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Tumour suppressor gene *CDKN2A/p16* germline mutations in melanoma patients with additional cancer and cancer in their family history

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Abstract

Germline mutations of the tumour suppressor gene *CDKN2A/p16* have been reported in association with familial melanoma, sporadic melanoma and pancreatic cancer. We studied the possibility of mutations in the *CDKN2A/p16* gene in patients with melanoma and additional unrelated cancer and in patients with additional unrelated cancer in their family history as well. Twenty five melanoma patients with additional cancer and twenty six melanoma patients with additional unrelated cancer in their family history were studied. The entire coding region of the *CDKN2A/p16* gene was screened by single stranded conformation polymorphism analyses and direct DNA sequencing. No germline mutations were detected in the observed melanoma patients. The previously described Ala148Thr and 500 C/G polymorphism were detected. It appears that the cancer development in the patients studied is related to a combination of low-risk susceptibility genes and environmental factors.

Key words: Cancer, *CDKN2A/p16*, melanoma, mutations, polymorphism, tumour suppressor gene.

Introduction

During the past several decades the incidence of malignant melanoma has increased throughout the world. The main risk factors for melanoma development are sunlight exposure (Geller, Annas 2003) and hereditary predisposition (Bataille 2000). Cytogenetic, linkage and molecular analyses of familial and sporadic melanoma provided evidence for the involvement of *CDKN2A* or tumour suppressor gene on chromosome 9p21 (Cannon-Albright et al. 1996). This gene encodes a cyclin-dependent kinase (CDK) inhibitor p16 (Serrano et al. 1993) that arrests progression of the cell cycle at the G1 checkpoint prior to S phase. A non-functional p16 protein has lost its regulatory capacity and can not constrain cells from passing through the cell cycle (Ruas and Peters 1998). Somatic *CDKN2A/p16* mutations have been registered in a great variety of human tumours (Foulkes et al. 1997), providing evidence for *CDKN2A/p16* involvement in the development of these malignancies. Germline mutations of the *CDKN2A/p16* tumour suppressor gene have been reported in association with familial melanoma (Hussussian et al. 1994; Yakobson

et al. 2000; Mantelli et al. 2002) and in sporadic melanoma with multiple primary lesions (Auroy et al. 2001). Additional pedigrees with individuals carrying *CDKN2A/p16* mutations who exhibit both melanoma and other cancers, including pancreatic cancer (Bartsch et al. 2002; Lynch et al. 2002), breast cancer (Borg et al. 2000), neurofibroma (Petronzelli et al. 2001), and glioma (Tachibana et al. 2000), have been described. The presence of *CDKN2A/p16* germline mutations in melanoma kindreds with coexisting additional cancers suggest an association of these mutations with increased risk for various malignancies (Platz et al. 2000). It was shown that about 3 % of melanoma patients developed another melanoma or additional unrelated cancer (Retsas et al. 2000). There is a hypothesis that patients with melanoma and additional unrelated cancers may harbour mutations in the *CDKN2A/p16* gene (Alao et al. 2002).

According the data of the Latvian Cancer Registry, the incidence of malignant primary melanoma in Latvian population is about 150 individuals a year. Same of these patients developed another additional cancer and several have an additional coexisting cancer in their family history.

The aim of the present study was to screen for *CDKN2A/p16* germline mutations in patients with melanoma and additional unrelated cancer developed in the same individual, as well as in patients with a family history of melanoma unrelated cancer. Some patients belonged to both groups.

Materials and methods

Patients

Melanoma patients were recruited through the Latvian Oncological Center, where they completed a brief family history, indicating any first and second degree relatives with a clinically confirmed cancer. The including criteria for examined melanoma patients was clinically and histologically confirmed malignant melanoma with i) additional non-melanoma cancer (n=25), or ii) cancer in family history (n=26). All participants enrolled in this study received a brief 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.

Mutation detection

Genomic DNA was extracted from blood samples by the standard phenol-chloroform extraction method followed by ethanol precipitation.

Polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) analyses of the entire p16 coding region and an untranslated 3' portion of the gene was carried out by amplifying exon 1ß, exon 2 fragments 2A, 2B, 2C and exon 3 using the primers given in Table 1 and described earlier (Hussussian et al. 1994). The PCR reactions were performed according to the manufacturer instructions (Fermentas). Briefly, the 30 μl reaction mixture consisted of 10 x PCR buffer (100 mM Tris-HCl, pH=8.8 and 500 mM KCl), 0.2 mM of each deoxynucleoside triphosphate, 1.5 mM magnesium chloride, 0.5 U Taq polymerase, 0.5 μM forward and reverse primers, and 100 ng genomic DNA. For exon 1ß and the exon 2 fragments 2A, 2B, and 2C, amplification 5 % DMSO was included. Each sample was denatured at 94 ºC for 5 min and subjected to 30 amplification cycles: denaturation at 94 ºC for 30 s, annealing (at temperatures specific for each exon as

Table 1. Primers used for PCR amplification of exons of the *CDKN2A/p16* gene. Primer names beginning with X are derived from the intron sequence. The other primers are numbered according to the cDNA sequence. Primer names ending with F and R denote forward and reverse primers, respectively

Exon	Primer	Primer sequences (5'-3')	Annealing temperature (ºC)	Product size (bp)
1ß	X1.31F	GGGAGCAGCATGGAGCCG	63	204
	X1.26R	AGTCGCCCGCCATCCCCT		
2A	X2.62F	AGCTTCCTTTCCGTCATGC	56	204
	286R	GCAGCACCACCAGCGTG		
2B	200F	AGCCCAACTGCGCCGAC	56	147
	346R	CCAGGTCCACGGGCAGA		
2C	305F	TGGACGTGCGCGATGC	56	189
	X2.42R	GGAAGCTCTCAGGGTACAAATTC		
3	X3.90F	CCGGTAGGGACGGCAAGAGA	64	169
	530R	CTGTAGGACCTTCGGTGACTGATGA		

given in Table 1) for 30 s and extension at 72 ºC for 30 s. The final extension was at 72 ºC for 5 min. Following PCR, 10 μl of each reaction product was run on 1.5 % agarose gels to verify the presence of an amplification product. For single stranded conformational polymorphism analysis, 1 to 2 μl PCR product was mixed with 5 μl loading buffer (95 % formamide, 10 mM NaOH, 0.25 % bromophenol blue, and 0.25 % xylene cyanol), denatured at 95 ºC for 5 min and chilled on ice. Products were run on 6 % polyacrylamide gel in at least three different conditions (7 ºC, 10 ºC, 15 ºC). The DNA bands were visualised by silver staining.

All samples with aberrant migrating bands found in PCR-SSCP analyses were further investigated by direct DNA sequencing. PCR product was cut out from agarose gel, purified by using a DNA extraction kit (Fermentas, Lithuania) according the manufacturer instructions and subjected to 25 cycles of sequencing reaction by using a terminator cycle sequencing kit (BigDay; Applied Biosystems) and forward and reverse primers separately (Table 1). The precipitated sequencing reaction products were analysed in an automated sequencer (ABI PRISM 3100 Genetic Analyser; Applera).

Results

We analysed 47 cases of melanoma for germline mutations in the *CDKN2A/p16* gene. The characteristics of the patients studied and the results of the *CDKN2A/p16* germline mutation analyses are shown in Tables 2 and 3.

No *CDKN2A/p16* germline mutations were detected in patients with melanoma and additional cancer (Table 2), nor in patients with melanoma and additional non-melanoma cancer in their family history (Table 3).

