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QUARTERLY

Analysis of mutations in the *p16/CDKN2A* gene in sporadic and familial melanoma in the Polish population★

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Mutations in *CDKN2A* have been found in sporadic cutaneous malignant (CMM), in familial CMM and in other syndromes associated with melanoma. In this study DNA was obtained from 207 individuals and five cell lines. There were 157 CMM patients and 50 healthy members of melanoma patients families. The CMM group included patients with one or two melanoma cases in the family, families with dysplastic nevus syndrom (DNS) and patients with a spectrum of other types of cancers in the family. PCR-SSCP analysis and sequencing identified: six substitutions in codon 58 CGA/TGA (Arg/Stop), 16 substitutions GAC/GAT in codon 84 (Asp/Asp), six substitutions CGA /TGA in codon 148 (Arg/Thr), 14 substitutions G/C in 3′UTR and 4 double changes (two in codon 84 and 3′UTR; two in codon 148 and 3′UTR). The mutation identified in codon 58 was found in tissue only. Other substitutions were polymorphisms found in DNA from tissue and blood samples. Most of them were identified in sporadic CMM (six in codon 148 Ala /Thr, 12 in codon 84 Asp/Asp and six in 3′UTR). The frequency of the polymorphisms was also high in DNS and CMM/DNS families (four in codon 84 Asp/Asp and six in 3′UTR). No mutations or polymorphisms were found in CMM patients with one or two melanoma cases and CMM patients, with other cancers in family history.

The analysis of the *CDKN2A* gene mutations in the Polish population demonstrated: (i) no germline mutations; (ii) a relatively high number of genetic changes in sporadic melanoma; (iii) a high number of polymorphisms in DNS and CMM/DNS families.

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Abbreviations: CDK, cyclin-dependent kinases; CMM, cutaneous malignant melanoma; DNS, dysplastic nevus syndrome; FMM, familial malignant melanoma.

The progression of mammalian cells from G_1 to the S phase is regulated by a cascade of protein interactions and protein phosphorylation. One of the regulatory proteins is p16, an inhibitor of cyclin-dependent kinases (CDK) 4 and 6 (Sherr, 1996). p16 by binding to CDKs complexes inhibits their ability to interact with cyclin D (Lukas *et al.*, 1995). Cyclin D/CDK4-6 complex phosphorylates the retinoblastoma protein (pRb), which leads to the activation of a group of transcription factors essential for cell cycle progression (Serrano *et al.*, 1993) and DNA replication (Stone *et al.*, 1995). The p16 gene (*CDKN2A*) was mapped to chromosome region 9p21 (Kamb *et al.*, 1994b). Later studies demonstrated that from this locus two distinct gene transcripts are derived from different promoters. The second transcript, referred to as p14/ARF, arises from the alternative exon 1 (exon 1 beta) and is spliced into common (with p16) exons 2 and 3 but is translated in a different reading frame (Quelle *et al.*, 1995).

A common feature of cancer cell is unrestrained division, that might be related to changes in genes encoding components of the cell cycle regulatory machinery. Mutations in the *p16* locus, whose protein products are involved in the Rb pathway, have been implicated in the pathogenesis of many tumor types. The high frequency of mutations observed in melanoma cell lines suggested an important role of p16 in carcinogenesis. Further studies revealed a relatively lower frequency of *CDKN2A* mutations in sporadic melanoma (0–15%) (Ruiz *et al.*, 1998; Fujimoto *et al.*, 1999) compared to cell lines (16–40%) (Castellano *et al.*, 1997). Moreover, a number of other mechanisms of *CDKN2A* inactivation, such as point mutations, small deletions, large hetero- and homozygous deletions, and silencing by methylation of CpG islands in the promoter region have been characterized. They have been analyzed in melanoma patients, but their frequency and role in pathogenesis and progression of melanoma are still unclear.

Genetic predisposition plays an important role in the development of nearly 10% of cutaneous malignant melanomas (CMM) (Holuska & Hodi, 1998). Inherited and acquired deletions or point mutations in *CDKN2A* increase the likelihood that a mutagenic DNA damage would escape repair before cell division. The best known genetic syndrome predisposing to melanoma is referred to as familial malignant melanoma (FMM) (Koph *et al.*, 1986). Beyond melanoma other types of cancer, such as breast, pancreas and colon were observed in FMM (Ciotti *et al.*, 1996). However, only 20% of FMM patients were shown to carry germline mutations in *CDKN2A*. Other genetic syndromes associated with melanoma are familial atypical mole-malignant melanoma syndrome-pancreatic cancer (FAMMM-PC) (Lynch *et al.*, 2002), dysplastic nevus syndrome (DNS) (Lee *et al.*, 1997), and melanoma-astrocytoma syndrome (Hayward, 2000). In addition, melanoma may appear in the cancer spectrum of other syndromes such as Lynch syndrome (Kamb *et al.*, 1994a) and Li-Fraumeni syndrome (Burt *et al.*, 1999). In these syndromes mutations in the *CDKN2A* gene were also reported.

