.0	0.0	Fargnoli et al., 2006
France	6.2	0.9	7.1	0.0	1.9	0.9	2.4	2.4	1.4	Matichard et al., 2004
The Netherlands	8.3	0.9	6.9	0.8	4.8	-	10.5	4.9	0.7	Kennedy et al., 2001
USA	14.0	0.8	9.8	1.1	6.4	1.7	6.2	3.9	2.8	Kanetsky et al.,2004
Ireland	19.7	3.9	14.1	0.0	23.9	1.4	15.5	5.6	6.9	Smith et al., 1998
Australia	12.2	1.2	9.7	0.4	11.0	0.9	7.0	4.7	2.7	Duffy et al., 2004
Asia	1.0	0.0	4.0	0.0	0.0	0.0	0.0	39.0	0.0	Harding et al., 2000
Asia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	70.0	0.0	Nakayama et al., 2006
Africa[c]	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4	0.7	Harding et al., 2000

Red hair color "R" variants are designated in ***bold;*** [a] *- compound heterozygotes and homozygotes are grouped together as "MC1R variant";* [b] *- the controls from Landi et al., 2005 in this group are from a melanoma case-control study;* [c] *- all variants were detected in African Americans.*

After adjusting for hair colour and skin type this risk gradient was abolished in those with fair skin but persisted in individuals with medium (OR 2.2; 95% CI: 1.2-4.1) or olive/dark (OR 10.8; 95% CI: 1.2-95.6) complexion (Palmer et al., 2000). The later indicates a significant risk conferred by *MC1R* variants even in those who have dark skin or are able to tan well. Very similar findings were reported also by Kennedy et al., 2001 with one variant allele to give a 2.9-fold risk (95% CI: 1.6-5.4) of melanoma, whereas two variants conferred a 4.8-fold (95% CI: 2.5-9.4) relative risk. Notably, the D84E variant was most significantly associated with melanoma (OR 16.1; 95% CI: 2.3-139.0). Once again susceptibility was largely found to be independent of hair colour and skin type. The D84E variant association with melanoma risk was also recently found in the study carried by Mossner et al., 2007. A strong association between *MC1R* variants with compromised function and melanoma, independent of clinical and UV exposure risk factors, was recently reported in a French population (Matichard et al., 2004). In an Italian population, the association between "R" variants and melanoma risk was confirmed (Landi et al., 2005; Fargnoli et al., 2006) and was shown to be stronger in phenotypically low risk individuals (Landi et al., 2005). Similar association between "R" variants and melanoma was recently shown also in a largest population-based study carried out to date (Kanetsky et al., 2006). The association between melanoma and *MC1R* variants was found also in a Polish population (Debniak et al., 2006a) and more notable also in a southern European populations with a relatively low incidence of the disease (Pastorino et al., 2004; Stratigos et al., 2006a), confirming that *MC1R* is a low-penetrance susceptibility locus for melanoma. There are also evidences that *MC1R* variants are a predisposing factors for non-melanoma skin cancers such as basal and squamous cell carcinomas (Bastiaens et al., 2001b; Box et al., 2001b). However, in contrast, *MC1R* variants have not been shown to predispose individuals to uveal melanoma (Metzelaar-Blok et al., 2001; Vajdic et al., 2003; Hearle et al., 2003).

Interestingly, apart from acting as independent risk factor for melanoma, *MC1R* variants have also been shown to act as modifier alleles, increasing the penetrance of mutation in the *CDKN2A*. In a study of 15 Australian melanoma families in which *CDKN2A* mutation were segregating with the disease, the presence of an *MC1R* variant significantly increased (p=0.01) the penetrance of *CDKN2A* mutation from 50% to 84% and this increase in penetrance was associated with a significantly decreased mean age of onset from 58.1 years in individuals with wild type for *MC1R* to 37.8 years in those who carried an *MC1R* variant (Box et al., 2001a). In a Dutch study, germline carriers of the *CDKN2A* $p16^{INK4a}$-Leiden deletion mutation again showed an increased risk of melanoma when they carried *MC1R* variant alleles. In this study the melanoma penetrance in *MC1R* wild-type individuals was 18%, which increased to 35% or 55% in those with one or two variant alleles respectively (p=0.01). The R151C variant of *MC1R* was responsible for the majority of the observed effect on melanoma risk (van der Velden et al., 2001a). A recent study, including 20 French melanoma kindreds, further supported the suggestions that *MC1R* "R" variants modify penetrance of *CDKN2A* mutations (Chaudru et al., 2005). Moreover, an association between multiple *MC1R* variants and the development of MPM in patients with *CDKN2A* mutations has been suggested in a study of 16^{INK4a} American melanoma families with *CDKN2A* mutations (Goldstein et al., 2005).

Given the limited number of reported families with mutations in *ARF* and *CDK4*, determining changes in penetrance of these genes due to *MC1R* variants is not possible to date.

Mechanisms underlying the increased risk of melanoma in carriers of *MC1R* gene variants are unclear. *MC1R* "R" variants have been causally associated with fair skin and/or fair hair, which are known melanoma risk factors, and functional studies have confirmed an impaired function for the "R" *MC1R* receptors (Schioth et al., 1999; Robinson and Healy 2002; Righolm et al., 2004). In addition, melanocytes expressing high-penetrance *MC1R* variant receptors exhibit higher sensitivity to the cytotoxic effect of UV radiation, thus

increasing skin cancer risk (Scott et al., 2002). The presence of *MC1R* variants might alter the protective $p16^{INK4a}$ mediated cell cycle response to UV and, thus, increase melanoma risk in carriers both *CDKN2A* mutations and *MC1R* variants (Pavey and Gabrielli 2002). However, an alternate nonpigmentary mechanism for MC1R variants influencing melanoma susceptibility has also been suggested. Modifications in the behavior of melanoma cells carrying *MC1R* variants, with regard to proliferation and binding to the cellular matrix proteins have been reported (Robinson and Healy 2002).

1.8.2. Other potential low-penetrance melanoma susceptibility genes

CDKN2A polymorphisms

Since inactivating mutations within the *CDKN2A* gene have been shown significantly associated with risk of developing melanoma, it has been suggested that there may be other variants with more modest affects. Conflicting data have been published on the association of melanoma risk with three most common *CDKN2A* polymorphisms, including one coding variant (c.442G>A) at the end of exon 2 and two noncoding variants (c.*29C>G, c.*69C>T) in the 3'UTR of the gene.

The c.442G>A variant converts an alanine (GCG) to a threonine (ACG) residue at codon 148 (A148T), located in the fourth ankyrin repeat domain and has no recognised effect on $p16^{INK4a}$ (Ranade et al., 1995; Reymond and Brent 1995; Lilischkis et al., 1996). Several studies have identified the A148T polymorphism in melanoma family members with no segregation of this variant with the disease (Hussussian et al., 1994; Kamb et al., 1994a, Harland et al., 1997; Yakobson et al., 1998; Ghiorzo et al., 1999). The A148T variant has been found to be present with varying frequencies also in general populations, showing no evidence for the A148T variant as a melanoma susceptibility allele. In association studies, however, using candidate gene approach different results have been obtained (Table 1.11). In an English study, the A148T variant was detected in 4.9% of individuals from 179 families with the atypical nevus phenotype and/or family history of melanoma, and in 5.2% of controls (Bertram et al., 2002). In another study, the frequency of A148T polymorphism in the Italian population was 6.72% in melanoma patients versus 6.2% in control (OR 1.1; 95% CI: 0.51-2.37) and in the French population 3.4% in melanoma patients versus 4.2% in controls (OR 0.77; 95% CI: 0.39-1.52). Although no significant differences of the A148T polymorphism frequencies were observed between melanoma patients and controls in the Italian and French populations, the variant allele frequency was significantly different between these populations under the study (p=0.008) (Spica et al., 2006). In the large cohort of Polish melanoma patients, the A148T variant was demonstrated to be associated with an increased risk of cutaneous malignant melanoma. It was detected in 7% of melanoma patients compared with 2.89% of the control individuals (p=0.0003; OR 2.53; 95% CI: 1.55-4.12), especially in patients under 50 years of age (p=0.0002; OR 3.44; 95% CI: 1.86-6.37) (Debniak et al., 2005b). Another work from the same group showed that the risk of melanoma seems to be increased additively for patients harbouring the A148T variant in *CDKN2A* and the R151C variant in *MC1R* (Debniak et al., 2006a). Recently Puig et al., 2005 has also shown an increased frequency of the A148T polymorphism also in melanoma patients with MPM compared to the control population (p=0.05). In the Polish population, there are also evidences for the A148T variant association with an increased risk for other malignancies, e.g. breast cancer, lung cancer, and colorectal cancer (Debniak et al., 2006a; 2006b; 2006d), which should be confirmed in other populations and larger studies.

Table 1.11. *Frequency (%) of CDKN2A A148T polymorphism in melanoma patients and controls of different populations*[a]

Reference	Geographic origin	Study population	n	A148T (%)	p-value
Bertram et al., 2002	UK	AN/FM patients (AMS≥2 or familial melanoma or MPM patients)	488	4.9	0.95
		Population – based samples	599	5.2	
Debniak et al., 2005b	Poland	Melanoma patients (56 familial and 415 sporadic melanoma patients)	471	7.0	0.0003
		Controls	1,210	2.9	
Spica et al., 2006	France	Melanoma patients (57 familial, 38 MPM, and 405 sporadic melanoma patients)	500	3.4	0.46
		Controls	143	4.2	
	Italy	Melanoma patients (sporadic melanoma patients)	119	6.7	0.81
		Controls	121	6.2	

[a] *- both genotypes (heterozygotes and homozygotes) are grouped together as "polymorphism"; AMS – atypical mole syndrome; AN – atypical nevus; FM – familial melanoma; MPM – multiple primary melanoma*

Similarly different results were obtained concerning 3'UTR polymorphisms, c.*29C>G and c.*69C>T . In a population-based study Aitken et al., 1999 showed that the frequencies of c.*29C>G and c.*69C>T polymorphisms were higher in melanoma patients compared to controls, being 14.2% versus 11.1% and 10.9% versus 9.5%, respectively. These values were not significantly different between groups (Table 1.12), however, when the melanoma cases were stratified on the basis of strength of familial risk of melanoma, there was a significant trend (p=0.02) for the c.*29C>G polymorphism to increase with increasing familial risk and was highest in the group that also carried intragenic mutations of *CDKN2A*. A similar but nonsignificant trend was also observed for the c.*69C>T polymorphism. After adjustment for ethnic origin the relation between the c.*29C>G polymorphism and risk was weakened (p=0.25) indicating that this effect was, at least in part, because of population stratification, most likely representing predominantly Celtic ancestry. This association may therefore primarily reflect a highly susceptible genetic background (e.g. fair skin, red hair, poor tanning ability, and easy of sun-burning). In the subsequent study, Kumar et al., 2001 similarly found that the frequency of both polymorphisms, c.*29C>G and c.*69C>T, was higher in melanoma patients than that in controls, although it only reached statistical significance (p=0.01) for the c.*69C>T polymorphism (14% in melanoma patients versus 8% in controls) and for the c.*29C>G polymorphism being non-significant. The authors estimated that this difference in c.*69C>T polymorphism frequency conferred a 1.7-fold (95% CI: 1.11-2.66) relative risk to carriers of the variant allele (T). Further in support of the role for 3'UTR polymorphism in melanoma development prior work from the same group (Sauroja et al., 2000) had shown that carriers of either of these variants had a significant (p=0.007) shorter progression time from diagnosis of the primary tumour to the appearance of metastasis.

Moreover, in tumours that showed loss of heterozygosity the variant allele was usually retained. In contrast, Straume et al., 2002 showed again that the presence of the c.*69C>T polymorphism was associated with an improved survival in multivariate analysis (hazard ratio 2.6; p=0.02) and suggested that the c.*69C>T polymorphism has a role in the initiation or early progression of a subset of cutaneous melanomas with less aggressive behaviour. It should be noted that the work of Sauroja et al., 2000 was performed on melanoma metastases but the work of Straume et al., 2002 was done on the primary melanomas in the vertical growth phase. There are evidences that the polymorphisms in the 3'UTR of the gene are associated also with the tumour invasiveness in bladder cancer patients (Sakano et al., 2003).

Table 1.12. *Frequencies (%) of CDKN2A 3'UTR polymorphisms in different melanoma risk groups and control subjects* [a]

Reference	Study/group	n	c.*29C>G	c.*69C>T
Aitken et al., 1999	High-risk melanoma cases (*CDKN2A* positive)	34	30.4	18.9
	High-risk melanoma cases (*CDKN2A* negative)	133	16.8	10.7
	Intermediate-risk melanoma cases	214	13.9	11.1
	Low-risk melanoma cases	201	12.7	10.5
	All melanoma cases	514	14.2	10.9
	Controls	200	11.1	9.5
Kumar et al., 2001	All melanoma cases	229	15.1	13.8
	Controls	235	11.7	8.5
Debniak et al., 2005b	All melanoma cases	471	23.6	14.2
	Controls	1,210	20.2	12.6

[a] *-both genotypes (heterozygotes and homozygotes) are grouped together as "polymorphism".*

Recent evidence strongly suggests that the 3'UTR of mRNA is involved in the regulation of gene expression by controlling nuclear export, polyadenylation status, subcellular targeting, translation rates, and mRNA degradation (Conne et al., 2000). However, the mechanism underlying the predisposing role of the 3'UTR polymorphisms of the *CDKN2A* gene to melanoma are unclear. They might alter the stability of the *CDKN2A* transcripts or the level of transcription, or, alternatively, these polymorphisms might be in linkage disequilibrium with an unidentified variant responsible for the increased melanoma susceptibility. Indeed, the A148T polymorphism has been shown to be in linkage disequilibrium with the c.*29C>G polymorphism (Aitken et al., 1999) and the c.-493A>T *CDKN2A* promoter variant (Harland et al., 2000), which is known to affect gene expression.

BRAF

The most important molecular finding in melanoma research over the last few years has been the identification of a high frequency of mutations in the *BRAF* proto-oncogene in melanoma and other tumour types (Davies et al., 2002). *BRAF*, mapping to 7q34, is the archetype of 3 *RAF* genes in the human genome: *ARAF1, BRAF*, and *RAF1* (also known as *CRAF* or *c-RA*F). *RAF* proteins are serine/threonine kinases and the primary mediators of *RAS* signalling, which links extracellular mitogenic stimuli to transcription of growth related genes via the mitogen-activated protein kinase (MAPK) pathway.

A high prevalence of somatic mutations in the *BRAF* oncogene has been reported in 40-88% of melanomas (Davies et al., 2002; Brose et al., 2002a; Pollock and Meltzer 2002;

Dong et al., 2003; Gorden et al., 2003; Satyamoorthy et al., 2003). *BRAF* mutations are also very frequent (74-82%) in benign melanocytic nevi (Pollock et al., 2003; Yazdi et al., 2003), suggesting a critical role for *BRAF* in initiating melanocytic neoplasia. To date, the majority of documented somatic *BRAF* mutations cluster in the kinase domain of *BRAF*, either in the glycine-rich loop encoded by exon 11 or the activation domain segment encoded by exon 15 (Davies et al., 2002). Changes at V600E (previously reported as position 599) account for approximately 80% of all somatic *BRAF* mutations, resulting in constitutive activation of *BRAF* (Davies et al., 2002).

These findings led to the speculation that *BRAF* might also be a candidate melanoma susceptibility gene. Initially, mutation analysis of the *BRAF* gene in the germline yielded a number of negative findings (Lang et al., 2003; Laud et al., 2003; Meyer et al., 2003b), however, the number of cases in these studies was relatively small (total of 296 cases, range 44-172), the analysis was limited to exon 15 (Lang et al., 2003; Meyer et al., 2003b), or recruitment included patients who were known to carry germline mutations in *CDKN2A* (Lang et al., 2003). Evidence for a role of *BRAF* in melanoma susceptibility has come from a recent finding of three germline mutations in 4/569 (0.7%) Italian melanoma patients (Casula et al., 2004). One of the patients was reported to carry the most common somatic *BRAF* mutation V600E, but this finding has since been retracted (Casula et al., 2005). The significance of the two other germline changes found, M116R and Q608H, is unclear, particularly the M116R change, since it falls outside of both the activation segment and glycine-rich loop (G-loop), which constitute the kinase domain of *BRAF*. The Q608H substitution, however, does residue in the activation segment of *BRAF* and is therefore likely to be functionally important.

Association between polymorphic variants of the *BRAF* gene and melanoma risk has been assessed in several case-control studies to investigate the role of the *BRAF* gene as a low-penetrance melanoma susceptibility gene (Laud et al., 2003; Meyer et al., 2003a; James et al., 2005). Although, the first of these (Laud et al., 2003) identified several novel intronic, 5'UTR or silent coding region variants in French melanoma patients, the study was underpowered (80 patients and 91 controls) and no statistical significance could be attributed to these polymorphisms. In a larger German case-control study, six out of 12 intronic *BRAF* variants demonstrated significant association with melanoma in males, but not in females or when both sexes were combined (Meyer et al., 2003a). Contribution to melanoma susceptibility of *BRAF* promoter variants as well as the presence of intron 11 polymorphism (rs1639679), strongly correlated with the haplotype reported to be associated with melanoma in males (Meyer et al., 2003a), was also investigated in two incident melanoma series in England, showing no statistical difference in genotype or allele frequencies of this intronic *BRAF* variant between cases and controls or between males and females (Jackson et al., 2005). However, again the notion of *BRAF* as possible low-risk melanoma predisposing gene was recently supported by a large Australian association study including 755 melanoma patients and 2239 control persons that were genotyped for 16 SNP within the *BRAF* gene and 5 in neighbouring genes (James et al., 2005). This study estimates that risk of melanoma due to variants in *BRAF* gene is 1.6%, but the causal variant has yet to be determined.

EGF

Epidermal growth factor (*EGF*) gene was considered as a reasonable candidate for conferring melanoma risk because of its role in mitogenesis and wound healing. Recently, Shahbazi et al., 2002 sought to determine whether there existed differences in variants of the *EGF* gene between melanoma patients and controls and suggested that A/G polymorphism at position 61 in the 5'UTR of the gene (c.-61A>G) is a melanoma susceptibility allele. They found that this substitution is responsible for modulating the level of *EGF* transcription *in vitro* in cultured peripheral blood mononuclear cells after stimulation with phorbol myristate

and liposaharide. Cells from individuals homozygous for the G allele produced significantly more *EGF* than those from individuals homozygous for A allele (p=0.0004) or heterozygous for this SNP (p=0.001). Although the mechanism by which this SNP is related to levels of *EGF* expression is not known, this functionally relevant polymorphism was also found to be associated with melanoma risk. Overall, the frequency of the G allele was significantly more common in melanoma patients compared to control individuals (66% versus 44% respectively, p<0.0001). This difference conferred a 2.7-fold relative risk (95% CI: 1.9-4.0) of developing melanoma. When genotype was considered a 4.9-fold relative risk (95% CI: 2.3-10.2) was associated with homozygosity of the G (G/G). There was also a marginally significant association (p=0.045) between homozygous G genotype and tumour presenting with Breslow thickness of >3.5 mm versus tumours <3.5 mm (OR 3.7; 95% CI: 1.0-13.2). However this difference was no longer significant when skin type, hair colour, and eye colour were included in the analysis. Taken together these results appear to indicate that increased expression of *EGF* is associated with melanoma progression. Since than, several studies have reported conflicting results. Several reports failed to confirm the association of the *EGF* c.-61A>G phenotype with melanoma risk (McCarron et al., 2003; Amend et al., 2004; James et al., 2004; Randersoon-Moor et al., 2004), although two confirmed the association of the G/G homozygote phenotype with thicker tumours (McCarron et al., 2003; James et al., 2004) and one demonstrated that *EGF* c.-61A>G polymorphism is associated with survival, where G/G genotype represented a significant risk factor for both shorter disease-free period (hazard ratio of 2.246, 95% CI: 1.06-4.78, p=0.036) and overall malignant melanoma specific survival (hazard ratio of 3.8, 95% CI: 1.5-9.5, p=0.004) compared with A/A genotype, while the heterozygous A/G phenotype demonstrated an intermediate risk (Okamoto et al., 2006).

GST

Homozygous germline deletion in the genes encoding two isozymes of glutathione-S-transferase (*GST*), *GSTM1* and *GSTT1*, occur in the large proportion of the population. About half of the Caucasian population is genotypically null for these genes. Since *GSTM1* and *GSTT1* are enzymes expressed in the skin that detoxify products of oxidative stress reactions caused by UV radiation, individuals who lack this gene might be expected to be at increased risk of melanoma. Initial studies comparing melanoma cases with controls in the predominantly Anglo-Celtic populations from UK and Australia found the same frequency (52%) of *GSTM1* null-zygotes in groups, melanoma patients and controls, analysed (Heagerty et al., 1994; Shanley et al., 1995). In contrast, there was a significantly increased frequency of *GSTM1* null phenotype in melanoma patients compared with controls (58% versus 41%, p=0.002) from a hospital-based study in Spain (Lafuente et al., 1995). The most noticeable feature of this study was the much lower frequency of null-zygotes in the Spanish controls compared to that observed in Caucasian control population. More recently, in the study of *GST* polymorphisms and melanoma risk Kanetsky et al., 2001 found no significant increase in frequency of both either *GSTM1* or *GSTT1* null-zygotes in the melanoma patient cohort compared with controls from USA. However, when they stratified by pigmentation characteristics they found that among individuals with red or blond hair melanoma patients were 2.2-fold more likely (95% CI: 1.2-4.2) than controls to be *GSTM1* null, and 9.5-fold more likely (95% CI: 1.2-73.0) to be nullzygous for both *GSTM1* and *GSTT1*. As red/blond hair colour is determined to a large extent by variants of *MC1R* (discussed above), these findings may indicate that *MC1R* genotype acts to modify melanoma risk from *GST* nullzygsity in a manner similar to the way it acts as a modifier of *CDKN2A* penetrance, but these suggestions were not confirmed (Mossner et al., 2007).

CYP2D6

Since cytochrome P450 enzymes have the interesting potential to act in opposing biological ways, namely either detoxify certain lipophilic compounds or to activate them to carcinogenic forms, polymorphisms of P450 family members are likely to be associated with genetic susceptibility to a wide variety of cancers, including melanoma. There are three well-documented inactivating polymorphisms at the cytochome P450 debrisoquine hydroxylase locus (*CYP2D6*), gene deletion, a splice site mutation, and a nucleotide deletion in the coding region (reviewed in Wolf et al., 1992). Homozygotes for non-functional alleles are termed poor metabolisers and have greatly reduced capacity to metabolise certain chemicals. If *CYP2D6* is responsible for activating of various carcinogens then poor metaboliser phenotype would be expected to be associated with reduced cancer susceptibility. However, Wolf et al. 1992 found no significant difference in the frequency of poor metabolisers between melanoma patients and controls (5.3% versus 4.3% respectively) and observed a significant increase ($p=0.02$) in mutant alleles in the melanoma cohort. It could indicate that rather than activating certain carcinogens, *CYP2D6* is mainly involved in detoxifying potentially carcinogenic compounds. This observation was also supported by Strange et al., 1999, who in a case-control study found a significantly higher frequency of mutant alleles (28% versus 21%, $p=0.039$) and poor metabolisers in melanoma patients (10.8% versus 4.5%, $p=0.001$). In contrast Dolzan et al., 1995 found no increase in mutant *CYP2D6* alleles in melanoma patients versus controls, but rather a non-significant lower frequency of poor metabolisers in the melanoma cohort (5% versus 6.5%).

VDR

A role for the vitamin D receptor (*VDR*) gene in melanoma susceptibility has been hypothesised since calcitriol, the *VDR* ligand, inhibits proliferation and induces differentiation of melanocytes and melanoma cells and polymorphisms of this gene have been associated both with serum calcitriol levels and risk of several other types of cancers (Hutchinson et al., 2000). In a case-control study comprising 316 melanoma patients and 108 controls the rarer allele of a *FokI* polymorphism, which results in a novel *VDR* translation start site, was more common in melanoma patients than controls (39.9% versus 31.5, $p=0.029$). This difference is marginal and was not confirmed (Santonocito et al., 2007). Similarly, the A allele of a novel c.-1012A>G polymorphism of the *VDR* promoter region was over-represented in melanoma patients, and was related to the development of melanoma metastasis and increased tumour thickness (Halsall et al., 2004). However, this initial report of a role for this polymorphism in melanoma development has not been confirmed (Santonocito et al., 2007). In addition, significant associations have been found between the *BsmI* bb genotype frequency and melanoma ($p=0.02$) along with Breslow thickness ($p=0.001$). Although the biological meaning of the effect exerted by *BsmI* polymorphism is still under debate, additional work should be done to verify this variant as a possible risk marker for malignant melanoma and its aggressiveness (Santonocito et al., 2007).

EDNRB

The endothelin signalling pathway is known to play an important role in melanocytes differentiation and migration. Recently, germline mutation of the endothelin receptor B (*EDNRB*) gene, involved in Hirschsprung disease, have been associated also with melanoma risk in a French population (Soufir et al., 2005). This single report, again, remains to be repeated. Similarly, the *OCA2* gene involved in the most prevalent form of oculocutaneous has recently been demonstrated to influence melanoma susceptibility, with the risk likely

being the result of the interaction of several intragenic *OCA2* variants (Jannot et al., 2005) or also interaction with other genes e.g. *MC1R* (Duffy et al., 2004).

1.9. Genes underlying other syndromes and confer substantial risk of melanoma

RB1

The *RB1* gene was the first tumour suppressor gene to be cloned. Knudson's classical 2-hit hypothesis is based on the observation that germline mutation carriers develop retinoblastoma when the remaining wild-type copy is lost somatically. Germline mutations in the *RB1* gene cause retinoblastoma in approximately 85-95% of cases, usually bilaterally. The retinoblastoma protein pRb is a well characterised regulator of cell proliferation, regulated by $p16^{INK4a}$ via its negative effect on the activity of the cyclin D-CDK4/CDK6 complexes which phosphorylate and thereby inactivate the transcriptional suppressor function of Rb (Figure 1.10).

An excess risk of cancers other than retinoblastoma has been reported in *RB1* germline mutation carriers. A recent cohort study of retinoblastoma survivors showed a cumulative incidence of adult cancer of approximately 70% (Fletcher et al., 2004). In a review of 5 large case series of more than 1800 survivors of retinoblastoma, Traboulsi et al., 1988 reported 201 second malignant neoplasms, 14 (7%) of which were melanomas. A retrospective study on mortality among 1603 long-term retinoblastoma survivors showed a relative risk of 30 for second primary tumours, melanoma being among the tumours with a statistically significant excess mortality (Eng et al., 1993). In a more extensive review of the literature, Moll et al., 1996 summarised 11 cohorts of 5856 survivors of retinoblastoma, in which 243 second malignant neoplasms developed. Melanoma arose in 18 cases (7.4%) and was the second most common tumour type after osteosarcomas. The point estimates for the relative risk (RR) of developing melanoma in *RB1* mutations carriers are extremely high, but the confidence intervals are broad due to the low absolute numbers of cases, e.g. 2 melanoma cases/117 mutations carriers (RR>73) (Sanders et al., 1989) or 2 melanoma cases/144 mutation carriers (RR>23) (Fletcher et al., 2004). The co-occurrence of melanoma and retinoblastoma in the same individual was also observed (Albert et al., 1990). Thus, the numerous reports of an association between melanoma and retinoblastoma support an underlying common genetic susceptibility.

TP53

TP53 is mutated in many sporadic tumours, a finding which led to the identification of germline mutations in Li-Fraumeni syndrome (LFS), a cancer syndrome characterised by predisposition to a diverse range of tumours (Malkin et al., 1990). Classical LFS is defined by the proband with sarcoma diagnosed before 45 years, with a first degree relative aged under 45 years with any cancer plus additional first- or second-degree relative in the same familial line with any cancer aged less than 45 years or a sarcoma at any age (Li et al., 1988). An additional definition has been proposed which is referred as Li-Fraumeni-like syndrome (LFL), which is defined by a proband with any childhood tumour, sarcoma, brain tumour or adrenocortical tumour diagnosed under age 45 years, with a first- or second-degree relative in the same familial line with typical LFS tumour at any age, plus a first- or second-degree relative in the same familial line with any cancer diagnosed under 60 years (Birch et al., 1994).

Mutations in *TP53* were found in 77% of classical LFS families and in 40% of LFL families and many studies have indicated an association with a wider range of cancers, including melanoma (Malkin et al., 1990; Birch et al., 1994; 2001; Nichols et al., 2001; Chompret et al., 2001; Olivier et al., 2003), but since the absolute number of melanoma cases

is low, there is some debate regarding whether melanoma is truly a rare manifestation of LFS/LFL. Indeed, in some studies of LFS there have been no documented cases of melanoma (Li et al., 1988; Frebourg et al., 1995; Birch et al., 1998) and the tumour spectrum in *TP53* mutation-positive families seems to be restricted to that originally described for classical LFS (Birch et al., 2001). However, in support of the argument that germline *TP53* mutation carriers at increased risk of melanoma is the young age of onset of some of the cases – some developing melanoma as young as 16 years of age (Birch et al., 1994; Nichols et al., 2001). Recently in one study, an increased risk of melanoma has also been associated with the p5372Arg allele, particularly in the subgroup of individuals older than 50 years and of individuals with skin type 3 or 4 (Shen et al., 2003). In another study an increased risk of melanoma was found to be associated with *p53* codon 72 Pro/Pro homozygosity and, again, especially in the subgroups with darker skin pigmentation, as well as among non-carriers of the *MC1R* gene red hair polymorphic variants (Stefaneki et al., 2007). However, these later findings need further confirmation.

CHEK2

Candidate gene searches looking for other LFS genes have led to the identification of a recurrent 1100delC mutation in some LFS patients in the *CHEK2* gene (Bell et al., 1999). This mutation has also been shown to be a low penetrance breast cancer susceptibility allele (Meijers-Heijboer et al., 2002) as well as a predisposition allele for families with hereditary breast and colon cancer (Meijers-Heijboer et al., 2003). Interestingly, the proband of one of the first 3 families in which a germline *CHEK2* mutation was identified had developed melanoma in addition to breast and colon cancer (Bell et al., 1999). The role of the *CHEK2* 1100delC mutation in melanoma susceptibility has been more directly studied by germline mutation analysis in sporadic as well as in familial melanoma cases and the only mutation detected was in a patient whose medical history was suggestive of LFS, with the occurrence of brain tumours and a sarcoma in addition to the melanoma (Debniak et al., 2004b).