We detected a nucleotide 500 polymorphism C/G (500 C/G) in the 3' untranslated region of exon 3 in eight patients of the 44 examined, three of whom had additional non-

Table 2. Patients with melanoma and additional cancers screened for *CDKN2A/p16* germline mutations. 500 C/G, *CDKN2A/p16* 3' untranslated region nucletide C at position 500 substituted with G. [a], benign form neoplasm

Case no.	Gender	Additional cancer	*CDKN2A/p16* variants	Cancer in family history (affected relative, cancer)
1	Female	Papilloma[a]		
2	Female	Meningioma		
3	Male	Basalioma[a]		
4	Female	Pancreas		
5	Female	Basalioma[a]		
6	Female	Breast		
7	Female	Uterine		
8	Female	Uterine		Aunt, pancreas
				Uncle, pancreas
9	Female	Colorectal	500 C/G	Daughter, ovarian
10	Female	Breast, uterine		
11	Male	Lipoma, melanoma satellites	500 C/G	Father, lung
12	Female	Non-Hodgkin's lymphoma		Daughter, breast
13	Female	Ovarian		Father, lung
14	Female	Adenoma, mioangioadenoma		
15	Male	Submandibularis		
16	Female	Breast		
17	Female	Ovarian		
18	Female	Breast		
19	Female	Mioma[a]	500 C/G	Cousin, breast
			Ala 148Thr	
20	Female	Papilloma[a]		Father, prostate
21	Female	Dermatofibrioma		
22	Female	Angiolipoma		
23	Female	Melanoma satellites		Aunt, breast
24	Female	Melanoma satellites		Mother, breast
				Sister, renal
25	Male	Melanoma dissemination		

melanoma cancer and seven additional non-melanoma cancer in their family history. All three patients with a 500 C/G polymorphism developed additional cancer had a relative with non-melanoma cancer as well (Table 2). One patient with additional non-melanoma primary cancer (case 19; Table 2) and two patients with a non-melanoma cancer in their family history (cases 2, 3; Table 3) were found to have the Ala148Thr polymorphism in addition to 500 C/G. One patient with a cancer in their family history had also a rare G/C polymorphism at position –33 in the 5' untranslated portion of the gene (Table 3).

Table 3. Patients with melanoma and a first-degree relative with non-melanoma cancer screened for *CDKN2A/p16* germline mutations. 500 C/G, *CDKN2A/p16* 3' untranslated region nucletide C at position 500 substituted with G. -33 G/C, *CDKN2A/p16* 5' untranslated region nucleotide G at position -33 substituted with C. [a], patients also have an additional non-melanoma cancer

Case no.	Gender	Affected realtives	Relatives cancer	*CDKN2A/p16* variants
1	Female	Father	Nase	
2	Male	Brother	Hepatis	500 C/G; Ala148Thr
3	Female	Sister	Mieloleukemia	500 C/G; Ala148Thr
4	Male	Father	Cancer (where?)	-33 G/C
5	Male	Mother	Gastric	
6	Female	Mother	Lympholeicosis	500 C/G
7	Female	Mother	Breast	
8	Female	Mother	Skin	
9	Female	Mother	Gastric	
10	Female	Mother	Skin	500 C/G
11	Female	Sister	Skin	
		Father	Bladder	
12	Male	Father	Gastric	
13[a]	Female	Daughter	Ovarian	500 C/G
14	Female	Mother	Breast	
15	Female	Father	Gastric	
16	Female	Mother	Breast	500 C/G
17[a]	Male	Father	Lung	500 C/G
18[a]	Female	Daughter	Breast	
19[a]	Female	Father	Lung	
20	Female	Brother	Gastric	
21	Male	Father	Lung	
22	Female	Father	Larynx	
23	Female	Father	Bladder	
		Brother	Colorectal	
24	Female	Sister	Leucosis	
25	Female	Father	Prostate	
26	Male	Father	Gastric	

