Mutational analysis of the CDKN2 gene in metastases from patients with cutaneous malignant melanoma

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Summary We analysed 26 metastases from 25 patients with sporadic cutaneous malignant melanoma for alterations in the CDKN2 gene by a combined polymerase chain reaction/single-strand conformation polymorphism (PCR/SSCP)/nucleotide sequencing approach. Eleven alterations (one in exon 1, five in exon 2 and five in the 3' non-coding sequence of the exon 3 region) were concordantly and independently detected by both SSCP and nucleotide sequence analysis. Two of the exon 2 changes and the five changes in the non-coding exon 3 region are likely to represent natural polymorphism. Four (15%) of 26 metastases thus had CDKN2 mutations and belonged to 3 (12%) of 25 patients. Semi-quantitative PCR furthermore revealed no sign of homozygous deletions of the CDKN2 exon 2 region. The results support an involvement of the CDKN2 product in the development of a subgroup of sporadic melanomas and encourage the search for alterations in additional genes of the 9p21 region.

Keywords: CDKN2 mutation; human melanoma; metastases; polymerase chain reaction/single-strand conformation polymorphism; sequence analysis

Allelic losses on chromosome 9p21 are very frequent in many types of tumours and cell lines and thus suggest that 9p21 may contain at least one tumour-suppressor locus involved in the genesis of different human tumours, including malignant melanoma. A putative tumour-suppressor gene CDKN2 maps to 9p21 (Serrano et al., 1993; Kamb et al., 1994a; Nobori et al., 1994) and encodes a protein, p16 ink4, identical to a previously identified CDK4 inhibitory protein (Serrano et al., 1993). CDKN2 contains three coding exons of 126, 307 and 11 bp (Serrano et al., 1993; Nobori et al., 1994).

Five to ten per cent of human malignant melanomas seem to occur as a result of autosomal dominant inherited predisposition (Greene et al., 1985) and at least two loci linked with familial melanomas (on chromosome 1p and 9p) have been identified (Cannon-Albright et al., 1992; Goldstein et al., 1993). Germline mutations in the CDKN2 gene have now been detected in affected members from melanoma families with 9p21 linkage (Hussussian et al., 1994; Kamb et al., 1994b), suggesting that they indeed may represent melanoma-predisposing alterations. The concept of cancer heredity predicts the presence of a crucial primary alteration in the germ line as being the only mechanistic difference between the hereditary and the sporadic form of the disease. Mutational alterations of CDKN2 detected in the germ line of familial melanoma cases (Hussussian et al., 1994; Kamb et al., 1994b) should therefore also occur as somatic alterations in tumours of its sporadic counterparts.

The present report describes a successful mutational analysis, carried out independently by polymerase chain reaction (PCR) combined with single-strand conformation polymorphism (SSCP) and by direct nucleotide sequence analysis, of the CDKN2 gene in DNA from 26 melanoma metastases of 25 patients with sporadic cutaneous malignant melanoma.

Materials and methods

Tumour samples and DNA isolation

Fresh tumour tissues (26 metastases from lymph nodes and skin) were obtained from 25 melanoma patients. The tumour tissues were trimmed from surrounding non-tumour tissue and dissected into small pieces and immediately frozen and stored at −70°C. Representative parts of the frozen tissue pieces were formalin fixed and 4 μm sections were hematoxylin stained. Patient and tissue data are summarised in Table I. The frozen tissues were crushed to a fine powder under liquid nitrogen and DNA extracted according to standard procedures. The obtained DNA samples were stored as precipitations under ethanol at −20°C.

PCR / SSCP analysis and nucleotide sequence analysis (SA)

The genomic regions containing exons 1 and 2 of CDKN2 were primarily amplified by PCR from genomic DNA using the flanking primer sets 2F/1108R and 42F/551R (Kamb et al., 1994d). The PCR products (1000 bp) were electrophoresed on a non-denaturing 5% polyacrylamide gel and subsequently transferred to a Hybond N+ membrane (Amersham) by the capillary method. The hybridisation protocol was as described elsewhere (Popp et al., 1992) with the following modifications: (i) hybridisation was carried out at 60°C for 16 h and (ii) the 32P-labelled probe was diluted 1:1000 in a solution of 0.25 M NaOH and 0.6 M NaCl.