BRCA2* and *BRCA1

Female *BRCA2* mutation carriers have cumulative lifetime risk of 84% and 30%, respectively for developing breast or ovarian cancer. Males mutation carriers are not merely carriers of the genetic defects, but have also an increased risk of developing breast cancer (estimated cumulative lifetime risk of 6%), as well as prostate cancer (10-30%) (Ford et al., 1998). Melanoma has been reported in a number of *BRCA2*-linked (Grimmond et al., 1996) or *BRCA2* mutation-positive families (The Breast Cancer Linkage Consortium, 1999), and is one of a few other cancers (e.g. pancreas, stomach, and gall bladder) for which *BRCA2* mutations carriers have an increased, albeit modest, risk (RR 2.58, 95% CI=1.28-5.17) (The Breast Cancer Linkage Consortium, 1999). Some *BRCA2* breast cancer families have been reported with cases of ocular melanoma (Easton et al., 1997), but the total number of cases is too small to calculate an attributable risk with any degree of accuracy (Liede et al., 2004).

Female *BRCA1* mutation carriers have a cumulative lifetime risk of developing breast cancer of 85% and approximately of 65% of developing ovarian cancer (Easton et al., 1995). The wider spectrum of tumours for which there is an increased risk in *BRCA1* mutation carriers appears to be confirmed to only slight increases in colon, pancreas, and uterus cancer (Easton et al., 1997; Brose et al., 2002b). Rarely, melanoma has been reported in *BRCA1* mutation-positive families (Johannsson et al., 1996; Debniak et al., 2003), but overall, the risk of melanoma in *BRCA1* mutation carriers is not significantly evaluated (The Breast Cancer Linkage Consortium, 1999; Liede et al., 2004).

XP

Xeroderma pigmentosum (XP) is an autosomal recessive trait, consisting of a heterogenenous group of defects in the nucleotide excision repair (NER) genes. Classical XP has been subdivided into 7 subtypes, representing 7 different genes in NER (XPA, XPB/ERCC3, XPC, XPD/ERCC2, XPE/DDB2, XPF/ERCC4, XPG/ERCC5, XPV).

XPC is the most common form of XP and mutations are associated with an impaired repair of UV radiation induced DNA lesions, leading to the increase of UV associated skin tumours, predominantly basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). XP patients have approximately 1000-fold risk of developing skin cancers, often arising before the age of 1o years. Melanoma is seen in 5%-20% of patients (reviewed in Kraemer et al., 1994). However, there is a striking difference in melanoma type distribution with those found in general population. Instead of superficial spreading melanoma (SSM), the majority of melanomas found in XP patients are lentigo maligna melanoma (LMM), a melanoma type normally associated with older age. Also the body sites of the melanomas found in XP patients are similar to those of LMM found in older population, i.e. on chronically sun-exposed skin (Kraemer et al., 1994; Spatz et al., 2001). These findings are in keeping with the more complex association of UV radiation and melanoma induction.

More, a single case-control study described also the contribution of three polymorphic alleles of the *XPC* gene (intron 9 PAT+, intron 11-6A and exon 15 2920C) to melanoma susceptibility (Blankenburg et al., 2005), however, these findings should be further validated.

There are also reports of the NER gene *XPD* association with melanoma. Since Tomescu et al., 2001 have found that polymorphisms in exons 6, 22, and 23 of the *XPD* gene were over-represented in melanoma patients, several studies have been performed analysing *XPD* gene polymorphism association with melanoma risk. Two common non-synonymous polymorphisms – D312N and K751Q – of the *XPD* gene have been found to be associated with melanoma risk (Han et al., 2005; Millikan et al., 2006), especially in subjects older than 50 years of age and with the lack of dysplastic nevus (Baccarelli et al., 2004). Similar findings have also been reported by Debniak et al., 2006c, who showed that polymorphisms in the *XPD* gene were significantly over-represented among late-onset melanoma patients and that a combination of at least two SNP (Lys751Gln_CC and Gly156Gly_CC or Asp312Asn_AA and Lys751Gln_CC) inherited as a haplotype was associated with the disease. However, again, in one of the later studies there was no association of the K751Q polymorphism with melanoma risk, but the finding of NER genes *ERCC1* and *XPF* in association with melanoma, with the strongest association for melanoma cases aged 50 and under, remains to be repeated (Povey et al., 2007). However, suggestions that a polymorphism (C18067T) in the DNA repair gene *XRCC3* predisposed to melanoma (Winsey et al., 2000) were not confirmed by two later studies including lager populations (Duan et al., 2002, Bertram et al., 2004).

Clearly, large-scale epidemiological studies are needed to determine the relative risk of developing melanoma associated with different changes in NER genes.

WRN

The Werner syndrome (WRN) gene encodes a DNA helicase which appears to be involved in DNA repair. WRN is an autosomal dominant disease, characterised by premature aging. An excess of different cancers has repeatedly been reported, usually occurring from age 25-65 years. As summarised by Goto et al., 1996, apart from sarcoma, meningioma and thyroid cancer, the incidence of melanoma is markedly increased in patients with WRN in Japanese patients (21/124) as well as in patients outside the Japan (3/34). The association of melanoma with WRN in the Japanese is particularly remarkable, since melanoma in this population is a rare phenomenon (Tanaka et al., 1999).

A common thread provided by BRCA2, XP gene family, and WRN is that they encode proteins involved in DNA repair, thus establishing aberrations of this cellular process as important for melanoma development.

1.10. Genetic counselling and testing for hereditary cutaneous malignant melanoma

Clinical cancer genetic is becoming an integral part of the care for cancer patients. The discovery of genes that, when mutated in the germline, are associated with specific inherited cancer syndromes has stimulated the development of genetic testing for predicting cancer susceptibility. Multiple endocrine neoplasia type 2, von Hippel-Lindau disease, adenomatous poliposis coli are examples of syndromes for which genetic testing to identify at-risk family members is considered a standard of care (Eng et al., 2001). Genetic testing for these syndromes is sensitive and affordable, and it will change medical management. Cancer genetic counseling and testing is probably beneficial in other syndromes, such as the hereditary breast cancer syndromes, hereditary nonpolyposis colorectal cancer syndrome, Peutz-Jeghers syndrome, and juvenile polyposis. There are also hereditary cancer syndromes for which testing is controversial, including hereditary malignant melanoma.

Although commercial testing is available in North America and some European countries (e.g. the Netherlands) (Riedijk et al., 2005), the clinical utility of genetic analysis for hereditary CMM is uncertain and an ongoing issue of debate (Kefford et al., 1999; 2002; Hansen et al., 2004; Bishop et al., 2007).

The Melanoma Genetic Consortium "GenoMEL", until recently, held that gene testing is premature except in rare circumstances, and encouraged all patients to enrol in research protocols (Kefford et al., 1999; 2002). The reasons behind this position were multiple: i) mutations have yet not been detected in the majority of hereditary CMM families, ii) the understanding of CMM risk (penetrance) to carriers is limited, iii) other factors (including environmental factors) appear to influence the risk, iiii) negative testing may provide false reassurance, given that the rate of CMM in non-carriers in *CDKN2A*-mutation-positive families has been reported to be as high as 9%, and iiiii) the risk of other cancers is not yet known (Kefford et al., 2002). Therefore, GenoMEL's view on genetic testing remains under review and families should be counselled about the advantages and disadvantages of testing.

To date, only three potential genes, as summarised above, have been implicated in hereditary CMM: *CDKN2A, CDK4* and a locus on *1p22*, with genetic tests available for *CDKN2A* and *CDK4* (Niendorf and Tsao 2006).

The management strategy for hereditary CMM families is proposed (Figure 1.15) and this recommended that at risk-patients receive appropriate counselling services, and that the decision regarding performance of genetic testing outside the clinical research protocols occur sparingly and be judiciously decided on an individual basis (Niendorf and Tsao 2006).

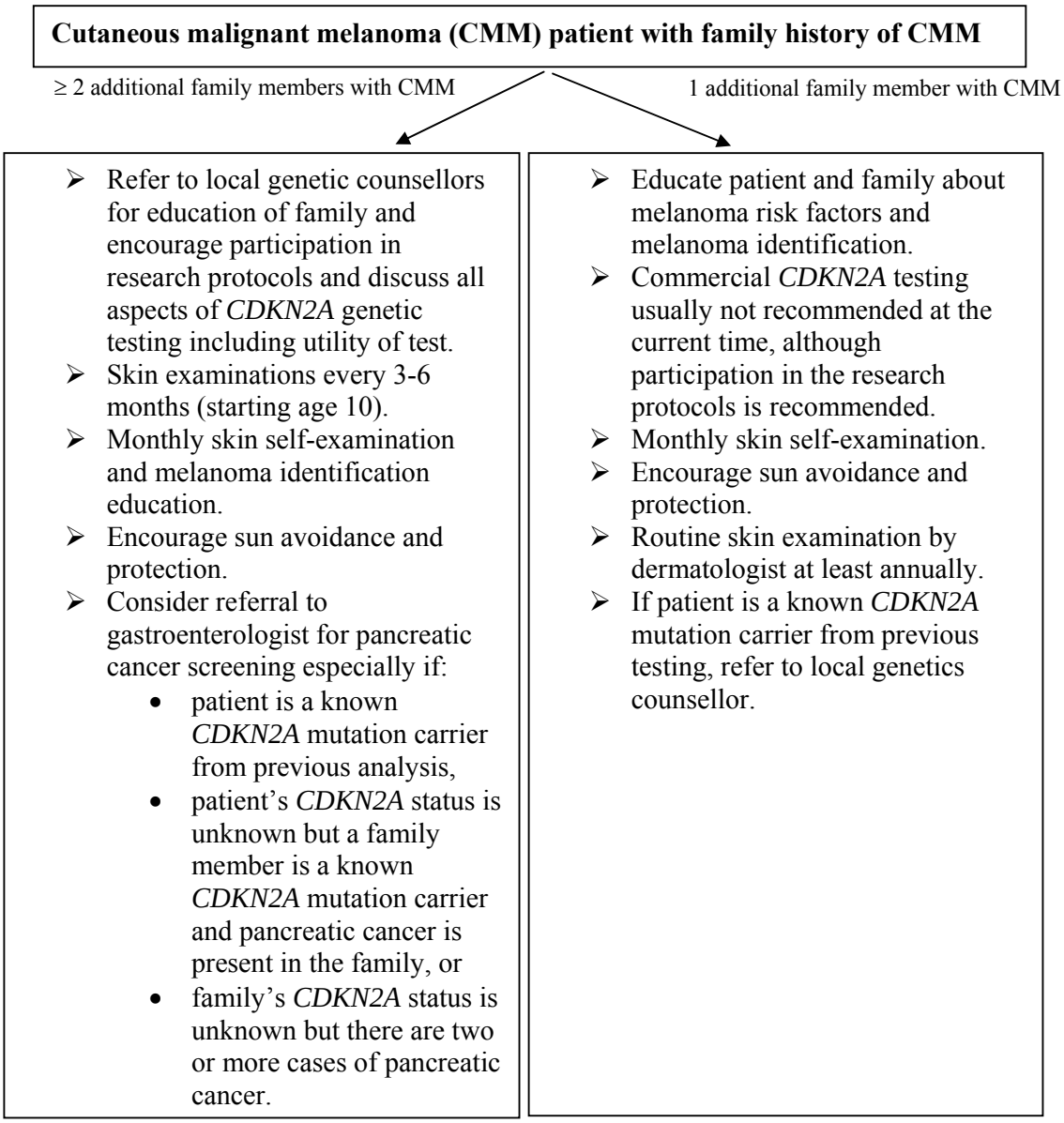


Figure 1.15. *Proposed management scheme for family members of CMM patients (redrawn from Niendorf and Tsao 2006).*

1.11. Sporadic alterations of melanoma susceptibility genes

After having discussed genes involved in melanoma susceptibility above, the next section summarises information about *CDKN2A* and *CDK4* somatic alterations.

CDKN2A/ARF

The *CDKN2A* gene was the first melanoma susceptibility gene to be cloned, and since *ARF* was only found later to be transcribed from the same locus, these two genes have to a large extent been regarded mutually. There are plentiful mutation reports of the *CDKN2A/ARF* locus in melanoma as well in different other tumour types as periodically and comprehensively summarised in a list of reviews (Smith-Sorensen and Hovig 1996; Foulkes

et al., 1997; Ruas and Peters, 1998; Piepkorn 2000; Rocco and Sidransky, 2001). At present, there are 106 different *CDKN2A* mutations mentioned in the public HGMD (Human Gene Mutations Database) (http://www.hgmd.cf.ac.uk/ac/index.php, 31.03.2007.). The literature review presented here just highlights a few salient points regarding somatic inactivation of the *CDKN2A/ARF* locus in melanocytic neoplasia.

Mutations and deletions affecting the *CDKN2A/ARF* locus are found frequently in melanoma cell lines (Kamb et al., 1994b; Hussussian et al., 1994; Liu et al., 1995; Pollock et al., 1995), but rarely in primary melanoma (Ohta et al., 1994; Healy et al., 1996a; Kumar et al., 1998a; 1999; Ruiz et al., 1998; Cachia et al., 2000; Pollock et al., 2001b; Soto et al., 2005). This difference reflects a highly selective event imposed by cell culturing, which is most likely explained by critical role of $p16^{INK4a}$ in cellular senescence (reviewed in Bennett 2003). Cells lacking $p16^{INK4a}$ thus escape senescence and can readily become immortalised. The preferential way of $p16^{INK4a}$ inactivation in cell lines is usually thought homozygous deletion of part, or all, of *CDKN2A* locus.

The role of *CDKN2A* in primary melanoma is however more complex. While the locus shows LOH in about 50% of primary melanomas, mutations in the *CDKN2A* or *ARF* genes are found only in a small proportion of tumours, including those with loss of this region (Pollock et al., 2001b). The later finding may suggest that $p16^{INK4a}$ or $p14^{ARF}$ haploinsufficiency is adequate for melanogenesis. However, the low mutation detection rate may also reflect the difficulty in assessment of other mechanisms of inactivation such as promoter methylation or homozygous deletion. Indeed, there are several reports showing $p16^{INK4a}$ inactivation by promoter methylation (Merlo et al., 1995). However, in melanoma promoter methylation is a rare event (less than 10%) (Gonzalgo et al., 1997; von Eggeling et al., 1999; Straume et al., 2002). When deletions have been assessed they have really been found to increase the overall proportion of tumours showing inactivation of either *CDKN2A* or *ARF* (Gonzalgo et al., 1997, Ruiz et al., 1998, Kumar et al., 1998b, Walker et al., 1998, Fujimoto et al, 1999). It should be noted that no intragenic mutation specifically affecting $p14^{ARF}$ alone, and not also $p16^{INK4a}$ has been reported in melanoma, although there are tumours in which *ARF* only is inactivated through homozygous deletion (Kumar et al., 1998b).

LOH of the *9p21* region is a frequent event also in nevi, thus supporting their role as melanoma precursor lesions (Cowan et al., 1988; Healy et al., 1996b; Birindelli et al., 2000; Hussein et al., 2003). Several reports have compared the frequencies of *9p* LOH between various types of nevi and have generally found lower ranges in banal nevi compared with to dysplastic nevi (Park et al., 1998; Birindelli et al., 2000; Rubben et al., 2002; Hussein et al., 2003). The number of mutations in nevi is low however (Healy et al., 1996b; Lee et al., 1997). Further delineation of the timing of LOH and mutations of the *CDKN2A* gene during melanocytes tumorigenesis may help to clarify the importance of $p16^{INK4A}$ and $p14^{ARF}$ in melanoma progression. There have also been strong suggestions that other tumour suppressor genes involved in melanoma development are located on the short arm of chromosome 9. This hypothesis is strengthened by several studies that have shown common regions of LOH flanking both sides of the *CDKN2A/ARF* locus, while the locus itself maintains heterozygosity (Pollock et al., 2001b; Puig et al., 1995; Ohta et al., 1996; Kumar et al., 1999).

CDK4

A somatic mutation in the *CDK4* gene in melanoma was first identified through analysis of tumour-specific antigens recognised by autologous cytotoxic T lymphocytes from a patient with melanoma (Wolfel et al., 1995). The C to T transition led to the above mentioned R24C substitution in the protein. This amino acid change prevents binding of the inhibitor $p16^{INK4a}$ and thus leads to the constitutive active kinase, with the consequent inactivation of pRB and cell cycle progression (Figure 1.10). Several groups have used

mutagenesis analyses to determine the key residues in the *CDK4* that are involved in binding $p16^{INK4a}$ (Coleman et al., 1997; Byeon et al., 1998; Ceha et al., 1998) and showed that amino acids K22 and R24, found to be mutated somatically, are critical in this process (Table 1.13).

Table 1.13. *CDK4 mutations in melanoma samples*

Mutation type	n mutated/n total	Reference
R24C	2/29	Wolfel et al., 1995
R24C	1/22	Bartkova et al., 1996
R24C	1/60	Castellano et al., 1997
R24C	3/45	Walker et al., 1998
R24C (2x) R24H (2x) K22Q	5/48	Tsao et al., 1998
R24C	1/17	Rizos et al., 1999
K22R	1/41	Daniotti et al., 2004

1.12. The genomics of melanoma

Genomic profiling of human melanoma has revealed a highly rearranged melanoma genome, attesting molecular heterogeneity of this disease. The molecular heterogeneity of human melanoma has long been apparent on clinical grounds. For example, melanomas arising at different sites of the body may exhibit markedly distinct biological and clinical behaviours. Lentigo maligna melanomas are indolent tumours that develop over decades on chronically sun exposed skin (e.g. face). In contrast, acral lentigenous melanomas, which develop on sun-protected regions, tend to be more aggressive. Thick and thin primary melanomas as well as superficial spreading melanomas (SSM) and nodular melanomas (ND) provide additional examples (Poetsch et al., 2003).

Recent advances in genome-wide technologies enable documentation of tumour heterogeneity at high resolution. Transcriptional profiling studies have provided evidences for distinct molecular subclases of melanoma (Bittner et al., 2000; Segal et al., 2003; Tschentscher et al., 2003; Onken et al., 2004; Haqq et al., 2005). At the genomic DNA level, the non-random nature of the chromosomal alterations characteristic to melanoma likely also dictates disease behaviour. Thus, patterns of alteration detectable either at DNA or RNA level may segregate melanoma tumours in to subtypes with distinct clinical behaviour and possible therapeutic responses. Indeed, a recent genome-wide CGH profiling and targeted re-sequencing on primary melanomas carried out by Curtin et al., 2005 showed that distinctive patterns of genomic alterations can be identified in melanomas arising in different anatomic sites and with varying UV exposure (Figure 1.16). Melanomas from skin with evidence of chronic sun damage, intermittent sun exposure without evidences of damage, and no significant exposure exhibit distinct genomic patterns of gain/amplifications and loss/deletions and different *BRAF/NRAS* mutational spectrums. *BRAF* and *NRAS* mutations were mutually exclusive, as were *BRAF/NRAS* activating mutations and increased copies of *CCND1* and *CDK4*. Gain/amplification of *CDK4* was more commonly seen in melanomas from protected skin than in melanomas from sun-exposed skin, as was deletion/loss of *CDKN2A* locus, which was observed exclusively in samples without *CDK4* amplification.

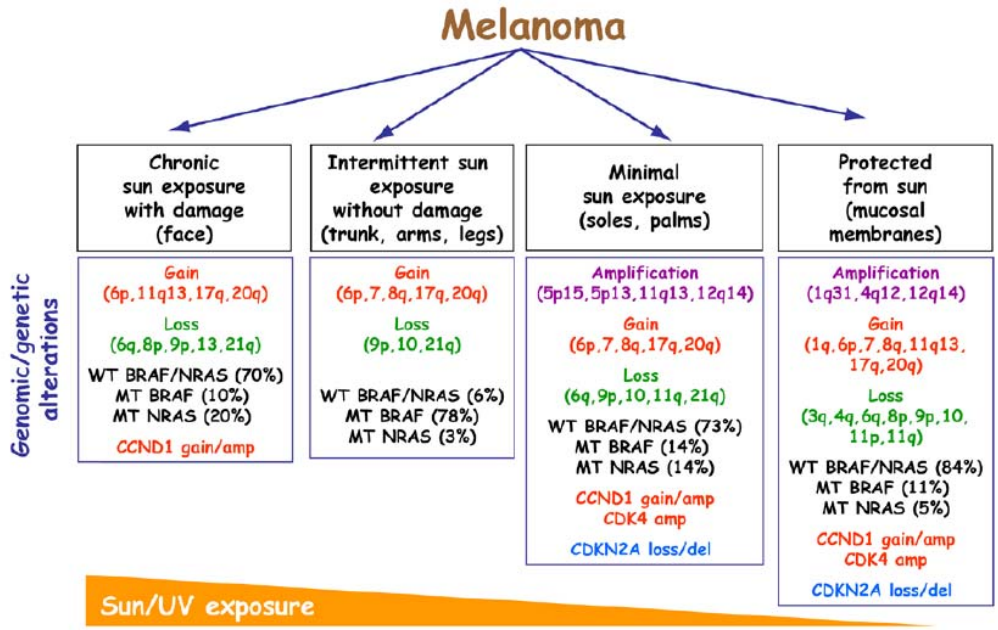


Figure 1.16. *Summary of genetic alterations in melanomas from patients with varying degrees of sun exposure as reported by Curtin et al., 2005: WT, wild type; MT, mutant; amp, amplification; del, deletion; CCND1, cyclin D1 (Kabbarah and Chin 2005).*

The overview above provides an outline of the most important genes and loci reported to be involved in melanoma susceptibility as well as in melanoma progression. It is evident that almost all of the genes, which were associated with melanoma development so far encode products that play a role in one of the three main cellular processes: cell cycle regulation, DNA repair, and receptor-mediated signal transduction.

To date, the only effective melanoma treatment is surgery which is effective for melanomas in stage I and II. It means that early detection is very important. Often the result depends not only from appropriate diagnosis but also from the biological features of cancer including its characteristics at genetic level. Adding the information about genetic alterations in the classification of melanomas might help to choose the appropriate therapy in the future.

AIM OF THE WORK

The aim of the present study was to characterise the *CDKN2A* and *CDK4* gene status in CMM patients and their tumours, relate these changes to melanoma susceptibility and pathogenesis, and assess the criteria for identification of individuals at evaluated risk for CMM in Latvia.

For these reasons there were the following tasks:

- To screen consecutive consenting CMM patients for germline mutations in *CDKN2A* and *CDK4* exon 2.
- To compare the frequencies of the three most common *CDKN2A* polymorphisms (A148T, c.*29C>G and c.*69C>T) between melanoma patients and control individuals from the same population.

- In order to expand on the role of the A148T polymorphism in modulation of melanoma risk in different populations, to asses the A148T carrier's haplotype from Latvia and Poland and compare this to the predominant haplotype of A148T carriers in England.
- To analyse familial melanoma patients for germline deletion at *9p21* chromosome region and for *MC1R* variants.
- To analyse primary melanoma tumours for *CDKN2A/p16*^{INK4a} intragenic mutation, promoter hypermethylation, and LOH on *9p21* chromosome region.

2. MATERIALS AND METHODS

2.1. Study subjects

Patients

Cutaneous malignant melanoma (CMM) patients were recruited at the Latvian Oncology Center (LOC) during the period from 2001 through 2004. For all patients involved in the study histopathology reports were comfirmed. A total of 176 unrelated primary melanoma (invasive or *in situ*) patients were included in the genetic testing and blood samples were obtained from them (Table 2.1). All individuals enrolled in the study completed a brief family history questionnaire, indicating any first- or second-degree relatives with a diagnosis of cancer and most of them answered questions about their phenotype (skin type, eye and hair colour, freckling, nevus count) as well. A more detailed history of familial melanoma patients were then obtained by personal interviews and medical confirmation was sought for all relatives reported to have had melanoma. In the studied cohort, 174 were consecutive consenting melanoma patients including two multiple primary melanoma and four familial melanoma patients. Two additional familial melanoma patients were joined to the study later because of multiple melanoma cases within the family.

For familial melanoma patients, where possible, members of the corresponding family either affected or unaffected were also analysed. To detect the mutational status of the deceased patients within families, archival material of their melanoma tissues, where available, was analysed as well. Before the analysis, the original diagnoses were reconfirmed by review of the hematoxylin and eosin stained tissue section.

There was also a small subgroup of 27 patients (Table 2.1) to whom diagnosis "melanoma" after the inspection of histopathology reports was not confirmed. This subgroup consisted of 14 individuals with atypical nevi and 13 individuals with other types of skin neoplams both malignant and benign: keratosis (5), squamous cell carcinoma (3), basalioma (2), adenoma (1), sarcoma (1), and xeroderma pigmentosum (1). These patients were analysed separately.

A further 252 unselected cancer patients with different types of cancer were included in the study (Table 2.1). All these individuals are participants in GenomeData Base of Latvian population and were recruited to the Data Base in different studies investigating each particular disease.

Controls

Two hundred and three control individuals were investigated in the present study (Table 2.1). The control DNA samples were derived from three separate collections, of patients living in Latvia. The first comprised 62 healthy persons (general practise group) (females 24, males 38) randomly selected from general population during regular health examination. The second consisted of 93 randomly selected individuals with cardiovascular diseases (females 22, males 71; average age 58.1 years, range 34 – 71 years) recruited in a study investigating heart diseases, and the third included 48 males Chernobyl accident clean-up workers. These convenience controls were sub-optimal and approaches to this problem are discussed subsequently.

Tumour samples

Twenty-seven primary melanoma tissues from unrelated patients treated at the Latvian Oncological Center were investigated. The main histopathological characteristics of the

tumours studied are shown in Table 3.19. Blood samples were available from 10 patients, whose tumours were also analysed (Table 3.19).

Table 2.1. *Study subjects*

Groups studied		Number
Melanoma patients[a]	SPM patients	168
	MPM patients	2
	FM patients[b]	6
	Total	**176**
Patients with skin neoplasm's other than melanoma and nevi	Patients with atypical nevi	14
	Skin neoplasm's other than melanoma	13
	Total	**27**
Other cancer patients [b]	Breast cancer patients	72
	Ovarian cancer patients	59
	Gastric cancer patients	48
	Colon cancer patients	47
	Rectum cancer patients	23
	Total	**252**
Controls	Healthy individuals	62
	Individuals with cardiovascular disease[c]	93
	Chernobyl accident clean-up workers	48
	Total	**203**
Tumour samples	Primary tumours	27
	Total	**27**
Altogether		**685**

SPM - sporadic primary melanoma; MPM - multiple primary melanoma; FM - familial melanoma; [a] - for all melanoma patients involved in the study histopathology reports were confirmed; [b] - including family with atypical nevi; [c] - participants in GenomeData Base of Latvian population.

2.2. Research ethics

All participants enrolled in the study received an explanation of the aims of the study, agreed to participate, and signed an informed consent form approved by the Central Medical Ethical Committee of Latvia.

2.3. DNA isolation

DNA from peripheral blood lymphocytes was isolated using standard phenol-chloroform extraction method followed by ethanol precipitation or "salting out" method described by Miller et al., 1988.

Prior to the DNA isolation from tumours, dissection of melanoma tissues from normal tissues was done on fresh-frozen samples. Tumour tissues were then homogenised in liquid nitrogen and DNA was isolated using standard phenol-chloroform extraction method.

DNA extraction from archival paraffin-embedded tissues was done according to the procedure of Mailman et al. 1999 with a little modification. Briefly, several 10 μM thick sections were cut from a block of paraffin-embedded tumour tissue and placed in a 1.5 ml microcentrifuge tube. To dissolve the paraffin, the sections were immersed in 1ml of xylene and incubated for 10 min at room temperature followed by 5 min centrifugations at 13 000 g. This procedure was repeated twice. The tissues were rehydrated gradually repeating the above step with 1ml of ethanol (twice), then with 80% ethanol, and then with 50% ethanol. At the end 1 ml water was added and the tube was refrigerated at 4 °C for 24 h to complete the rehydration. The cells were than lysed with 1 ml of nuclei lysis buffer (10 mM Tris, 400 mM NaCl, 2 mM Na_2EDTA, pH=8.4) and incubated at 37 °C for 24 h with 70 μl of proteinase K (1 mg/ml) and 30 μl of 20% sodium dodecyl sulfate. Precipitation of degraded proteins was accomplished by adding 233 μl of saturated NaCl solution, followed by 5 min centrifugation at 13 000 g. The supernatant (containing the DNA) was combined with 1 ml of ethanol and inverted to precipitate DNA.

2.4. Overview of molecular analysis

The scheme of analyses done in melanoma patients and primary tumours is outlined in Figure 2.1. The whole of the *CDKN2A* coding region (exons 1α, 2 and 3), 5'- and 3'-flanking intronic sequences of each exon, including *CDKN2A* alternatively spliced exon 1β of *p14ARF*, and *CDK4* exon 2 as well were screened for germline mutations in all melanoma patients included in the initial screening of consecutive consenting melanoma patients (n=174) by the use of a combination of single-strand conformational polymorphism (SSCP) analysis or/and high-resolution melting curve analysis (MCA) and sequencing, as detailed below.

Familial melanoma patients included in the initial screening (n=4) were also analysed for germline deletion at chromosome region *9p21* using multiplex ligation-dependent probe amplification (MLPA) approach, as detailed below.

Familial melanoma patients recruited to the study later (n=2) as well as relatives of familial melanoma patients both affected and unaffected were screened for mutations in all above mentioned genes by direct DNA sequencing. In addition, familial melanoma patients and their relatives, depending on DNA availability, were analysed for *MC1R* variants also using direct DNA sequencing. In kindred were sequence variation was found in the initial sample investigated, the segregation of the mutation with the disease was confirmed by direct sequencing using DNA from paraffin-embedded melanoma tissues of deceased individuals (archival material kindly provided by Prof. R. Kleina from Centre of Pathology).

In addition, unselected cancer patients (breast, ovarian, gastric, colon, rectum), individuals with atypical nevi, non-melanoma skin neoplasms and control individuals as well were analysed for the three most common *CDKN2A* variants[*], A148T (c.442G>A, rs371249), c.*29C>G (rs11515), c.*69C>T (rs3088440) by the use of restriction fragment length polymorphism (RFLP) analysis, as stated below.

