Environmental and genetic factors in the pathogenesis of melanoma and melanoma associated other primary malignancies

Ph.D. Thesis

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I. INTRODUCTION

Malignant melanoma (MM) is a multifactorial cancer with identified genetic and environmental predisposing factors. Prevalence rates and gene-environment interactions vary along geographical locations upon latitude. Major environmental predisposing factor is ultraviolet (UV) radiation. Next to UVB, the significance of UVA is also emerging based on epidemiologic data and also by findings on fibroblasts showing that there are weaker DNA damage response mechanisms after UVA than after UVB. Other extrinsic predisposing factors such as electromagnetic radiation, ionization radiation or firefighters' environment are also characterized among many others. In immunosuppressed status (e.g. organ transplant recipients: OTR) MM risk may be elevated by 3-8-fold.

Genetic factors, especially in multiple primary melanoma (MPM) patients and in familial MM aggregations are of a great importance. *CDKN2A* and *CDK4* are high-penetrance high risk genes. Melanocortin 1 receptor (*MC1R*) gene variants confer an intermediate risk to MM. *MC1R* 'R' variants (R151C, I155T, R160W, D294H) are in strong association with a phenotype of red hair and

fair-, UV sensitive skin; in contrast with the ‘r’ variants (V60L, V92M, R163Q) whose role in this phenotype is much weaker. MM risk is associated at most by the ‘R’ variants, albeit the significance of ‘r’ variants, moreover irrespectively of the type-, the number of carried variants also influences the risk to MM. *MC1R* variant frequencies show geographical differences. In microphtalmia-associated transcription factor (*MITF*) a germline point mutation (p.E318K) has been verified as an intermediate risk factor for MPM, familial MM, and for MM and renal cell cancer (RCC) co-occurrence.

MM survivors are at an increased risk of developing other malignancies, most frequently subsequent MM; however non-melanoma skin cancer (NMSC) mainly basal cell cancer (BCC), pancreatic cancer (PaC), and breast cancer occurs also at a higher rate among them. Furthermore as associated malignancy MM is also reported in a number of cancer predisposing syndromes, like in Breast-ovarian cancer syndrome (*BRCA1* and *BRCA2*), and rarely in PTEN hamartoma tumor syndromes (PHTS), Cowden syndrome (CS).

II. AIMS

As no genetic data was available about the major MM predisposing gene mutations among Hungarian MM (MPM) patients so far, our aim was to analyze:

II.1. MPM patients (MPM study) by

a. Collecting clinicopathological features, including patients' phenotypes, medical history, family history of malignancies; MPMs' characteristics with detailed histology and disease course with outcome;

b. Germline sequence analysis of the major MM predisposing genes: *CDKN2A, CDK4, MC1R*, and *MITF* E318K; and further analysis of possible associations between available clinical, pathological and genetic data focused on *MC1R* variants using statistical methods. We also discussed a comparison of our *MC1R* variant frequencies and distributions to other countries' results.

c. Analysis of *MC1R* variant status regarding 'r' carriers (00, r0, rr) and 'R' carriers (R0, rR, RR), and to see whether these genotypes influence any of the clinical or pathological characteristics of MPM patients.

II.2. Unique MM-associated cases

During patient care and data analysis, some patients presented with unique clinical course and highly positive medical -, and family history of benign and malignant tumors. In three unique MM-related cases we separately obtained clinical exploration of the detected tumor constellations and pedigrees together with

a. sequence analysis of the most suspicious genes

b. discussion of the relevant etiological factors including the identified genetic results, documented environmental factors, and further suspicious, herein not sequenced genetic events.

III. METHODS

III.1. Clinical data, patients' characteristics

III.1.1. MPM study

From patients diagnosed with primary MM at Semmelweis University Department of Dermatology Venereology and Dermatooncology in an 11-year long period, 108/1855 developed subsequent MMs (5.8%); 43 participated in the genetic studies. They developed altogether 106 MMs (with an average of 2.47 per capita). Mean follow-up time was: 6.7 (0-34.2) years. Besides the

routinely recorded data of medical self-, and family history, we also included pigmentation phenotype, approximate number of common and dysplastic nevi, smoking habit and occupation. Routine MM histologic parameters and disease course with propagations, treatments and MM-related deaths were also documented.