Here we summarize the results of the *CDKN2A* analysis carried out in sporadic and familial melanoma patients in the Polish population.

MATERIALS AND METHODS

Materials

Genomic DNA obtained from 207 individuals was analyzed. There were 157 patients with CMM and 50 healthy members of the melanoma patients families. Moreover DNA from five melanoma cell lines (WM9, WM35, WM239, WM902b and A375) was also studied. DNA was extracted from melanoma tissue only (31 patients), from tissue and peripheral blood samples (24 patients), or from peripheral blood only (78 patients). The CMM

group included 16 patients with one or two melanoma cases in family history, six CMM patients from DNS families, five families with DNS and a group of CMM patients with a spectrum of other types of cancers in family history. The latter group included: five – BRCA2, probably one Lynch syndrome and one Li-Fraumeni syndrome. Moreover, in 14 families also lung, stomach, breast, colon and liver cancer were reported.

Blood samples taken from 100 healthy blood donors served as a control.

Methods

DNA extraction. DNA was extracted from tissue samples and cell lines using a standard method with proteinase K, several phenol/chlorophorm extractions and ethanol precipitation.

DNA from blood samples was isolated with the Wizard genomic extraction kit (Promega, WI, U.S.A.) according to the manufacturer's instructions.

PCR-SSCP analysis. The following pairs of primers for PCR-SSCP analysis were used: for exon 1 – 1F/16: GGGAGCAGCATGGAGCCG and 1R/16: AGTCGCCCGCCATCCCCT; for exon 2 – 2AF/16: AGCTTCCTTTCCGTCATG and 2AR/16: GCAGCACCACCAGCGTG, 2BF/16: AGCCCAACTGCGCCGAC and 2BR/16: CCAGGTCCACCGGCAGA; 2CF/16: TGGACGTGCGCGATGC and 2CR/16: GGAAGCTCTCAGGGTACAAATTC; for exon 3 – 3F/16: CCGGTAGGGACGGCAAGAGA and 3R/16: CTGTAGGACCCTCGGTGACTGATGA. Primers were labeled on the 5′ end with [γ-^{32}P]ATP (30 00 Ci/mmol, Amersham). The reaction volume was 5 μl and included the following reagents: 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.8 mM dNTPs, 6% Me_2SO, 1 μM of each primer, 0.125 U of Taq polymerase (Promega WI, U.S.A.) and 50 ng of genomic DNA. The amplification programs included 35 cycles and 3-steps amplification cycle. The annealing temperature was 55°C for exons 1 and 3 and 58°C for fragments of exon 2. Following amplification, the obtained products were mixed with 1/9 volume of formamide dye mixture, heated at 95°C/5′ and loaded into 5% polyacrylamide gel containing 10% glycerol. Electrophoresis was carried out at room temperature at 15 W. Then the gels were transferred onto Whatman 3MM paper, dried and autoradiographed.

Sequencing analysis. Bands showing different mobility shift were cut out from the gel, eluted into water and amplified. Products of the reamplification were purified and served as templates in the cycle sequencing procedure using the fmol DNA sequencing system (Promega, WI, U.S.A.) and end labeled amplimers as sequencing primers.

Methylation assay. Genomic DNA was modified using 3 M sodium bisulfite with 10 mM hydroquinone according to a previously described method (Herman *et al.*, 1996). Modified DNA was purified with the Wizard purification resin (Promega, WI, U.S.A.). The reaction was terminated by NaOH treatment, followed by ethanol precipitation. The isolated DNA served as template in PCR reaction with primers and reaction conditions as described previously (Herman *et al.*, 1996). PCR products were analyzed by electrophoresis in 2% agarose gel. The accuracy of the procedure was confirmed by using known methylated p16 promoter region DNA template as a control of all steps.

RESULTS

PCR-SSCP analysis and sequencing identified the following genetic alterations: six substitutions in codon 58 CGA/TGA (Arg/ Stop), one G/T substitution in codon 69 Glu/Stop in the WM902b cell line, 16 substitutions GAC/GAT in codon 84 (Asp/Asp), 6 substitutions CGA/TGA in codon 148 (Arg/Thr), 14 substitutions G/C in 3′UTR and four double changes (two GAC/GAT in codon 84 and 3′UTR; two polymorphisms in codon 148 and 3′UTR) (Fig. 1). Methylated CpG islands in