In melanoma patients carrying the *CDKN2A* A148T variant the haplotype analysis was done and these individuals were analysed for a total of six SNPs (c.-3705Ex1bT>C (rs2811712), c.-493A>T, c.-191A>G (rs3814960), IVS1+1255C>A (rs2811708), IVS1-782G>C, c.*29C>G (rs11515)) in the *CDKN2A* region, one of which (c.*29C>G (rs11515))

[*] - variants here and further in the work are given following the standard nomenclature for sequence variation (den Dunnen and Antonarakis 2001).

was assessed by RFLP analysis and the other five (c.-3705Ex1bT>C (rs2811712), c.-493A>T, c.-191A>G (rs3814960), IVS1+1255C>A (rs2811708), and IVS1-782G>C) by allele-specific PCR, as detailed below. In addition to A148T variant carriers from Latvia, there were analysed 19 melanoma patients and 20 control individuals A148T carriers from Poland (samples were kindly provided by Dr. T. Debniak) and haplotypes were compared between populations.

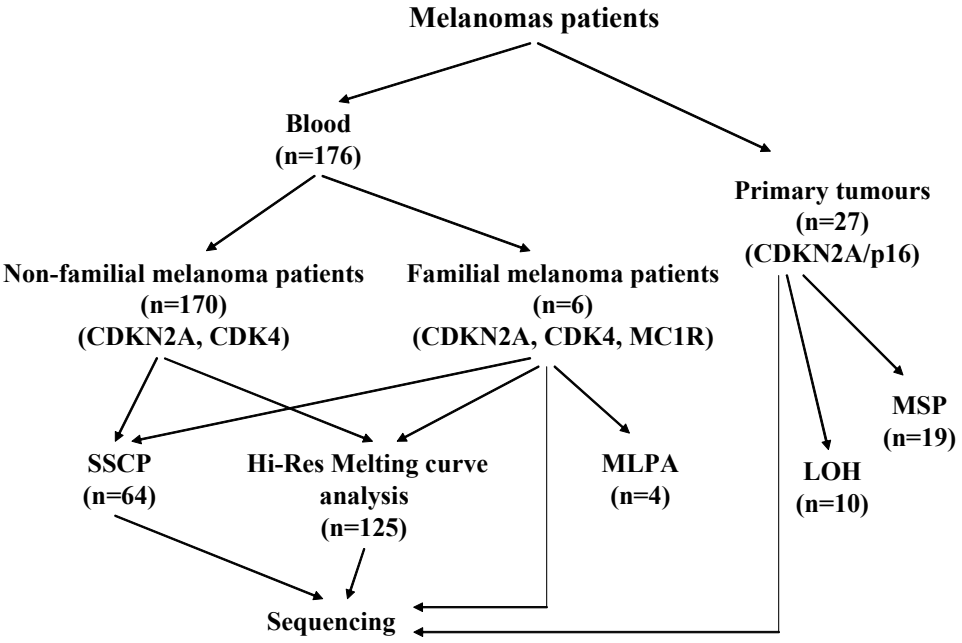


Figure 2.1. *The scheme of analyses done in melanoma patients and primary tumours. Several patients were analysed by more than one method.*

Primary melanoma tumours (n=27) were analysed for mutations in the *CDKN2A* (exons 1α, 2 and 3) using direct DNA sequencing. Tumours were also studied for inactivation of the *CDKN2A* by methylation of CpG islands using methylation-specific PCR (MSP), as detailed below. LOH at *CDKN2A* locus using four microsatellite markers (*D9S974, D9S942, D9S1870,* and *D9S171*) was assessed as well. The type of mutation analysis was limited by the amount of DNA available from each tumour and the availability of tumour-lymphocyte paired samples (Figure 2.1).

2.5. Polymerase Chain Reactions (PCR)

The three exons of *CDKN2A* of p16INK4A (1α, 2, and 3) were amplified from genomic DNA using the primers described by Kamb et al., 1994a and also those described by Hussussian et al., 1994. The alternatively spliced exon 1β of p14ARF was amplified using primers as follows: 1βF CACCTCTGGTGCCAAAGGGC, 1βR CCTAGCCTGGGCTAGAGACG. *CDK4* exon 2 was amplified using the primers described by Zuo et al., 1996. PCR reaction were carried out using reagents from MBI Fermentas (Vilnius, Lithuania) in a 25 μl volume, containing 2.5 μl 10xPCR buffer (1x = 10 mM Tris-

HCl, pH=8.8 and 50 mM KCl), 0.5 µl dNTP (200 µM each dGTP, dCTP, dATP, dTTP), 1.5 µl $MgCl_2$ (25 mM) 0.25 µl forward primer (50 µM) 0.25 µl reverse primer (50 µM), 1.25 µ dimethyl sulfoxide (DMSO) (10%), 0.125 µl Taq polymerase (5 U), and 1 µl genomic DNA (~ 100 ng). PCR amplification conditions were as follows. An initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds and extension at 72 °C for 30 seconds, with a final 7 min extension at 72 °C. To verify the amplification, PCR products were run on 1.5 % agarose gels and visualised by ethidium bromide staining.

CDKN2A exons 1α, 2, and 3 from melanoma tissues were amplified using primers and PCR conditions described above with a few modifications. To reduce the inhibitory effect of melanin on polymerase (Eckhart et al., 2000), these reactions contained less DNA (dilution range from 1:10 till 1:100) and the number of cycles was increased to 40 – 45.

For the amplification of *CDK4* exon 2 from paraffin-embedded tissues, it was necessary to choose primers giving shorter PCR product (212 bp). The primers were *CDK4* exon 2F GCTGCAGGTCATACCATCCT and *CDK4* exon 2R CTCCTCCATTGGGGACTC.

Prior to sequencing, PCR fragments were either isolated by agarose gel electrophoresis and purified using the DNA Extraction Kit (MBI Fermentas) or treated with 20 U exonuclease I and 4 U shrimp alkaline phoshatase (both MBI Fermentas) at 37 °C for 30 min to degrade residual oligonucleotides and to dephosphorylate trinucleotides followed by the inactivation of the enzyme at 80 °C for 15 min.

2.6. Sequencing

Sequencing reactions were carried out using the ABI PRISM Dye Terminator Cycle Sequencing Kit (Applied Biosystems, UK) and following cycling conditions: 25 cycles of denaturation at 94 ºC for 30 seconds, annealing at 55 ºC for 30seconds, extension at 60 ºC for 4 minutes. Samples were then purified by sodium acetate and ethanol precipitation and analysed on an ABI 3100 Sequencer (Applied Biosystems, UK). Sequencing chromatograms were reviewed manually and with the Vector NTI Suite v.6.0 software. DNA sequencing was performed in both directions, initiated from the forward and reverse primers used in the initial PCR amplification of each fragment.

2.7. Polymerase Chain Reaction – single strand conformational polymorphism (PCR-SSCP) analysis

To maximise the sensitivity of mutation detection by subsequent SSCP, primers were chosen to obtain PCR products of 207 bp or less for all primer sets, except *CDKN2A* exon 1α, which prior to SSCP was digested with the restriction endonuclease PdiI (MBI Fermantas, Vilnius, Lithuania). Primers used were as given in Table 2.2. PCR reactions were carried out using reagents from MBI Fermentas as described above. Cycling conditions were as follows: initial denaturation at 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing (at temperatures specific for each exon as given in brackets after each primer set) for 30 seconds, and extension at 72 °C for 30 seconds, with a final 7 min extension at 72 °C.

For SSCP, 1 to 3 µl of each PCR product were mixed with 5 µl of loading buffer (95% formamide, 10 mM NaOH, 0.25% bromphenol blue, and 0.25% xylene cyanol), heat denatured at 95 ºC for 5 min, and chilled on ice. Products were run overnight on 6 % polyacrylamide gel (BIO-RAD, California, USA) in 1xTBE buffer (0.09 M Tris-HCl, 0.09 M Boric Acid, 2 mM EDTA, adjusted to pH=8.3) at constant 100-180V depending on fragment

size and in at least three different temperatures (7 ºC, 10 ºC, 15 ºC). The DNA bands were visualised by silver staining (Bassam et al., 1991) and documented using GSP UVP-system device. PCR fragments that showed aberrant migrating bands were then isolated by agarose gel electrophoresis and purified prior to sequencing by the DNA Extraction Kit (MBI Fermentas) and sequenced in both directions, initiated from the forward and reverse primers used in the initial PCR amplification of each exon as described above.

Table 2.2. *Primers used for PCR amplification of the CDKN2A locus and the CDK4 exon 2 for SSCP analysis*

Exon		Primer sequences (5'-3')	Annealing temperature (°C)	Reference
CDKN2A exon 1α	F	GAAGAAAGAGGAGGGGCT	55 °C	This study
	R	GCGCTACCTGATTCCAATTC		Kamb et al., 1994a
CDKN2A exon 2A	F	AGCTTCCTTTCCGTCATGC	56 °C	Hussussian et al., 1994
	R	GCAGCACCACCAGCGTG		
CDKN2A exon 2B	F	AGCCCAACTGCGCCGAC	56 °C	
	R	CCAGGTCCACGGGCAGA		
CDKN2A exon 2C	F	TGGACGTGCGCGATGC	56 °C	
	R	GGAAGCTCTCAGGGTACAAATTC		
CDKN2A exon 3	F	CCGGTAGGGACGGCAAGAGA	56 °C	Hussussian et al., 1994
	R	CGAAAGCGGGGTGGGTTGT		This study
CDKN2A exon 1βA	F	CTCAGGGAAGGCGGGTGC	56 °C	This study
	R	CGCACGCGCGCCGAATCC		
CDKN2A exon 1βB	F	CTGGAGGCGGCGAGAAC	56 °C	
	R	CCTTTCCTACCTGGTCTTC		
CDK4 exon 2	F	GCTGCAGGTCATACCATCCT	55 °C	Zuo et al., 1996
	R	CTCTCACACTCTTGAGGGCC		

F and R denote forward and reverse primers respectively.

2.8. High-Resolution Melting Curve Analysis (MCA)

High-resolution melting curve analysis (MCA) has recently been described as a novel mutation screening method (McKinney et al., 2004; Dobrowolski et al., 2005). Incorporation of a fluorescent saturating ds-DNA binding dye, LCGreen+, during PCR allows measurement of the melting kinetics of the amplicon as the rate of decrease of fluorescence due to the release of dye during thermal denaturation of the PCR product. The presence of heteroduplex (mismatched) DNA in the amplicon pool due to the presence of a heterozygous mutation in the template DNA modifies the melting characteristics of the amplicon and hence a modified melting curve is obtained.

Primers used for MCA PCR reactions were as follows:

- ***CDKN2A* exon 1αF** CAGCACCGGAGGAAGAAAG,
  ***CDKN2A* exon 1αR** GCGCTACCTGATTCCAATTC;
- ***CDKN2A* exon 2F** AGCTTCCTTTCCGTCATGC,
  ***CDKN2A* exon 2R** GGAAGCTCTCAGGGTACAAATTC;
- ***CDKN2A* exon 3F** CCATTGCGAGAACTTTATCC,
  ***CDKN2A* exon 3R** TGGACATTTACGGTAGTGGG;

- ***CDKN2A* exon 1βF** CACCTCTGGTGCCAAAGGGC,
  ***CDKN2A* exon 1βR** CCTAGCCTGGGCTAGAGACG;
- ***CDK4* exon 2F** GCTGCAGGTCATACCATCCT,
  ***CDK4* exon 2R** ATCATCACACCCCACCTATAGG.

Ten-µl PCR reactions contained 20 ng of genomic DNA, 200 µM each dNTP, 1x PCR reaction buffer (Idaho Technology Inc., http://www.idahotech.com/), 1x LCGreen+ (Idaho Technology Inc., Utah, USA), 0.25U Thermostart Taq polymerase (ABGene, http://www.abgene.com/), 2 pmol each primer, and 10% DMSO. Reactions were carried overlaid with 15 µl of mineral oil (Sigma, Taufkirchen, Germany). Cycling conditions were as follows: initial denaturation at 94 ºC for 12 minutes followed by 45 cycles of denaturation at 95 ºC for 10 seconds, annealing at 55 ºC for 15 seconds (60 ˚C for *CDKN2A* exon 1β), extension at 72 ºC for 15 seconds. After final denaturation at 94 ºC for 30 seconds, reactions were held at 25 ºC.

MCA was carried out using a beta test LightScanner (Idaho Technology Inc.) operating LightScanner v1.0.364 software. After completion of PCR, each microtitre plate was transferred directly to the LightScanner and fluorescence data was collected as samples were melted. Data was collected over the temperature range 70 to 97 ºC.

Data was analysed using the 'manual scanning' setting of LightScanner v1.0.364. For DNA fragments with more than one melting domain, as well as examining the entire melting curve, each individual melting domain was also analysed individually. PCR fragments that showed modified melting curves were purified prior to sequencing using the MinElute PCR Purication Kit (Qiagen) and sequenced in both directions, initiated from the forward and reverse primers used in the initial PCR amplification of each exon as described above.

2.9. Analysis of *MC1R*

A specific amplification of *MC1R* coding sequence was done with the primers previously described by van der Velden et al., 2001a. This primer set is located in the noncoding region enclosing the *MC1R* gene and resulting in a 1,019 bp PCR fragment. PCR reactions were done using reagents from MBI Fermantas in a final volume of 25 µl with 50 ng of genomic DNA, 10xPCR buffer (100 mM Tris-HCl, pH=8.8 and 500 mM KCl),1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.5 µM of each primer, 10% DMSO, and 0.5 U Taq polymerase. The reaction of PCR was initially denaturated for 10 min at 94 °C, followed by 35 cycles of 50 seconds at 94 °C, 30 seconds at 59 °C, and 1 min at 72 °C, with a final extension step at 72 °C for 10 min.

To identify *MC1R* variants, PCR products were sequenced with the same primers used for the initial PCR amplification and an additional internal primer set. The sequences of the internal primers were

- **MC1R_InF** ACCGCTACATCTCCATCTTC,
- **MC1R_InR** TAGCCAGGAAGA AGACCACG.

Sequencing was carried out as described above.

Analysis of *MC1R* variants was conducted only in familial melanoma patients and their relatives, depending on DNA availability.

2.10. Multiplex Ligation-dependent Probe Amplification (MLPA)

MLPA was carried out according to the supplied protocol using the *9p21* MLPA probe kit of MRC-Holland, Amsterdam, the Netherlands. The technique and preparation of the probes used in this kit are described elsewhere (Schouten et al., 2002). The *9p21* MLPA kit

contains 12 probes for *CDKN2* locus sites, 11 probes for other *9p* gene sites, and 16 control probes specific for DNA sequences outside the *9p21* region. The genes included in this analysis were *TEK, ELAV2, CDKN2B, CDKN2A, MTAP, KIAA, INFW1, IFNB1, MLLT3,* and *FLJ00026.*

Briefly, 25 ng/μl genomic DNA (in volume 2.5 μl) was denatured at 98 °C for 5 min, cooled to 25 °C, and 1.5 μl of the supplied SALSA probe mix and MLPA buffer were added. The mixture was re-heated to 95 °C and then the hybridisation was carried out at 60 °C for 16 hr. The hybridised probes were ligated with the ligase-65 mix (ligase-65 enzyme and ligase-65 buffers) at 54 °C for 10-15 min. The ligase-65 enzyme was inactivated by incubation at 98 °C for 5 min and the ligation products were then amplified by PCR according to the manufacturer's protocol using one primer labelled with 6-FAM. The amplification was performed on an GeneAmp9700 Thermal Cycler (Applied Biosystems, UK) with a hot-start PCR program beginning with the addition of the polymerase mix (SALSA primers, SALSA enzyme dilution buffer, SALSA polymerase) to the PCR reaction (ligation products premixed with SALSA PCR buffer) at 60 °C. PCR was carried out for 33 cycles at 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 60 sec, and incubation at 72 °C for 20 min.

PCR products (1μl) were then mixed with 8.5 μl of de-ionised formamide (HiDi formamide, Applied Biosystems) and 0.5 μl fluorescent size standard (GeneScan-ROX 500, Applied Biosystems) and analysed on the ABI3100 Automated Capillary DNA sequencer with a 36 cm capillary array and ABI POP-4 polymer (Applied Biosystems). Analysis was automated using the ABI PRISM GeneScan and Genotyper software. Specific peaks corresponding to each product were identified according to their migration relative to the size standards and exported to a Microsoft Excel spreadsheet. To obtain gene dosage quotients (DQ), peak heights were taken as the quantitative measure of DNA content and peak fractions were calculated by dividing the peak area of a certain probe by the sum of peak areas of all 16 control probes in a certain sample. Subsequently, this relative peak area of each probe was compared to the average relative area of this probe in control samples. The means and standard deviations of the DQ provide quality control of the assay. Results from samples with standard deviation (SD) more than 0.2 were considered as false results and analysis was repeated in accordance with the manufacturers recommendations.

Theoretically, gene dosage quotients close to 1.0 indicate two copies present (i.e. wild-type), 0.5, one copy absent (i.e. hemizygous), and 0.00, both copies absent (i.e. homozygous deletion). Quotients were scored according to observation made from large number of previous samples (Mistry et al., 2005): wild type if the quotient exceeded 0.75, hemizygous deletion if the quotient was between 0.43 and 0.68, and homozygous deletion if the quotient was between 0.00 and 0.19.

2.11. Restriction Fragment Length Polymorphism (RFLP) analysis

RFLP analysis was used to assess the frequency of the three most common *CDKN2A* variants, A148T (c.442G>A, rs3731249), c.*29C>G (rs11515), and c.*69C>T (rs3088440) in unselected cancer patient, individuals with atypical nevi, non-melanoma skin neoplasms and in control individuals as well. After PCR amplification of *CDKN2A* exons 2 and 3, products were digested with the enzymes Cfr42I, MspI, and BsuRI (MBI Fermantas) for the A148T (c.442G>A, rs3731249), c.*29C>G (rs11515), c.*69C>T (rs3088440) variants respectively. Restriction digests were carried out in 15 μl reactions: 10 μl of PCR product was digested using 5 U of appropriate restriction enzyme at 37 °C for 2 hr. Products were then run on 2% agarose gel and visualised by ethidium bromide staining. Sequencing and restriction data were initially cross-checked to ensure the validity of the restriction tests.

2.12. A148T carriers haplotype analysis

DNA samples from individuals carrying the *CDKN2A* A148T variant were further analysed for six SNPs (c.-3705Ex1bT>C (rs2811712), c.-493A>T, c.-191A>G (rs3814960), IVS1+1255C>A (rs2811708), IVS1-782G>C, c.*29C>G (rs11515)) in the *CDKN2A* region. All individuals included in the analysis were retested for A148T (c.442G>A, rs3731249) variant to confirm the appropriateness of the sample for the study. The c.-3705Ex1bT>C (rs2811712), c.-493A>T, c.-191A>G (rs3814960), IVS1+1255C>A (rs2811708), and IVS1-782G>C polymorphisms were detected by PCR using allele-specific primers. PCR tests were designed for these variants using wild-type and mutation-specific inner primers and with fluorescent dyes either 6-FAM or NED labelled outer primers as shown in Figure 3.6. The primers used (Eurogentec, Belgium) were as follows:

- **c.-3705Ex1bT>C (rs2811712)**, forward (outer) CTGCATACTGCAACT GAAAG, reverse (outer) TATAATCATTTTCTCTCCACA, forward (wild type allele) GAACAGAGAGCTTACTATATAATTGTT, reverse (mutant allele) GAATGCCATCTGAATAATAGG (annealing temperature 53 °C);
- **c.-493A>T**, forward (outer) GACTTAGTGAACCCCGCGCTCCTGAAAA, reverse (outer) CGTGTTTGAGTGCGTTCACTCTGTTA, forward (mutant allele) CAAACACCCCGATTCAATTTGGCAGTT T, reverse (wild type allele) CTGTTCCTTCCTCCGCGATACAACCTTTCT (annealing temperature 59 °C);
- **c.-191A>G (rs3814960)**, forward (outer) AGACCCAACCTGGGGCGACTT, reverse (outer) CCGCCCGCTGCCTGCTCTCCCCCTCT, forward (wild type allele) AGCACCTCCTCCGAGCACTCGCTCACA, reverse (mutant allele) CCGCGGTATCTTTCCAGGCAAGGGGACGCC (annealing temperature 66 °C);
- **IVS1+1255C>A (rs2811708)**, forward (outer) CGGGAGTTTGGCTTTGTAGT, reverse (outer) AATGTTCCTATATTAAACACC, forward (wild type allele) GTTTTGACAATTTTTAATGGGGAC, reverse (mutant allele) CCTTTTCTACATGTTCTTCTACCT (annealing temperature 53 °C);
- **IVS1-782G>C**, forward (outer) TGGAATTGGAGAGCATTATGCTAAATG, reverse (outer) TGTCAAATGAGAGCATTTGGGATTTACT, forward (wild type allele) GGAGGAATGGGGAGATGGTAATCGAG, reverse (mutant allele) CCTCCCATCTTAACTGAGGCTTTATTTG (annealing temperature 58 °C).

Twenty-five μl of PCR reactions were carried out using 100 ng genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 50 mM each primer, 5% DMSO, and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) in the reaction buffer supplied by the manufacturer. Thermal cycling consisted of an initial denaturation at 94 °C for 10 min, followed by 30 cycles of denaturation at 94 °C (30 seconds), annealing at temperatures specific for each SNP as indicated in brackets above (30 seconds) and extension at 72 °C (1 min) with a final 7 min extension at 72 °C. PCR products were then analysed on an ABI3100 Automated sequencer (Applied Biosystems, UK) with Genescan-500 Rox (Applied Biosystems) as a size standard. Sequencing and PCR data were initially cross-checked to ensure the validity of the PCR tests.

The A148T (c.442G>A, rs3731249) and c.*29C>G (rs11515) polymorphisms were assessed by restriction digest with enzymes Cfr42I and MspI respectively as described above.

2.13. Haplotype analysis for the *CDK4* mutation positive family

In order to compare the mutated haplotype of *CDK4* mutation positive family from Latvia with co-segregating haplotypes in other *CDK4* mutation positive families, three

markers upstream of the *CDK4* gene (*D12S305*, CDK4M4, CDK4M5) one marker within *CDK4* (CDK4M7), and two markers downstream (CDK4M1, *D12S1691*) were analysed. Sequences for the *D12S305* and *D12S1691* markers are available from the Genome Database at http://www.gdb.org. CDK4M4, CDK4M5, CDK4M7, and CDK4M1 markers were as described by Molven et al., 2005. Three SNPs in *CDK4* (rs2270777, rs2069502, rs2069506) and one SNP in the promoter region (rs2072052) were identified with the heterozygosity greater than 0.25 in the SNP database (http://www.ncbi.nlm.nih.gov/SNP/) and analysed by sequencing as detailed by Molven et al., 2005.

2.14. Promoter metylation studies

Primary melanoma tumours were studied for inactivation of *CDKN2A* gene by promoter CpG islands methylation using methylation specific PCR (MSP) and sequencing.

The MSP method and primer sequences as described by Herman et al., 1996 were used. First, genomic DNA was modified with sodium bisulfide using the EZ DNA Methylation Kit (Zymo Research, California, USA) according to the recommendations of the manufacturer. As a result of this modification, cytosine residues are deaminated and changed into uracil residues, but a methylated cytosine is protected and will remain unmodified. Hence, sequence differences are introduced that allow for amplification with methylated (M) and unmethylated (U) DNA-specific primers. The efficiency of the bisulfide modification was determined by primers specific for the wild-type p16 promoter region (W primers). All cases analysed were amplified by M, U, and W primers. The products were electrophoresed on a 2% agarose gel containing ethidium bromide and visualised by UV light. Cases not showing amplification with any of the primers were regarded to have insufficient quality of DNA. DNA from lymphocytes and untreated DNA were used as controls as well.

To confirm the methylation of the CpG islands in the *CDKN2A* promoter region sequencing of bisulfide modified DNA was performed. First, bisulfide modified DNA was amplified using primers and conditions as described by Herman et al., 1996 and then sequenced in both directions using the same primers as for initial PCR amplification.

2.15. LOH analysis at *9p21*

Loss of hetorozygosity (LOH) analysis has been performed for 10 tumours derived from patients from whom blood DNA was also available. Five microsatellite markers on chromosome region *9p21*, namely *D9S1846*, *D9S974*, *D9S942*, *D9S1870*, and *D9S171*, were amplified by PCR using primer sequences described in the Genome Data Base (http://www.gdb.org). One primer in each set was labelled at the 5' end with the fluorescent dye: PET, 6-FAM, VIC or NED (Applied Biosystems, UK). The PCR reactions were carried out in a final volume of 15 µl using 0.5 U Taq polymerase (MBI Fermentas), 10xPCR buffer (100 mM Tris-HCl, pH=8.8 and 500 mM KCl), 0.2 mM of each deoxynucleoside triphosphate (dGTP, dATP, dCTP, dTTP), 1.5 mM $MgCl_2$, 0.5 µM forward and reverse primers, and 50 ng genomic DNA. PCR amplification conditions were as follows: an initial denaturation at 94 °C for 10 min followed by 35 cycles of denaturation at 94 °C for 15s, annealing at 50 °C (*D9S1870*), 54 °C (*D9S1846*, *D9S974*, *D9S942*), and 59 °C (*D9S171*) for 15s, and extension at 72 °C for 30s, with a final 10 min extension at 72 °C. PCR products were then separated on the ABI PRISM 310 Genetic Analyser (Applied Biosystems, UK). In the case of informative markers, LOH was scored when a reduction of at least 50% was seen in one allele in DNA isolated from the tumour tissue compared with DNA isolated from the respective blood sample.

2.16. Statistical analysis

All statistical comparisons were performed using either two-sided Fisher's exact or χ^2 tests. There was a small proportion of individuals with the rare (GG) genotype for the *CDKN2A* polymorphism c.*29C>G (rs11515), therefore, the CG and GG genotypes were combined in statistical analyses. All statistical tests were carried out using calculator on http://home.clara.net/sisa/index.htm.

3. RESULTS

3.1. Screening of *CDKN2A* and *CDK4* genes for germline mutations in cutaneous malignant melanoma patients from Latvia

3.1.1. Characteristics of melanoma patients involved in the study

Altogether 176 cutaneous malignant melanoma (CMM) patients were involved in the genetic testing. Clinical data of patients analysed and histopathological features of their tumours removed are summarised in Table 3.1. The phenotypic characteristics were available for 65 melanoma patients analysed and are shown in Table 3.2.

Table 3.1. *Clinical data of cutaneous malignant melanoma patients analysed in the present study and histopathological features of their tumours*

Total number:		**176**
Gender:	Males	42 (24 %)
	Females	134 (76 %)
Age at diagnosis (years):	Range	20 – 89
	Mean	60.0
Breslow thickness (mm)[a]:	Range	0.1 - 20
	Mean	4.6
Clark invasion level:	I	2 (1 %)
	II	23 (13 %)
	III	62 (35 %)
	IV	41 (23 %)
	V	22 (13 %)
	No data	26 (15 %)
Ulceration:	No	37 (21 %)
	Yes	59 (34 %)
	No data	80 (45 %)
Pigment:	No	12 (7 %)
	Yes	132 (75 %)
	No data	32 (18 %)
Anatomical localisation:	Head/neck	14 (8 %)
	Trunk	70 (40 %)
	Extremities	85 (48 %)
	Nail/palm/sole	6 (3 %)
	Metastasis[b]	1 (1 %)

[a] *- information on tumour Breslow thickness was available from 113 patients;* [b] *- primary tumour (melanoma) not detected.*

In the studied cohort, 174 were consecutive consenting melanoma patients including four familial melanoma patients (two additional familial melanoma patients were joined to the study later) and 48 patients diagnosed with malignant melanoma before the age of 50 years (Table 3.3). As shown in Table 3.3, only two patients (1%) studied had a strong family history of melanoma (both 5 melanoma cases within the family) applying the generally accepted criteria for a melanoma family (≥ 3 affecteds). Four (2%) patients had melanoma or atypical nevi in the first-degree relatives. Two patients had melanoma in mother, one had melanoma in sister and one patients had sister with the removed atypical nevus. Sixty-three (36%) patients

reported 78 cases of other cancers in their family. Gastric (17 cases), breast (15 cases), uterus (10 cases) cancers were reported more frequently in families of patients analysed. Lung (6 cases), colon (5 cases), brain (3 cases), skin (other than melanoma) (3 cases), prostate (3 cases), pancreas (2 cases), bladder (2 cases), kidney (2 cases), liver (2 cases), ovarian (1 case), colorectal (1case), larynx (1 case), nose (1case) cancers and leucosis (3 cases), leukaemia (1 case) were reported as well. Eight of the reported tumours were unknown localisation.

Table 3.2. *Self-reported phenotypic data of cutaneous malignant melanoma patients analysed*[a]

Melanoma patients	
Total number:	**65**
Gender:	
Males	19 (29%)
Females	46 (71%)
Age at diagnosis (years):	
Range	20-82
Mean	56.9
Skin type[b]:	
I	5 (8%)
II	15 (23%)
III	7 (11%)
IV	38 (58%)
Hair colour:	
Red	6 (9%)
Blond	23 (35%)
Brown	36 (56%)
Eye colour:	
Blue	50 (77%)
Brown	9 (14%)
Other	6 (9%)
Freckling:	
Yes	8 (12%)
No	57 (88%)
Increased number of moles (>100; ∅ 2mm):	
Yes	12 (18%)
No	53 (82%)

[a] *– information on phenotype was available from 65 patients analysed;* [b] *– skin typing was performed according to the Fitzpatrick skin typing system: I – very pale skin, very sensitive to UV light, never tans, always burns; II – pale skin, very sensitive to UV light, sometimes tans, usually burns; III – pale skin; sensitive to UV light, usually tans, sometimes burns; IV – light brown skin, moderately sensitive to UV light, always tans, never burns (Fitzpatrick 1988).*

In addition, two patients in the studied cohort had multiple primary melanomas (MPM) (two primary melanomas both) and a total of 17 additional malignant non-melanoma primary cancers occurred in 15 individuals. There were also present 18 additional benign neoplasms in malignant melanoma patients analysed (Table 3.4).