III.1.2. Unique MM-associated cases

III.1.2.1. Two cancer prone families

Family A and B with two common offspring also with early onset malignancies have been examined.

In family A, with many heavy smokers and firefighters, two non-twin brothers (one of them is index person) both had a unique constellation of four similar primary malignant tumors: MPMs (2 each), BCCs, PrC and LC. Furthermore within family A, LC and neural crest tumors occurred more times, while PaC, RCC, and gastric cancer incidentally. Index persons' wife was member of family B that was highly positive for PaC in a dominantly inherited manner. One of their offspring had early onset RCC, while the other died at age of 37 at PaC.

III.1.2.2. Six primary MMs

We analyzed a 59 year old female renal OTR patient, who developed six primary MMs under 7 years of immunosuppressive therapy. She had MM vulnerable cutaneous pigmentation characteristic (red hair, fair skin), a harmful sun tanning habit for years and bad compliance regarding dermatological follow-ups. Despite the revision of her immunosuppressive therapy after the 4th MM to a more favorable combination in terms of malignant tumor development (tacrolimus to sirolimus), she further developed two MMs and deceased of MM propagation one year later.

III.1.2.3. MM and phenotype suggesting PHTS/CS:

We examined a female patient from a cancer prone family (LC, BCC, uterine cancer) who presented with significant body deformities (severe scoliosis, skull hyperostosis, dolichocephaly, disproportionate overgrowths and hemihypertrophies of certain body areas with atrophies of other sites) and the following diversified oncological history: lentigo maligna, verified breast cancer, myoma uteri, thyroid and parathyroid adenomas, lipomas, connective tissue nevi together with

suspicious RCC and genitourinary malformations raising the possibility of PHTS, especially of CS.

III.2. Mutation analysis

Sanger sequencing: DNA was isolated from peripheral lymphocytes (Roche Magna Pure System) and in some cases from formalin fixed paraffin embedded (FFPE) tissue sections (Qiagen). For PCR amplification primers were either obtained from previous publications, or self-designed. Quality and quantity of PCR reaction have been checked with agarose gel electrophoresis on 2% agarose gel and GelGreen® (Biotium) staining. Enzymatic system (ExoShap-IT, USB) was used for purification of PCR products that was followed by sequencing reaction (BigDye Terminator v3.1 Cycle Sequencing Kit, Abi) and purification with gel filtration technology (NucleoSEQ, Macherey-Nagel GmbH). Finally Sanger sequencing in both directions was obtained using ABI PRISM 3100 Genetic Analyzer. Sequence results were visualized in FinchTV Softver, than were compared to the actual NCBI reference sequences. The following selected genes were analysed.

In the **MPM study** all patients have been genotyped for the whole coding regions with extensions to either ends, and exon intron boundaries of *CDKN2A*, *MC1R* together with exon 2 of *CDK4* and *MITF* E318K (in exon 10). Regarding **Unique MM-associated cases**, in all the three cases the MM predisposing genes have been genotyped. Additionally: In the **Two cancer prone families** in certain family members hot spot containing fractions of *BRCA 1* (exon 2, segments of exons 11 and 20) and of *BRCA2* (segment of exon 11) were sequenced; while in some members the whole genes of *BRCA1* and *BRCA2*, and *PTEN* gene were genotyped.

In **the OTR with six primary MMs** the whole coding *PTEN* gene has been genotyped.

In the case of **MM and phenotype suggesting PHTS/CS** *SDHB* and *SDHD* have been genotyped.

Mutation verification and control analysis were obtained using locus specific restriction endonuclease enzymes: *MC1R* D117G with PsyI (Tth111I) (200 alleles); *MITF* V320A with BsrBI (MbiI) (100 alleles) (both Fermentas Life Science).

III.3. Statistical methods

Comparisons of continuous variables between groups in MPM study were performed using independent Student's t-test. Categorical variables were compared using the Chi-square test or the Fisher-exact test whichever appropriate. The levels of significance were set to $p<0.05$.

IV. RESULTS

IV.1. Results of MPM study

IV.1.1. Clinicopathological attributes

Among the 43 MPM patients, the mean age at initial diagnosis was 61 years, which was significantly lower in women than in men (55.3 versus 65.4 years; p=0.016) and decreased with i) the history of other malignancy, ii) family history of MM and iii) higher final MM number. Family history of MM was positive in 4 patients (9.3%).