Discussion

In our study 25 melanoma patients with additional non-melanoma cancer and 26 patients with cancer in their family history were examined for mutations in the *CDKN2A/p16* gene. The *CDKN2A/p16* is emerging as an important determinant of susceptibility to the development of a malignant melanoma. Genetic alteration of the *CDKN2A/p16* gene has been identified in several different tumour types (Foulkes et al. 1997). Germline mutation of this gene may predispose some family members to an excess risk of melanoma and other

cancers (Borg et al. 2000). It is hypothesised that patients with melanoma and additional cancer may also harbour germline mutations in the *CDKN2A/p16* gene (Alao et al. 2002). However, no such mutations were detected among patients with melanoma and additional non-melanoma cancer in our study. Observation that the presence of *CDKN2A/p16* germline mutations in melanoma kindred with coexisting additional cancers is associated with increased risk for various malignancies (Platz 2000) would indicate that patients with melanoma and unrelated additional cancer in their family history may also have germline mutations in the *CDKN2A/p16* gene. No such mutations were detected among patients with melanoma and an additional non-melanoma cancer in family history in the present study. The determined 500 C/G and Ala148Thr polymorphisms could not be fully evaluated in this study as we have not studied these frequencies in healthy persons. The investigation of the role of Ala148Thr as a low penetrance melanoma susceptibility allele did not support this hypothesis (Bertram et al. 2002). The incidence of 500 C/G polymorphism was associated with increasing familial risk of melanoma (Hayward 2000) and a shorter disease-free survival in melanoma (Kumar et al. 2001). This association was not studied in our cases. In bladder cancers the polymorphism in the 3' untranslated region of the *CDKN2A* gene was related to the mechanism of tumour invasiveness (Sakano et al. 2003). The 500 C/G polymorphism in melanoma patients may be associated with tumour invasiveness as well, through an unknown mechanism (Pjanova, unpublished data). The concurrence of both 500 C/G and Ala148Thr polymorphisms detected in three patients is of uncertain significance. In our study the combination of both polymorphisms in all three cases was obtained in patients with melanoma and an additional cancer in their family history.

There is evidence that patients with two or more primary melanomas appear to have a better prognosis than patients with a single primary lesion (Burden et al.1994). The same would be true in the case with patients with melanoma and unrelated additional primary cancers (Retsas et al. 2000). The impact of mutated genes and polymorphisms on the evolution of the disease is an area of future investigation.

The studied cohort is too small for definite conclusions. However, it would appear that melanoma and additional, apparently unrelated, cancers developing in the same individual and melanoma in context with additional cancer in the family history are related to a combination of low-risk susceptibility genes and environmental factors.

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Audzēju supresorgēna *CDKN2A/p16* iedzimtās mutācijas melanomas pacientiem ar citu audzēju un pacientiem ar audzēju ģimenes anamnēzē

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Kopsavilkums

Audzēju supresorgēna *CDKN2A/p16* mutācijas novēro ģimenes locekļiem ar pārmantotu melanomu un aizkuņģa dziedzera audzēju, kā arī iegūtās melanomas pacientiem. Dotajā darbā meklēja iespējamās *CDKN2A/p16* gēna mutācijas asins paraugos divās melanomas pacientu grupās: 1) 25 melanomas pacientiem, kuriem diagnosticēts cits audzējs, 2) 26 melanomas pacientiem, kuriem ģimenes anamnēzē vienam vai vairākiem pirmās pakāpes radiniekiem ir kāds audzējs. Analīzes veica, izmantojot polimerāzes ķēdes reakcijas, vienpavediena konformācijas polimorfisma analīzi un gēna sekvenēšanu. Nevienā gadījumā melanomas pacientu asinīs mutācijas *CDKN2A/p16* gēnā nenovēroja. Atrada gēna polimorfismu - 500. nukleotīda C nomaiņu ar nukleotīdu G (500 C/G) un 148. kodona aminoskābes alanīna nomaiņu ar aminoskābi treonīnu (Ala148Thr). Rezultāti liecina, ka pētījumā iekļautajiem pacientiem audzēju attīstība nav saistīta ar mutācijām *CDKN2A/p16* gēnā.