Table 3.3. *Self-reported family history of cancers of melanoma patients analysed*[a]

Age at diagnosis	Family history of cancers[b] Number of patients analysed (%)					
	> 2CMM +/- other cancer	1 CMM +/- other cancer	Only other cancers	None cancer	No information	Total
≤ 50 years	2 (1 %)	-	16 (9 %)	19 (11 %)	11 (6 %)	48 (27 %)
> 50 years	-	4 (2 %)[c]	47 (27 %)	47 (27 %)	30 (17 %)	128 (73 %)
Total	2 (1 %)	4 (2 %)	63 (36 %)	66 (38 %)	41 (23 %)	176 (100 %)

[a] *– for familial melanoma patients, medical confirmation was sought for all relatives reported to have had melanoma;* [b] *- proband not included;* [c] *- including melanoma patient with relative, who had atypical nevus.*

3.1.2. Analysis of the *CDKN2A* gene

First, analysis of 64 patients (selected because of the cancer of any type in their family history or additional primary cancer in the same patient) was done by single strand conformational polymorphism analysis (SSCP) and yielded 25 potentially mutations positive PCR fragments (Figure 3.1.a,c,e). Sequencing of PCR fragments with aberrant migrating bands in SSCP confirmed the presence of four different *CDKN2A* variants (Figure 3.1.b,d,f and Table 3.5). Sequencing of the remaining PCR fragments without detectable changes in SSCP did not yield any changes.

Subsequent screening of 125 additional melanoma patients by high-resolution melting curve analysis (Figures 3.2 and 3.3) and sequencing of DNA samples with aberrant melting curves identified five different *CDKN2A* variants, four of which were identical to that detected previously by SSCP and one was novel *CDKN2A* variant situated in the 3' UTR of the gene, c.*42C>A (Table 3.5).

Fifteen samples were analysed by both approaches and similar results were obtained indicting that both approaches are sensitive and suitable for mutation screening.

Taking results together, the screening of 174 consecutive consenting cutaneous malignant melanoma patients for *CDKN2A* germline mutations with above mentioned methods identified five different *CDKN2A* gene variant. Three variants were common *CDKN2A* polymorphisms, A148T, c.*29C>G, and c.*69C>T, one rare *CDKN2A* polymorphism, c.-33 G>C, and one *CDKN2A* variant of uncertain significance situated in the 3' UTR of the gene, c.*42C>A. Strong linkage disequilibrium was found between the A148T polymorphism at the end of the second
exon and the c.*29C>G polymorphism in the 3' UTR of the gene (Fisher exact test, $p<0.0001$). No functionally deleterious mutations of the *CDKN2A* were detected in patients analysed (Table 3.6).

Table 3.4. *Patients with CMM and additional neoplasms*

Case no	Lab. code	Age at diagnosis (years)	Gender	Additional cancer[a]	Cancer in family history (affected relative, cancer)	*CDKN2A* variants[c]
1.	M2	57	Female	Papillomab	-	-
2.	M3	76	Female	Meningiomab	-	-
3.	M7	61	Male	Basaliomab Papillomab	-	*c.*69C>T*
4.	M8	82	Male	Keratosisb	Brother, liver	*A148T;* *c.*29C>G*
5.	M16	56	Female	Pancreas	nd	-
6.	M21	64	Female	Keratosisb	-	*c.*29C>G* ; *c.*69C>T*
7.	M23	72	Female	Basaliomab	-	*c.*69C>T*
8.	M30	70	Female	Breast	nd	*c.*69C>T*
9.	M46	61	Female	Uterus	-	*c.*69C>T*
10.	M62	54	Female	Uterus	Aunt, gastric Uncle, gastric	-
11.	M65	83	Female	Breast Uterus	nd	-
12.	M87	52	Male	Lipomab	Father, lung	*c.*29C>G*
13.	M91	77	Female	Non-Hodgkin's lymphoma	Daughter, breast	-
14.	M97	51	Female	Ovarian Lipomab	Father, lung	-
15.	M98	55	Female	Dermatofibromab	nd	-
16.	M111	71	Male	Breast Melanoma	-	*c.*69C>T*
17.	M119	67	Female	Keratosisb	nd	*c.*69C>T*
18.	M120	59	Female	Breast	-	-
19.	M121	62	Female	Ovarian	-	*c.*69C>T*
20.	M149	76	Female	Breast	-	-
21.	M150	62	Female	Miomab	Cousin, breast	*A148T;* *c.*29C>G*
22.	M155	46	Female	Papillomab	Father, prostate	-
23.	M186	70	Female	Basaliomab	Sister, uterus	*A148T;* *c.*29C>G*
24.	M200	59	Male	Papillomab Melanoma	-	-
25.	M202	66	Female	Colon Breast	-	-
26.	M210	79	Male	Prostate	Brother, gastric	-
27.	M212	29	Female	Basaliomab (2) Ovarian	Father, leucosis Grandmother, uterus Aunt, uterus Uncle, gastric Father cousin, uterus Father cousin, uterus	-
28.	M222	52	Female	Basaliomab	-	*c.*29C>G*
29.	M223	79	Male	Leucosis Basaliomab	-	-

[a] - primary melanoma not included; [b] - benign form neoplasm; [c] – locations of base changes are given following the standard nomenclature for sequence variation (den Dunnen and Antonarakis 2001); in italics are indicated CDKN2A polymorphisms; nd – no data.

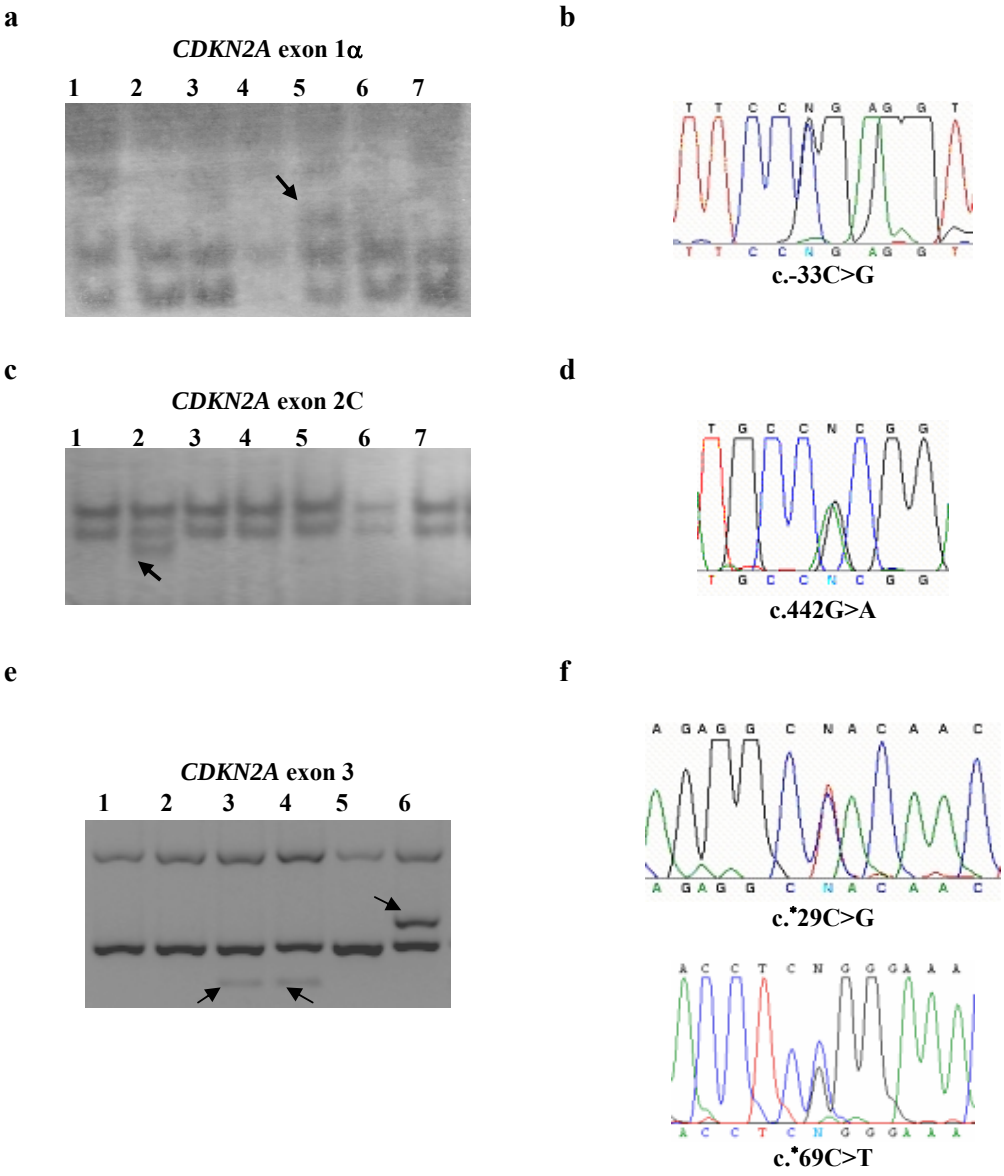


Figure 3.1. *Examples of aberrant shifts (arrows) detected in SSCP analysis of CDKN2A and sequence chromatograms demonstrating corresponding changes in DNA:* ***(a),*** *CDKN2A exon 1α. Prior to SSCP analysis PCR fragment of exon 1α was digested with the restriction endonuclease PdiI; shift in sample 5 represents c.-33C>G polymorphism;* ***(b),*** *chromatogram showing the c.-33 C>G change;* ***(c),*** *CDKN2A exon 2C; shift in sample 2 represents c.442 G>A (A148T) polymorphism;* ***(d),*** *chromatogram showing the c.442 G>A change;* ***(e),*** *CDKN2A exon 3; shifts in sample 6 represents c.*29C>G polymorphism and those in samples 3, and 4 represent c.*69C>T polymorphism;* ***(f),*** *chromatograms showing the c.*29C>G and c.*69C>T changes.*

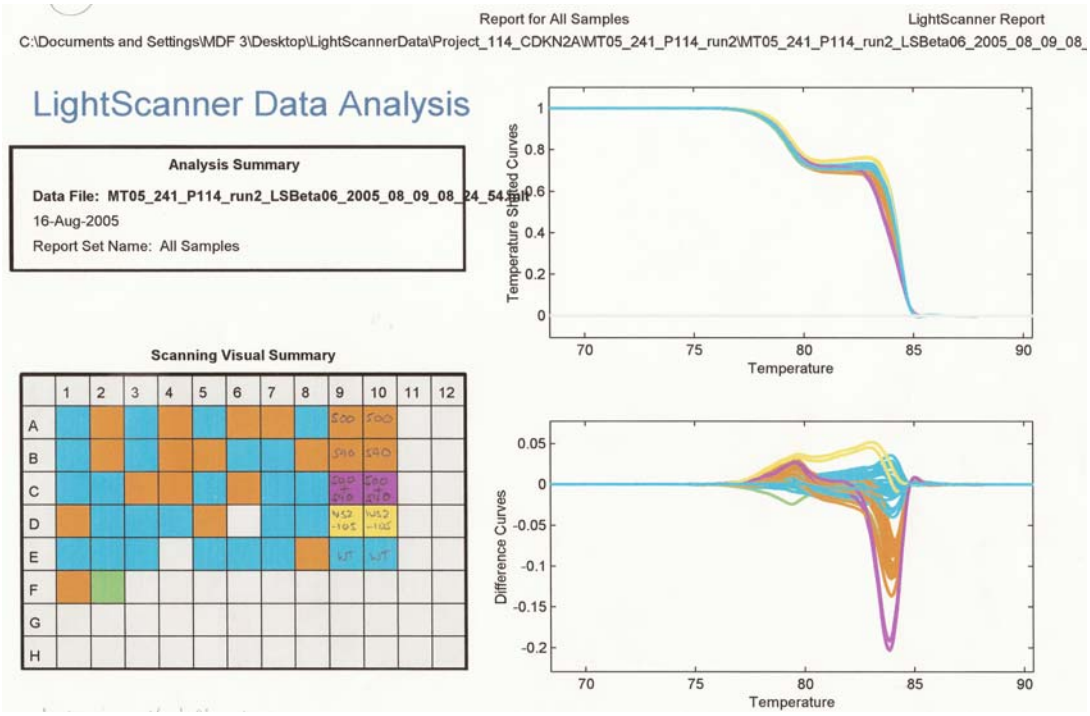


Figure 3.2. *An example for the LightScanner data output of high-resolution melting curve analysis. Analysis of the CDKN2A exon 3. In scanning visual summary (left panel) columns 9 and 10 represent control DNA samples (kindly provided by Dr. C. Taylor): A9, A10 – c.*29C>G polymorphism (light brown); B9, B10 – c.*69C>T polymorphism (light brown); C9,C10 – both c.*29C>G and c.*69C>T polymorphisms (purple); D9, D10 – IVS2-105A>G mutation (yellow); E9, E10 – wild type sequence (blue). Green square represents novel variant not presented as control. Melting curves (right panel): temperature shifted curves (upper graph), difference curves (lower graph).*

Table 3.5. *The number and frequency (%) of the CDKN2A variants identified in the present study by SSCP and high-resolution melting curve analyses separately and totally*

CDKN2A variants	Method of the analysis			Total (%) (n=174)
	SSCP + sequencing (n=49)	High-resolution meting curve analysis + sequencing (n=110)	Both methods (n=15)	
c.-33G>C	-	1	1	2 (1 %)
c.442G>A (A148T)	3	6	2	11 (6 %)
c.*29C>G	9	17	5	31 (18 %)
c.*42C>A	-	1	-	1 (1 %)
c.*69C>T	3	25	2	30 (17 %)

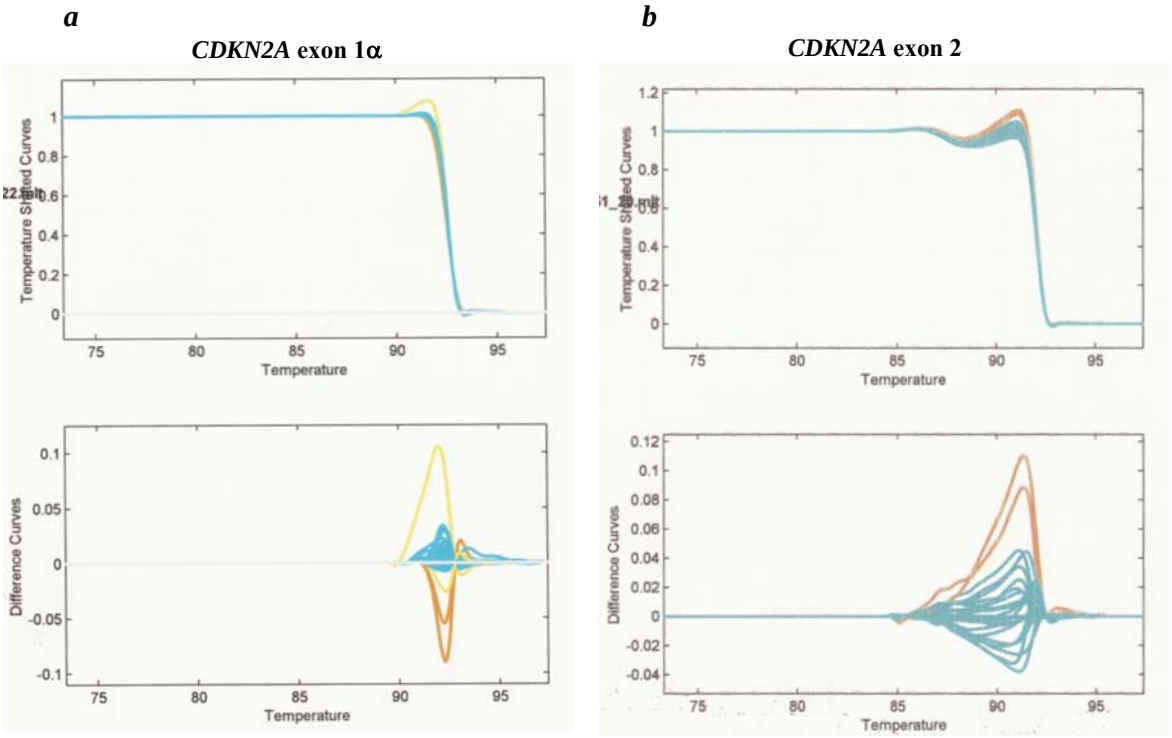


Figure 3.3. *Representative examples of high-resolution melting curve analysis of the CDKN2A gene. Upper graphs temperature shifted curves, lower graphs difference curves.* ***(a),*** *CDKN2A exon 1α; modified melting curves obtained from control DNA sample (c.-34G>T) (yellow graph) and DNA samples with c.-33C>G polymorphism (light brown graphs) as later confirmed by sequencing;* ***(b),*** *CDKN2A exon 2; modified melting curves obtained from DNA samples with A148T polymorphism (light brown graphs) confirmed by sequencing; control DNA samples were kindly provided by Dr. C. Taylor.*

Table 3.6. *Variants of the CDKN2A identified in the present study and their effect for both $p16^{INK4a}$ and $p14^{ARF}$*

***CDKN2A* variant**	**Codon**	**Effect on $p16^{INK4a}$ protein**	**Effect on $p14^{ARF}$ protein**
c.- 33 G>C	-	None	None
A148T	148	A148T	None
c.*29C>G	-	None	None
c.*42C>A	-	None	None
c.*69C>T	-	None	None

3.1.3. Analysis of the $p14^{ARF}$ transcript

To screen for mutations in the $p14^{ARF}$ transcript, its unique first exon, known as exon 1β, was analysed in the same manner as the rest of *CDKN2A* gene. All 174 consecutive cutaneous malignant melanoma patients were analysed using SSCP or high-resolution melting curve analyses and sequencing. No variants of exon 1β were detected. The second exon of the $p14^{ARF}$ is identical to that of the *CDKN2A*, but its codon reading frame is shifted following splicing to exon 1β (box in Figure 1.10). So, the changes in *CDKN2A* exon 2 could also influence the *$p14^{ARF}$* transcript, however, the A148T polymorphism detected in the *CDKN2A* exon 2 does not have effect on $p14^{ARF}$ protein (Table 3.6).

3.1.4. Analysis of the *CDK4* exon 2

Despite the rarity of the *CDK4* mutations, exon 2 of the gene was screened in all 174 consecutive cutaneous malignant melanoma patients involved in the study. Analysis of the *CDK4* exon 2 was done in the same manner as described above using SSCP or high-resolution melting curve analyses and sequencing.

An aberrant melting curve by high-resolution melting curve analysis was detected in one sample, corresponding to a missence mutation at codon 24, changing an Arg to a His (R24H) that was conformed by sequencing (Figure 3.4). The mutation was detected in a patient with the strongest family history of melanoma (5 case family). The same mutation was also found in another familial melanoma patients included in the study later and analysed separately (for details see chapter 3.4).

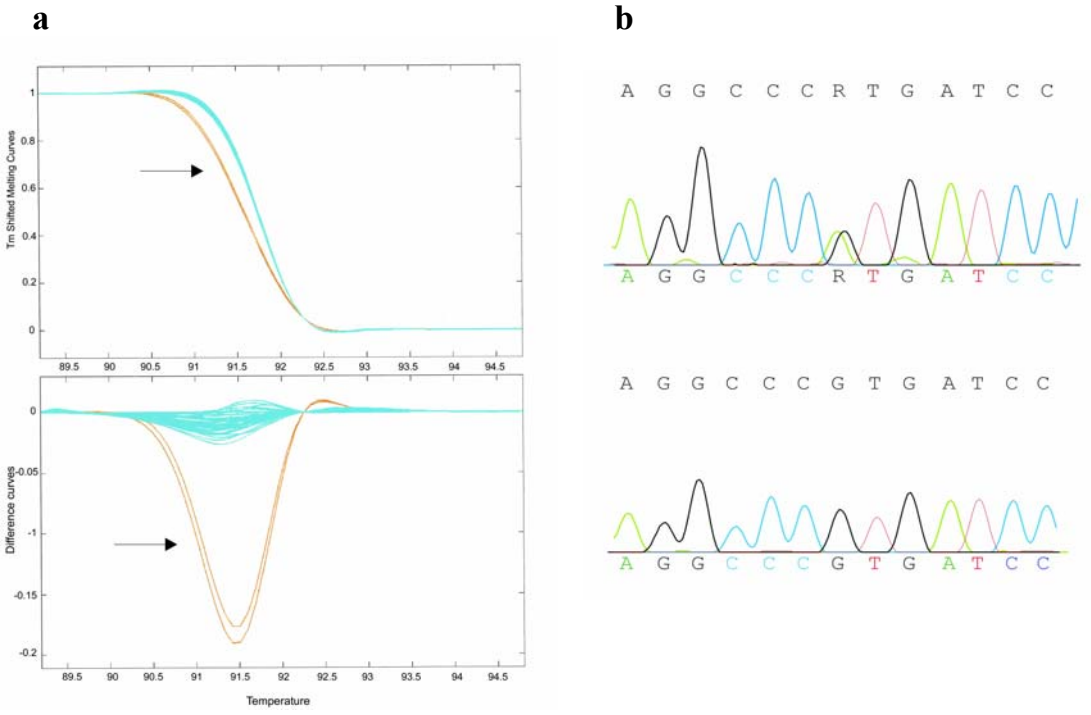


Figure 3.4. *Screening of the CDK4 exon 2 for germline mutations.* ***(a),*** *high-resolution melting curve analysis: temperature shifted melting curves (upper panel), difference curves (lower panel); modified melting curves (arrows) corresponding to the mutated allele were detected for control DNA sample with CDK4 R24H mutation (kindly provided by Dr. C. Taylor) and for melanoma patient under the analyses;* ***(b),*** *chromatogram confirming the heterozygous CDK4 codon 24 mutation CGT > CAT (Arg > His) (upper panel), and chromatogram of the wild type DNA sequence around codon 24 of CDK4 (lower panel).*

3.2. The role of *CDKN2A* common variants in melanoma risk and progression

Because there were detected a relatively high frequencies 6%, 18%, and 17% for the *CDKN2A* polymorphisms A148T, c.*29C>G, and c.*69C>T respectively, these polymorphisms were analysed as a putative low penetrance melanoma susceptibility alleles and melanoma progression alleles as well.

3.2.1. *CDKN2A* common variants association with melanoma risk in Latvian population

In order to assess the role of the three most common *CDKN2A* polymorphisms, A148T, c.*29C>G, and c.*69C>T, in melanoma risk, a comparison of polymorphisms frequencies between melanoma patients analysed and 203 control persons from the same population was done. The frequencies of polymorphisms in the control group were assessed using the RFLP analysis (Figure 3.5). The control group consisted of three separate subgroups (general practise group, individuals with cardiovascular disease, and Chernobyl clean-up workers). The frequencies of polymorphisms in each subgroup are given in Table 3.7. These frequencies were not statistically different and were in Hardy-Weinberg equilibrium. The control groups were therefore combined for the purposes of statistical analysis.

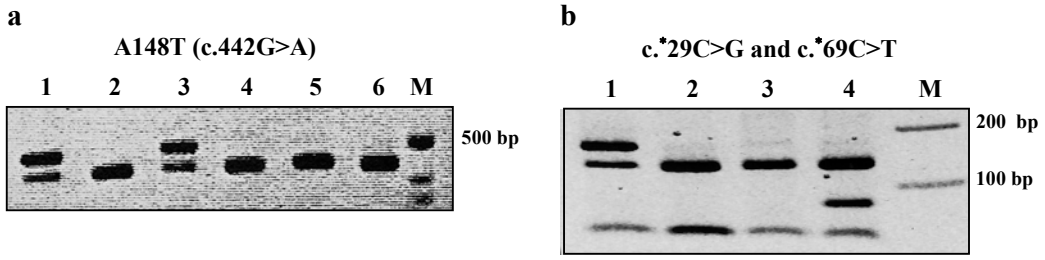


Figure 3.5. *Representative examples of RFPL analysis of the three most common CDKN2A polymorphisms, A148T, c.*29C>G, c.*69C>T. After PCR amplification of CDKN2A exon 2 and 3 products were digested with appropriate restriction endonucleases and separated on 2% agarose gels.* ***(a)****, A148T polymorphism was assessed by treating CDKN2A exon 2 with Crf42I (SacII) restriction endonuclease which cleaves the wild type allele and does not cleave the mutant allele; lines 1 and 3 heterozygous A148T carriers; lines 2, 4-6 wild type sequence; M, DNA marker (100 bp);* ***(b)****, c.*29C>G and c.*69C>T polymorphisms were assessed by treating CDKN2A exon 3 with restriction endonucleases MspI and BsuRI respectively which both cleave the wild type allele and does not cleave the mutant allele; line 1, heterozygous c.*29C>G carrier; lines 2-3, wild type sequence for both polymorhisms; line 4, heterozygous c.*69C>T carrier; M, DNA marker (100 bp).*

Table 3.7. *Comparison of the CDKN2A polymorphisms frequencies (%) between different control groups*

CDKN2A polymorphisms	Controls n (%)				
	General practise group (n=62)	Individuals with heart disease (n=93)	Chernobyl Clean-up workers (n=48)	Total (n=203)	p-value (two-sided Fisher's exact)
A148T (c.442 G>A)					
GG genotype	62 (100 %)	91 (98 %)	47 (98 %)	200 (99 %)	0.52 (0.52)[a]
GA genotype	0 (0 %)	2 (2 %)	1 (2 %)	3 (1 %)	0.44 (0.42)[b]
Variant allele (A) frequency	0/124 (0 %)	2/186 (1 %)	1/96 (1 %)	3/406 (1 %)	1.00 (1.00)[c]
c.*29C>G					
CC genotype	52 (84 %)	77 (83 %)	40 (83 %)	169 (83 %)	0.99 (0.69)[a]
CG+GG genotype	10 (16 %)	16 (17 %)	8 (17 %)	34 (17 %)	0.99 (1.00)[b]
Variant allele (G) frequency	10/124 (8 %)	18/186 (10 %)	8/96 (8 %)	36/406 (9 %)	1.00 (0.83)[c]
c.*69C>T					
CC genotype	53 (85 %)	81 (87 %)	41 (85 %)	175 (86 %)	0.81 (0.82)[a]
CT genotype	9 (15 %)	12 (13 %)	7 (15%)	28 (14 %)	0.99 (1.00)[b]
Variant allele (T) frequency	9/124 (7 %)	12/186 (6 %)	7/96 (7 %)	28/406 (7 %)	0.80 (0.80)[c]

[a] *- p- value for the differences in polymorphism (variant allele) frequencies between general practice group and individuals with heart disease;*
[b] *- between general practice group and Chernobyl clean-up workers;*
[c] *- between individuals with heart disease and Chernobyl clean-up workers.*

In the melanoma population under the study the frequency of the A148T polymorphism was significantly higher compared with the control population (6% vs. 1%; p = 0.01) as shown in Table 3.8 and it was associated with an increase in the odds ratio (OR = 4.50) of developing melanoma. The difference between groups was weakened by comparing the frequency of variant allele (A), however, its frequency was still significantly higher in melanoma patients than that in controls (3% vs. 1%; p = 0.02) (Table 3.8). Although the variant allele frequencies for c.*29C>G and c.*69C>T polymorphisms were also higher in melanoma patients than controls, there were no statistically significant differences in the c.*29C>G and c.*69C>T polymorphisms distribution between melanoma patients and controls (Table 3.8).

To further evaluate the importance of polymorphisms in melanoma risk, their frequencies were assessed in patients who were ≤50 years of age and compared with those >50 years (Table 3.9). The expected greater frequencies of polymorphisms in younger group was observed neither for A148T nor c.*29C>G polymorphisms. There was marginally higher proportion of older patients with c.*29C>G polymorphism compared with the younger patients (p = 0.07 for polymorphism frequency; p = 0.004 for variant allele frequency). Only the frequency of c.*69C>T polymorphism was greater in the < 50-years-old group compared with the older group. However, the differences between polymorphism frequencies were not statistically significant for groups analysed (Table 3.9).

Similarly, the mean age at the time of diagnosis was lower in polymorphism non-carriers than carriers for both A148T (59.5 vs 65.9) and c.*29C>G (59.2 vs 62.9) polymorphisms and higher for c.*69C>T (60.0 vs 59.3) polymorphism, however, statistically non-significant.

Table 3.8. *Frequencies (%) of the CDKN2A polymorphisms in melanoma patients and controls*

CDKN2A polymorphisms	Melanoma patients (n=174)	Controls (total) (n=203)	p-value (two-sided Fisher's exact)	OR (95% CI)
A148T (c.442 G>A)				
GG genotype	163 (94 %)	200 (99 %)	0.01[a]	4.50 (1.23-16.40) [a]
GA genotype	11 (6 %)	3 (1 %)	0.02[b]	4.38 (1.21-15.85) [b]
Variant allele (A) frequency	11/348 (3 %)	3/406 (1 %)		
c.*29C>G				
CC genotype	143 (82 %)	169 (83 %)	0.79[a]	1.08 (0.63-1.84) [a]
CG+GG genotype	31 (18 %)	34 (17 %)	0.71[b]	1.11 (0.68-1.82) [b]
Variant allele (G) frequency	34/348 (10 %)	36/406 (9 %)		
c.*69C>T				
CC genotype	144 (83 %)	175 (86 %)	0.39[a]	1.30 (0.74-2.28) [a]
CT genotype	30 (17 %)	28 (14 %)	0.41[b]	1.27 (0.75-2.18) [b]
Variant allele (T) frequency	30/348 (9 %)	28/406 (7 %)		

OR – odds ratio; CI – confidence interval;
[a] - p- value and OR for the differences in polymorphism frequencies between control group and melanoma patients;
[b] - p- value and OR for the differences in variant allele frequencies between control group and melanoma patients.

Table 3.9. *Comparison of the CDKN2A polymorphism frequencies (%) between melanoma patients ≤50 years of age and melanoma patients >50 years of age*

CDKN2A polymorphisms	Melanoma patients age ≤50 years (n=47)	Melanoma patients age >50 years (n=127)	p-value (two-sided Fisher's exact)	OR (95 % CI)
A148T (c.442 G>A)				
GG genotype	46 (98 %)	117 (92 %)	0.29[a]	0.25 (0.03-2.04) [a]
GA genotype	1 (2 %)	10 (8 %)	0.30[b]	0.26 (0.03-2.08) [b]
Variant allele (A) frequency	1/94 (1 %)	10/254 (4 %)		
c.*29C>G				
CC genotype	43 (91 %)	100 (79 %)	0.07[a]	0.34 (0.11-1.04) [a]
CG+GG genotype	4 (9 %)	27 (21 %)	0.04[b]	0.33 (0.11-0.97) [b]
Variant allele (G) frequency	4/94 (4 %)	30/254 (12 %)		
c.*69C>T				
CC genotype	38 (81 %)	106 (83 %)	0.66[a]	1.20 (0.50-2.84) [a]
CT genotype	9 (19 %)	21 (17 %)	0.67[b]	1.17 (0.52-2.67) [b]
Variant allele (T) frequency	9/94 (10 %)	21/254 (8 %)		

OR – odds ratio; CI – confidence interval;
[a] - p- value and OR for the differences in polymorphism frequencies between melanoma patients ≤50 years of age and melanoma patients >50 years of age;
[b] - p- value and OR for the differences in variant allele frequencies between melanoma patients ≤50 years of age and melanoma patients >50 years of age.

