Genes involved in cell cycle G1 checkpoint control are frequently mutated in human melanoma metastases

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Summary A common characteristic of cancer cells is unrestrained cell division. This may be caused by mutational changes in genes coding for components of cell cycle-controlling networks. Alterations in genes involved in G1 checkpoint control have been registered in many human tumours, and investigations from several laboratories show that such alterations, taken together, are the most frequent changes detected in cancer cells. The present paper describes mutational analysis by polymerase chain reaction—single-strand conformation polymorphism (PCR/SSCP) and nucleotide sequence analysis of the genes coding for the p15, p53 and N-ras proteins in 26 metastases from 25 melanoma patients. The registered mutation frequencies add together with previously registered mutations in p16 in the same patient samples to a substantial total frequency of 44% of patients with mutation in at least one of the investigated genes. These results show the occurrence of heterogeneous defects among components of the cell cycle controlling machinery in a human melanoma tumour sample collection and demonstrate that the total frequency of detected alterations increases with the number of cell cycle controlling genes included in the screening panel.

Keywords: CDKN2A; CDKN2B; p53; N-Ras mutation; human melanoma metastases; polymerase chain reaction—single-strand conformation polymorphism; nucleotide sequence analysis

The p16 protein, product of the CDKN2A (MTS-1) suppressor gene, is a specific inhibitor of the CDK4 and CDK6 interactions with cyclin D (Serrano et al., 1993; Grana and Reddy, 1995). Mutational alteration or inactivation of p16 may result in disturbance of the cell cycle G1 checkpoint control and deregulation of cell growth (Clurman and Roberts, 1995; Cordon-Cardo, 1995). Linkage studies among members of melanoma/dysplastic naevus syndrome (DNS) families have pointed out the chromosome 9p21 region as the likely site for at least one tumour-suppressor gene locus responsible for inherited melanoma predisposition (Cannon-Albright et al., 1992; Goldstein et al., 1994). The CDKN2 gene has recently been mapped to this region (Kamb et al., 1994; Nobori et al., 1994) and germline mutations in CDKN2 segregating with melanoma have been registered in families from the United States, Holland and Sweden (Hussussian et al., 1994; Kamb et al., 1994b; Platz et al., 1995a), indicating that this gene may be involved in melanoma heredity in at least one subgroup of melanoma-prone families. A structurally highly related protein, p15, has recently been identified (Hannon and Beach, 1994). The gene CDKN2B (MTS-2) encoding p15 is located on the short arm of human chromosome 9 adjacent to the p16 gene and may be a second suppressor gene critical for the development of human tumours. Functional studies of binding and inhibitory capability of cyclin-dependent kinases were carried out for both p15 and p16. They revealed specific binding to CDK4 and CDK6 as well as inhibition of the cyclin D–CDK4 and cyclin D–CDK6 enzyme complexes. Both proteins therefore play a functional role in the control of the cell cycle G1 restriction point, and alterations of the CDKN2B gene may thus take part in the molecular pathogenesis of both hereditary and sporadic melanoma. The p53 protein, product of a third tumour-suppressor gene, binds to the promoter of the WAF1 gene and activates its transcription, leading to the accumulation of its protein product p21 and to inhibition of CDK–cyclin D interactions and G1 cell cycle arrest (Levine et al., 1991; Harris and Holstein, 1992; El Diery et al., 1993; Hunter and Pines, 1994). Thus, mutational alterations of p53 constitute an additional genetic defect leading to cell cycle deregulation and a tumorigenic phenotype (Soussi et al., 1994).

The ras genes, H-ras, Ki-ras 2 and N-ras, encode 21 kDa proteins belonging to a large family of GTP-binding proteins that play a key role in signal transduction leading from cell-surface receptors to the interior of the cell, thereby forming a functional part of proliferation control. Controlled proliferation, however, is disrupted by mutational alterations of the ras genes (Barbacid, 1978). The ras genes can become activated via point mutations in codon 12, 13 or 61. The mutated forms act as oncogenes and have commonly been detected in many types of tumours (Bos, 1989). In addition, ras alterations may affect cell cycle control. Recently published data point to an effect of activated H-ras p21 leading to overexpression of cyclin D1 (Filimur et al., 1994). Altered N-ras genes, predominantly with mutations in codon 61 and/or altered N-ras gene expression levels have been registered in human melanomas (Albino et al., 1989; Van’t Veer et al., 1989; Ball et al., 1994; Platz et al., 1994, 1995b).