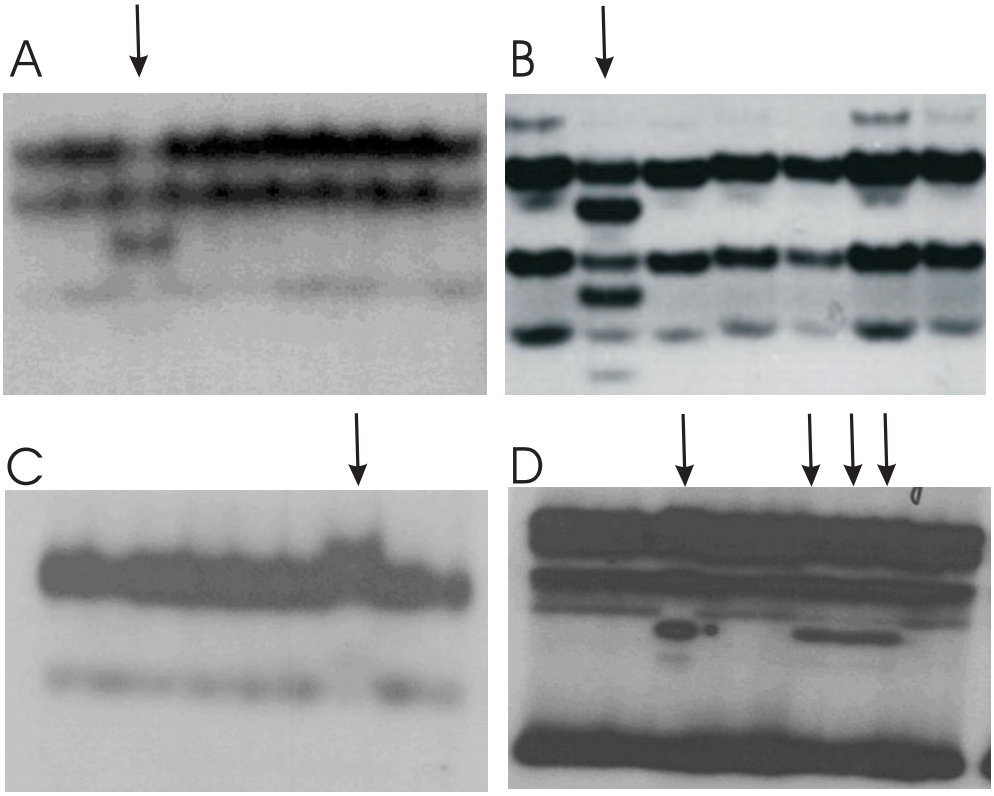

Figure 1. Examples of PCR-SSCP analysis, arrows indicate mutated bends.

A, fragment A of exon 2, mutation in codon 58; B, fragment B of exon 2, polymorphism in codon 84; C, fragment C of exon 2, polymorphism in codon 148; D, fragment of exon 3 with 3'UTR, G/C substitutions in 3′UTR.

the promoter region of *CDKN2A* were not found. The mutation identified in codon 58 (Arg/Stop) was found only in DNA isolated from melanoma tissue samples obtained from CMM patients without family history. The somatic character of this change was confirmed by analysis of DNA from blood samples derived from the same patients.

The polymorphism in codon 148 Arg/Thr was found in six CMM cases both in blood and tissue samples. The most frequently seen substitution was found in codon 84 GAC/GAT (Asp/Asp), both in tissue and blood samples. In 12 cases the genetic alteration appeared in CMM patients without family history, and in one DNS family but only in four healthy persons (one analyzed CMM member of the family did not have this genetic change). The nucleotide substitution in 3′UTR was also frequently found (18 cases). The G/C change was localized six nucleotides from the last codon. The substitution was found in eight CMM patients: six of them were sporadic and two reported stomach, breast and lung cancer in the family. Healthy persons with the 3′UTR substitution were members of the families with one reported melanoma case. Moreover, in four individuals double genetic changes were identified: G/C in 3′UTR and GAC/GAT in codon 84 (two cases), G/C in 3′UTR and Ala/Thr in codon 148 (two cases). The above alternations were seen in two CMM patients and their healthy children. The frequency of the genetic alterations found was verified on DNA isolated from 100 healthy blood donors. All the detected genetic changes in the p16 gene are summarized in Table 1.

DISCUSSION

The analysis of the *CDKN2A* gene mutations in Polish population demonstrated: (i) no germline mutations; (ii) a relatively high num-

Table 1. Genetic alternations found in *CDKN2A* gene

Patient's characteristics	Number of cases	Codon 58 Arg/Stop	Codon 69 Glu/Stop	Codon 84 Asp/Asp	Codon 148 Ala/Thr	C/G in 3'UTR
CMM sporadic	109	6	–	12	6	6
CMM + one or two melanoma cases	16	–	–	–	–	–
CMM + other cancers	14	–	–	–	–	2
CMM/DNS	6	–	–	4	–	2
DNS	5	–	–	–	–	4
BRCA2	5	–	–	–	–	–
Li-Fraumeni	1	–	–	–	–	–
Lynch	1	–	–	–	–	–
Healthy members of patients' families	50	–	–	2	2	4
Healthy controls	100	–	–	1	3	4
Cell lines	5	–	1	–	–	–

ber of genetic changes in sporadic melanoma; (iii) a high number of polymorphisms in DNS and CMM/DNS families.