3.2.2. Comparison of the Latvian, Polish and English A148T carriers haplotypes

To further define the role of the A148T variant in modulation of melanoma risk, giving conflicting results between groups, the haplotype analysis (for SNPs in the *CDKN2A*: c.-3705Ex1bT>C (rs2811712), c.-493A>T, c.-191A>G (rs3814960), IVS1+1255C>A (rs2811708), IVS-782G>C, c.*29C>G (500C/G, rs11515)) was performed in 10 Latvian A148T variant carriers and 19 Polish melanoma patients and in 20 Polish control individuals carrying the A148T variant as well (DNA samples kindly provided by Dr. T. Debniak). Haplotype analysis was done either by allele-specific PCR tests for most of the SNPs (Figure 3.6) or RFLP analysis for SNP c.*29C>G as shown above. Subsequently, the obtained Latvian and Polish haplotypes were compared to the predominant A148T carriers haplotype in England. In England the A148T variant segregates on a distinctive haplotype (Figure 3.7), with the minor allele present at c.-493A>T, c.-191A>G (rs3814960), IVS1+1255C>A (rs2811708), IVS-782G>C, and c.*29C>G (500C/G, rs11515) (Figure 3.7A), and in the majority of haplotypes also at c.-3705Ex1bT>C (rs2811712) (Figure 3.7B) (Bishop, personal communications). The analysis of Latvian melanoma patients presented here and the study of Debniak et al., 2005b suggest that the T allele is associated with an increased risk of melanoma in Latvia and Poland, which was not seen in an English study. Therefore, we sought to determine whether the A148T haplotype in Latvia and Poland is the same as the predominant haplotype in England.

Among all of the samples, predominantly, (Table 3.10) individuals do carry the minor allele at the tested loci with the occasional exception at c.-3705Ex1bT>C (rs2811712), which is the most distant from A148T. Among the Latvian samples, the only exception with consistency with England population was for M150 for which individual only carry the common allele at c.-493A>T, IVS1+1255C>A (rs2811708), and IVS-782G>C (Figure 3.7C). The Polish melanoma samples are also all entirely consistent with this perspective. Among the Polish controls again all the samples appear to have the England A148T haplotype except for sample 54068 whose allele distribution matches that of the Latvian sample M150 and carries the common allele at c.-493A>T, IVS1+1255C>A (rs2811708), and IVS-782G>C (Figure 3.7C). The predominant impression, however, is that the A148T haplotypes in Latvia and in Poland are the same as that in England.

Table 3.10. *Results of CDKN2A SNP analyses*

Sample	c.-3705Ex1BC>T	c.-493A>T	c.-191A>G	IVS+1255C>A	IVS1-782G>C	c.442AG>A (A148T)	c. '29C>G
	rs2811712		rs3814960	rs2811708		rs3731249	rs11515
Latvian Melanoma							
M8	T/T	A/T	A/G	C/A	G/C	G/A	C/G
M17	T/C	A/T	A/G	A/A	G/C	G/A	C/G
M19	T/C	A/T	A/G	C/A	G/C	G/A	C/G
M38	C/C	A/T	A/G	C/A	G/C	G/A	C/G
M78	T/C	A/T	A/G	A/A	G/C	G/A	C/G
M110	T/T	A/T	A/G	C/A	G/C	G/A	C/G
M150	T/T	A/A	A/G	C/C	G/G	G/A	C/G
M165	T/C	A/T	G/G	A/A	C/C	G/A	G/G
M215	T/T	A/T	A/G	C/A	C/C	G/A	C/G
M217	C/C	A/T	A/G	C/A	G/C	G/A	C/G
Polish Melanoma							
22387	T/C	A/T	A/G	C/A	G/C	G/A	C/G
58725	T/C	A/T	G/G	A/A	C/C	G/A	G/G
58726	T/C	NR	A/G	C/A	G/C	G/A	C/G
58742	T/T	A/T	G/G	A/A	G/C	G/A	G/G
69898	NR	A/T	G/G	A/A	C/C	G/A	G/G
69899	C/C	A/T	G/G	A/A	C/C	G/A	G/G
69911	T/C	A/T	G/G	A/A	C/C	G/A	G/G
70090	T/T	A/T	A/G	C/A	G/C	G/A	C/G
70096	T/C	A/T	A/G	C/A	G/C	G/A	C/G
70098	T/C	A/T	G/G	A/A	G/C	G/A	C/G
70231	T/C	A/T	G/G	C/A	C/C	G/A	G/G
71836	T/C	A/T	G/G	C/A	G/C	G/A	C/G
71845	T/C	A/T	G/G	A/A	G/C	G/A	C/G
71926	T/C	A/T	A/G	C/A	G/C	G/A	C/G
72616	T/C	A/T	A/G	C/A	G/C	G/A	C/G
96369a	T/C	A/T	G/G	A/A	G/C	G/A	C/G
96369b	T/C	A/T	G/G	A/A	C/C	G/A	G/G
96372	T/T	A/T	G/G	C/A	G/C	G/A	C/G
96381	T/C	A/T	G/G	C/A	G/C	G/A	C/G
Polish Controls							
48	T/C	A/T	A/G	C/A	G/C	G/A	C/G
54	T/C	A/T	G/G	C/A	G/C	G/A	C/G
87	T/C	A/T	A/G	C/A	G/C	G/A	C/G
297	T/C	A/T	G/G	A/A	G/C	G/A	C/G
321	C/C	A/T	G/G	C/A	G/C	G/A	G/G
366	T/T	A/T	G/G	C/A	G/C	G/A	C/G
430	C/C	A/T	G/G	A/A	C/C	G/A	G/G
562	T/T	A/T	A/G	C/A	G/C	G/A	C/G
572	T/T	A/T	G/G	A/A	G/C	G/A	C/G
2289	T/C	A/T	G/G	C/A	G/C	G/A	C/G
2710	T/T	A/T	A/G	C/A	G/C	G/A	C/G
2880	T/C	A/T	A/G	C/A	G/C	G/A	C/G
54068	T/T	A/A	A/A	C/C	G/G	G/A	C/C
54353	T/C	A/T	G/G	C/A	G/C	G/A	C/G
58719	T/C	A/T	A/G	C/A	G/C	G/A	C/G
58884	T/T	A/T	A/G	C/A	G/C	G/A	C/G
62705	T/C	A/T	A/G	C/A	G/C	G/A	C/G
63331	T/C	A/T	A/G	C/A	G/C	G/A	C/G
77857	C/C	A/T	G/G	A/A	C/C	G/A	G/G

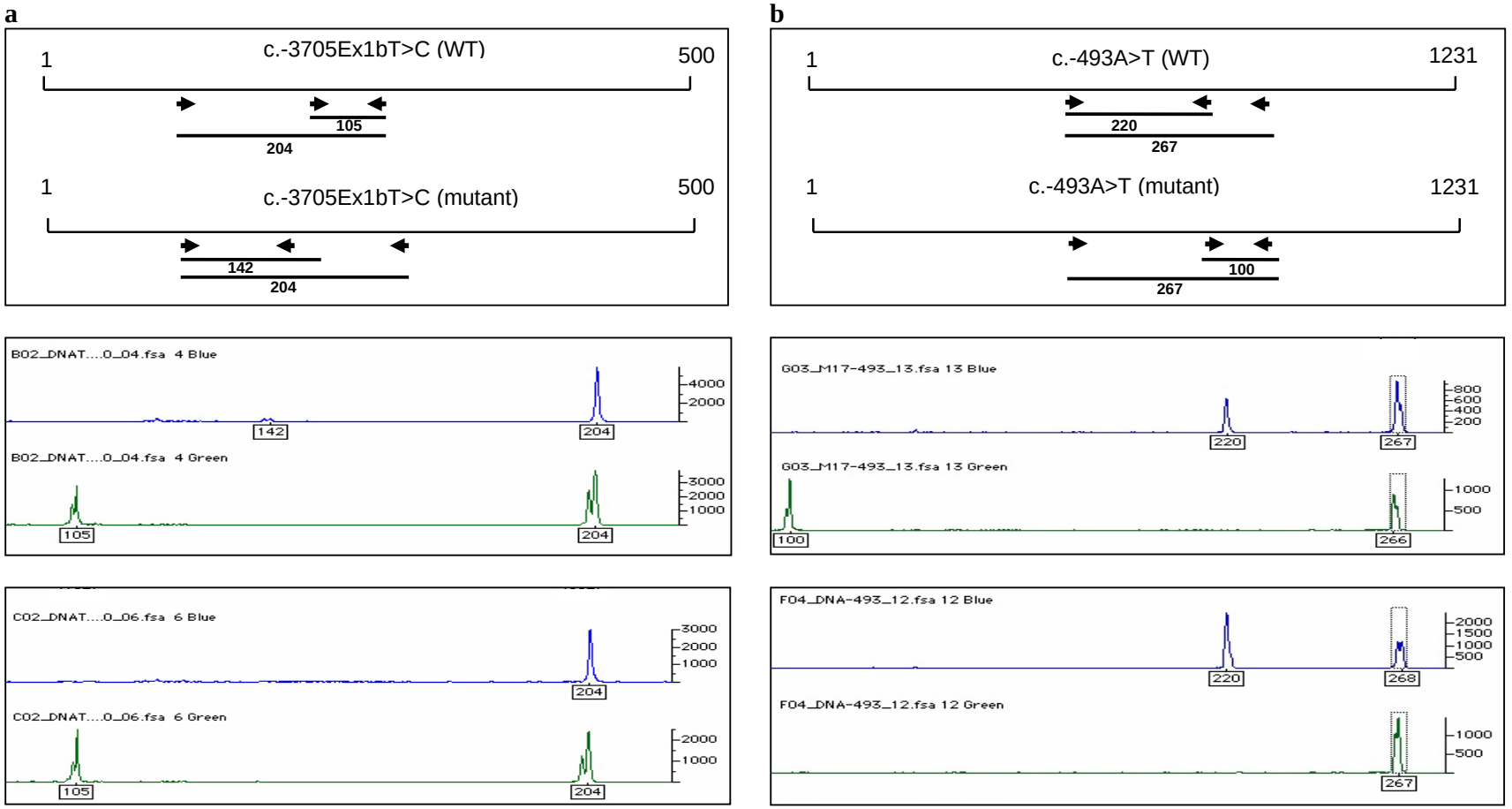


Figure 3.6. *Allele-specific PCR. Upper panel always schematic representation of test design; middle and lower panel's representative examples of analyses done:* ***(a)****, c.-3705Ex1bT>C;* ***(b)****, c.-493A>T. The figure is continued on the next page.*

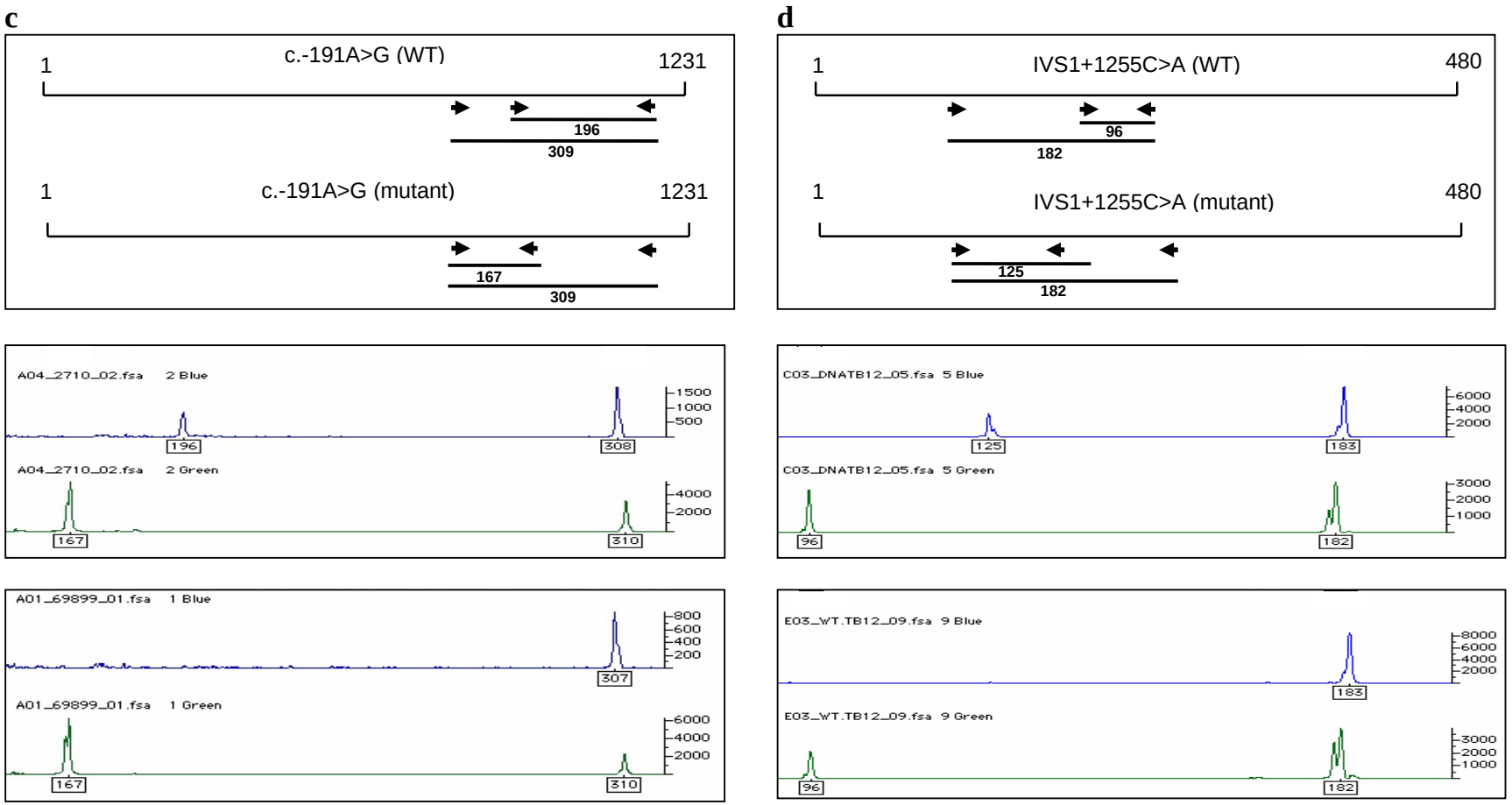


Figure 3.6. *(continuation). Allele-specific PCR. Upper panel always schematic representation of test design; middle and lower panels representative examples of analyses done:* ***(c)****, c.-191A>G;* ***(d)****, IVS1+1255C>A. The figure is continued on the next page.*

e

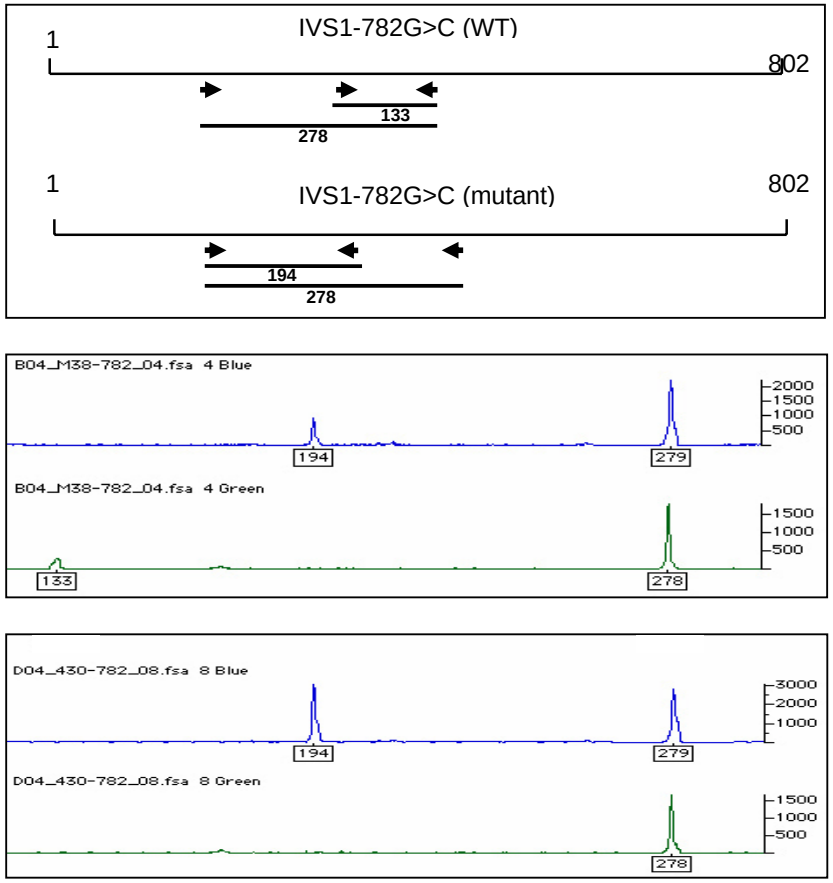


Figure 3.6*. (continuation). Allele-specific PCR. Upper panel always schematic representation of test design; middle and lower panels representative examples of analyses done:* ***(e)****, IVS1-782G>C.*

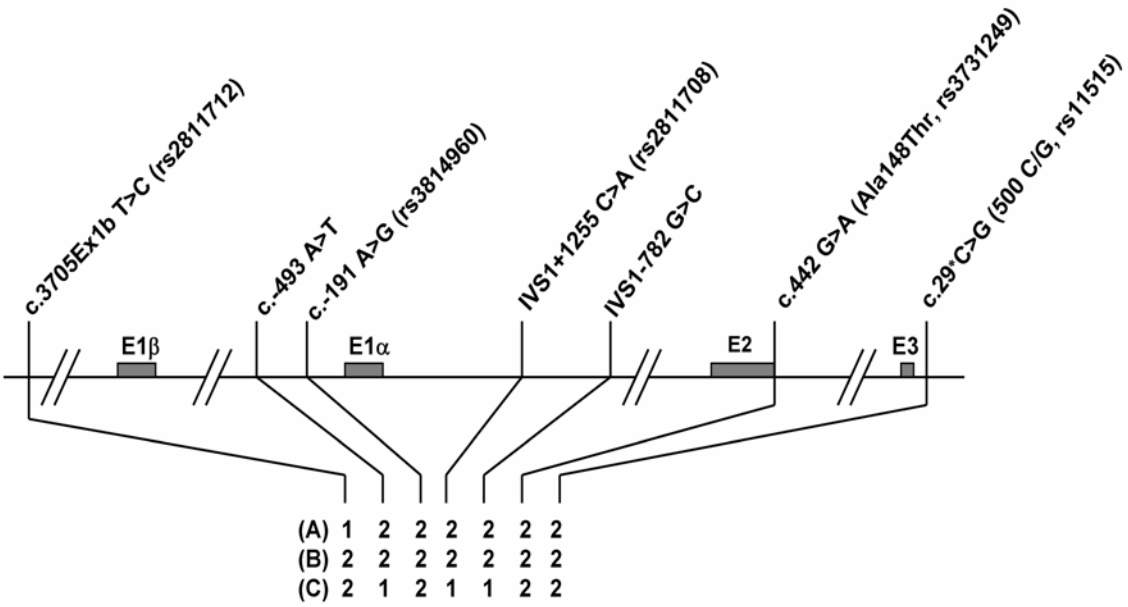


Figure 3.7*. Distribution of CDKN2A SNPs analysed in A148T carriers:* ***(A)*** *and* ***(B)*** *common haplotypes in English and Latvian/Polish populations;* ***(C)*** *rare haplotype found in Latvian/Polish population; 1, major (common) allele; 2, minor (rare) allele.*

3.2.3. *CDKN2A* common variants association with melanoma prognostic factors

Taking into account published estimations of the role for *CDKN2A* 3' UTR polymorphisms in melanoma (Sauroja et al., 2000, Straume et al., 2002) and other tumours, particularly bladder cancer (Sakano et al., 2003), invasiveness and progression, and in order to define the relation of *CDKN2A* polymorphisms to the clinical course of melanomas, the main histopathological features of melanoma tumours removed from patients with polymorphisms were compared to those from patient without polymorphisms. No statistically significant differences were found between variables analysed (Table 3.11). The distribution of polymorphisms between patients with different clinical stages of malignant melanoma also seems to be random with no statistically significant differences between groups (Figure 3.8).

Table 3.11. *Comparison of the CDKN2A polymorphism (%) frequencies between melanoma patients with different histopathological features of their tumours*[a]

	Histopathological features of tumours from patients analysed											
CDKN2A polymorphisms	Clark I-III n=87	Clark IV-V n=61	χ^2 test (p)	Breslow ≤1.5mm n=29	Breslow >1.5mm n=84	χ^2 test (p)	Ulceration absent n=36	Ulceration present n=58	χ^2 test (p)	Pigment absent n=11	Pigment present n=131	χ^2 test (p)
A148T (c.442 G>A)												
GG genotype	79 (91 %)	59 (97 %)	0.16[b]	29 (100 %)	78 (93 %)	0.12[b]	35 (97 %)	54 (93 %)	0.39[b]	9 (82 %)	122 (93 %)	0.18[b]
GA genotype	8 (9 %)	2 (3 %)	0.17[c]	0 (0 %)	6 (7 %)	0.14[c]	1 (3 %)	4 (7 %)	0.39[c]	2 (18 %)	9 (7 %)	0.19[c]
Variant allele (A) frequency	8/174 (5 %)	2/122 (2 %)		0/58 (0 %)	6/168 (4 %)		1/72 (1 %)	4/116 (3 %)		2/22 (9 %)	9/262 (3 %)	
c.*29C>G												
CC genotype	69 (79 %)	50 (82 %)	0.69[b]	24 (83 %)	66 (79 %)	0.63[b]	31 (86 %)	45 (78 %)	0.31[b]	8 (73 %)	106 (81 %)	0.51[b]
CG+GG genotype	18 (21 %)	11 (18 %)	0.94[c]	5 (17 %)	18 (21 %)	0.49[c]	5 (14 %)	13 (22 %)	0.2[c]	3 (27 %)	25 (19 %)	0.67[c]
Variant allele (G) frequency	19/174 (11 %)	13/122 (11 %)		5/58 (9 %)	20/168 (12 %)		5/72 (7 %)	15/116 (13 %)		3/22 (14 %)	28/262 (11 %)	
c.*69C>T												
CC genotype	74 (85 %)	49 (80 %)	0.45[b]	25 (86 %)	70 (83 %)	0.72[b]	29 (81 %)	48 (83 %)	0.79[b]	10 (91 %)	106 (81 %)	0.41[b]
CT genotype	13 (15 %)	12 (20 %)	0.47[c]	4 (14 %)	14 (17 %)	0.47[c]	7 (19 %)	10 (17 %)	0.8[c]	1 (9 %)	25 (19 %)	0.44[c]
Variant allele (T) frequency	13/174 (7 %)	12/122 (10 %)		4/58 (7 %)	14/168 (8 %)		7/72 (10 %)	10/116 (9 %)		1/22 (5 %)	25/262 (10 %)	

[a] *- in the analysis were included only patients with known histopathological features of their tumours;* [b] *- p value for polymorphisms frequencies;* [c] *- p vale for allele frequencies.*

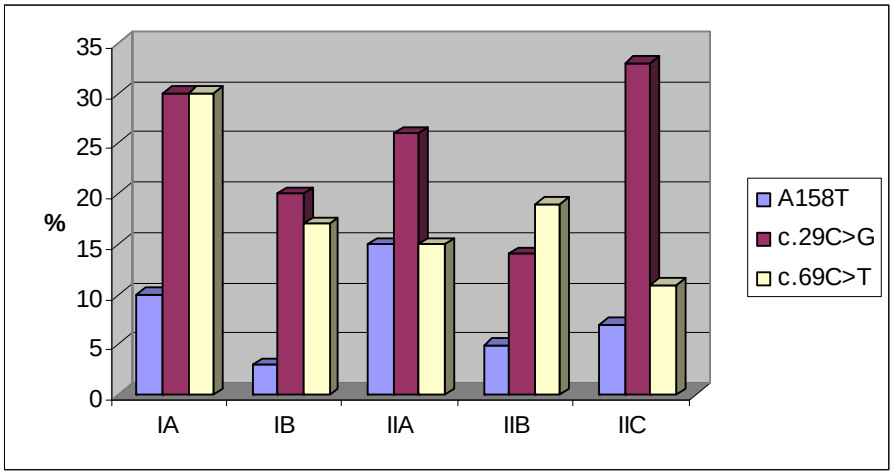


Figure 3.8. *CDKN2A polymorphisms distribution in melanoma patients with different clinical stages of melanoma. Stage III and IV patients were not included in the analyses because of the small number of patients available for the analyses (staging explanation see in table 1.3).*

3.2.4. *CDKN2A* common variants association with cancer risk

On the basis of observation that 63 out of 176 (36 %) (Table 3.3) melanoma patients analysed had additional coexisting cancer in their family history and 50 of them (29%) had additional cancer in the first-degree relatives, and 16 patients (9%) had more than one relative with cancer, the association between polymorphisms and cancers of any type in the family was analysed. More frequently observed cancers in the family histories of polymorphism carriers were: breast (8 cases), gastric (4 cases) uterus (4 cases), lung (3 cases), and brain (2 cases). Single cases of leucosis, leukaemia, kidney, colon, pancreas, skin, and liver cancers were observed as well. Familial melanoma cases were excluded from this evaluation, because of suggested predisposition to melanoma.

Although polymorphisms were more frequently observed in melanoma patients with cancers in their family history, except c.*69C>T polymorphism, which was more common in melanoma patients without cancers in the family (Table 3.12), the analysis revealed again no statistically significant differences in polymorphisms frequencies between melanoma patients with and without cancers in their family history. There were also no correlation between polymorphisms and cancer number in the genealogy (Table 3.13).

There was also a group of melanoma patients, who in addition to melanoma, had unrelated additional primary malignant tumour (n=15) or benign neoplasm (n=17), developing prior to, concurrently with, or after the diagnosis of melanoma (Table 3.4). Although all three *CDKN2A* common polymorphisms were also found between these patients, no association between polymorphisms and additional neoplasm in the same individual was noted.

Table 3.12. *Comparison of the CDKN2A polymorphism frequencies (%) between melanoma patients with and without cancers in their family history*

CDKN2A polymorphisms	Melanoma patients with cancers in their family history (n=63)	Melanoma patients without cancers in their family history (n=66)	p value (two-sided Fisher's exact)
A148T (c.442 G>A)			
GG genotype	57 (90 %)	61 (92 %)	0.76[a]
GA genotype	6 (10 %)	5 (8 %)	0.77[b]
Variant allele (A) frequency	6/126 (5 %)	5/132 (4 %)	
c.*29C>G			
CC genotype	46 (73 %)	56 (85 %)	0.13[a]
CG+GG genotype	17 (27 %)	10 (15 %)	0.44[b]
Variant allele (G) frequency	17/126 (13 %)	13/132 (10 %)	
c.*69C>T			
CC genotype	54 (86 %)	53 (80 %)	0.49[a]
CT genotype	9 (14 %)	13 (20 %)	0.51[b]
Variant allele (T) frequency	9/126 (7 %)	13/132 (10 %)	

[a] *- p- value for the differences in polymorphisms frequencies between melanoma patients with and without cancers in their family history;*
[b] *- p- value for the differences in variant allele frequencies between melanoma patients with and without cancers in their family history.*

Table 3.13. *CDKN2A polymorphism frequencies (%) in melanoma patients with different number of cancers in their family history*[a]

CDKN2A polymorphisms	1 additional cancer in the family (n = 47)	2 additional cancers in the family (n = 12)	3 and more additional cancers in the family (n = 4)
A148T (c.442 G>A)			
GG genotype	41 (87%)	12 (100%)	4 (100%)
GA genotype	6 (13%)	0 (0%)	0 (0%)
Variant allele (A) frequency	6/94 (6%)	0/24 (0%)	0/8 (0%)
c.*29C>G			
CC genotype	32 (68%)	11 (91%)	3 (75%)
CG+GG genotype	15 (32%)	1 (9%)	1 (25%)
Variant allele (G) frequency	15/94 (16%)	1/24 (4%)	1/8 (13%)
c.*69C>T			
CC genotype	39 (83%)	11 (91%)	4 (100%)
CT genotype	8 (17%)	1 (9%)	0 (0%)
Variant allele (T) frequency	8/94 (9%)	1/24 (4%)	0/8 (0%)

[a] *- statistical evaluation of the differences between groups was not done because the number of samples in each group was small.*

In order to show or straight deny the association of polymorphisms with malignancy of any type, the genotyping was extended to an additional DNA samples from unselected cancer patients with breast (72 patients), ovarian (59 patients), gastric (48 patients), colon (47 patients), and rectum (26 patients) cancers. The A148T polymorphism was observed more frequently in patients from all cancer groups, however the difference from the control group was small and statistically insignificant (Figure 3.7). Additional larger studies are required to determine whether this particular change can be associated with an increased risk of malignancy. The frequencies of c.*29C>G and c.*69C>T polymorphisms varies between different groups of cancer patient but these frequencies were not statistically significant neither between different cancer patients groups nor between cancer patients and control group (Figure 3.7).