Mutated variants of the p53 and CDKN2A genes are commonly found in tumour cell lines and have also been detected in a number of human tumour tissues (Holstein et al., 1991; Caldas et al., 1994; Okamoto et al., 1994; Mori et al., 1994; Zhou et al., 1994). Mutational analyses of both p53 and CDKN2A in tumour samples from sporadic human malignant melanoma have been carried out by several investigators, but conflicting results have been obtained (Volkenandt et al., 1991; Castresana et al., 1993; Floresen et al., 1994; Ohta et al., 1994; Gruis et al., 1995; Platz et al., 1996). No report on mutational alteration of the p15 gene CDKN2B in sporadic human malignant melanoma has yet been published.

We have recently registered the presence of somatic mutations in the CDKN2A gene in melanoma metastases from 3/25 (12%) of patients with sporadic cutaneous malignant melanoma (Platz et al., 1996). The present report describes the mutational analysis of the same patient samples for CDKN2B exons 1 and 2, for p53 exons 4–9 and for N-ras exon 2, by polymerase chain reaction—single-strand-conformation polymorphism (PCR/SSCP) and their final characterisation by nucleotide sequence analysis. In addition, p53 was studied by immunohistochemical analysis using the mouse monoclonal antibody DO-1.
Materials and methods

Patients, tumour samples and DNA extraction

All patients included in the study had a histologically verified cutaneous malignant melanoma. Twenty-six metastases from 25 patients were studied. Patient and tumour details as well as histopathological analyses, sample preparation and DNA extraction have been described previously (Platz et al., 1996).

Antibodies and immunohistochemistry (IHC) procedures

A mouse monoclonal antibody DO-1 (diluted 1:200) recognising an N-terminal epitope (residues 37–45) on

![Figure 1](image-url)

*JI*, *BT*, *JA*, *TN*, *, wild-type references.

(b) SSCP analysis of p53 exonic regions. Examples of obtained bandshifts in exon 4 (primers C1/C2), patient BT. Exon 5–6 (primer pair D1/E2), patient SB. Exon 5 (primers E1/E2), patients MP, TN and JIR. EXON 6 (primers E6S/E6A), patients MP, EE and GL. *, Wild-type references. JA and WN, patient samples with bandshifts. JA shows loss of the wild-type allele.

Table I Primers used for PCR and sizes of amplified fragments

| Primer | Sequence | Size of amplified fragment (bp) |
|--------|----------|--------------------------------|
| N-ras  |          |                                |
| 5'     | CAAGTGGTTATAGATGGTGA | 118 |
| N2b    | ATACACAGAGGAGCCCTTCG |
| CDKN2B |          |                                |
| Exon 1 | AAGAGTGTCTGTTAAGTTTAG | 310a |
| p151A  | CATCGGCCGATCTAGGGTCA |
| Exon 2 | TGAGTTTAAGCTGAAGGGTG |
| 89F    | GGTGGGGAATTGGTAAG |
| 89R    | TCTGACCACCTTGCTCTCTC |
| Exon 2 | TCTGACCACCTTGCTCTCTC |
| p152S  | CATCGGCCGATCTAGGGTCA |
| p152A  | CATCGGCCGATCTAGGGTCA |
| Exon 5 | CTGCCTCTATGGAATATGTT |
| S0R    | TCTGACCACCTTGCTCTCTC |
| Exon 5 | TCTGACCACCTTGCTCTCTC |
| E5S    | ACTGACCACCTTGCTCTCTC |
| ESA    | GGTGGGGAATTGGTAAG |
| Exon 6 | TCTGACCACCTTGCTCTCTC |
| E6S    | TCTGACCACCTTGCTCTCTC |
| E6A    | TCTGACCACCTTGCTCTCTC |
| Exon 7 | ACTGACCACCTTGCTCTCTC |
| E7S    | TCTGACCACCTTGCTCTCTC |
| E7A    | TCTGACCACCTTGCTCTCTC |
| Exon 8 | TCTGACCACCTTGCTCTCTC |
| E8S    | TCTGACCACCTTGCTCTCTC |
| E8A    | TCTGACCACCTTGCTCTCTC |
| Exon 9 | TCTGACCACCTTGCTCTCTC |
| E9S    | TCTGACCACCTTGCTCTCTC |
| E9A    | TCTGACCACCTTGCTCTCTC |
| Exon 5 | TCTGACCACCTTGCTCTCTC |
| D1     | TCTGACCACCTTGCTCTCTC |
| D2     | TCTGACCACCTTGCTCTCTC |
| Exon 6 | TCTGACCACCTTGCTCTCTC |
| D1     | TCTGACCACCTTGCTCTCTC |
| D2     | TCTGACCACCTTGCTCTCTC |