Original studies on melanoma cell lines suggested an important role of mutated p16 in arising and progression of melanoma. Later studies demonstrated that *CDKN2A* is rather not the first target in the development of CMM. Studies of p16 mRNA and p16 protein expression in different stages of melanoma progression demonstrated a gradual loss of p16 expression during malignant transformation of melanocytes (Morita *et al.*, 1998). Moreover, the loss of p16 protein expression may be involved in the acquisition of metastatic phenotype of melanoma (Reed *et al.*, 1995). In our studies, five cell lines derived from melanoma at various stages of progression, such as horizontal phase, radial phase and metastases were analyzed. Only in one cell line (derived from metastatic tumor) one *CDKN2A* mutation was found. Such mutation in codon 69 was described earlier and is considered as typical for cell lines (Smith-Sorensen & Hoving, 1996).

Variable frequencies of p16 germline mutations have been reported in different collections of melanoma families in Sweden, Australia, Great Britain, Israel, U.S.A. and other countries (Hashemi *et al.*, 2001; Holland *et al.*, 1995; MacKie *et al.*, 1998; Yakobson *et al.*, 2000; Goldstein *et al.*, 2000). It is also known that the incidence of other cancers, such as pancreatic adenocarcinoma, is higher in CMM families with the 358delG mutation (Holuska & Hodi, 1998). On the other hand, *CDKN2A* mutations occurred rarely and did not play an important role in genetic melanoma predisposition (Tsao *et al.*, 2000). Very low number of genetic changes identified in melanoma cases with reported other cancers or one/two melanoma cases in family history showed that *CDKN2A* is not a target for mutational changes in such groups of patients (Shennan *et al.*, 2000). In our CMM patients with either sporadic CMM or with one/two melanoma incidents, or other cancer cases in the family no *CDKN2A* gene germline mutations were found. Unfortunately, there were no classical FMM families, thus the lack of germline mutations need not necessarily be representative of the Polish population.

In contrast, in sporadic CMM a relatively high number of genetic alterations was found. In six out of 55 (10.9%) tissue samples studied the somatic mutation CGA/TGA creating a

stop codon was identified. In addition several other polymorphisms were seen. They included a silent change in codon 84, Arg/Thr substitution in codon 148 and G/C substitution in 3′UTR. The substitution identified in codon 148 is the most frequently reported polymorphisms in the *CDKN2A* gene. However, more frequent in our studies is the polymorphism identified in 3′UTR. Such polymorphism is regarded as common in melanoma (Kumar *et al.,* 2001). The most frequently identified substitution was GAC/GAT in codon 84. The silent mutation, which has not been described before, was found in 11% of sporadic CMM. Surprisingly, it was detected in one healthy control only.

In over 90% of DNS and CMM/DNS families *CDNK2A* polymorphisms were seen. In 2/3 of CMM/DNS families the codon 84 silent mutation was observed. Interestingly, in one of the CMM/DNS families all the members except the CMM patient demonstrated the C/G substitution in 3′UTR. This further underlines the genetic diversity between DNS and CMM (Puig *et al.,* 1997).

The discovery of the p14/ARF gene may radically change our understanding of genetic alterations linked to the *CDKN2A* gene so far. Studies of the alternative exon 1 beta demonstrated a 16 bp insertion specifically altering p14/ARF, which in consequence fails to stabilize p53 and arrest growth of a p53 expressing melanoma cell line. This 60ins16 mutation was also found in an individual with multiple primary melanomas (Rizos *et al.,* 2001b). However, most of the mutations appear in the common exon 2. In fact more than 40% of all mutations described were localized there and affected both the p16 and p14/ARF genes. Nevertheless, a clear distinction between p16 and p14/ARF mutations and their function exists since studies of five definitive mutations inactivating the p16 gene demonstrated correct p14/ARF transcripts in melanoma patients (Stott *et al.,* 1998). In other studies three of seven *CDKN2A*/ARF mutations tested altered the subcellular distribution of p14/ARF and diminished the ability of p14 to activate the p53 pathway. All these facts indicate that p14/ARF might also be involved in melanoma predisposition (Rizos *et al.*, 2001a).

Accordingly, the substitution C/T in codon 84 of *p16/CDKN2A* (silent) corresponds to a change in codon 99 of p14/ARF-substitution CGC/TGC (Arg/Cys). Thus, the functional target of the above nucleotide alteration might be rather p14/ARF than p16. However, the biological consequences of the alteration have to be confirmed by functional analysis of this p14/ARF variant.

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