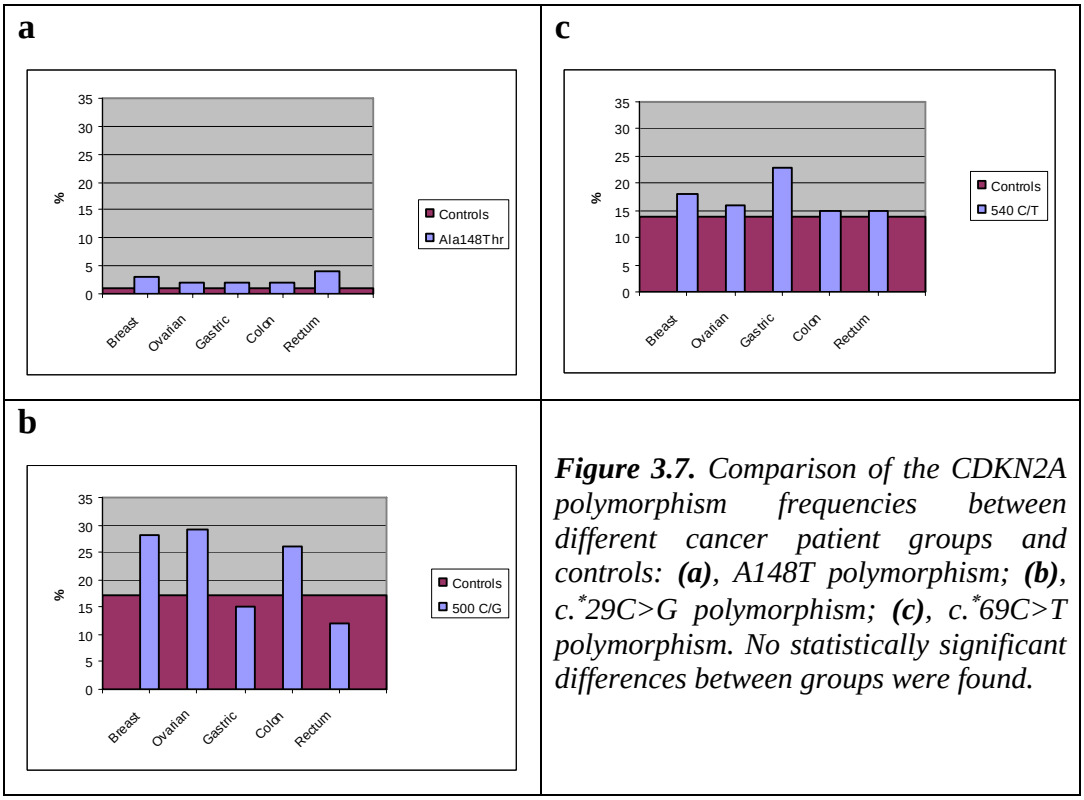


Figure 3.7. *Comparison of the CDKN2A polymorphism frequencies between different cancer patient groups and controls:* ***(a)****, A148T polymorphism;* ***(b)****, c.*29C>G polymorphism;* ***(c)****, c.*69C>T polymorphism. No statistically significant differences between groups were found.*

3.3. Analyses of the *CDKN2A* gene in individuals with atypical nevi and skin neoplasms other than melanoma

The *CDKN2A* germline mutation analysis of 14 individuals with atypical nevi and 13 individuals with skin neoplasms other than melanoma was performed using the combination of the SSCP and sequencing. Only the previously described *CDKN2A* polymorphisms, A148T, c.*29C>G, and c.*69C>T, were detected in these individuals with frequencies similar to other groups analysed (Table 3.14).

Table 3.14*. CDKN2A polymorphism frequencies (%) in individuals with atypical nevi and other skin neoplasms than melanoma*

CDKN2A polymorphisms	Individuals with atypical nevi n=14	Individuals with other skin neoplasms than melanoma n=13
A148T (c.442 G>A)		
GG genotype	14 (100%)	12 (92%)
GA genotype	0 (0%)	1 (8%)
Variant allele (A) frequency	0 (0%)	1 (4%)
c.*29C>G		
CC genotype	11 (79%)	11 (85%)
CG+GG genotype	3 (21%)	2 (15%)
Variant allele (G) frequency	3 (11%)	2 (8%)
c.*69C>T		
CC genotype	12 (86%)	12 (92%)
CT genotype	2 (14%)	1 (8%)
Variant allele (T) frequency	2 (7%)	1 (4%)

3.4. Familial melanoma patients analysis

Familial melanoma patients were analysed for *CDKN2A*, *p14ARF*, *CDK4* exon 2 germline mutations, germline deletions at *9p21*, and *MC1R* variants as well depending on the DNA availability. Altogether six familial melanoma patients were mutation tested (Table 3.15). Testing of *CDKN2A*, *p14ARF*, and *CDK4* exon 2 in four of these patients was described previously in this work (Chapter 3.1) using either SSCP and sequencing (M66, M162, M199) or high-resolution melting curve analysis and sequencing (M66, M199, M247), and *CDK4* exon 2 mutation Arg24His was detected in the patient with the strongest family history of melanoma (M247) (Table 3.15 and Figure 3.4). Two additional familial melanoma patients (M260, M268) were analysed for *CDKN2A*, *p14ARF*, and *CDK4* exon 2 germline mutations using direct DNA sequencing. In one of these patients (M268), the same *CDK4* exon 2 germline mutation Arg24His was identified (Table 3.15). The close relationship between these two familial melanoma patients and wherewith families were excluded by the paternity test which includes 15 different loci (Table 3.16). No other mutations were detected.

Three common previously found *CDKN2A* polymorphisms were identified in the analysis of familial melanoma patients (Table 3.15). A148T was found to be present in one patient and c.*29C>G and c.*69C>T polymorphisms both were identified in two of the patients analysed.

In the family M260 it was also possible to test patient's relative with atypical mole. Mutations were found neither in *CDKN2A*, *p14ARF* nor *CDK4* exon 2. This individual does not also have *CDKN2A* polymorphisms detected in melanoma patient from this family.

Four familial melanoma patients (Table 3.15) were also analysed for hemizygous and homozygous germline deletions at chromosome arm *9p* using multiplex ligation-dependent probe amplification (MLPA). This quantitave technique enables fine-scale mapping at the *CDKN2A* locus and allows other flanking genes to be screened (23 sites of interest on *9p* in total). The MLPA assay was robust and reliable in samples analysed, as seen by peak heights

across the multiplex (Figure 3.8) and standard deviation in dosage quotients (Table 3.17). No deletions were observed for any *9p* probes in samples analysed (Table 3.17).

In addition, where possible, *MC1R* variants were analysed in familial melanoma patients and those found in two of them (Table 3.15). The *MC1R* variant, R160W, detected in the patient M260 was also found in its relative with atypical mole.

Table 3.15. *Melanoma families included in the study and the results of genes testing*

Kindred	**Number of cases**[a]	**Cases with multiple primaries**	***CDKN2A***	***ARF***	***CDK4* exon 2**	***9p* germline deletion**	***MC1R variants***
M 247	5 (3)[b]	Yes	*c.*29C>G*	WT	R24H	-	WT
M268	5 (1)	Yes	WT	WT	R24H	nd	R160W
M 66	2 (1)	nd	WT	WT	WT	-	nd
M 162	2 (1)	nd	*c.*69C>T*	WT	WT	-	nd
M 199	2 (1)	nd	*c.*69C>T*	WT	WT	-	nd
M260	1 (1)[c]	No	*A148T* *c.*29C>G*	WT	WT	nd	R160W

[a] *- numbers in parentheses indicate the number of samples from cases analysed;* [b] *– in addition to proband, archival paraffin-embedded melanoma tissues from two deceased melanoma patients were analysed;* [c] *– in this family only one individual was affected with melanoma, but have relative with atypical mole and therefore was included in the analysis; WT – wild type; nd – no data; CDKN2A polymorphisms are indicated in italics.*

Table 3.16. *Paternity tests done using AmpF STR Identifier (Applied Biosystems, UK)*[a]

Locus	**M247**	**M268**
D8S1179	13/14	12/12
D21S11	29/30.2	30/32.2
D7S820	12/13	9/10
CSF1PO	10/11	9/11
D3S1358	16/17	15/16
THO1	6/9.3	7/9.3
D13S317	8/11	11/12
D16S539	11/13	11/14
D2S1338	17/25	17/23
D19S433	13/13	12/14
vWA	16/18	14/15
TPOX	8/8	11/11
D18S51	16/20	15/15
Amel	x/x	x/x
D5S818	11/11	12/13
FGA	20/26	23/23

[a] *- markers analysed are listed and the results are presented as allele sizes for the markers tested.*

Table 3.17. *MPLA gene dosage quotients of patients from Latvian melanoma-prone families*

	Probe[a] number	Probe	Fragment lenght (bp)	Controls				Patients							
				WT		Heterozygous deletion		M66		M162		M199		M247	
				DQ	SD	DQ	SD	DQ	SD	DQ	SD	DQ	SD	DQ	SD
Telomeric Centromeric	1	*TEK*	436	1.10	0.07	1.19	0.17	1.10	0.10	0.85	0.10	1.49	0.26	1.36	0.16
	2	*ELAVL2*	418	1.16	0.07	1.00	0.14	1.13	0.10	0.91	0.11	1.27	0.22	1.07	0.13
	3	***CDKN2B* promoter**	**202**	**1.11**	**0.07**	**0.93**	**0.13**	**0.99**	**0.09**	**1.14**	**0.14**	**1.09**	**0.19**	**0.91**	**0.11**
	4	***CDKN2B* exon 1**	**211**	**0.96**	**0.06**	**0.95**	**0.13**	**0.96**	**0.09**	**1.17**	**0.14**	**1.01**	**0.18**	**0.87**	**0.10**
	5	***CDKN2B* intron**	**454**	**1.05**	**0.07**	**1.44**	**0.20**	**1.22**	**0.11**	**0.91**	**0.11**	**1.45**	**0.25**	**1.04**	**0.12**
	6	***CDKN2A* 1β CpG island**	**265**	**0.98**	**0.06**	**0.83**	**0.12**	**0.93**	**0.09**	**1.03**	**0.12**	**0.96**	**0.17**	**1.04**	**0.12**
	7	***CDKN2A* 1β promoter**	**157**	**1.03**	**0.06**	**0.49**	**0.07**	**1.03**	**0.10**	**1.15**	**0.14**	**1.00**	**0.17**	**0.96**	**0.11**
	8	***CDKN2A* exon 1β**	**427**	**1.07**	**0.07**	**0.49**	**0.07**	**1.22**	**0.11**	**0.84**	**0.10**	**1.03**	**0.18**	**1.22**	**0.14**
	9	***CDKN2A* intron**	**148**	**1.13**	**0.06**	**0.68**	**0.15**	**1.05**	**0.09**	**1.12**	**0.12**	**1.02**	**0.20**	**1.08**	**0.12**
	10	***CDKN2A* intron**	**355**	**0.98**	**0.07**	**0.53**	**0.10**	**1.02**	**0.10**	**1.15**	**0.13**	**0.97**	**0.18**	**0.95**	**0.13**
	11	***CDKN2A* 1α promoter**	**391**	**1.04**	**0.06**	**1.29**	**0.18**	**1.18**	**0.11**	**0.81**	**0.10**	**1.28**	**0.22**	**1.08**	**0.13**
	12	***CDKN2A* exon 1**	**238**	**1.01**	**0.06**	**0.98**	**0.14**	**1.09**	**0.10**	**1.08**	**0.13**	**1.06**	**0.18**	**0.98**	**0.11**
	13	***CDKN2A* exon 2**	**256**	**0.98**	**0.06**	**0.83**	**0.12**	**0.93**	**0.09**	**1.03**	**0.12**	**0.96**	**0.17**	**1.04**	**0.12**
	14	***CDKN2A* exon 3**	**274**	**0.98**	**0.06**	**0.92**	**0.13**	**0.92**	**0.08**	**0.96**	**0.11**	**0.92**	**0.16**	**0.92**	**0.11**
	15	*MTAP* exon 7	292	1.00	0.06	0.97	0.14	0.97	0.09	1.10	0.13	1.02	0.18	1.04	0.12
	16	*MTAP* exon 6	310	1.00	0.06	0.96	0.13	1.07	0.10	0.99	0.12	0.99	0.17	0.87	0.10
	17	*MTAP* exon 1	328	1.00	0.06	0.82	0.11	0.94	0.09	0.93	0.11	0.84	0.15	1.11	0.13
	18	*KIAA*	364	1.03	0.06	1.07	0.15	0.96	0.09	0.99	0.12	1.16	0.20	1.00	0.12
	19	*IFNW1*	382	1.02	0.06	1.02	0.14	1.08	0.10	0.94	0.11	1.29	0.22	1.13	0.13
	20	*IFNB1*	400	1.10	0.07	1.12	0.16	1.09	0.10	0.91	0.11	1.21	0.21	1.05	0.12
	21	*MLLT3* exon 2	184	0.90	0.06	0.82	0.11	1.01	0.09	1.13	0.13	0.89	0.16	0.90	0.11
	22	*MLLT3* exon 8	166	0.97	0.06	0.97	0.14	1.09	0.10	1.14	0.14	1.08	0.19	0.81	0.09
	23	*FLJ00026*	319	0.98	0.06	0.82	0.12	0.98	0.09	1.07	0.13	0.93	0.16	1.06	0.12
		Mean SD			0.06		0.13		0.10		0.12		0.19		0.12

[a] *- the probes are ordered from top to bottom across 9p21, probes for CDKN2 locus are highlighted in bold; bp - base pairs; DQ - dosage quotients; values close to 1 indicate both copies of the fragment present and close to 0.5 loss of one copy of the fragment; SD - standard deviation, used for quality control; only results with SD ≤ 0.2 were taken into account; WT - wild type control; DNA used as heterozygous control was derived from melanoma family with a heterozygous deletion at CDKN2A 1β (highlighted red) described by Randerson-Moor et al., 2001.*

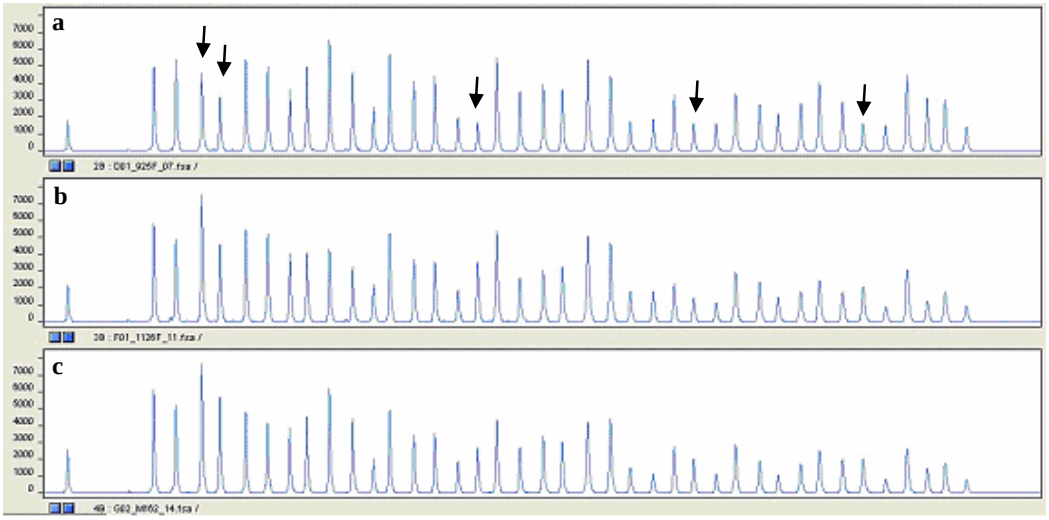


Figure 3.8. *GeneScan traces of MPLA products carried out on an automated capillary sequencer (ABI3100, Applied Biosystems, UK):* ***(a)****, heterozygous deletion at CDKN2A exon 1β (control) previously reported in an English melanoma family by Randerson-Moor et al., 2001; arrows indicate the fragments delete;* ***(b)****, wild type control;* ***(c)****, representative sample of familial melanoma patient from Latvia (wild type).*

3.4.1. Segregation analysis of the R24H mutation in *CDK4* mutation positive families

The segregation analysis of the R24H mutation in both families was limited by the availability of melanoma cases (Figure 3.9). However, in the kindred M247 (Figure 3.9a), it was possible to analyse paraffin-embedded melanoma tissues from two deceased patients with melanoma (individuals II8 and III10) and three healthy family members (individuals III11, III20, and IV26). In this kindred, malignant melanoma appeared to be inherited as an autosomal dominant trait and the occurrence of multiple primary melanomas was noted (Figure 3.9a). The proband (individual III17) presented with a cutaneous malignant melanoma at the age 42 years. Subsequently, it was noted that probands mother died at the age of 61 from malignant melanoma and two of her brothers and niece had the same diagnosis. All melanomas within this family were mainly located on patients back or face. One out of five melanomas occurred in individual less than 30 years old, two melanomas were diagnosed when the patients were between 42 and 45 years of age, and two melanomas occurred in individuals after 50 years of age. The mean age of onset for primary melanomas was 43.8 years, which means that the patients in this family develop skin melanoma at an earlier age than usually do patients with sporadic melanomas.

All three unaffected family members were analysed for *CDK4* exon 2 germline mutations and *CDKN2A*, and *p14ARF* germline mutations as well and none was found. Except, individual III11 carry both *CDKN2A* polymorphisms, A148T and c.*29C>G, and individual IV26 carry *CDKN2A* c.*29C>G polymorphism.

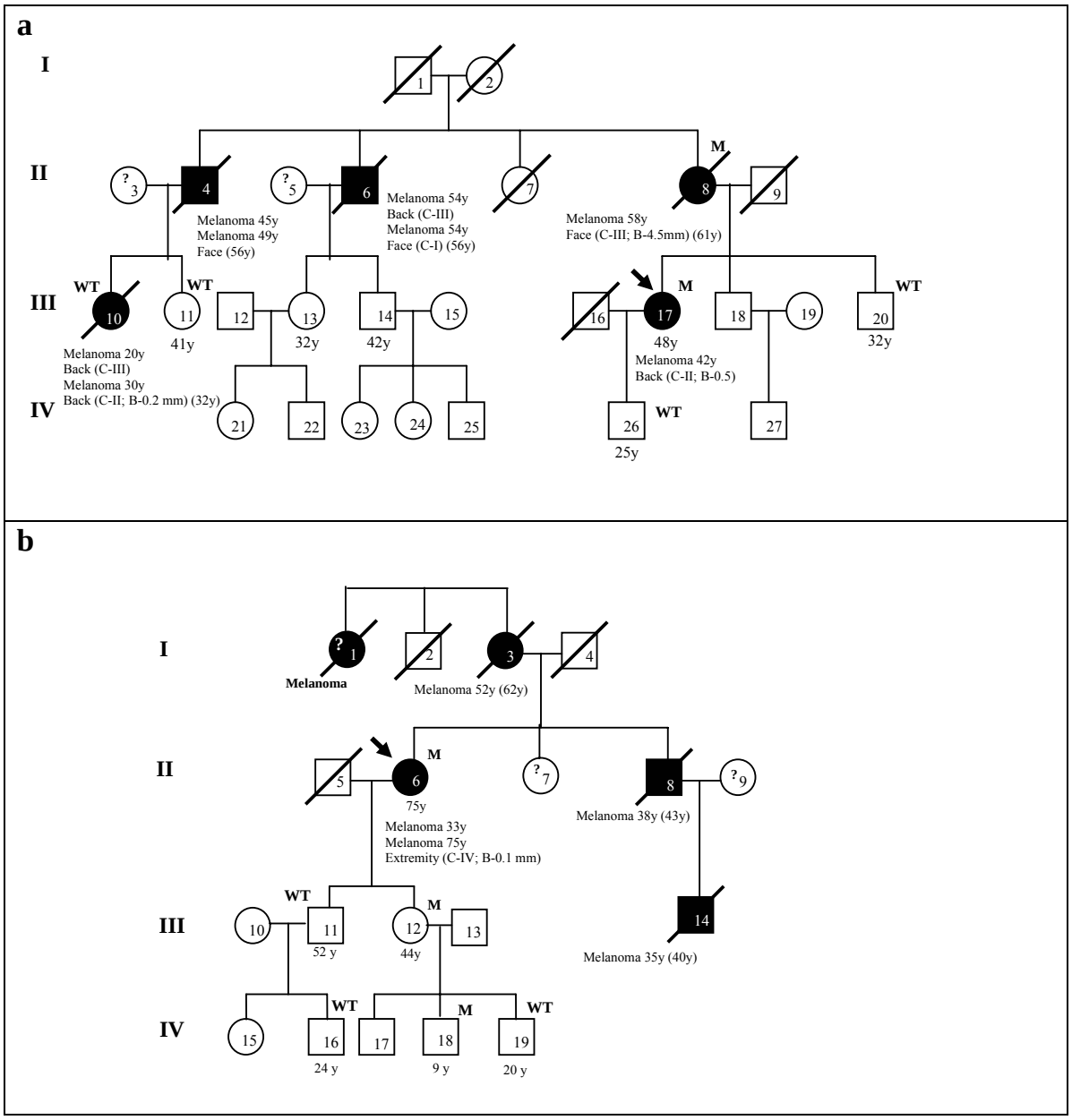


Figure 3.9. *Pedigree diagrams of the families with malignant melanoma and CDK4 codon 24 mutation (R24H): affected family members are shown by filled symbols; ages of melanoma onset, anatomic sites, and histopathological features of melanomas (C – Clark level, B – tumour Breslow thickness) are indicated; arrow denotes proband; numbers in parentheses indicate age of death and numbers bellow the symbol current age of living individuals; question marks mean – no data available; CDK4 mutation status is indicated as M (carrier of the R24H mutation) or WT (wild type sequence);* ***(a),*** *family M247;* ***(b),*** *family M268.*

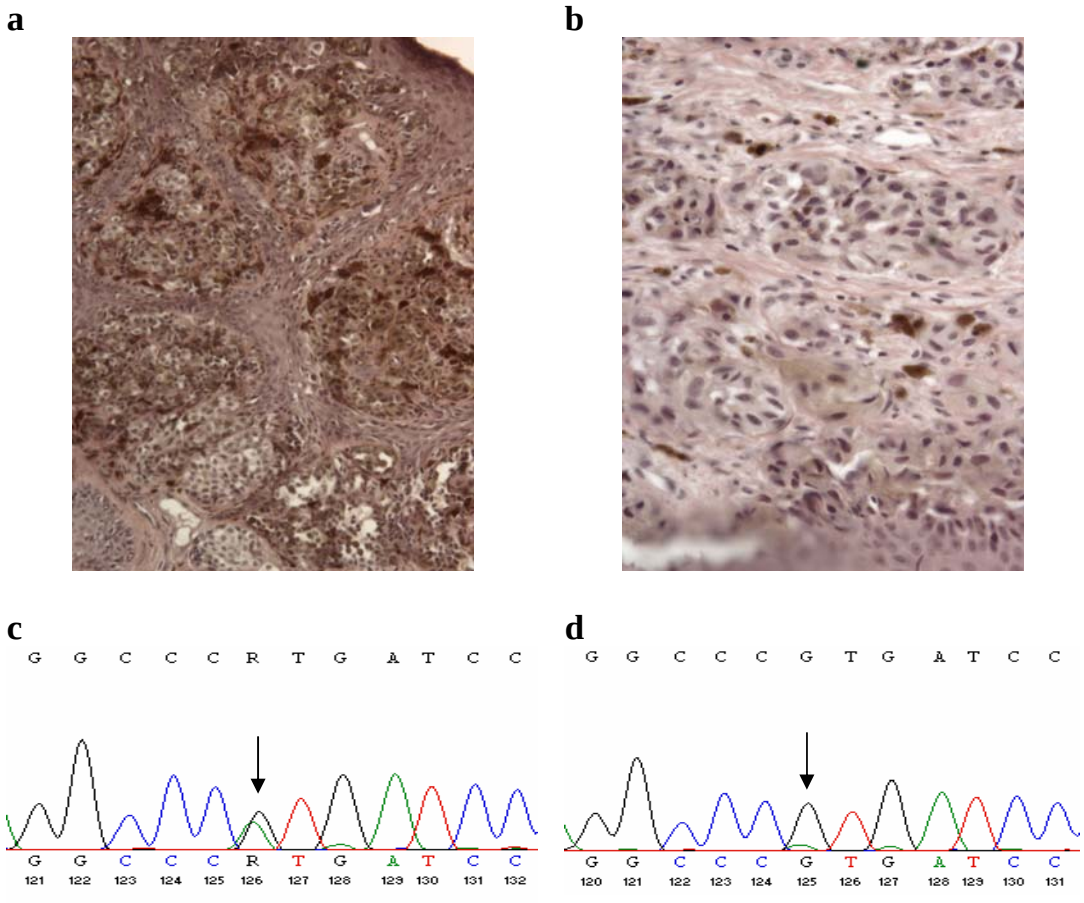


Figure 3.10. *Melanoma tissues analysis:* ***(a-b),*** *haematoxylin and eosin (H&E) stained histologic section of melanoma tissues (magnification 400x);* ***(a),*** *melanoma tissue from probands mother;* ***(b),*** *melanoma tissues from probands cousin;* ***(c),*** *chromatogram confirming the heterozygous CDK4 codon 24 mutation CGT > CAT (Arg > His) in DNA from probands mother melanoma tissue (arrow);* ***(d),*** *the sequence around CDK4 codon 24 of DNA extracted from melanoma specimen of probands cousin without the mutation (arrow).*

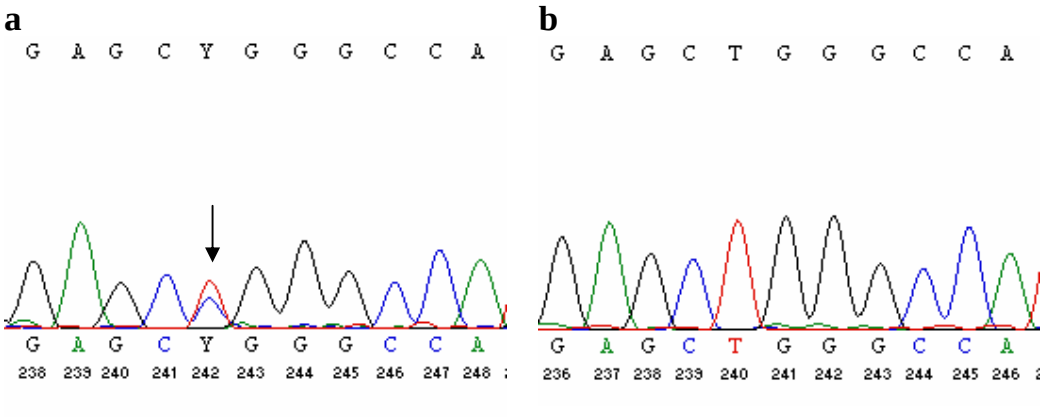


Figure 3.11. *Chromatograms showing CDKN2A codon 121 mutation CTG>CCG (c.362T>C) causing a substitution of leucine to proline at amino acid 121 (L121P):* ***(a),*** *heterozygous mutation L121L (arrow);* ***(b),*** *wild type sequence.*

Two unaffected family members (III20 and IV26) were also analysed for *MC1R* variants and those found in both of them. Individual III20 carried three different *MC1R* variants: g.464 T>C, I155T; g.478 C>T, R160W; g.942 A>G, T314T, and individual IV26 two different variants: g.274 G>A, V92M; g.942 A>G, T314T (Figure 3.12). All these variants are common *MC1R* variants with frequencies 0.28, 0.02, 0.06, and 0.31 for V92M, I155T, R160W, and T314T variants respectively (http://www.ncbi.nlm.hih.gov/SNP/).

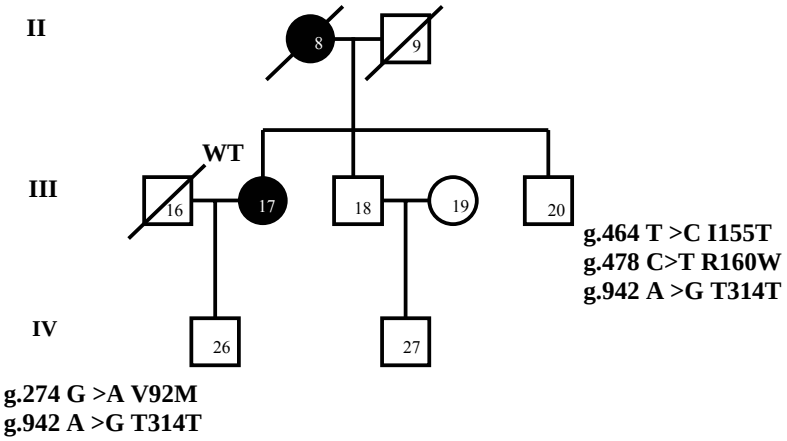


Figure 3.12. *MC1R analysis performed on family members from whom blood samples were available: the pedigree is an extract of figure 3.9a with the same numbering system as in figure 3.9; MC1R variants found were indicated next to each symbol; WT – wild type sequence.*

Phenotypic data of family members analysed were similar, all predominantly having brown hair, blue eyes, and fair skin. The presence of slightly increased total number of moles was also noted in all individuals examined with the highest mole number in individual III20 (Figure 3.9a). From individuals analysed, the presence of an atypical nevi was observed only in the proband.

In the kindred M268 (Figure 3.9b), malignant melanoma also appeared to be inherited as an autosomal dominant trait. The proband (individual II6) presented with the primary melanoma at age 33 years and the presence of increased number of moles was noted as well. In the subsequent years adenoma recti, two synchronous basaliomas cuti, atypical nevi, and second melanoma (at age 75 years) were detected during regular follow-up examination. It was also noted that probands mother, brother, aunt and nephew had the same diagnosis of cutaneous malignant melanoma. All melanomas within this family were diagnosed when the patients were between 33 and 52 years of age with the mean age of onset for primary melanomas being 39.5 years.

In addition to the proband, five healthy family members (individual III11, III12, IV16, IV18, and IV19) were analysed as well and two additional R24H mutation carriers were found – probands daughter (III12) and her son (IV18) (Figure 3.9b). At present, daughter is 44 years old and seems to be non-penetrant for the disease. Clinical examination of this individual revealed a slightly increased mole number, however, without any signs of atypia. Other unaffected carrier (IV18) is only 9 years old and may still develop melanoma since the average age of diagnosis in this family is 39.5 years. Other family members analysed were proven to be mutation non-carriers (Figure 3.9b).

Within this family, the analysis of *MC1R* gene was done in all individuals available for analysis and the segregation of *MC1R* variant R160W was noticed. All family members tested carry this variant except one who is also *CDK4* gene R24H mutation carrier (Table 3.18).