Second MMs were observed synchronously in 49% (21/43) of the patients. Phenotype with more than 20 common nevi or any dysplastic naevus was presented in 51% of patients; they had significantly more MMs (2.8 versus 2.4MMs; p=0.026). Twenty patients (47%) had the first and second MMs on the same body site. More in

situ tumors were observed among the second (37%)-, or subsequent MMs (25%) than among first MMs (9%). Superficial spreading MM was the most common subtype (60.4%), while nodular MM occurred only in four cases (3.8% of all MMs). First MMs exhibited significantly thicker Breslow (2.16 versus 1.16 mm; p=0.013) than subsequent ones.

Subsequent primary malignancies among MPM patients: Of the 43 MPM patients, 18 (42%) had non-MM tumors from which 83% had cutaneous, 17% extra-cutaneous, while 28% had both.

IV.1.2. Genetic results:

***CDKN2A*:** Two out of 43 MPM patients (4.7%) had *CDKN2A* mutations (c.296G>C-p.R99P and c.206A>G-p.E69G). The well-known c.-191 G>A SNP was present in 88.3% of MPM patients. In the 3'UTR, SNPs c.*29 C>G was observed in eleven patients (25.6%) while the c.*69 C>T in 7 (16.3%) The p.A148T SNP was carried only by 2 patients (4.6%).

***CDK4*:** None of the patients carried any of the previously described mutations (R24H, R24C).

***MITF*:** The c.1075G>A-p.E318K mutation was not detected in any of our patients. We identified the SNP c.1082T>C-p.V320A (rs2055006) in the same exon in one patient, but not in 50 healthy controls.

***MC1R*:** Nine different variants (8 non-synonymous and 1 synonymous) were detected in 37/43 (86%) patients. The overall allele frequency was 30% for 'r' variants and 33% for 'R' variants. The most common variant was R151C (30%). One novel non-synonymous variant (c.350A>G-p.D117G) was identified in one MPM patient but not in 100 healthy controls. The R163Q was observed in 16.2% of patients with an allele frequency of 8.1%.

IV.1.3. Analysis of MC1R variant status

By comparing clinicopathological features of *MC1R* 'R' and 'r' carrier patients, 'R'carriers were more prone to develop non-melanoma malignancies and multiple BCCs. Regarding MM histology, we found in 'R'carriers a non-significant association with more i) invasive second MMs, ii) ulcerated first MMs, iii) and non-ulcerated second MMs, but correlated significantly with tumor infiltrating lymphocytes (TILs) in second MMs (67 versus 27%; p=0.035). Regression or mitotic rate showed no considerable differences in 'R' and 'r'carriers.

Although propagation and death were more frequent among ‘R’carriers, 5-year overall survival showed no signicant difference.

IV.2. Results of Unique MM-associated cases

IV.2.1. Two cancer prone families

In family A, index person carried a germline *CDKN2A* mutation (p.R99P) that was segregated also to his offspring with early onset fatal PaC at somatic level in PaC sample. The index person also carried two *MC1R* ‘r’variants (V60L and V92M) and a synonymous variant (T314T). He also harbored a number of SNPs along *CDKN2A* (c.-191 G>A, c.*69 C>T), *PTEN* (c.80-96 A>G, c.1026+32 T>G), and *BRCA1* (c.1067 A>G; p.Q356R) genes. In family B, we found no mutations, but number of SNPs in *CDKN2A* (c.-191 G>A, c.193+174 A>G), in BRCA1 (c.2311 T>C; p.L771L) and in *BRCA2* (c.-26 G>A, c.631+183 T>A, c.3396 A>G; p.K1132K, c.3807 T>C; p.V1269V, c.5744 C>T; p.T1915M, c.7242 A>G; p.S2414S, c.7806-14 T>C, c.8755-66 T>C, c.*105 A>C), from which *BRCA1* L771L and *BRCA2* K1132K were those, carried only by a PaC affected family member.

IV.2.2. Six primary MMs

No mutations were detected in the high-risk MM predisposing genes (*CDKN2A*, *CDK4*). We found some SNPs in *CDKN2A* (c.-191 G>A, c.*29 C>G) and she carried a *MC1R* 'R' variant (R151C).

