CDKN2A gene mutations and genetic interactions in the pathogenesis of melanoma

Summary of PhD thesis

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ABBREVIATIONS

ACTH	Adrenocorticotrop hormone
alpha-MSH	Alpha-melanocyte stimulating hormone
ARF	Alternate reading frame
ASIP	Agouti signaling protein
BOLD	Bleomycin-Vincristine-Lomustine-Dacarbazine chemotherapy
bp	Base pair
BRCA1	Breast cancer type 1 susceptibility gene
BRCA2	Breast cancer type 2 susceptibility gene
CDK4	Cyclin dependent kinase 4
CDKN2A	Cyclin dependent kinase inhibitor 2A
DNA	Desoxyribonucleic acid
FAMMM	Familial atypical multiple mole and melanoma syndrome
FAMMM-PC	Familial atypical multiple mole melanoma and pancreatic cancer syndrome
MC1R	Melanocortin-1 receptor
MIM	Mendelian inheritance in man
mRNA	Messenger ribonucleic acid
NRHC	Non-red hair colour
nt	Nucleotide
p16 / $p16^{INK4A}$	Protein product of the CDKN2A gene
p14 / $p14^{ARF}$	Protein product of the CDKN2A gene with an alternative reading frame
PCR	Polymerase chain reaction
Rb	Retinoblastoma gene/protein
RHC	Red hair colour
RNA	Ribonucleic acid
UV	Ultraviolet

I. Introduction

Melanoma is a malignancy developing from cutaneous or ectopic melanocytes. Its incidence has been growing worldwide over the last decades. The prevalence of melanoma is relatively low compared to all cancers. However, it has the highest mortality rate among skin cancers which underlines the importance of the awareness of the predisposing factors and prevention, as well as early diagnosis and treatment. If diagnosed and treated early, most patients have the potential for full recovery and remain tumor free. Unfortunately, the number of patients who see the dermatologist with advanced melanoma has not significantly decreased yet, which can explain the relatively high mortality rate in our country.

It is well known that the major extrinsic predisposing factor for melanoma is UV exposure, in particular the UVA spectrum. Intrinsic predisposing factors are fair skin, multiple or large congenital nevi and dysplastic nevi. Approximately two third of the Hungarian population belongs to Fitzpatrick skin type II/III which means that their skin burns easily, tans poorly hence they are more susceptible to developing skin cancer including malignant melanoma. Personal or family history of melanoma, the familial atypical multiple mole and melanoma (FAMMM) syndrome along with certain associated gene mutations and polymorphisms are known contributors to melanoma susceptibility. Familial melanoma accounts for approximately 10% of all melanoma cases. The association of pancreatic cancer is not uncommon in familial melanoma; it is often referred to as the FAMMM-PC syndrome.

The major locus for melanoma predisposition is the cyclin dependent kinase inhibitor 2A (CDKN2A) gene on chromosome 9p21. This cell-cycle regulatory gene constitutes of four exons and has different transcript variants as a result of alternative splicing. The two main variants are p16 and $p14^{ARF}$. The main function of these proteins is tumor suppression via two separate molecular pathways. p16 acts by inhibiting the CDK4 kinase in the retinoblastoma (Rb) pathway. The $p14^{ARF}$ is structurally unrelated and is linked to the p53 pathway. Mutations of the CDKN2A gene are rare but CDKN2A mutation prevalence in familial melanoma is approximately 20-40%. Most of the so far detected CDKN2A mutations affect the coding regions of the gene. However, variations in the intronic sequences have also been reported in association with melanoma either or not directly affecting the splice sites. The penetrance of CDKN2A mutations is highly variable and may be modified by other genes or environmental factors.