Table 3.18. *MC1R gene variants and their relationship to the CDK4 exon 2 mutation in the melanoma family M268*

Individual	Melanoma status	Age (years)	*CDK4* exon 2	*MC1R* variant
II6	Affected	75	R24H	R160W
III11	Unaffected	52	WT	R160W
III12	Unaffected	44	R24H	R160W
IV16	Unaffected	24	WT	R160W
IV18	Unaffected	9	R24H	WT
IV19	Unaffected	20	WT	R160W

Phenotypic data of family members analysed were similar, all predominantly having brown or black hair, eye colour other than blue or brown, and fair skin. There was only one exception – individual IV18 in addition to the *CDK4* R24H mutation has also blond hair fair skin and blue eyes. Similarly as in above described family, the presence of slightly increased total number of moles was noted in all individuals examined with the highest mole number in individual III11 (Figure 3.9b). However, again the presence of atypical nevi was observed only in the proband.

3.4.2. *CDK4* mutation positive family haplotype

CDK4 mutation positive families found in the present study both had the same previously reported R24H mutation (Soufir et al., 1998; Molven et al., 2005). In order to determine whether this mutation was of an ancient and common origin, in one of the reported families (M247) haplotype analysis was performed.

Five microsatellite markers surrounding the *CDK4* gene and five SNPs within or close to the transcribed region of the gene were analysed (Figure 3.13). With the current information from samples available, it was not possible to deduce the SNP haplotype for the mutation within this family. It has occurred either on the GGAT or on the TAGG haplotype. An additional marker CDK4-M7 which was originally thought to be a SNP (rs2069504) but turned out to be a tetranucleotide repeat with observed alleles of 8, 10 or 11 repeats (Molven, personal communication) also did not allow to deduce the SNP haplotype for the mutation running in the family. However concerning the microsatellite markers, it was possible to deduce that the mutated haplotype is 113-194-206-gene-174-201 for the markers tested (Figure 3.13). This haplotype does not coincide with any of the other known haplotypes reported to date (Figure 4.1).

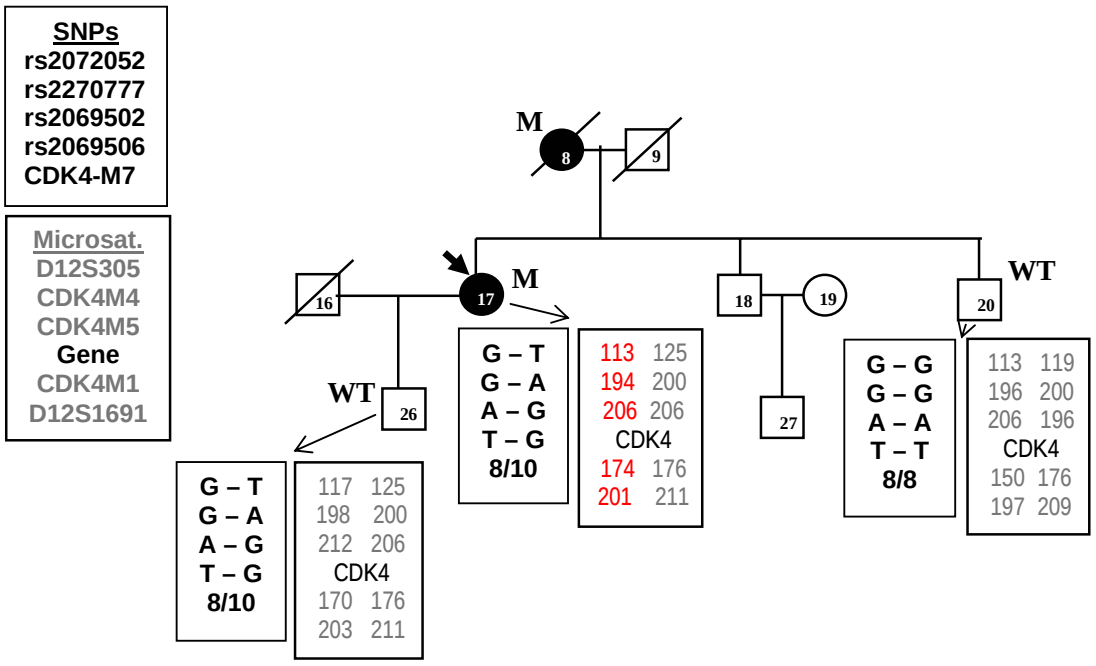


Figure 3.13. *Haplotype analysis performed on family members from whom blood samples were available: the pedigree is an extract of figure 3.9a with the same numbering system as in figure 3.9a; in boxes (SNPs – black characters, mirosatellites – grey numbers) are shown results of haplotype analysis; numbers represent allele sizes for microsatellite markers; the deduced microsatellite haplotype of the allele carrying the mutation is shown in red colour; SNP haplotype was not possible to deduce from the presented results.*

3.5. Analysis of *CDKN2A* gene in primary melanoma tissues

To clarify the role of *CDKN2A* gene in primary melanoma development, 27 primary melanoma tumours from sporadic (n=26) and familial melanoma patients (n=1) were analysed for mutations in the gene, studied for inactivation of the gene by promoter methylation and analysed for LOH at the *CDKN2A* locus. As shown in Table 3.19, most of the tumours (n=23) were thick, i.e. exceeding 1.5 mm in thickness with average Breslow thickness 8.56 mm and Clark invasion level III or more.

3.5.1. *CDKN2A* mutations in primary tumours

All 27 primary melanomas were analysed for *CDKN2A* mutations using direct sequencing. No disease-related mutations were detected in the *CDKN2A* gene in tumours analysed. Three common polymorphisms, A148T, c.*29C>G, and c.*69C>T, found also in melanoma patients blood samples and control individuals as described previously, were identified in this study. The A148T polymorphism was found to be present in two tumour samples (T4 and T25) from 27 (7.4 %) (Table 3.19). A blood sample was available from only one patient of the two A148T carriers and also presented the variant, indicating that it did not originate in the tumour. Seven tumour samples (26.0 %) presented the c.*29C>G and six tumour samples (22.2 %) the c.*69C>T polymorphisms (Table 3.19). In all cases samples

were heterozygous for the variant except one sample (T9), which was homozygous for the c.*29C>G change (genotype GG) (Table 3.19). For this tumour sample the corresponding blood sample was not available, so it is not possible to comment whether the sample is homozygous for this change or there is one allele lost.

Table 3.19*. Characteristics and sequencing results of primary melanomas analysed*

Nr.	Code	Breslow thickness (mm)	Clark level	Ulceration	Pigment	Mts	Blood sample	*CDKN2A* variant
1	T2	10	V	nd	+	-		
2	T3	8	IV	nd	-	+	+	*c.*29C>G*
3	T4	nd	nd	nd	-	+	+	*A148T; c.*29C>G*
4	T5	1.5	III	+	+	-		*c.*69C>T*
5	T6	10	V	+	+	+		*c.*69C>T*
6	T7	8	IV	+	+	+	+	
7	T8	10	V	+	+	-		
8	T9	0.5	V	+	+	-	+	*c.*29C>G*[a]
9	T10	40	V	+	-	+		*c.*69C>T*
10	T11	2	III	+	+	-	+	
11	T13	8	V	+	+	-		*c.*69C>T*
12	T17	0.8	IV	+	+	+		*c.*29C>G*
13	T18	5	IV	+	+	-		
14	T21	8	V	nd	-	-		
15	T22	3	V	nd	+	+		
16	T23	30	V	-	nd	+		
17	T24	5	V	nd	+	-	+	*c.*69C>T*
18	T25	3.5	III	+	+	-		*A148T; c.*29C>G*
19	T28	2	III	nd	+	-	+	
20	T29	20	IV	nd	+	+		*c.*29C>G*
21	T34	0.3	I	-	+	-	+	
22	T35	20	III	+	+	-		*c.*29C>G*
23	T36	2	III	-	+	-	+	*c.*69C>T*
24	T37	6	III	-	-	-	+	
25	T39	10	IV	+	+	-		
26	T41	5	III	+	-	-		
27	T42	4	IV	+	+	-		

[a] *– homozygous for c.*29C>G change (genotype GG); "+" - feature present, blood sample available; "-"- feature absent; Mts – metastases; NM – nodular melanoma; nd – no data.*

3.5.2. *CDKN2A* methylation studies

Methylation of the *CDKN2A* promoter region was assessed by methylation-specific PCR (MSP) using methylated (M), unmethylated (U) and wild type (W) specific primers. Cases not amplifying with any of the primers were regarded to have insufficient quality of DNA. As the result, nineteen primary tumours showed amplification with the U- and/or M-primers. Seventeen tumours (90%) were unmetilated, one tumour (5%) showed bands with both the U- and M-primers, and one tumour (5%) had positive M-bands only (Figure 3.14a). In addition the methylation status of tumour with positive M-band was confirmed by sequencing (Figure 3.14b; c).

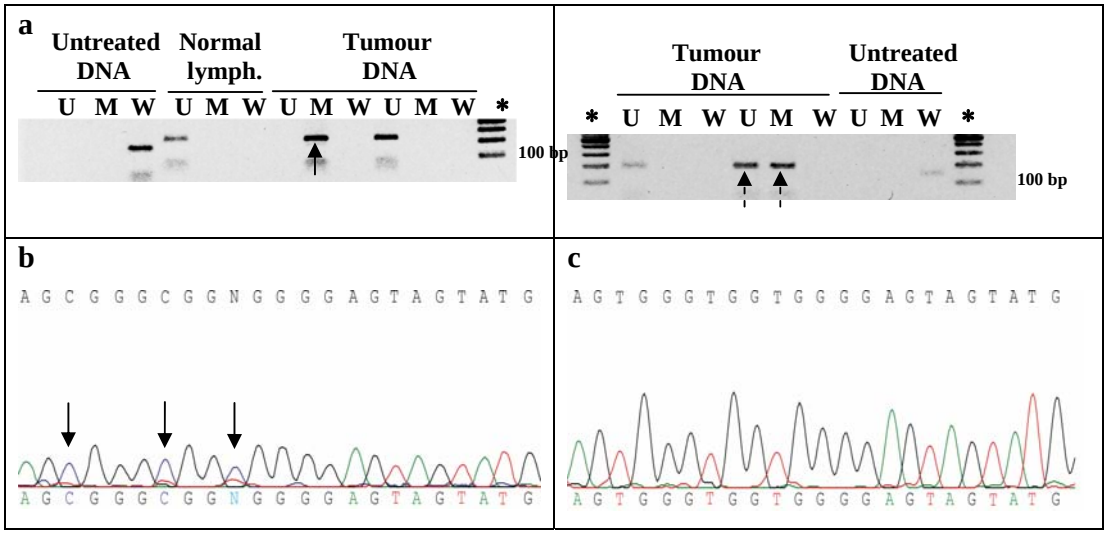


Figure 3.14. *Promoter methylation analysis of CDKN2A:* ***(a)****, MSP of CDKN2A; primer sets used for amplification are designated as unmethylated (U), methylated (M), or unmodified/wild type (W); ∗ - molecular weight marker (100 bp); untreated DNA and DNA from lymphocytes were used as a control; tumour sample showed positive M-band (→); tumour sample with positive both M- and U-bands (--►);* ***(b)****, chromatogram confirming the methylation of CpG islands (arrows) in the CDKN2A promoter region;* ***(c)****,chromatogram showing unmethylated CDKN2A promoter region and the efficiency of the bisulfide modification as well.*

3.5.3. LOH at *CDKN2A* locus

Loss of heterozygosity was determined at four different microsatelite markers on chromosome *9p21* in 10 tumour-lymphocyte paired samples. These markers cover the *CDKN2A* locus as well as flanking centromeric loci on chromosome *9p21* (Figure 3.15). The fifth marker (*D9S1846*) chosen for the analysis was located telomeric from *CDKN2A* locus, however, the specific PCR product for this marker was not obtained and the marker was excluded from the analysis.

Three out of 10 primary melanomas (30%) showed LOH for at least one microsatellite marker studied (Figure 3.15). One melanoma tissue sample (T24) showed allelic losses at both markers, *D9S974* and *D9S942*, within *CDKN2A* locus (Figure 3.16a;b) and was obtained from the patient with the family history of the disease. This sample had also the c.*69C>T polymorphism in the 3' UTR of the gene. One melanoma tissue sample (T28) had LOH confirmed to the marker *D9S974* (Figure 3.16c) being uninformative for the other *CDKN2A* marker *D9S942*. No information was available about family history of cancers from this patient. One melanoma tissue sample (T3) obtained from the patient with metastases had LOH at the marker *D9S171* (Figure 3.16d), which is located centromeric to the *CDKN2A*. This tissue sample also had the polymorphism in the 3'UTR of the gene, c.*29C>G. None of these cases with LOH at *CDKN2A* locus were methylated at the promoter region.

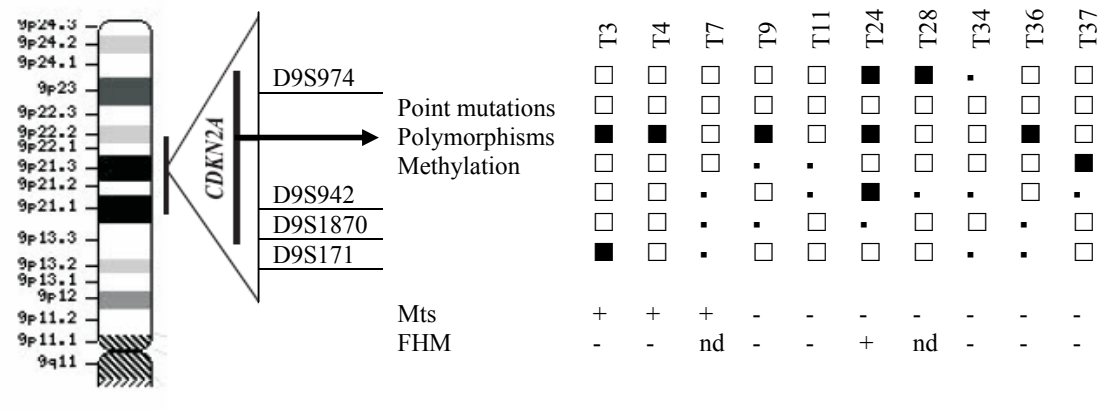


Figure 3.15. *Schematic representation of the 9p21 region showing the positions of the microsatellite markers studied, the CDKN2A gene, mutational, methylation and loss of heterozygosity (LOH) analyses result: □ - both alleles present, absence of CDKN2A mutation or promoter methylation; ■ - LOH, CDKN2A mutation or polymorphism or promoter methylation; ▪ - uninformative marker; nd - no data; patients who had developed metastases (Mts) or have a family history of melanoma (FHM) are identified by "+" in the two final rows.*

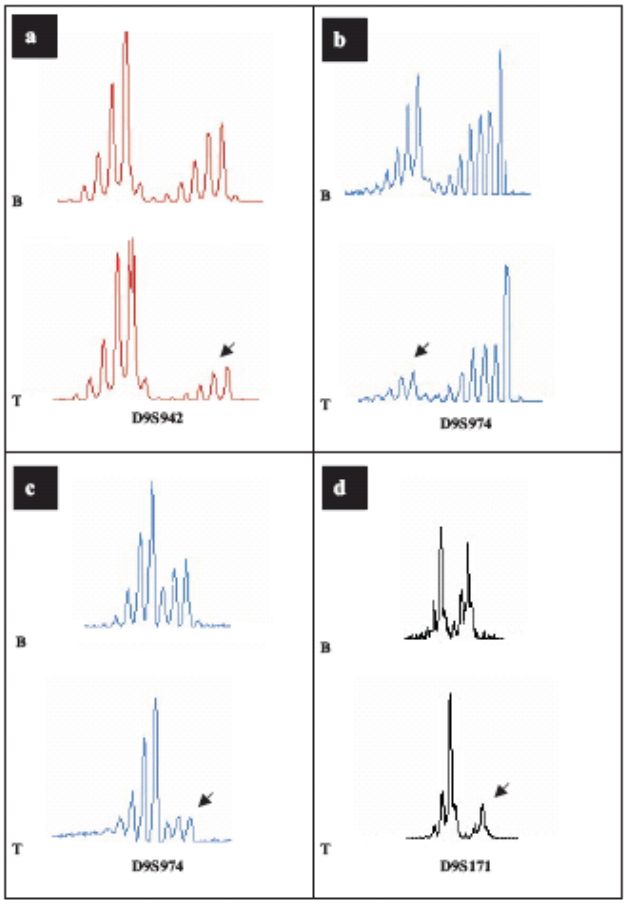


Figure 3.16. *Melanomas showing LOH at microsatellite loci detected using an automated sequencer ABI PRISM 3100:* ***(a,b)****, melanoma T24;* ***c****, melanoma T28,* ***d****, melanoma T3; B indicates PCR products amplified from peripheral blood; T indicates PCR products amplified from tumour; one allele reduction of at least 50% was scored as the LOH (arrows).*

4. DISCUSSION

The present study estimates the prevalence of *CDKN2A* and *CDK4* exon 2 germline mutations in clinically based population of melanoma patients. Many previous reports have determined the incidence of such mutations in various cohorts of CMM cases focused on patients with high probabilities of carrying the mutation – those individuals with a family history of melanoma (Table 1.7), multiple CMMs (Table 1.9) or early age of onset of the disease (Whiteman et al., 1997; Youl et al., 2002; Berg et al., 2004; Soufir et al., 2004; Nagore et al., 2005; Stratigos et al., 2006b; Hocevar et al., 2006). There have, to date, been only few published estimations of the prevalence of *CDKN2A* germline mutation in sporadic melanoma patients or in general population (Aitken et al., 1999; Begg et al., 2005; Berwick et al., 2006; Orlow et al., 2007), and, according to the author knowledge, no published estimation of *CDK4* germline mutations in those patients. In the first population based study of melanoma cases from Queensland, Australia, Aitken et al. 1999 estimated that just 0.2% of all melanoma cases are caused by *CDKN2A* mutations. Subsequently, Begg et al., 2005 using a population-based study design found *CDKN2A* mutations in 1.8% of patients, extrapolating that 1.3% of sporadic primary melanoma patients had *CDKN2A* mutations. Similarly, the population based study of Berwick et al., 2006 found a *CDKN2A* mutation frequency to be 1.2% in incident cases of first primary melanoma. This differences between studies might be probably explained by the fact that the latest two studies undertook direct sequencing to identify all possible mutations in the coding region after DHPLC (denaturing high-performance liquid chromatography) screening of all participants and found that mutation occur in many locations along the gene, while in the study of Aitken et al., 1999 sequencing was performed only in the high risk patients and among medium risk patients, low risk patients, and control subjects only six relatively common candidate variants were examined. Therefore, sequencing seems to be essential for studies of prevalence of *CDKN2A* mutations.

The study design of the present investigation by analysing consecutive consenting melanoma patients treated at Latvian Oncological Center is closer to population studies than the estimation of mutation spectra in Latvian hereditary melanoma patients. So, according to the previous observations mentioned above and similar investigation strategies with combination of pre-screening and sequencing used in the study, it was hypothesised that the *CDKN2A* mutation rate in the studied cohort of clinic ascertained melanoma patients could be 1-3% (1-3 mutations). Owing to the relatively low incidence of melanoma in Latvia, ~ 7.5 per 100 000 per annum, it was postulated that here may be a slightly higher incidence of germline mutations compared to areas of the world with higher melanoma incidence rates. However, no *CDKN2A* germline mutations, also those in alternatively spliced product *ARF*, were identified in the 176 patients analysed, including two multiple primary melanoma patients (MPM), 48 patients diagnosed with malignant melanoma before the age of 50 years (of these 11 patients were early-onset melanoma patients (i.e. ≤35 years) and six melanoma patients who have a family history of melanoma or atypical nevi.

CDKN2A germline deletions have also been excluded as a causal event in four out of six familial melanoma patients analysed as shown by MLPA analysis. Although some reports (Bauhau et al., 1998; Randerson-Moor et al., 2001) have detected *CDKN2A* germline deletions in melanoma-prone families, such deletions explain susceptibility only in a small proportion of the families. In the first comprehensive study carried out to date only 3% of melanoma families (3 families out of 93), in which *CDKN2A* germline mutations have not been found, have been shown to carry *CDKN2A* germline deletion. One family was reported to have a hemizygous deletion involving *CDKN2A* exons 1α, 2, and 3, and two families had hemizygous deletions of *CDKN2A* exon 1β (Mistry et al., 2005). The absence of *9p21* (*CDKN2A*) germline deletions in Latvian melanoma-prone families could be partly explained by the rarity of such a deletions.

In two familial melanoma patients the development of melanoma could be explained by a *CDK4* mutation (discussed later).

The absence of detectable *CDKN2A* germline mutations in four other familial melanoma patients may indicate the presence of an alternative mechanism to coding region mutations for *CDKN2A* inactivation. Indeed, Liu et al. 1999 have demonstrated a mutation in the 5' untranslated region of the *CDKN2A* (c.-34G>T) that creates an aberrant transcription initiation codon and have also been found among GEM (Genes Environment and Melanoma) study participants (Berwick et al., 2006). Harland et al. 2001 have reported a deep intronic mutation (IVS2-105A>G), which was found to be common in English melanoma families and identified by lower frequency also by other groups (Loo et al., 2003, Majore et al., 2004). Subsequently, Harland et al., 2005a reported the identification of other intronic mutations (IVS1+1104C>A, IVS1-1104C>G), which were found to be associated with an increased risk of melanoma. Two of these mutations, c.-34G>T and IVS2-105A>G, were also assessed in this study but none was found. Just recently, there has been also reported the identification of a cluster of five different germline mutations at the *p14^{ARF}* exon 1β splice donor site (g.192A>T; g.193G>C; g.193+1G>A; g.193+2T>C; g.193+3A>G) which all are showed evidence of being causal mutations (Harland et al., 2005b). The primers in the present study were designed to cover 5'- and 3'-flanking intronic sequences of each *CDKN2A* exon, including exon 1β, however, no splice site mutations were found.

Moreover, Knappskog et al., 2006 described a novel large germline deletion, including exon 1α and half of exon 2, which is not detectable through the direct exon sequencing and may also escape identification by use of MLPA analysis. The defect has been identified through detection of a truncated $p14^{ARF}$ mRNA and loss of $p16^{INK4a}$ mRNA expression from the affected allele. This finding is the first reported large *CDKN2A* germline deletion with a breakpoint located within an exon and suggests that atypical, large deletions in the *CDKN2A* gene may explain linkage to the *9p21* chromosome band without identified gene mutations among melanoma prone families. It shows also the need to include $p14^{ARF}$ and $p16^{INK4a}$ transcript analysis when searching for unknown mutations within *CDKN2A* locus in melanoma prone families (Knappskog et al. 2006).

Alternatively, the absence of detectable *CDKN2A* germline mutations in familial melanoma patients analysed here may reflect the existence of as yet unidentified high penetrance melanoma susceptibility gene(s), one of which may be at 1p22 (Gillanders et al. 2003). Finally, there is the possibility that the development of melanoma in these four familial melanoma patients is related to a combination of low-risk susceptibility genes and a shared environment.

Some patients (families) overestimate the number of melanoma cases within the family and confuse melanoma with non-melanoma skin cancers. So, it is important to confirm the diagnosis of relatives reported to have melanoma. In the present work the diagnosis is confirmed for most patients but not all.

Malignant tumours in genetic cancer syndromes are generally characterised by an early onset. In addition, a higher proportion of germline mutation carriers have been found in young cancer patients. Thus, young colon cancer patients frequently have mutations in *hMSH2* and *hMSH1* (Farrington et al, 1998; Marcos et al., 2006), and patients with early-onset breast cancer carry germline *BRCA1* and *BRCA2* mutations (FitzGerald et al., 1996b; Krainer et al., 1997). Similarly, relatively high frequency (26.0%) of germline BRCA1 mutations was observed also in Latvian breast cancer patients diagnosed before 48 years (Tikhomirova et al., 2005). However, with regard to melanoma, a low prevalence of *CDKN2A* germline mutations with the range 1.6 – 5.6% was found in previous studies in patients diagnosed with cutaneous melanoma at an early age and often these individuals have had also a family history of melanoma (Whiteman et al., 1997; Tsao et al., 2000; Youl et al., 2002; Berg et al., 2004; Soufir et al., 2004; Nagore et al., 2005; Stratigos et al., 2006b). Similarly no germline *CDKN2A* mutations were detected in the present study in melanoma patients less

than 50 years of age including also a small group of patients (n=11) with early-onset melanoma (e.g. ≤ 35 years). More, of these patients only two had the family history of melanoma and both were also found to carry the *CDK4* mutation (discussed later). Such a low prevalence of mutation in early-onset melanoma, with mutations found mostly in a familial setting, supports the fact that the penetrance of *CDKN2A* and probably also *CDK4* mutations is sufficiently high not to consider age as a single factor to select patients for mutation testing, even in lower incidence populations. Early-onset melanoma may thus be explained by other mechanisms, the most plausible being the contribution of other low-penetrance melanoma susceptibility genes, although environmental modulation, i.e. sun exposure, should also be considered. It was not assessed in the present study, but it has been shown that the presence of a large number of nevi and atypical nevi indicates increased susceptibility to melanoma in an early age (Youl et al., 2002; Nagore et al., 2005). These clinical features were not found to be strongly correlated with the presence of *CDKN2A* mutations (Gruis et al., 1995b; Puig et al., 1997; Wachsmuth et al., 1998; Goldstein et al., 2000a; Newton-Bishop et al., 2000). Thus, yet unidentified nevus gene might be probably related to the melanoma in early-onset melanoma group. However, an early onset might be still useful to predict *CDKN2A* mutations in populations in which founder mutations are prevalent (Mantelli et al., 2002, 2004).

In another group that one would expect to be more genetically predisposed to melanoma, namely cases that have developed more than one primary tumour, the mutation frequency has also proved to be relatively low (Table 1.9). In this context, the absence of *CDKN2A* mutations in two MPM patients analysed is not surprisingly.

Apart from above mentioned patients, the chosen strategy for the recruitment of patients for the present study collected mainly sporadic melanoma patients and showed that *CDKN2A* germline mutations have a limited role, if any, in sporadic melanoma development in Latvia. It is likely that the development of melanoma in these patients is rather related to a combination of low-risk melanoma susceptibility genes (e.g. *MC1R*), phenotype and environmental factors: putative associations which were not assessed in the present study. However, the phenotype (skin type, freckling, eye and hair colour) of melanoma patients analysed here do not significantly differ from that in general population (data not shown), once again indicating that there might be different genetic background.

The common way for the characterising the mutations spectra in genes connected to the hereditary diseases or syndromes is to screen probands with several cases of the disease in their family. In the melanoma case, the presence of the atypical nevus syndrome is also often a helpful criterion in mutation screening. Using such a screening strategy, probably, more mutations can be found. This assumption has also been further supported in the present study. Focusing on individuals who had evidence of predisposition to melanoma in terms of multiple cases in the family, mutations were found in 2 out of 6 (33%) familial melanoma patients both with a strong family history of melanoma (5 cases in the family) indicating that mutations in Latvian melanoma families exist. Interestingly, both patients have mutation in the *CDK4* gene (discussed later). Overall in our six melanoma families, none of the families with two affected relatives and 100% of families with more then 3 affected relatives had identifiable melanoma specific mutations. Because of the *CDK4* mutation rarity (previously reported only six *CDK4* mutation positive melanoma families worldwide), the comparison of the prevalence of such mutations is difficult to make. Withal, such a high mutation rate in families with more than 3 affected relatives may have arisen because of the small sample number of familial melanoma patients in the present study or plausible that dense clustering of melanoma cases within a family in Latvia (country with a relatively low melanoma incidence rates) could indicate the presence of a mutation in melanoma susceptibility genes. In other studies on the larger sample size (estimation mostly made for *CDKN2A* gene) (see Table 1.7) lower prevalence of mutations have been reported. However, the difference in the probability of identifying mutations in families of different size found in the present study is consistent with that reported worldwide and appears to be dependent on the strength of family history, as would

be also expected for an inherited susceptibility (Bishop et al., 2007). For *CDKN2A* gene, however, there is also suggestion that the proportion of identifiable mutations is greater in areas of the world with lower incidence, e.g., Europe compared with Australia, as clustering of cases by chance is predicted to be more likely in sunny areas of the world with higher baseline rates. If the characterised germline mutation is not present there are no clear cut criteria for "familial melanoma", but as working definition researchers generally use this term to describe families in which there are at least three cases of melanoma. However, in low sunlight areas such a northern Europe "familial melanoma" is often defined when melanoma occurs in as few as two first-degree relatives. It is important to note that one of the previously reported causal *CDKN2A* mutations (23ins24) was detected in the English melanoma family with only two cases of melanoma (Harland et al., 1997), highlighting the importance of investigating families with lower numbers of cases in countries with a lower incidence of melanoma. In the more recent study of Mantelli et al., 2004, *CDKN2A* mutations have been found to be more likely in small families when one of the two affected members carries multiple primary melanomas. This finding can account for the absence of mutations found in our families with two affected members, as these were free of clinical features predictive of mutations.

While in some studies an increased incidence of pancreatic cancer in *CDKN2A* mutation positive families has been observed and also showed by recent collaborative studies carried out by GenoMEL (Goldstein et al., 2006; 2007), the two melanoma patients in our study who had pancreatic cancer in their family history did not have *CDKN2A* germline mutations. However, these both patients were sporadic melanoma patients and it is more likely that the observed coexistence of these two tumour types in the family represents clustering of sporadic tumours rather than genetic predisposition. Similarly, no germline mutations in the *CDKN2A* as well as *ARF* part of the gene have been found in pancreatic cancer patients from Latvia (Stāka et al., 2006). However, in this study a positive family history of pancreatic cancer was also reported only in three patients (one first degree relative in each case) showing that mutations in the *CDKN2A* gene do not contribute significantly to pancreatic cancer in the general population of Latvia.

In another study aimed at investigating whether familial susceptibility to melanoma increases the risk of other tumour types, increased risk of breast carcinomas have been found in *CDKN2A* mutations carriers (Borg et al., 2000). In the present study, there were 15 melanoma patients with breast cancer in their family history, but none have *CDKN2A* mutation. However, again, all these patients were sporadic melanoma patients and the occurrence of breast cancer in their family history may be because of chance. More, breast cancer is the most common of all cancers in Latvia. The number of melanoma families analysed in the present study, the absence of *CDKN2A* mutations, and the observed number of non-melanoma tumours in these families is far too small to draw more conclusions. However, a recent large population-based study carried out by Larson et al., 2007 has estimated that in addition to the increased risk for melanoma there is also an increased risk, ranging from 32% to 72%, for prostate, breast, and colon cancers, non-Hodgkin's lymphoma, and multiple myeloma among first-degree relatives of melanoma patients. Among second-degree relatives, in addition to increased risk for melanoma there is an increased risk for prostate cancer and multiple myeloma (27% and 53% increase, respectively). Such an increased risk for additional cancer types in first- and second-degree relatives of melanoma cases suggests that for individuals with a family history of melanoma screening for all cancers is recommended.