IV.2.3. MM and phenotype suggesting PHTS/CS

No pathogenic mutation was detected along the examined genes, but she carried a number of SNPs in *CDKN2A* (IVS1β+174 A>G, c.*69 C>T), *PTEN* (c.80-96 A>G, c.1026+32 T>G), *SDHB* (c.18 C>A), *SDHD* genes (c.52+136 G>T, c.314+15 T>A) and a *MC1R* 'R'variant (R160W), from which the alteration located in the IVS3 of *SDHD* c.314+15 T>A is a new finding.

V. CONCLUSIONS

V.1. MPM study

With this work, as a first study on Hungarian MPM patients from clinical, histologic and genetic point of view we can conclude, that

1. The following results were in accordance with previous data from the literature:

- Rate of MPM occurrence among MM patients
- Higher number of common nevi or presence of dysplastic naevus are susceptibility factors for MPM development
- Younger age of onset in women than in men
- Subsequent MMs in are thinner, more likely Mis than invasive MM, and develop commonly on the same body site with the first MM
- Rate of *CDKN2A* mutations among MPM patients
- Frequency of certain *CDKN2A* SNPs (c.*29 C>G, c.*69 C>T)
- Frequency of *MC1R* non-synonymous variants
- Frequency of 'R' variants among MPM patients

2. The following of our observations differed from previous reports, or brought novel results:

- Less frequent positive family history of MM
- A higher rate of synchronous first and second MMs
- More frequent non-melanoma malignancy occurrence
- Two Hungarian *CDKN2A* mutation carrier identification (p.E69G, p.R99P)
- Lower frequency of *CDKN2A* A148T SNP

- Higher allele and carrier frequency of SNP *CDKN2A* c.-191 G>A
- The *MC1R* R163Q variant to be exceptionally common among Hungarian MPM patients, a variant otherwise frequent in Asia, but not in Europe, supporting the previous findings on geographical differences regarding *MC1R* variant occurrence.
- Identification of a new *MC1R* variant firstly in humans (c.350 A>G;p.D117G)
- some new potentially unfavorable predictive observations among *MC1R* 'R' carriers compared to *MC1R* 'r' carriers
    - younger age of onset
    - MPM co-occurrence with more non-melanoma primary tumors, or with multiple BCCs
    - more ulcerated first MMs, less ulcerated second MMs
    - TILs in second MMs (significantly).

Therefore we hypothesize that as already suspected in terms of other MM predisposing genes (*CDKN2A*), *MC1R* genotype details may also carry additional useful

information concerning patient survival and prognosis if confirmed on bigger sample sizes.

V.2. Unique MM-associated cases

V.2.1. Two cancer prone families

In family A, an unfavorable coincidence of inherited and environmental risk factors induced the new constellation of 4 primary malignancies (MPM, BCC, PrC, LC) in two non-twin siblings. In family B with a dominantly inherited PaC aggregation we could identify only a number of SNPs with unknown significance regarding PaC development. The inheritance of predisposing genetic events from the two families resulted in early onset malignant tumor formation in both of the offspring, in one of them even with fatal outcome. These data also indicate the relevance of identification of such cancer prone families and the necessity of a very close follow-up of the uninvolved family members too.

V.2.2. Six primary MMs

Despite the immunosuppressed state in the OTR patient, the development of six primary MMs raised the possibility of a genetic susceptibility to MM. in Besides

the skin tumor formations there was a complex interplay between diverse environmental (UV/sun tan habit; combined long term immunosuppression; bad compliance) and genetic (*MC1R* 'R' variant) predisposing factors. This case points out the importance of the careful dermatological follow up of OTRs under immunosuppressive therapy, and also of their education about the subsequent environmental predisposing factors.

V.2.3. MM and phenotype suggesting PHTS/CS

As a rare co-aggregation, MM with phenotype highly suspicious for PHTS/CS has been explored. However the involvement of a particular signal transduction pathway (*PIP3K*, *PTEN*, *AKT1*) and other genetic loci (*SDHB*, *SDHD*) were highly suspicious, we could not identify the exact underlying germline genetic disturbance. Analysis of *AKT1* somatic mutations suspicious for Proteus syndrome, or next-generation techniques would be rational future options.

In conclusions of the three Unique MM-associated cases we hypothesize the relevance of parallel inherited common gene alterations and gene-environment

interactions in malignant tumor development and aggregations.

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