* aFragment cleaved with BamHI into 132 bp and 178 bp before SSCP. *bFragment cleaved with Smal into 208 bp and ~ 320 bp before SSCP. *cFragment cleaved with Smal into 195 bp and 234 bp for SSCP.
human wild-type and mutant p53 protein (Oncogene Science, Manhasset, NY, USA) was used on 4 μm sections of
formalin-fixed tumour tissue as described earlier (Platz et al., 1995b). The sections were pretreated with microwaves in a
Microwave Tender Cooker® (Nordic Ware, Minneapolis,
MN, USA) placed in a household microwave oven. The treatment was for 15 min at maximum power and for an
additional 10 min at 65% power setting. The lower limit for
p53 immunopositivity was arbitrarily set at 1% of positive
cells.

Polymerase chain reaction (PCR) and single strand
conformation polymorphism analysis (SSCP)
Amplification of CDKN2A exon regions and their SSCP
analysis has been reported earlier (Platz et al., 1996). The
CDKN2B exon 1 and 2 regions were separately amplified.
Exon 1 was amplified as a 310 bp fragment using the primers
p151S and p151A and cleaved with BamHI into two
fragments of 132 bp and 178 bp. Exon 2 was amplified
either with the primers p152S and p152A resulting in a
fragment of 429 bp which was cleaved with Smal into two
fragments of 195 bp and 234 bp, or with the primers 89F and
50R (Kamb et al., 1994a) resulting in a fragment of ~530 bp
which was cleaved into fragments of 208 and ~320 bp. The
PCR programmes were 30 cycles at 94°C for 30 s, 60°C for
30 s, 90°C for 30 s. SSCP runs were always carried out with
both the intact and the cleaved PCR fragments.

The genomic regions containing the p53 exons 4 to 9
were separately amplified using primer combinations
resulting in fragment sizes from 171 to 325 bp, suitable for
efficient SSCP (Naito et al., 1992). The genomic region
including exon 5 and exon 6 was also amplified as one single
fragment, including the intervening sequence, by the primer
combination D1/E2, exon 5–6, or as two overlapping
segments using the primer combinations D1/D2, exon 5–6,
or E1/E2, exon 5–6 (Kishimoto et al., 1992). The N-ras
exon 2 region was amplified as a 118 bp fragment using the
primer pair N-ras 5’/N2b, as previously described (Platz et al.,
1994). All primer sequences are summarised in Table I.
The PCR products were labelled by incorporation of
[x-32P]dCTP and the SSCP gel runs were carried out as
described by Mashiyama et al. (1990). SSCP was performed
both in the presence of 5% glycerol at 18°C or in the
absence of glycerol at 5°C.