The melanocortin-1 receptor (MC1R) gene is a key determinant of normal human pigmentation and sun sensitivity. It is located on chromosome 16, has only on exon and encodes a

G-protein coupled receptor protein, located in cutaneous and hair follicle melanocytes. Binding of alpha-MSH initiates intracellular signaling which leads to melanin production. Eumelanin (brown/black pigment) is photoprotective while pheomelanin (yellow/red pigment) generates free radicals upon UV radiation therefore may contribute to UV-induced skin damage. The balance of melanin production is determined by the several existing MC1R polymorphisms but in individuals with a wild type MC1R the predominant pigment is eumelanin. MC1R is a low-penetrance melanoma susceptibility gene and has been shown to modify the penetrance of other melanoma predisposing gene mutations including CDKN2A.

II. Aims

The frequency of CDKN2A mutations and the proportion of families with the most frequent founder mutations of each locale vary remarkably across geographical areas. Our aims were:

- to gather data on the frequency and pattern of CDKN2A mutations and polymorphisms in Hungarian melanoma prone families,
- to compare CDKN2A mutation detection rates in Hungarian patients to those in the European and world population,
- to contribute to the understanding of the genetic factors and the gene-environmental interactions – including the role of MC1R polymorphisms – in the pathogenesis of melanoma.

III. Patients and methods

Enrolment of patients to the study

Patients and their relatives were recruited from the Dermato-Oncology clinics of the department. Those patients were offered participation in genetic testing who presented with (1) melanoma affecting at least one first- or second-degree relative, (2) melanoma and at least one first- or second degree relative with atypical moles and/or high total body nevi count and/or melanoma (FAMMM), (3) multiple primary melanoma and (4) melanoma with presentation of other malignancies in the patient or family members, especially pancreatic cancer (FAMMM-PC syndrome).

Over 120 samples from melanoma prone families were tested for germline genetic alterations in the CDKN2A gene. The four exons and flanking intronic sequences of the CDKN2A gene were

amplified. Three cases will be highlighted in which the results of genetic analysis were found remarkable in view of current data on CDKN2A mutations.

Case history 1 and details of the genetic analysis

A 30 year-old Caucasian male with multiple atypical nevi presented with three primary melanomas. Despite surgical removal of the lesions, consequent radical lymph node dissection, and later chemo and radiotherapy, he died of metastatic disease. The presentation of multiple primary melanomas at a relatively young age and the presence of numerous atypical nevi suggested a genetic predisposition to melanoma.

2 ml of venous blood was taken from the patient. Genomic DNA was isolated by using the Genomic DNA Purification Kit of Gentra (Minneapolis, MN, USA) and exons 1A, 1B, 2 and 3 of the CDKN2A gene were amplified. After detection of a rare mutation in exon 1A of the CDKN2A gene, oral swabs were taken from the family members and genomic DNA was isolated from the samples with the MagNA Pure Compact system (Roche, Mannheim, Germany). In case of the family members, only exon 1A was sequenced with the Resequencing Amplicon probe system (http://www.ncbi.nlm.nih.gov/genome/probe/reports/probereport), probe ID: RSA001284450. We also performed sequence analysis of the MC1R in all the family members. The whole length of the only exon of MC1R gene was amplified using methods detailed in the paper of Szell et al. All PCR products were purified using the Quantum Prep PCR Kleen Spin Columns of Bio-Rad (Hercules, CA, USA) and the sequence analysis was carried out using the BioEdit software.

Case history 2 and details of the genetic analysis

A 33-year-old female patient developed melanoma, consecutive (metachronous) ductal adenocarcinoma of the breast and primary pancreas adenocarcinoma. The three tumors developed independently of each other. Family history revealed gastric and laryngeal carcinoma in her father while her mother had no malignancy. The paternal aunt had died from breast cancer at a young age decades ago therefore her genetic investigation could not be performed.