Table I Patient and tumour characteristics

| Patient no. | Sex | Anatomical site of primary tumour | Histogenetic type | Location of analysed metastases |
|-------------|-----|----------------------------------|-------------------|---------------------------------|
| AM          | Female | Upper extremities | NM | Skin |
| MA          | Female | Lower extremities | SSM | Lymph node |
| SB          | Male | Unknown primary | Unknown | Lymph node |
| AA          | Female | Lower extremities | Unclassified | Lymph node |
| LS          | Male | Trunk | NM | Lymph node |
| IN          | Male | Lower extremities | SSM | Lymph node |
| JI          | Female | Trunk | SSM | Skin |
| MP          | Male | Trunk | NM | Skin |
| AI          | Female | Trunk | NM | Lymph node |
| BT          | Male | Trunk | SSM | Skin |
| LL          | Male | Trunk | SSM | Skin |
| JA          | Male | Trunk | Unclassified | Skin |
| HE          | Male | Trunk | SSM | Lymph node |
| GK          | Male | Trunk | Unclassified | Skin |
| GL          | Male | Trunk | Unclassified | Skin |
| DO          | Male | Lower extremities | Unclassified | Skin |
| TN          | Male | Lower extremities | NM | Lymph node |
| DJ          | Male | Lower extremities | Unclassified | Lymph node |
| HW          | Male | Lower extremities | SSM | Skin |
| HY          | Male | Trunk | SSM | Lymph node |
| JJ          | Female | Trunk | NM | Lymph node |
| WN          | Male | Lower extremities | SSM | Lymph node |
| RO          | Male | Trunk | SSM | Skin |
| JH          | Female | Lower extremities | Unclassified | Lymph node |
| EE          | Male | Trunk | SSM | Skin |

NM, nodular melanoma; SSM, superficial spreading melanoma.
For amplification of the third coding exon, a 346 bp fragment was generated with the 5’ primer TTTTCTTCTCGCCTCTGC and the 3’ primer CCA- CATGATTGCGCGT. The following PCR conditions were used. For exon 1, 4 min at 94°C, followed by 30 cycles consisting of 30 s at 94°C, 60 s at 63°C, 60 s at 72°C and finally 7 min at 72°C. For exons 2 and 3, the same amplification conditions were used, but with an annealing temperature of 60°C. AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, CT, USA) was used in standard PCR buffer at 1.5 mM magnesium chloride and in the presence of 5% dimethyl sulphoxide (DMSO). The purity of the PCR products was checked by electrophoresis on 4% NuSieve agarose gels and ethidium bromide staining (PCR is covered by US patents 4,683,195 and 4,683,202 owned by Hoffman-La Roche).

The sequencing reactions were carried out by direct sequencing of the PCR products using Pharmacia Biotech’s (Uppsala, Sweden) combs solid phase DNA sequencing (Autoload kit) (Lagerkvist et al., 1994). One of the PCR primers was biotinylated for the isolation of single-strand DNA and FITC-modified sequencing primers were used for the sequencing reactions. The electrophoretic separations were performed on Pharmacia Biotech’s ALF DNA sequencer. Aliquots of 40 μl of the PCR products were used for capture on streptavidin-coated plastic combs. They were incubated at room temperature for at least 30 min, then transferred to a fresh plate for strand denaturation. The combs were washed. FITC-modified sequencing primers (4 pmol) were used. After incubation at 55°C for 5 min, then at room temperature for at least 10 min, the combs were transferred to the sequencing-mixes containing T7 DNA Pol. The combs were incubated at 37°C for 5 min and kept on ice until loading. The electrophoretic gel was prewarmed at 45°C and the well filled with formamide. The combs, specifically designed to fit the gel, were inserted and incubated for 10 min before being carefully removed and the run started. For exon 2, the sequencing was performed using two overlapping pieces obtained by the internal primers p16-1B (CACGCTGGTTGTCGTGCA) and p16-2B (CAGGTGC- CACGGGAGACG) in combination with two M13 tail-modified primers, 42FU (CUGACCGCAGCTTGTAATTGACGAG/CCCTGAGGCCGAGTCAG) and 551RU (CUGACCGCAGCTTGTAATTGACGAG/ CAGTCTGAGCTTTGAAGCTCT) respectively. The sequencing was performed using the M13 Universal FITC primer. The sequencing of the exon 3 region was carried out using the primer PF3a1R (F-TGATCTAAGTTTCCGGAGGT) or PF3a2R (F-CCTTAGGACCTCCTCCGTGAC).

