High Prevalence of Germline CDKN2A Mutations in Slovenian Cutaneous Malignant Melanoma Families

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Aim To prospectively determine the prevalence of germline CDKN2A mutations in the Slovenian cutaneous malignant melanoma (CMM) families.

Methods From January 2001 till the end of 2003 we prospectively screened 19 individuals from 11 CMM families, as well as 3 children with CMM aged from 6 to 13 years, with a negative family history.

Results Five distinct mutations were detected in 5 out of 11 screened families (10/19 individuals) and a previously recognized polymorphism was detected in a single family. Detected mutations were functionally deleterious (T281A, G68A, G301T, G71C and IVS – 1g > a). No mutations could be detected in 3 children.

Conclusions The prevalence of CDKN2A mutations among Slovenian CMM families was high, indicating the need for genetic counseling.

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The CDKN2A gene is the most common cause of inherited cutaneous malignant melanoma (CMM) (1). The presence and frequency of CDKN2A gene mutations in familial CMM vary considerably in different populations. Yakobson (2) did not find even a single case of germline CDKN2A mutation among 31 Israeli CMM families. However, Mantelli (3) reported a high prevalence of 34% of the germline CDKN2A mutations in his study of 62 Italian CMM families. Overall, the CDKN2A mutations were detected in approximately 20% of the tested CMM families (1).

The aim of our study was to prospectively determine the prevalence of germline CDKN2A mutations in the Slovenian CMM families.

Patients and Methods

Patients

From January 2001 until the end of 2003, 11 patients with CMM and the history of at least two affected family members were treated at the Melanoma Clinic of the Institute of Oncology, Ljubljana. All these 11 index patients and their 8 healthy relatives who agreed to participate in the study were referred to the Familial Cancer Clinic for genetic counseling and testing (Table 1).

Additionally, 3 children with CMM aged from 6 to 13 years and with negative family history were also referred to the Familial Cancer Clinic for Genetic Testing. We did not examine the children's relatives since children were all mutation negative.

Methods

Genomic DNA of the patients was extracted from the peripheral blood using the salting-out procedure (4). Each of the three exons, together with their splicing sites, was amplified in the polymerase chain reaction (PCR). The fragment with the exon 1 included also a part of the promoter, the fragment with the exon 3 included a part of 3′-UTR, and on the other side, a part of the intron 2, included the possible mutation spot IVS2-105. The PCR reaction mixture, thermal cycling conditions, and primers were used as described elsewhere (5). Briefly, AmpliTaq Gold polymerase was used for amplification in 32 thermal cycles in all reactions. The presence of amplicons was verified by a 2% agarose gel electrophoresis.

For PCR fragments, the purification QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) was used. The sequencing was performed in both directions by the same forward and reverse primers that were used for the initial PCR amplification. Sequencing reaction was performed using the ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Norwalk, CT, USA). The products were analyzed on the ABI PRISM 310 DNA genetic analyzer (Applied Biosystems).

The obtained nucleotide sequences were compared to the genomic reference sequence in GenBank, accession number AF527803.

We used *t* test for comparison of the mean values between groups.

Results

Five distinct mutations (T281A, G68A, G301T, G71C and IVS – 1g > a) were detected in 5 out of 11 screened families (10/19 individuals) (Table 1). Additionally, a previously recognized polymorphism (A148T) was detected in one family. Five out of 8 relatives were also tested positive. In the family No. 2 (T281A mutation), all 4 rel-

Table 1. Clinical details and mutation analysis results of 11 Slovenian cutaneous malignant melanoma families tested for germline CDKN2A gene mutations

Family	Patient's sex	Age at diagnosis of first primary melanoma	No of primary melanomas	Affected relatives	Mutation	Protein	No. of tested family members/ No. of mutation carriers
1	female	27	3	two sisters	–	–	
2	female	57	5	son, nephew	T281A	L94Q	4/4
3	female	36	1	mother	–	–	
4	male	40	4	mother, sister	G68A	G23D	2/1
5	female	44	1	mother	–	–	
6	female	35	1	mother	–	–	
7	female	21	3	mother	IVS – 1g > a		1/0
8	female	50	3	mother	G301T	G101W	1/0
9	male	62	1	nephew	–	–	
10	female	35	6	mother	G71C	R24P	
11	female	62	1	brother	G442A	A148T	

atives were tested positive. Although clinically healthy (without CMM) at the time of the test, one already developed CMM at the age of 41.