DNA from venous blood was isolated using the QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and exons 1A, 1B, 2 and 3 of the CDKN2A gene were amplified with the Resequencing Amplicon probe system (probe IDs: RSA001284450, RSA000045423, RSA000942236, RSA000942233). The PCR products were purified using the Quantum Prep PCR Kleen Spin Columns (Bio-Rad, Hercules, CA, USA) as in our previous experiments. Because of the occurrence of breast adenocarcinoma in our patient's medical history, it was also tested whether she

carried mutations in the BRCA1 and BRCA2 genes. The 15 most common hot spot BRCA mutations were studied using external service (Delta Bio 2000 Kft.).

Case history 3 and details of the genetic analysis

Investigations in an extensive Hungarian family with FAMMM syndrome were carried out in collaboration with the Human Molecular Genetics Laboratory at the International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy.

Detailed analysis of the family tree revealed other malignancies beside melanoma. The genetic analysis of family members in branches B and C was carried out using the same methods as previously detailed under Case 2. Unfortunately, members in Branch A were not available for genetic screening. After detection of a rare intronic mutation, we planned to obtain skin specimens to facilitate the *in vivo* identification of potential alternative CDKN2A splice variants. However, the family members declined to provide skin biopsy specimens. We therefore conducted *in vitro* functional analysis to investigate whether the IVS1+37 G⁄C intronic mutation had any effect on splicing regulation.

Wild type and mutant minigenes were constructed. To investigate the effects of the mutation on splicing, HeLa cells were transfected with the wild-type and mutant minigenes. Transfection was carried out with the Turbo Fect reagent (Fermentas, Vilnius, Lithuania). The cells were cultured for 24 hours and total RNA was isolated using the TRI Reagent Solution (Applied Biosystems, Foster City, CA, U.S.A.). Reverse transcription was performed with the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, U.S.A.) and the splice variants were detected with PCR. T7 and Sp6 primers were used for the pcDNA3 vector in order to avoid the amplification of internal CDKN2A transcripts. PCR reactions were performed in GoTaq Hot Start mix (Promega, Madison, WI, U.S.A.). The PCR products were run on 2% agarose gel and photographed, and the bands were purified from the gel and sequenced.

In addition to CDKN2A studies in the family, the MC1R gene was also amplified and the 951 bp coding region was sequenced in two overlapping fragments.

IV. Results

1. Detection of the rare P48T mutation in the CDKN2A gene and melanoma susceptibility related MC1R polymorphisms

In case of the young male patient with multiple primary melanoma and unaffected family members, we detected a homozygote 142C>A transition in exon 1A, which causes a proline to threonine amino acid change at codon position 48 (P48T). The patient's father, mother and daughter are all heterozygote carriers of the mutation. His wife harbors the wild-type allele. There were no other mutations detected in the sequences examined in the patient or his relatives.

Three different well-known MC1R polymorphisms were found to run in the family. The parents and the widow of the patient harbor only one polymorphism in heterozygote form: R151C, V92M and R142H. The patient and his young daughter are compound heterozygotes and carry two melanoma susceptibility-related MC1R polymorphisms: V92M, R151C and V92M, R142H respectively.

2. Detection of the R24P CDKN2A mutation in association with multiple primary malignancies including melanoma

The investigation of the 33-year-old female patient with three consecutive primary malignancies and her parents revealed that the patient and her father carried a heterozygote missense mutation in exon 1A of CDKN2A. The mutation causes an arginine to proline amino acid change in codon 24 (R24P) affecting only the $p16^{INK4a}$ transcript variant.

According to the sequencing data of the BRCA1 and BRCA2 genes, none of the 15 most common BRCA mutations could be detected in the female patient. Having received these data, we did not perform BRCA gene analysis on her father.

3. Detection of the rare IVS1+37 G/C intronic mutation and its role in splicing regulation

In case of the family with FAMMM syndrome, a rare intronic CDKN2A mutation was detected in intron 1 (IVS1+37 G⁄C). In the following minigene experiments, sequence analysis and comparison of the mRNA arising from the wild-type and that from the mutant minigene revealed a differential splicing pattern. In contrast to the sequence expected as a result of normal splicing from the wild type minigene, the mRNA from the mutant minigene corresponded to an extended alternative splice product formed by the addition of a 120-nt sequence of intron 1 as an exon. This result was identically obtained in three independent transfection experiments on HeLa cells,

including work both at the ICGEB and the Department of Dermatology and Allergology laboratories, suggesting that the *in vitro* minigene approach applied was suitable for studying the effects of the identified intronic IVS1+37 G⁄C mutation on splicing.