PCR amplifications of exon 1 for SSCP were carried out as described above. PCR amplifications for SSCP of the exon 2 and exon 3 regions were carried out using the following primer combinations: exon 2, AP167 ACACAGCTCCTCTTCCGT and AP168 TCAAGATCATCAGCTTCCAC resulting in 392 bp fragments; and exon 3, 5’ primer TTTCCTTTCTGCCCCTGTC and 3’ primer TTGTG GCCCTGTAGAGCATCTTT. The sequencing was carried out by direct sequencing of the PCR products using Pharmacia Biotech’s ALF DNA sequencer. Aliquots of 40 μl of the PCR products were used for capture on streptavidin-coated plastic combs. They were incubated at room temperature for at least 30 min, then transferred to a fresh plate for strand denaturation. The combs were washed. FITC-modified sequencing primers (4 pmol) were used. After incubation at 55°C for 5 min, then at room temperature for at least 10 min, the combs were transferred to the sequencing-mixes containing T7 DNA Pol. The combs were incubated at 37°C for 5 min and kept on ice until loading. The electrophoretic gel was prewarmed at 45°C and the well filled with formamide. The combs, specifically designed to fit the gel, were inserted and incubated for 10 min before being carefully removed and the run started. For exon 2, the sequencing was performed using two overlapping pieces obtained by the internal primers p16-1B (CACGCTGGTTGTCGTGCA) and p16-2B (CAGGTGC- CACGGGAGACG) in combination with two M13 tail-modified primers, 42FU (CUGACCGCAGCTTGTAATTGACGAG/CCCTGAGGCCGAGTCAG) and 551RU (CUGACCGCAGCTTGTAATTGACGAG/ CAGTCTGAGCTTTGAAGCTCT) respectively. The sequencing was performed using the M13 Universal FITC primer. The sequencing of the exon 3 region was carried out using the primer PF3a1R (F-TGATCTAAGTTTCCGGAGGT) or PF3a2R (F-CCTTAGGACCTCCTCCGTGAC).

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amplifications of serial diluted genomic DNA from blood samples from normal individuals and from genomic DNA of tumours with hemizygote CDKN2 mutation, we constructed standard curves for analysis of the concentration-dependent amplification of the two gene regions. The multiplex PCR reactions were carried out in standard PCR buffer in the presence of 2 mM magnesium chloride and 5% DMSO (94°C, 30 s / 60°C, 30 s / 72°C, 30 s / 30 cycles). Co-amplification of

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Figure 2  Examples of obtained sequencing results. (a) Wild-type sequence, codons 63 to 69 of CDKN2 exon 2. (b) Mutant sequence (sample from patient EE) with stop codon in position 66. (c) Wild-type sequence, codons 145–151. (d) Polymorphic variant, codon 148 (sample from patient BT).
HSS26 and CDKN2 exon 2 was carried out at three dilution steps for each of the 26 tumour DNA samples and the band intensities obtained on ethidium bromide-stained agarose gels were scanned with an Ultrascan XL Densitometer (Pharmacia Biotech) and compared with the corresponding part of the standard curve in order to register a possible gene loss by differential loss of the CDKN2 exon 2 specific band.

Results

The histopathological investigation of haematoxylin–eosin-stained 4-µm-thick sections, representative for the 26 dissected metastases, showed large and homogeneous tumour cell populations. The majority of the sections represented a minimum of 90% of tumour cells. PCR for CDKN2 exon 1, exon 2 and exon 3 regions resulted in the expected fragment sizes for all 26 samples. Gel electrophoresis on 4% NuSieve Agarose gels revealed no sign of small deletions.

SSCP screening of the CDKN2 exon 1- and exon 2-containing fragments was carried out, both in the presence and absence of glycerol and revealed clear bandshifts for 6 of the 25 metastatic samples. One bandshift in exon 1 and 5 bandshifts in exon 2 were detected (Figure 1). The shortening of the analysed fragments containing exon 1 and exon 2 by Smal cleavage improved the recognised bandshifts. The wild-type band was very weak or absent in three of the samples with bandshifts in the exon 2-containing fragment, which most likely indicates hemizygosity and also demonstrates that normal cells are rare in the extracted tumour pieces. SSCP screening of the exon 3-containing fragments, including the 3'-untranslated region, revealed in 5/26 (19%) of the samples an identical bandshift, indicating the presence of a common polymorphism (Figure 1). Xu et al. (1994) reported a C to G polymorphism in the 3'-untranslated region in 7/37 (19%) of cases.

