Methylation analysis of several tumour suppressor genes shows a low frequency of methylation of **CDKN2A** and **RAR** in uveal melanomas

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Abstract

We have investigated the frequency of methylation of several tumour suppressor genes in uveal melanoma. As the loss of one copy of chromosome 3 (monosomy 3), which is found in about half of these tumours, is tightly associated with metastatic disease, a special emphasis was laid on genes located on this chromosome, including the fragile histidine triad (**FHIT**), von Hippel–Lindau (**VHL**), β-catenin (**CTNNB1**), activated leukocyte cell adhesion molecule (**ALCAM**) and retinoic acid receptor-β2 (**RAR**B) genes. In addition, the methylation patterns of the CpG-rich regions 5′ of the E-cadherin (**CDH1**), p16/cyclin-dependent kinase inhibitor 2 A (**CDKN2A**) and retinoblastoma (**RB1**) genes were analysed by bisulphite genomic sequencing or methylation-specific PCR (MSP). Furthermore, the **SNRPN** and **D15S63** loci, which are located in the imprinted region of chromosome 15, were included in the study. Aberrant methylation was detected in nine of 40 tumours analysed: The imprinted **SNRPN** and **D15S63** loci were hypermethylated in three tumours, all of which retained both copies of chromosome 3. Methylated **RAR**B alleles were detected in three tumours, whereas in three other tumours **CDKN2A** was found to be methylated. As we did not find **RAR**B and **CDKN2A** preferentially methylated in tumours with monosomy 3, which is a significant predictor of metastatic disease, we suggest that these genes may play a causative role in the formation of uveal melanoma but not in the development of metastases. Copyright © 2003 John Wiley & Sons, Ltd.

Keywords: uveal melanoma; metastatic disease; tumour suppressor gene; DNA methylation; chromosome 3

Introduction

Uveal melanoma is the most common form of primary eye cancer, with an incidence of six cases per million people per year. About 50% of these tumours show loss of an entire chromosome 3. This chromosomal aberration is significantly associated with metastatic disease. In long-term studies, approximately 70% of patients with monosomy 3 in the primary tumour died of metastases, whereas tumours having two normal copies of chromosome 3 (disomy 3) rarely gave rise to metastatic disease (Prescher et al., 1998; White et al., 1998a; Sisley et al., 1997). The molecular mechanisms underlying uveal melanoma development, progression and metastasis are yet unknown. Assuming that loss of one chromosome 3 is part of a two-step inactivation mechanism typical of tumour suppressor genes (TSGs), one would expect that metastasizing uveal melanoma depends on biallelic inactivation of one or more genes located on chromosome 3. This hypothesis is backed by the finding of common regions of deletion overlap in tumours that have
lost only parts of chromosome 3 (Tschentscher et al., 2001). Alternatively, as uveal melanomas occasionally show isodisomy (two identical copies) of chromosome 3, the possible role of epigenetic mechanisms has been put forward (White et al., 1998b). However, no imprinted regions have been reported on chromosome 3 so far.

De novo DNA methylation is an epigenetic alteration that has been found to contribute to the development and progression of several tumours (for review, see Baylin et al., 2001). Aberrant methylation frequently targets CpG-rich regions (CpG islands) at the 5′ end of genes. With the exception of CpG islands associated with imprinted genes, or genes located on the inactive X chromosome, these regions are maintained free of methylation in normal cells. The RB1 gene, which was the first TSG shown to be methylated in tumours (Greger et al., 1989; Sakai et al., 1991), shows allele-specific epigenetic alterations in about 10% of retinoblastoma cells. The 5′ regions of eight genes known to be involved in tumourigenesis, and two imprinted loci, in 40 uveal melanomas. In view of the strong association of monosomy 3 with metastatic disease, the methylation status of genes on this chromosome was of particular interest. We investigated the fragile histidine triad gene (FHIT), a putative TSG located in chromosome region 3p14.2, the retinoic acid receptor-β2 gene (RARβ, on 3p24), the VHL gene (3p25), the activated leukocyte cell adhesion molecule gene (ALCAM, on 3q13) and the β-catenin gene (CTNNB1, on 3p21). We also included the E-cadherin gene (CDH1, on 16q22) in our study, as loss of function of the complex formed by β-catenin and E-cadherin occurs in a variety of epithelial tumours and is correlated with invasion and metastasis (Yap, 1998). In addition, CDH1 was found to be expressed in a variable manner in uveal melanoma (Anastassiou et al., 2001).