MC1R sequence analysis showed that all family members carry at least one frequent red hair colour variant. Four family members harbour the V92M variant in heterozygote form. The two most senior individuals with melanoma carry at least two MC1R variants, including the R160W variant in heterozygote form, which is a frequent RHC variant and associated with melanoma.

V. Discussion

Three cases from our cohort of patients and relatives are highlighted in which the detection of CDKN2A mutations was thought to strongly contribute to melanoma susceptibility and possibly contribute to other malignancies.

The **P48T mutation** has been reported to date in only four cases, all in patients with Italian ancestors. In these cases the P48T mutation was found in heterozygote form in association with familial and multiple primary melanoma, pancreatic carcinoma as well as with a case of MEN2A syndrome along with a RET proto-oncogene mutation and family history of melanoma, pancreatic carcinoma and several other malignancies. Our case lends further support to the multifactorial nature of melanoma predisposition, where the penetrance of a certain allele might depend on modifying factors and can be extremely variable across geographic areas, since the heterozygote mutant parents of the patient, at the age of 69 and 63 were free of any malignancies and atypical nevi despite the fact that both have had a rural lifestyle with extensive exposure to sunlight. Furthermore, it is extremely rare to discover individuals homozygous for CDKN2A mutations.

Our results support that the rare P48T mutation of CDKN2A is a melanoma-predisposing factor, but the heterozygous status itself is not causative of malignancy. The the patient carried two MC1R polymorphisms (V92M, R151C), both of which have been shown to be associated with melanoma and also thought to predispose to non-melanoma skin cancer, independently of the effect on skin type. This supports the role of certain MC1R polymorphisms as additional predisposing factors to melanoma. The fact that the daughter of the deceased patient carries both the P48T CDKN2A mutation, as well as two frequently detected, melanoma-associated MC1R variants (V92M and R142H), underlines the importance of long term dermatology follow-up. However, the risk of developing melanoma from carrying these genetic variants can not be quantified.

Although the patient's parents were not aware of consanguinity in their families, the fact that they both harbor the P48T germ line mutation suggests that there must have been such an event or

events a few generations ago. It is interesting that the only four previous reports of this mutation, and the one reported after our results were published, involved Italian patients and a Brazilian patient with Italian ancestors. The members of the mentioned Hungarian family are unaware of any Italian family relatives. The question whether this mutation is the result of an independent event or whether the founder mutation migrated some generations ago from Italy to Hungary or from Hungary to Northern Italy demands further investigation.

Many independent studies proved the melanoma-predisposing nature of the **R24P mutation** being one of the most widespread among the so far identified disease-associated mutations of the CDKN2A gene. To our best knowledge, ours is the first report on the identification of the R24P mutation in a Central-European family. Whether it is an ancient founder mutation that has spread to many geographical locales in the past, or independent mutation events happened, would be interesting to investigate.

We also tested the patient for the presence of BRCA1 and BRCA2 hotspot mutations but found no alterations in her case. Although we can not exclude the possibility that other predisposing gene variants may have contributed to the breast cancer of the patient, we suggest that the disclosed R24P CDKN2A mutation may have played a key role in the pathogenesis of her multi-organ primary malignancies. As CDKN2A mutation studies became more and more intensive with the enrolment of centers from all over the world, not only the genetic predisposition of familial melanoma but also its co-morbidities became recognised. This is a good example of how genetic investigations can inspire epidemiological studies and shed light to connections of different diseases and their common predisposing factors. Surveying the relevant literature clearly revealed that CDKN2A germline mutations are highly accepted as predisposing genetic factors for patients who suffer from co-existing pancreas carcinoma and malignant melanoma. However, no such consensus exists for the association of CDKN2A germline variants and the primary multiple occurrence of melanoma and breast cancer. Studies performed in relatively small cohorts of patients resulted in contradictory data: some of them support while others reject the notion of the breast cancer-predisposing nature of CDKN2A germline mutations. To resolve this problem, extended studies on a wide range of low- and high-penetrance genetic predisposing factors must be examined on a multicentre basis. We believe that single cases such as the one we presented here may contribute to the understanding of the role of genetic susceptibility and environmental factors in the pathogenesis of multiple primary malignancies.