### Table II Primers used for PCR and nucleotide sequence analysis of
N-ras, CDKN2B and p53 regions

| PCR primers | Sequencing primers | Sequence |
|-------------|-------------------|----------|
| N-ras       |                    |          |
| Exon 2      | N-ras 5’           | CAAG TTTAGAGGATGTTGGA |
| N2b         | N2a                | F-GGTGAACCTGTTTGGGA  |
| CDKN2B      |                    |          |
| Exon 2      | 5’                 | CCGGCCATCTCCATACCTG  |
| 3’          | seq                | F-CCCACCCCTGCTGTGAC |
| p53         | Exon 4             | B-TGAACAATGGTTCGACTGAGCC |
| RIT 438     |                    | TCAGGGCAACTGACCAGCAG |
| RIT 442     |                    | F-TCAGGGCAACTGACCAGCAG |
| Exon 5–6    | RIT 597            | B-TTCACCTGGGCCCTGACTT |
| RIT 595     |                    | AGTTGCGAAACCCGACCTC |
| RIT 600     |                    | F-GCTCATAGGGCACCACC |

B corresponds to biotinylated primer and F corresponds to
fluorescein isothiocyanate (FITC) labelled primer.

Figure 2 Examples of obtained sequencing results. (a) Point
mutation at codon 85 AAT Asn of CDKN2B, patient A1. (b)
Corresponding wild-type sequence. (c) Point mutation at codon
218 GGG Gly of p53, patient GL. (d) Corresponding wild-type
sequence. (e) Point mutation at codon 61 AAA Lys of N-ras,
patient JA. (f) Corresponding wild-type sequence.
Nucleotide sequence analysis

Nucleotide sequence analyses were carried out by direct sequencing of the exon-specific PCR products, using a biotinylated primer for single-strand DNA isolation and fluorescein isothiocyanate (FITC)-modified exon-specific sequencing primers with the comb solid-phase DNA-sequencing Autoload™ kit (Pharmacia Biotech, Uppsala, Sweden). Electrophoretic separation of the sequence ladders was done on an automatic ALF™ DNA sequencer (Pharmacia Biotech, Uppsala, Sweden) (Lagerkvist et al., 1994). The primers used for sequencing were given in Table II. The primers for p53 sequencing have been described previously by Hedrum et al. (1994). The sequencing of the CDKN2A regions has been reported earlier (Platz et al., 1996).

Results

Immunohistochemistry

Only the metastases of patients BT and GL showed high p53-specific nuclear immunopositivity at a level of 80% positive-staining tumour cells. Three additional patients (SB, AI and HW) showed immunopositivity for p53, but the content of positive cells was less than 10%.

DNA extraction, PCR and SSCP

DNA extracts were the same batches as previously used for mutational analysis of the CDKN2A gene region (Platz et al., 1996). The extracts have been established from dissected and trimmed frozen tissue pieces containing at least 90% tumour cells. The PCR amplification of the CDKN2B, p53, and N-ras exonic regions all resulted in the expected fragment sizes, as checked by electrophoretic analysis of NuSieve agarose gels, for all 26 metastatic samples. No sign of small deletions or homozygous gene losses could be recognised. SSCP analysis detected CDKN2B exon 2 band shifts in PCR samples from five metastases. In one sample a p53 exon 4 band shift was observed and in six samples band shifts for the exon 5–6 region were seen. In two more samples N-ras exon 2 band shifts were detected (Figure 1).

Nucleotide sequence analysis

Nucleotide sequence changes were with one exception exclusively detected in samples that also had SSCP band shifts. The remaining samples had the wild-type sequence, the sole exception being from patient AI, who had a point mutation in CDKN2B but no clear bandshift in SSCP. The registered mutations are summarised in Figure 2. Patients JI and AI had metastases with missense mutations in CDKN2B. A known natural polymorphism C/A (Otsuki et al., 1995) in the non-coding sequence 5′ to exon 2 of CDKN2B (at nucleotide position 74) was found by both SSCP and nucleotide sequence analysis in metastases from five (20%) of the patients. Patients BT, EE and GL with p53 mutations in their metastases showed no corresponding wild-type allele. Two metastases of patient MP and a metastasis of patient TN had silent mutations in the Arg-213 codon of p53. The wild-type N-ras allele was not detectable in the metastasis of patient JA who had a mutation in N-ras exon 2, whereas both a mutant and the wild-type N-ras allele was registered in the metastasis from patient WN.