Given that somatic mutations in the *CDKN2A* have been documented in a wide variety of different tumour types (Foulkes et al., 1997, Ruas and Peters 1998), it was hypothesised by Alao et al., 2002 that patients with melanoma and additional malignant cancer in the same individual may also harbour germline mutations in the *CDKN2A* gene. In the present study, there were 15 melanoma patients with additional malignant non-melanoma cancer, however, *CDKN2A* mutations were found neither in patients analysed here nor in the study of Alao et

al., 2002. So, it would appear that melanoma and additional, apparently unrelated cancers developing in the same individual are likely to be related to environment or again to a combination of low-risk susceptibility genes and environmental factors.

In the studied cohort, as mentioned above, there were detected two melanoma families with *CDK4* mutation R24H. This result is important, since these are the seventh and eighth melanoma families worldwide with *CDK4* mutations. In previous studies, segregation of the *CDK4* mutations has been shown to be compatible with an association with the disease (Zuo et al., 1996; Soufir et al., 1998; Molven et al., 2005).

In the present study, there was only one melanoma patient in each family alive at this moment. However, it was possible to study the segregation of the mutation with melanoma by analysing paraffin-embedded tissue blocks containing melanoma of the deceased family members in one out of two families (M247, Figure 3.9a). In this family the *CDK4* mutation was also found in melanoma tissue from probands mother showing the association of the mutation with the disease at least in one branch of the family (both unaffected family members analysed do not carry the mutation). Although the normal allele was also detected in the analysed tissue, it is not possible to exclude possible contamination by non-tumour cells.

CDK4 mutation was not detected in melanoma tissues from probands cousin. However, the theoretical possibility that the mutated allele had been specifically deleted in the tumour was not excluded as the precise microdissection was not done. Moreover, in the microscopic re-examination of the specimen it was not possible to evaluate whether it is a second primary melanoma developed 10 years after the diagnosis of the first primary melanoma (was not available for analysis), relapse or cutaneous melanoma metastases. Interestingly, this melanoma tissue had novel *CDKN2A* mutation (L121P), but again with the material available it is not possible to deduce whether this mutation was present in the germline or arose in the tumour during the development of melanoma. Other melanoma patients within this family analysed are proven not to have this mutation. Previously, one melanoma family in which both *CDK4* and *CDKN2A* mutations have been found to segregate with the disease was identified in Australia; however, the *CDK4* variant (S52N) found in this family is of uncertain significance (Holland et al., 1999). Analysis of more family members and long-term follow-up of the family may shed some light on this issue.

Segregation of the mutation with the disease in the second *CDK4* mutation positive family could not be established due to the limited number of patients samples available for the analysis. However besides the proband, mutation carrier, there were analysed five unaffected family members and the mutation was found in two of them. One of the mutation carriers (probands daughter) seems to be non-penetrant for the disease, since she is 44 years old and the average age at melanoma diagnosis in this family is 39.5 years. Other unaffected carrier is only 9 years old and may still develop the melanoma. Such a situation is possible, because the pattern of melanoma susceptibility is consistent with the inheritance of autosomal dominant genes (to which belong also *CDK4* gene) with incomplete penetrance (Anderson and Badzioch 1991).

The segregation of the *CDK4* codon 24 mutations with the disease in other melanoma pedigrees, however, suggests that these mutations are causal. Moreover, this amino acid is critical in the p16INK4a-cdk4 interaction, and when Arg at position 24 is substituted, p16INK4a can no longer bind to and inactivate cdk4 protein (Wölfer et al., 1995; Coleman et al., 1997).

Because of the limited number of families with *CDK4* mutations, there are also limited clinical data on these families. A comparison between 12 American *CDK4* mutation positive patients originated from two families and *CDKN2A*-positive melanoma patients carried out by Goldstein et al., 2000b found that the age of melanoma onset, the number of melanomas, and the total number of nevi were comparable. The overall median age of melanoma onset in the Latvian families (42 years) was a bit higher than in the American *CDK4* mutation positive

patients (34 years) (Goldstein et al., 2000b) but similar to median age of onset in the Norwegian family (41 years) (Molven et al., 2005). This could reflect the lower sun exposure in Latvia and Norway as well, or differences in low-penetrance modifier genes (Hayward 2003).

In families with germline *CDKN2A* mutations, malignant melanoma is the dominating tumour type. However, some *CDKN2A* mutations positive kindreds also include individuals with pancreatic cancer (as discussed above). In the *CDK4* mutations positive families reported here, neoplasms other than melanoma have not been recorded. No other tumour types were also reported in the previously described *CDK4* mutation positive melanoma families (Zuo et al., 1996; Soufir et al., 1998; Molven et al., 2005). Thus, as yet there is no support for an increased risk for cancers other than malignant melanoma in carriers of *CDK4* codon 24 mutations. Except for the predisposition to pancreatic cancer, there appears to be no other phenotypic criterion that can aid in differentiating *CDK4* from some *CDKN2A* mutations positive families.

The two American families with malignant melanoma because of *CDK4* mutation both carry a CGT > TGT change in codon 24, predicting an Arg > Cys substitution in the protein (Zuo et al., 1996). Later, it was suggested that this mutation is present on the same haplotype background (Goldstein et al., 2000b). The CGT > CAT mutation detected in Latvian melanoma families also affects codon 24, predicting Arg > His substitution in the protein. It is identical to the mutation in a French (Soufir et al., 1998), Australian, English, and Norwegian pedigrees (Molven et al., 2005). In the haplotype analysis done in one of the families (M247), it was sought to determine whether the Arg24His mutation had an ancient and common origin. With the current information, it was not possible to deduce the SNP haplotype for the Latvian mutation. However, the microsatellite analysis indicated that the Latvian mutated haplotype is unique and does not coincide with any of the other known haplotypes (Figure 4.1). Thus, if there is a common mutational event it must be quite ancient. In this respect, *CDK4* differs from *CDKN2A*, for which detailed haplotyping has suggested a common origin for some of the mutations, e.g. M53I, G101W, and V126D, found in melanoma families worldwide (Ciotti et al., 2000; Goldstein et al., 2001; Lang et al., 2007).

It seems that codon 24 serves as a mutational hot spot in the *CDK4* gene, probably because of methylation of its CG dinucletide. CG dinucletides are potential methylation sites and as such susceptible to mutations because the methylated cytosine can spontaneously deaminate to generate thymine. If the methylation has occurred in the *CDK4* codon 24 (CGT) on either DNA strand, both the TGT and the CAT mutations can be explained by this mechanisms. It is possible that negative selection may work to eliminate other *CDK4* mutations affecting the functions of the protein.

Because it is known that *MC1R* gene variants are associated with human pigmentation, act as low penetrance melanoma susceptibility alleles and have significant impact on penetrance of *CDKN2A* mutations in melanoma-dense pedigrees, the analysis of *MC1R* gene in familial melanoma patients as well as melanoma families was done. Although there were found *MC1R* gene variants (predominantly R160W variant) in familial melanoma patients and their relatives, with the current information, it was not possible to estimate the role of these variants in melanoma susceptibility in individuals analysed. From other studies it is known that the found R160W variants is co called “R” variant which is associated with red hair, fair skin, skin sensitivity to UV light, and predisposes individuals to melanoma (Box et al., 1997; Palmer et al., 2000; Matichard et al., 2004; Kanetsky et al., 2006). Additional studies on the prevalence of *MC1R* variants and their association with phenotype and melanoma risk in Latvian melanoma patients and population as well are needed. With the current information, it was also not possible to ascertain the impact of R160W variant to penetrance of *CDK4* mutation. More, because of the overall *CDK4* mutation rarity (few families worldwide), it is difficult to estimate the impact of *MC1R* gene variants on *CDK4* mutations penetrance and even the penetrance of *CDK4* mutations themselves. However, one study carried out in two

American *CDK4* mutation positive families estimates the penetrance of *CDK4* mutation to be 63% (Goldstein et al., 2002), which is close to the *CDKN2A* mutation penetrance.

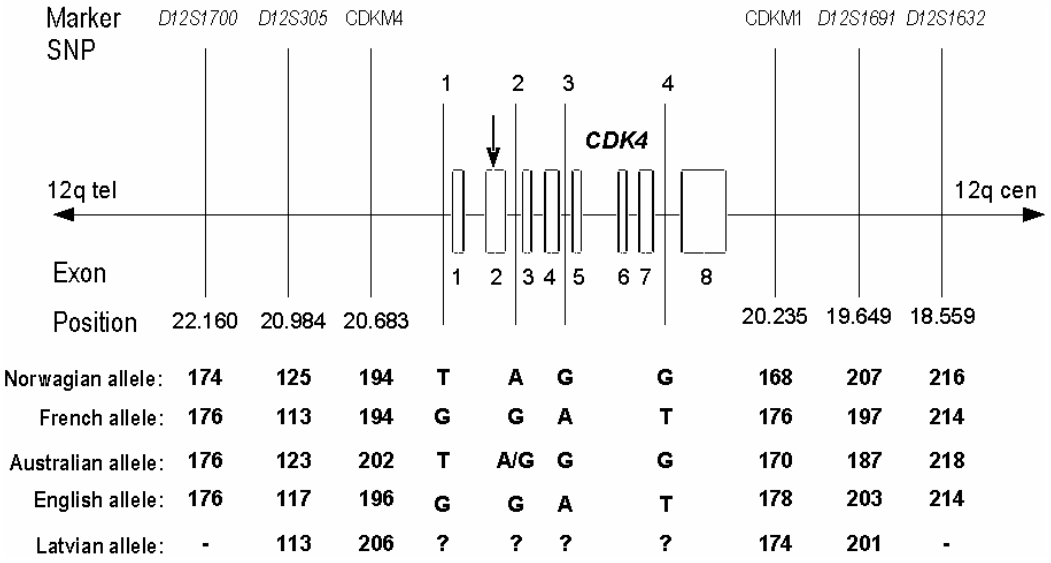


Figure 4.1. *Haplotypes of the CDK4 region from the Norwegian, French, Australian, English, and Latvian melanoma families harbouring the same R24H mutation. The positions of eight CDK4 exons on chromosome arm 12q are indicated by boxes, with the arrow pointing to the mutation in the exon 2. The relative positions of the microsatellite markers are listed below. Positions are not drawn to scale. SNP numbers 1-4 refer to ID numbers rs2072052, rs2270777, rs2069502, and rs2069506, respectively, in the SNP database (http://www.ncbi.nlm.nih.gov/SNP/). The haplotypes of the alleles carrying the mutation for each family are shown on the bottom of the picture as allele sizes for microsatellite markers and as nucleotides for the SNPs. Latvian SNP haplotype with the available information could not be resolved. Figure adopted from Molven et al., 2005 by adding information about Latvian CDK4 mutation positive family.*

Although the screening of consecutive consenting melanoma patients for *CDKN2A* germline mutation did not identified any disease related mutations, there were present several gene polymorphisms (Table 3.6). More, the *CDKN2A* A148T polymorphism was found to be increased among melanoma patients compared to the controls (6% vs. 1%, p=0.01). This polymorphism has been broadly cited as a common polymorphism on the basis of observation that i) functional studies indicate that this amino acid substitution does not appear to adversely affect the ability of this variant protein to inhibit *CDK4* enzymatic activity, with inhibition comparable to wild type protein (Ranade et al., 1995; Reymond and Brent 1995; Lilischkis et al., 1996) ii) this variant does not segregate with melanoma in melanoma-prone families (Hussussian et al., 1994; Walker et al., 1995; FitzGerald et al., 1996a; Erlandson et al., 2007), and iii) this variant has also been found to be present in general population in Utah (Kamb et al., 1994a), in CEPH parents (Hussussian et al., 1994), Queensland (Aitken et al., 1999), England (Bertram et al., 2002), France and Italy (Spica et al., 2006), and in an international multi-center population-based study of melanoma, the GEM (Orlow et al., 2007). Even A148T polymorphism is overexpressed in melanoma kindreds in comparison with general population, often this finding is not statistically significant (Aitken et al., 1999). In Spain, however, this polymorphism was found to be associated with an increased risk of MPM (Puig

et al., 2005). In series of consecutive consenting melanoma patients presented here, only two patients had more than one primary melanoma, however, both patients did not have the A148T polymorphism. Moreover, the evidence for the A148T variant's association with an increased risk of melanoma irrespective of primary melanoma number in the Polish melanoma population exist (Debniak et al., 2005b). Interestingly, when melanoma cells where transfeted with this variant, cells showed a predominantly nuclear staining by immunocytochemistry compared to an even nuclear/cytoplasmic staining observed in wild type $p16^{INK4a}$ transfected cells (Walker et al., 1999). Population stratification might explain the diverse impact of the A148T variant on melanoma risk in different populations.

Genetic studies of Latvian population have been mostly touched on the spread of mutations and polymorphisms in association with diseases (Pronina et al., 2003; Tikhomirova et al., 2005) and little is known about the population substructures within Latvia. However, it seems that there are no major population substructures. A recent study of mitochondrial DNA (mtDNA) variations in Latvians indicates the intra-population homogeneity and similarity to other neighbouring populations (Lithuanians, Estonians, Norwegians, Russians etc.) irrespective of their linguistic affiliation (Pliss et al., 2006). The control group in the present study has also been composed of three different randomly selected subgroups. Nevertheless, each group shows similar frequencies of the *CDKN2A* polymorphisms. Such a similarity between groups suggests homogeneity at least with respect to the *CDKN2A* polymorphisms. Additional studies based on larger number of samples and the distributions of genotypes at other loci are needed for population studies.

Because the A148T has not been shown to alter protein function yet seems to be associated with disease it is most likely that this polymorphism is in linkage disequilibrium with another alteration that does affect protein function. Indeed, there are several reports indicating that A148Tpolymorphism is in linkage disequilibrium with a change in the promoter region A-493T (Mantelli et al., 2002; Harland et al., 2000). Father examination of the haplotype, which is known to segregate with A148T variant in England (Bishop, personal communication), done in Latvian and Polish melanoma patients, and Polish control individuals with A148T polymorphisms showed no significant difference from the predominant haplotype in the English population, where this variant is not associated with an increased risk of melanoma. The appearance of a rare haplotype segregating with the A148T polymorphism found in one Latvian melanoma patient and one Polish control person does not explain the differences in modulation of melanoma risk in these populations. Therefore this analysis does not suggest that there is any reason why the A148T haplotype should be associated with increased risk of melanoma in both Latvia and Poland but apparently not in England. One explanation, however, might be that this polymorphism is in linkage disequilibrium with another alteration that does affect the protein function either by stable altering its function or by reducing its expression and is not assessed in this study, but when taken together result in malignancy. In the Polish population it was also shown that A148T polymorphism is associated with an increased risk of melanoma especially for patients diagnosed with melanoma less than 50 years of age (Debniak t al., 2005b) which was not observed in the present study. However, the difference in polymorphism frequencies between patients who were less than 50 of age compared to those more than 50 years of age was not statistically significant and the mean age of melanoma patients (60.0 years) in our studied cohort was somehow higher than in melanoma patients (54.5 years) studied by Debniak et al., 2005b.

The frequencies the two 3'UTR polymorphisms, c.*29C>G and c.*69C>T, were not statistically significant overrepresented in the melanoma patients under the study in comparison to control individuals and in Latvian control population are similar to those reported in Nordic and Polish healthy control population (Kumar et al., 2001; Debniak et al., 2005b). However, there were lower incidences of these polymorphisms among melanoma patients in comparison with Nordic patients affected with melanoma (Kumar et al., 2001). In

comparison with Polish melanoma patients, there were lower incidence for c.*29C>G polymorphism but higher incidence for c.*69C>T polymorphism (Debniak et al., 2005b). However, the results of statistical analyses are again consistent with data presented by Debniak et al., 2005b and Aitken et al., 1999 in that there was no significant increase of the both polymorphisms in melanoma patients and that for each polymorphism the rare allele frequency was higher in melanoma patients than in control individuals. The significance of the remaining previously unreported 3'UTR variant c.*42C>A is unclear. It does not change the coding region of *CDKN2A* and is therefore unlikely to be associated with the disease.

Finally, the c.- 33 G>C change in the 5'UTR of the gene has been reported not to affect the transcription of the gene and is therefore considered as a polymorphic variant (Soufir et al., 1998).

Recent evidence strongly suggests that 3'UTR region of mRNA is involved in regulation of gene expression by controlling nuclear export, polyadenylation status, subcellular targeting, translation rates, and mRNA degradation (Conne et al., 2000). Both c.*29C>G and c.*69C>T polymorphisms in the 3'UTR of the *CDKN2A* gene have been described as being associated with significantly shorter progression time from primary to metastatic disease (Sauroja et al., 2000) and significantly improved survival (Straume et al., 2002). In the present work, the correlation between the *CDKN2A* polymorphisms and melanoma prognostic factors was done. However, no statistically significant differences in polymorphisms frequencies between melanoma patients with better and worse prognostic factors were observed. Follow-up study for patients included in the genetic testing was not conducted and therefore the impact of polymorphism in disease invasiveness could not be fully evaluated.

There were also observed a trend toward the increased frequency of c.*29C>G polymorphism, however statistically non significant, among melanoma patients with a first-degree relatives with cancer of any type and a trend toward the decreased frequency of c.*69C>T polymorphism, however again statistically non significant, among these patients (Table 3.12). To determine whether *CDKN2A* polymorphisms could be associated with an increased or decreased risk of malignancies at different sites of origin, cancer patients with different tumour types were genotyped for the three common *CDKN2A* polymorphisms and the frequencies of changes observed in these patients compared to the control individuals. Multi-organ cancer predispositions are characteristic for other genes in DNA damage signalling pathways, including *BRCA1, BRCA2, P53, NBS1*, and *CHEK*. There are also evidences that *CDKN2A* A148T polymorphism could be associated not only with an increased risk of melanoma (Debniak et al., 2005b), but also with an increased risk of developing cancers of the breast (Debniak et al., 2005a), lung, and colorectal cancer (Debniak et al., 2006d). Although the frequency of A148T polymorphism in the present study was slightly higher for all cancer types tested, this increase was not significant. The numbers of cases were small and an association, if any, may have been missed as a result of limited study power. Much larger studies are needed to establish the full range of risks associated with different *CDKN2A* alleles, especially low penetrance alleles.

The frequencies of the 3'UTR polymorphisms, c.*29C>G and c.*69C>T, varies between different cancer patients and the predominant impression is that these polymorphisms are not associated with an increased risks of malignancy of any type.

In the present study, there was also performed mutational and promoter hypermethylation analyses of the *CDKN2A* gene and LOH analysis on *9p21* region as well. The results of the mutational analysis of the *CDKN2A* are in agreement with the findings of most others in that *CDKN2A* is mutated very infrequently in sporadic primary melanomas (Healy et al., 1996a; Ruiz et al., 1998; Fujimoto et al., 1999; Cachia et al., 2000; Soto et al., 2005). As *CDKN2A* mutations were not revealed in the analysed material, it is not possible to comment whether *CDKN2A* mutations is an early or late event in the development of melanomas in which it is present. Considering alternative mechanisms to mutation for

$p16^{INK4a}$ inactivation, certain factors may influence the production of functional $p16^{INK4a}$ protein at transcriptional or translational level. For example, mutation in 5'UTR of the gene that creates an aberrant transcription initiation codon and is associated with melanoma risk, as discussed above. Methylation of a CpG island in promoter region is frequent in several cancer types, being associated with transcriptional silencing of the *CDKN2A* gene, including melanomas (Merlo et al., 1995; Gonzalgo et al., 1997; Fujimoto et al., 1999; von Eggling et al., 1999; Straume et al., 2002). Primary melanomas in this study showed hypermethylation at the promoter region of *CDKN2A* gene in 10% of cases. This frequency is similar to that observed in the most other studies (Gonzalgo et al., 1997; Funk et al., 1998; Fujimoto et al., 1999; von Eggling et al., 1999). One primary tumour was heterogenous, as also described by others (Suh et al. 2000) showing both methylated and unmethylated alleles in tumour cells.

In order to relate the allelic loses to mutations in the *CDKN2A* and provide a rationale for the inactivation of tumour suppressor gene, LOH analysis was performed. Although 30% of tumour-lymphocyte paired samples showed LOH for at least one microsatellite studied, the expected *CDKN2A* "double hit" for tumour suppressor gene inactivation was not observed. None of the cases with LOH had mutation or were hypermethylated at the promoter region of the gene. A number of possible explanations might be put forward. The simplest is that the mutational analyses performed have not been sufficiently rigorous. For example, the study has focused on the $p16^{INK4a}$ coding regions and it remains conceivable that mutations occur in the promoter or alternatively spliced exon of $p14^{ARF}$. Another intriguing explanation for the absence of $p16^{INK4a}$ mutations is that the lack of one of the functional alleles may be sufficient to confer a growth advantage on the tumour cell. This is a difficult question to address and the only supporting evidence thus far is that the rare individuals who are homozygous for mutations of $p16^{INK4a}$ do not appear to be at greater risk of developing melanomas than their heterozygote relatives (Gruis et al., 1995a). On the other hand, it is conceivable that cells with half the normal amount of $p16^{INK4a}$ may have an increased lifespan, making them more susceptible to other genetic alterations. More, the presence of LOH at *9p21* found in the present study and other studies as well indeed indicates the involvement of this region in melanoma pathogenesis and also suggests the possibility of the existence of an additional tumour suppressor locus on chromosome region *9p* yet to be discovered.

Although the significance of *CDKN2A* as a melanoma susceptibility and tumour suppressor locus is not in doubt, controversy regarding the importance of *CDKN2A* inactivation in melanoma remains. At the heart of this lies discordance between the high rates (57 – 63%) of mutations in *CDKN2A* gene in melanoma cell lines, with homozygous mutation being the main mechanism of mutations (Kamb et al., 1994b; Pollock et al., 1995) and low rates (0 – 20%) of homozygous deletions in uncultured melanoma tumours (Ruiz et al., 1998; Fujimoto et al., 1999; Kumar et al., 1999). The importance of homozygous deletion as a method for *CDKN2A* inactivation may be that there is a concomitant requirement for inactivation for another closely linked gene to permit melanoma development or progression. This would favor a single multilocus deletional event over the simultaneous occurrence of inactivating point mutations occurring in two or more genes. The finding of no *CDKN2A* mutations but LOH for *D9S974, D9S942,* and *D9S171* markers in the present study does not prove the necessity of contiguous deletion of *CDKN2A* and neighbouring tumour suppressor gene(s) in melanoma progression but would be consistent with it.

Most melanomas (88%) analysed in the present study were thick exceeding 0.76 mm and it is not surprisingly that LOH was observed exclusively in thick tumours. However, Cachia et al., 2000 have reported different frequencies of *9p21* LOH between thick and thin melanomas. This finding may suggest a dosage effect from loss of one *CDKN2A* allele or of another putative tumour suppressor gene in this chromosomal region in advanced primary, but not early primary, melanoma. It seems that although *CDKN2A* is important in further melanoma progression, the earliest genetic event(s) that permit initiation of malignant phenotype occur outside the *9p21* region. Alternatively, because tumours with LOH analysed

by Cachia et al., 2000 were nodular (similarly, one tumour with LOH in the present study also was nodular melanoma whereas for other two tumours the melanoma subtype was not known) but none of the thin tumours were, it is possible that different genetic pathways are involved in the pathogenesis of nodular versus superficial spreading or lentigo maligna melanoma. The later is supported by observation that different genetic changes occur in the radial growth phase (RGP) tumours, represented mainly by superficial spreading melanoma, versus vertical growth phase (VGP) tumours, represented mainly by nodular melanomas. However despite these differences and a variety of genes tested, neither a tumour suppressor gene with importance in all malignant melanomas nor one clearly defining the transition from RGP to the VGP has been determined (Poetsch et al., 2003). Melanoma heterogeneity is a strong feature that has become progressively more apparent as techniques for the molecular profiling of cancers have improved. It has been studied extensively at the level of gene expression and is also apparent at the DNA level. However, a complete understanding of the genetics of melanoma remains a distant goal.

In summary, there was characterised, for the first time in Latvia, the *CDKN2A* and *CDK4* status in Latvian melanoma patients and found two families with melanoma and *CDK4* mutation, which are the seventh and eight families with *CDK4* mutation worldwide identified so far. The study does also provide some additional evidences for a role for the *CDKN2A* A148T polymorphism as a low penetrance melanoma susceptibility gene and reveal several directions (looking for high penetrance genes in patients predisposed to melanoma development as well as looking for lower penetrance melanoma genes) for future investigation.

CONCLUSIONS

1. The prevalence of *CDKN2A* and *ARF* germline mutations has been assessed in 176 consecutive consenting CMM patients, including six familial melanoma patients and two patients with MPM, and none was found, indicating that germline mutations at the *CDKN2A* locus are rare in sporadic melanoma in Latvia.
2. Age and multiple primaries *per se* do not also appear to be a strong indicator of *CDKN2A* mutations, however, the number of individuals studied was too small to draw definite conclusions and further research is needed.
3. In 4 out of 6 familial melanoma patients germline deletions at the *9p21* (*CDKN2A* locus) were also assessed and excluded as a causal event in melanoma development.
4. The screening of the *CDKN2A* gene identified five different *CDKN2A* polymorphisms – three common c.442G>A (A148T) (6%), c.*29C>G (18%), c.*69C>T (17%) polymorphisms, rare c.-33 G>C (1%) polymorphic variant, and c.*42C>A (0.6%) variant of uncertain significance
5. Only the frequency of the c.442G>A (A148T) polymorphism was found to be higher in melanoma patients compared to that in control individuals (p=0.01) showing some evidence for the for this polymorphism in melanoma development in Latvia.
6. No significant differences were found between A148T carriers haplotypes in Latvia, Poland and England, giving no indication that the discrepancy could be explained by population differences in linkage disequilibrium.
7. No significant positive association between *CDKN2A* polymorphisms and cancers of more common sites was found, however, there was a trend toward the increased frequency for polymorphism A148T for all cancers analysed. More extensive studies are needed to establish the full range of risks associated with A148T allele.
8. The screening of the *CDK4* exon 2 revealed the presence of germline *CDK4* R24H mutation in two melanoma patients both with the strong family history of melanoma.
9. The segregation of the *CDK4* R24H mutation with the disease was shown, verifying the causality of the R24H mutation in melanoma development.
10. The *CDK4* mutation positive haplotype was assessed and found to be 113-194-206-*CDK4*-174-201 for the microsatellite markers tested which is unique and does not coincide with any of the known haplotypes.
11. Familial melanoma patients and their corresponding families were analysed for *MC1R* variants and predominantly R160W variant (7 from 11) was found, however, additional studies on the prevalence of *MC1R* variants in melanoma patients as well as in Latvian population are needed.
12. No intragenic mutations in the *CDKN2A* gene were found in 27 primary melanoma tissues analysed. In 2 out of 19 (10%) tumours *CDKN2A* promoter hypermethylation was detected, suggesting that *CDKN2A* inactivation by point mutations or promoter methylation are rare events in primary cutaneous malignant melanomas.
13. LOH for at least one microsatellite marker in *9p21* region was found in 3 out of 10 (30%) tumours, indicating the involvement of this region in melanoma pathogenesis and suggesting the possibility of the existence of an additional tumour suppressor locus on chromosome region *9p* yet to be discovered.
14. The study reveals also definite directions for future investigation (e.g. looking for high penetrance genes in patients predisposed to melanoma development and looking for lower penetrance genes in others as well).

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all persons who contributed to the making of this thesis. I am afraid I can not mention all them here but wish to thank all for your help and support.

First of all, I would like to thank my supervisors, Dr. Olita Heisele and Dr. Ludmila Engele for giving me an opportunity to work at this project. Dr. Ruta Bruvere and Dr. Valts Loza – my first supervisors and persons who introduced me to the field of biology. I thank Ruta Bruvere for supporting me all the time and valuable comments concerning the work. Dr. Zinaida Somsteine and Professor Elmars Grens for support and for believing in me.

I am thankful to clinicians, especially Inta Bolkovska and Baiba Streinerte from Latvian Oncological Center for recruiting patients, Biruta Popena for referral families, and Prof. Regina Kleina for enthusiasm and patience in looking for archival material.

Many thanks to colleges in Riga – Dr. Eriks Jankevics, Dr. Inna Inaskina, Dr. Inta Vasiljeva and Dr. Andris Zeltins for technical support and valuable discussion inside and outside the lab. Dr. Liene Nikitina – Zake for support from Gennome Data Base, Kristine Viksne for DNA isolation, Dr. Uldis Apsalons for oligonucleotide synthesis, Prof. Eva Stankevica and Dr. Ligija Ignatovica for automatic DNA sequencing.

I am thankful to the people in the lab Vita Ignatovica, Ingrida Desjatnikova, and Ingrīda Belousova for work together. Thanks to people around – Gunda, Natalija, Villija, Ina, Gunta for enjoyable atmosphere and friendship. Aleksandrs for last minute computer works and supporting me throughout writing process.

I would like to thank Prof. Julia Newton Bishop and Prof. Tim Bishop from Genetic Epidemiology Division, Cancer Research UK Clinical Centre, St. James's University Hospital in Leeds. Without you, there would be no thesis. I really enjoyed the time spent in your lab.

Many thanks to the colleagues in Leeds – Dr. Claire Taylor for "LightScanning", Juliette Randerson-Moor, Mark Harland, Kairen Kukalizch for many inspiring discussions and explanations on why something went wrong and what to do. Special thanks to Jules for outstanding help in the everyday life and tour to Yorkshire Dales. All staff and people in lab – Sharon, Caroline, Jo, Sophy, Kelly, Tracy, Jane, Paul, Linda.

Thanks to all people from "GenoMEL" for their friendship.

Thanks to Dr. Tadeusz Debniak from International Hereditary Cancer Center, Szczecin, Poland for the allowance to use results obtained from their samples as well as to Dr. Anders Molven from the Gade Institute, Haukeland University Hospital, Bergen, Norway for help with haplotyping.

The study was supported by grants from Latvian Council of Science (no 23.06.01, doctoral students grant), FP6 project no 018702, and grant from the European Social Fund (ESF) program no ESS2004/3.

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