With regards to the frequency of melanoma prone families linked to 9p21, the detection of germline coding mutations of the CDKN2A gene is lower than expected. Our *in vitro* minigene experiments showed that the presence of the **IVS1+37 G/C mutation** results in the recognition of an otherwise intronic sequence as an exon and a 120nt long sequence is therefore included in the mRNA. If the aberrant mRNA was translated, the inclusion of the cryptic exon would result in a frameshift and an early stop codon would change the structure of the p16 protein; if the aberrant mRNA was not stable, it would indirectly reduce the quantity of functional p16. It is interesting that, regarding the pancreas-specific isoform, the mutation theoretically causes a p.Gly63Arg amino acid change. However, mutations in the coding region might also affect splicing regulation. Whether the aberrant mRNA is stable and translated or not, our results indicate that this mutation may play a pathogenetic role in familial melanoma, most likely involving alteration of the splicing of the CDKN2A primary mRNA. Identification and characterization of intronic mutations could significantly contribute to our understanding of so far relatively unexplored mechanisms in melanoma pathogenesis and explain why the frequency of germline coding mutations of the CDKN2A gene is lower than expected in melanoma-prone families linked to chromosome 9p21.

Melanoma is an example of multifactorial diseases. There are well known environmental predisposing factors as well as multiple genes linked to melanoma susceptibility, the effects of which are highly variable on each individual. Melanoma predisposing genes are categorised into rare high penetrance genes such as CDKN2A, and low penetrance genes, MC1R being the most common. Melanoma risk in CDKN2A mutation carriers is modified by multiple factors including MC1R variants, pigmentation and nevus phenotype. Carrying any one of the four most frequent MC1R variants (V60L, V92M, R151C, R160W) or an increase in number of MC1R variants in CDKN2A mutation carriers is associated with a statistically significantly increased risk for melanoma across all continents. These associations may have important implications in risk assessment of families with multiple cases of melanoma. However, we currently do not have sufficient data to quantify melanoma risk according to the genetic make-up.

Having detected three CDKN2A mutations as detailed out of more than 120 samples analysed, we concluded that CDKN2A mutation detection rates in Hungarian melanoma-prone families are comparable to the European and worldwide statistics. The results of our experiments are not directly fed back to our patients partially due to ethical considerations but most importantly because clinical testing for CDKN2A mutations still has a very limited role at present in the clinical management. The American Academy of Dermatology has set recommendations regarding the

selection of patients with familial melanoma for genetic testing. It suggests – depending on the melanoma incidence of the geographical area – that two or three melanoma and/or pancreatic cancer events in a family may be sufficient to consider a genetics referral. In contrast, the current consensus of the Melanoma Genetics Consortium is that it is premature to suggest gene testing routinely which may change as more is known of the genes predisposing to melanoma.

The above considerations and our documented cases well demonstrate that genetic testing for research purposes has an important contributory role to science, and potentially to evidence based medicine on the long term. Our cases also underline the standpoint that malignant melanoma even with a highly predisposing genetic background must be considered as a multifactorial disease where the straightforward genetic testing and counselling can not be performed as in the cases of monogenic diseases. This also highlights the importance of multidisciplinary team working between professionals of various specialties, sharing their research, up-to-date evidence based knowledge and experience for a better and holistic patient care.

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