Discussion

Disturbances of cell cycle control may result in unregulated cell growth and consequently in tumour formation. The cell cycle is governed by a complex network of interacting proteins which at this stage is partially understood (Hartwell and Kastan, 1994). Several major checkpoints have been identified. One of them, the GI restriction point, is the site at which the decision for cell division is taken. Defects in any of the participating proteins may lead to genomic instability and contribute to the development of malignancy. Mutations and altered expression patterns of genes coding for components of cell cycle regulation have been reported in a variety of human neoplasms (Cordon-Cardo, 1995).

The mutational analysis of metastases from 25 patients with sporadic melanomas in the present investigation shows the occurrence of mutated forms of the CDKN2B, p53 and N-ras genes. The same samples had previously been analysed for CDKN2A and mutant alleles found (Platz et al., 1996). The registered alterations comprise point mutations exclusively. No indication of small deletions could be recognised, since all samples efficiently yielded fragments of expected sizes by PCR. Histological examination of the tumour tissue pieces revealed the presence of highly homogeneous tumour cell populations. There was no sign of homozygous deletions involving the investigated gene regions within the limits of the employed PCR method (Platz et al., 1996). The presence of subclones with gene losses may, however, have escaped detection. Altogether, the obtained results show that 10 of 25 patients had metastases with single mutational changes in one of the four investigated genes. One patient had a metastasis which contained both a CDKN2A and a p53 mutation.

The DO-1 monoclonal p53-specific antibody selectively recognised the two p53 missense mutants in the metastases from patients BT and GL but, as expected, did not recognise the truncated protein product in the metastasis.

Table III Mutations of CDKN2A, CDKN2B, p53 and N-ras in melanoma metastases

| Patient | CDKN2A* | CDKN2B* | p53* | N-ras |
|---------|---------|---------|------|-------|
| JI      | Pro82Ala CGG/CGG | Arg213Arg CGA/CGG | Gln61Lys CAA/AAA |
| MP      | Arg112Gly CGT/GGT | Arg213Arg CGA/CGG |
| Metast.1| Arg112Gly CGT/GGT | Arg213Arg CGA/CGG |
| Metast.2| Asp85Asn GAT/AAT  | Pro82Leu CGG/CTG |
| AI      | Arg213Arg CGA/CGG | Gln61Arg CAA/CGA |
| BT      | Arg213Arg CGA/CGG | Val218Gly GTG/GGG |
| JA      | Arg213Arg CGA/CGG | His6stop CAC/ TAG |
| TN      | Arg213stop CGA/TGA |
| HW      | C insert codon 4; frame shift and stop at 14 TAG |
| JIR     | Arg213Arg CGA/CGG |
| WN      | Arg213stop CGA/TGA |
| EE      | Val218Gly GTG/GGG |
| GL      |                           |

*Numbering according to Platz et al. (1996). *Numbering according to Kamb et al. (1994a). *Numbering according to Buchman et al. (1988).
with an exon 6 nonsense mutation from patient EE. All sections from metastases with wild-type p53 showed no immunoreactivity, except for three sections (SB, AI and HM), which showed small subpopulations of immunopositive cells (less than 10% of the cells in 4 μm sections) and may indicate the presence of p53 mutations. The corresponding frozen tissue pieces used for DNA extraction may contain an even smaller fraction of cells carrying a p53 mutation and might therefore have escaped detection by SSCP and sequence analysis. Stabilisation of the p53 wild-type protein by interaction with other protein components may be an alternative explanation.

To sum up, this investigation registered, among a total of 25 patients, two subjects with an N-ras alteration, two with a CDKNav alteration, two with a previously described CDKNa alteration, two with a p53 and one with both a CDKNA and a p53 alteration, both resulting in a truncated protein product. Heterogeneous genetic changes may thus contribute to one and the same malignant phenotype. Several additional components of the cell cycle controlling network may actually be altered in the cases where we could not find any change in the four investigated genes.

The combined results of studies concerning mutations of cell cycle-related proteins in tumours and tumour cell lines reported by many investigators show that such alterations are the most common genetic changes in human tumours (Clurman and Roberts, 1995). The present mutation screening, while reporting relatively low frequencies of changes for any of the four genes alone, indicates a substantial total frequency of 44% (48% if two silent p53 mutations are counted) of patients with mutations and may hypothetically approach the 100% level when additional genes with functional connection to cell cycle control are included in forthcoming studies.

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