In agreement with Caldas et al. (1994) we found for all sequenced samples a different nucleotide sequence 5' to the ATG than the one published by Serrano et al. (1993). An additional ATG codon at position −8 followed by seven additional coding triplets was recognised. Furthermore codon 27 was found to be Gly instead of the originally published Val (Serrano et al., 1993). All following nucleotide and codon positions refer to the corrected sequence. Sequence changes were recognised in the same samples that showed mobility shifts in SSCP (Table II). The mutation in the exon 1 region in the metastasis from patient HW turned out to be a single C insertion at codon position 4 leading to a frameshift. Both the mutated and the wild-type sequence were present. The frameshift in the mutated sequence results in a nonsense triplet TAG at codon position 14. The registered mutations in exon 2 are (i) a missence mutation Arg12Gly (CGT/GGT) found in two skin metastases from patient MP, and (ii) a complex mutation converting His66CAC to nonsense TAG by a C/T transition and a single-base frameshift deletion or by deletion of codon 66 and its replacement by the dinucleotide TA in the sample from patient EE (Figure 2b). No wild-type sequence was detected in these three cases, thus most probably pointing to loss of the wild-type allele. Two additional mutations registered in metastases from patients BT and IA were the same Ala148Thr (GCC/ACG) change, earlier registered by others (Hussussian et al., 1994; Kamb et al., 1994a), and represent a natural polymorphism (Figure 2d). Five out of 26 (19%) metastases had a G to C change in base 499 of the 3'-untranslated region of exon 3 and represent most likely an additional common polymorphism (Xu et al., 1994).

The semiquantitative multiplex PCR assay, measuring the relative band intensities of the amplified fragment of the gene for ribosomal protein HSS26 and of the CDKN2 exon 2 region on ethidium bromide-stained agarose gels showed no obvious loss of the CDKN2-specific band in any of the 26 tested metastases.

Discussion

Homozygote deletions of CDKN2 have been recognised in many cell lines derived from various human tumours and subsets of cell lines retaining CDKN2 harboured structural changes such as missense, nonsense and frameshift mutations (Kamb et al., 1994a; Nobori et al., 1994). An increasing number of mutational analyses of CDKN2 in non-cultured primary and metastatic human tumours have been published (Cairns et al., 1994; Mori et al., 1994; Okamoto et al., 1994; Spruck et al., 1994; Zhang et al., 1994). Studies of somatic CDKN2 alterations in human melanoma samples generated conflicting results. Nucleotide sequence analysis of 30 surgically resected melanomas of cutaneous and uveal origins could not detect any CDKN2 mutations (Ohta et al., 1994). Five (15%) of 34 primary melanomas in a second report in contrast had CDKN2 point mutations (Gruis et al., 1995).

The present investigation reports the presence of CDKN2 mutations in metastases from 3 (12%) of 25 patients with sporadic cutaneous malignant melanoma.

The combined SSCP/nucleotide sequence analysis approach as used in the present investigation convincingly demonstrates that an SSCP prescreening of all three CDKN2 coding exon regions using a simple arrangement of just three primer pairs and reduction of fragment sizes by single restriction enzyme cleavage efficiently revealed the presence of all mutations in the covered regions. Two of the three types of registered mutations result in premature termination, structural disruption and certainly loss of function of the CDKN2 protein product p16.

The existence of homozygote CDKN2 losses among our tumour samples seems to be unlikely, since no obvious loss of the CDKN2-specific PCR signal was registered in any of the samples and since the percentage of contaminating normal cells was very small in the dissected tissue pieces. This does of course not exclude the possibility that homozygous deletions may be present in smaller subpopulations of the tumour cells, which would need in situ hybridisation or in situ PCR techniques for detection. The employed semiquantitative PCR test, however, is an indicator for possible allelic losses when used on DNA from early homogeneous cell populations and should certainly detect the presence of homozygous deletions when present in the majority of a given cell population.

The present finding of CDKN2 mutations in 12% of patients with sporadic melanomas supports an involvement of this gene in the development of a subgroup of sporadic melanomas and also suggests that additional genes, other than CDKN2, could be involved in melanoma development. An additional gene adjacent to the CDKN2 gene has been recognised at 9p21 that codes for the closely related protein p15 (Hannon and Beach, 1994) and may be an alternative site for alterations coupled to melanoma development. Mutational analysis of the p15-coding sequences in the same samples and in samples from primary tumour material is in progress.

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