All 5 families with the detected CDKN2A gene mutation had an index patient with multiple primary melanomas. There was only one family (No. 1) with multiple primary melanomas and three affected family members without the CDKN2A gene mutation.

There was no statistically significant difference in the mean age at diagnosis of index patients between the group of families with the CDKN2A gene mutation (40.6 years, range 21-57) and the group without the CDKN2A gene mutation (40.8 years, range 27-62) ($P<0.05$).

No mutations were detected in all three children (all <14 years) with CMM and a negative family history.

Discussion

The presence and frequency of CDKN2A gene mutations in familial CMM vary considerably among different continents and even among different countries on the same continent (1). Our study was the first study of the Slovenian population. Slovenia is a Central European country with two million inhabitants and an incidence of CMM of 11.3 per year. We found 5 different CDKN2A gene mutations in 5 out of 11 Slovenian CMM families (10/19 patients). This is a surprisingly high percentage of CDKN2A gene mutations since this was a clinic-based population. There were 3 affected family members in 3 separate families and 2 in other 8 families. A possible explanation for the high percentage in our population is the fact that the percentage of multiple primary melanomas in our CMM families was very high, 6 out of 11. In fact, CDKN2A gene mutations were detected only in CMM families with multiple primary melanomas (5/6). Multiple primary tumors are one of the key features of the genetic cancer syndromes. Hashemi (6) reported that 9 of 80 (11%) patients with multiple primary melanomas had the CDKN2A gene mutations. Seven of these patients belonged to CMM families. Additionally, Auroy et al (7) reported a series of 100 patients with multiple primary melanomas and a negative family history. The CDKN2A gene mutations were detected in 9 of them.

Mutational analysis was negative in all 3 children in our study (aged 6, 8, and 13 years), who had a negative melanoma family history. The reason why we included them in the study was the fact that the early age of onset is also one of the hallmarks of genetic cancer syndromes. In fact, the American Society of Clinical Oncology recommends that cancer-predisposition testing should be offered when the person has a strong family history or a very early age of cancer onset (8). The results of these 3 children are in agreement with the report by Whitemann et al (9) who found a single germline CDKN2A gene mutation in a large group of 61 children. Tsao et al (10), who analyzed the prevalence of germline CDKN2A gene mutations in patients with an early-onset CMM, also concluded that these mutations were not common and that, at the moment, genetic testing based solely on the age of onset could not be warranted.

All of the mutations detected in our series of patients were functionally deleterious. Two of the disease-causing mutations were novel and one of them was already described elsewhere (5).

Nucleotide substitution g.281T>A in the exon 2 causes the amino acid change L94Q. Its functional importance was presumed from the protein p16 structure. Hydrophobic properties of leucine are assumed to be important in keeping the correct 3-dimensional structure of the protein p16, which is probably needed in assuring the effectiveness of the binding site (5). The L94Q mutation co-segregates with the disease in the family.

IVS1-1g>a is a novel acceptor splice site mutation. A previously described substitution IVS1-1g>c in a family with melanomas, neuro-

fibromas, and multiple dysplastic nevi was demonstrated to lead to skipping the entire exon 2 in the proteins p16 and p14ARF. Both proteins were consequently inactive (11).

A g.68G>A mutation of the exon 1 in the CDKN2A gene causes the amino acid change G23D (12). This mutation was previously described in one French and one Italian family, with the co-segregation of the mutation with the disease in one of the families (12,13). To our knowledge, the biochemical activity of the G23D mutants has not been assessed. However, glycine in this place is absolutely conserved in human and murine p16 analogues (14).

In addition to 5 detected mutations, one patient without identified disease-causing mutation from our study was found to be heterozygous for previously recognized single nucleotide polymorphism g.442G>A (A148T).

In conclusion, the prevalence of CDKN2A mutations appears to be high (approaching 50%) among Slovenian CMM families. Genetic counseling is necessary for these families since they may benefit from preventive clinical programs.

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References

1 Bishop DT, Demenais F, Goldstein AM, Bergman W, Bishop JN, Bressac-de Paillerets B, et al. Geographical variation in the penetrance of CDKN2A mutations for melanoma. J Natl Cancer Inst. 2002;94:894-903. Medline:12072543

2 Yakobson EA, Zlotogorski A, Shafir R, Cohen M, Icekson M, Landau M, et al. Screening for tumour suppressor p16(CDKN2A) germline mutations in Israeli melanoma families. Clin Chem Lab Med. 1998;36:645-8. Medline:9806478

3 Mantelli M, Barile M, Ciotti P, Ghiorzo P, Lantieri F, Pastorino L, et al. High prevalence of the G101W germline mutation in the CDKN2A (P16(ink4a)) gene in 62 Italian malignant melanoma families. Am J Med Genet. 2002;107:214-21. Medline:11807902

4 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988;16:1215. Medline:3344216

5 Avbelj M, Hocevar M, Trebusak-Podkrajsek K, Krzisnik C, Battelino T. A novel L94Q mutation in the CDKN2A gene in a melanoma kindred. Melanoma Res. 2003;13:567-70. Medline:14646619

6 Hashemi J, Platz A, Ueno T, Stierner U, Ringborg U, Hansson J. CDKN2A germ-line mutations in individuals with multiple cutaneous melanomas. Cancer Res. 2000;60:6864-7. Medline:11156381

7 Auroy S, Avril MF, Chompret A, Pham D, Goldstein AM, Bianchi-Scarra G, et al. Sporadic multiple primary melanoma cases: CDKN2A germline mutations with a founder effect. Genes Chromosomes Cancer. 2001;32:195-202. Medline:11579459

8 Statement of the American Society of Clinical Oncology: genetic testing for cancer susceptibility, Adopted on February 20, 1996. J Clin Oncol. 1996;14:1730-6. Medline:8622094

9 Whiteman DC, Milligan A, Welch J, Green AC, Hayward NK. Germline CDKN2A mutations in childhood melanoma. J Natl Cancer Inst. 1997;89:1460. Medline:9326917

10 Tsao H, Zhang X, Kwitkiwski K, Finkelstein DM, Sober AJ, Haluska FG. Low prevalence of germline CDKN2A and CDK4 mutations in patients with early-onset melanoma. Arch Dermatol. 2000;136:1118-22. Medline:10987867

11 Petronzelli F, Sollima D, Coppola G, Martini-Neri ME, Neri G, Genuardi M. CDKN2A germline splicing mutation affecting both p16(ink4) and p14(arf) RNA processing in a melanoma/neurofibroma kindred. Genes Chromosomes Cancer. 2001;31:398-401. Medline:11433531

12 Fargnoli MC, Chimenti S, Keller G, Soyer HP, Dal Pozzo V, Hofler H, et al. CDKN2a/p16INK4a mutations and lack of p19ARF involvement in familial melanoma kindreds. J Invest Dermatol. 1998;111:1202-6. Medline:9856841

13 Soufir N, Avril MF, Chompret A, Demenais F, Bombled J, Spatz A, et al. Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France. The French Familial Melanoma Study Group. Hum Mol Genet. 1998;7:209-16. Medline:9425228

14 Byeon IJ, Li J, Ericson K, Selby TL, Tevelev A, Kim HJ, et al. Tumor suppressor p16INK4A: determination of solution structure and analyses of its interaction with cyclin-dependent kinase 4. Mol Cell. 1998;1:421-31. Medline:9660926