We also analysed the methylation status of the RB1 (13q14) and CDKN2A (9p21) genes, which are involved in tumourigenesis of several tumours, including uveal melanoma. In previous studies, CDKN2A methylation was reported in 6% (Merbs and Sidransky, 1999) to 34% (Van der Velden et al., 2001) of uveal melanomas. Recently, loss of heterozygosity (LOH) at the RB1 locus was found to be a common alteration (21%) in uveal melanomas (Scholes et al., 2001), suggesting a role for RB1, or additional loci close to RB1, in uveal melanoma tumourigenesis.

Finally, we investigated the SNRPN and D15S63 loci, which are located in the imprinted region of chromosome 15. This region, which is affected in patients with Prader–Willi or Angelman syndromes, is not known to play a role in tumour formation but we were interested to know whether imprint maintenance is affected in tumour cells.

Materials and methods

Patients and biopsy specimens

Diagnosis of uveal melanoma was established following current ophthalmological and histological criteria. Vital tumour samples were obtained from patients treated by primary enucleation without prior radiation or chemotherapy. Peripheral blood and tumour material were obtained at the time of operation and stored at −80°C.

Analysis of chromosome 3 status

DNA extractions from blood and tumour samples and PCR-based diagnosis of chromosome 3 loss were carried out as described previously (Tschentscher et al., 2000; 2001). In brief, fluorescently labelled primers were used for amplification of the microsatellites D3S3050, D3S2406, D3S3045, D3S1744, D3S2421, D3S1311 and D3S1272 from the DNA of tumours and corresponding blood samples, in individual reactions. Reaction products were analysed using an ABI 3100 automated capillary genetic analyser and the GeneScan™ and Genotyper® software (Applied Biosystems, Foster City, CA). According to the report by Tschentscher et al. (2000), uveal melanomas showing LOH for all informative markers on chromosome 3 were considered to have monosomy 3.

Bisulphite treatment

Bisulphite modification was performed as described (Clark et al., 1994). Briefly, tumour DNA (5 µg
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Methylation-specific PCR (MSP)

Established MSP protocols were used to analyse the methylation status of the VHL, CDH1, CDKN2A (primers p16-U2 and p16-M2; for sequence, see Herman et al., 1996) and RBL promoter/exon 1 regions, as well as the imprinted SNRPN and D15S63 loci (Herman et al., 1996; Zeschnigk et al., 1997, 1999; Runte et al., 2001). The RARB-MSP was modified from Widschwendter et al. (2000). PCR was performed in a 25 μl reaction volume containing 2 μl bisulphite-treated DNA. The conditions were 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 50 μM of each dNTP and 0.5 U AmpliTaq (Applied Biosystems, Foster City, USA). For the detection of methylated alleles, 1 μM each primer (RAR–MAS and RAR–MES) was used; for the detection of unmethylated alleles, we used 1 μM each primer (RAR–UAS and RAR–USE) in a separate reaction. After the initial denaturation step at 94°C for 5 min, 35 cycles of denaturation (at 95°C for 15 s), annealing (at 60°C for 30 s) and extension (at 72°C for 30 s) were performed, followed by a final extension (at 72°C for 5 min). MSP products were separated on 2.5% agarose gels and visualized by ethidium bromide staining. The primer sequences used were as follows: RAR–MSE: 5’-ATGGTGAGAAGCGCGAGCGATTC-3’ (nt 102–123); RAR–MAS: 5’-CTCGACCATCCAACCGAAACG-3’ (nt 232–253); RAR–USE: 5’-GGATGTTGAGATGTTGATT-3’ (nt 100–123); RAR–UAS: 5’-TACTCAACATTACCAACCAAACA-3’ (nt 232–255). The nucleotide positions of the primers are given according to Widschwendter et al. (2000).

Genomic sequencing

PCRs for the ALCAM analysis were performed with the primers ALCAM5’ and ALCAM3’ (Table 1), which were designed to bind to the putative promoter/exon 1 region (GenBank Accession Nos L38608 and AC078806) in bisulphite-treated DNA. The primer sequences for CTNNB1 (CTNNB5’ and CTNNB3’) and FHIT (FHITas5’ and FHITas3’) amplification were generated on the basis of published sequence data (GenBank Accession Nos X89448 and U76262/U76263, respectively). These primer sequences are all given in Table 1. PCRs for methylation analysis of these genes were performed under the conditions given above for the

Table 1. The primers used for genomic sequencing

| Primer       | Sequence                                | Accession      | Position (nt) | Annealing (°C) |
|--------------|-----------------------------------------|----------------|---------------|----------------|
| FHITas5’     | GTTTTATTTTTTTAGGATTTGATAGTTG            | U76262         | 1413–11442    | 60             |
| FHITas3’     | AAATCTATACACTATAAACCCTTCTAATTACACC      | U76263         | 129–158       | 60             |
| CTNNB5’      | TATTTTAAATCCAGGGTTTTGGTGATAGTGAG       | X89448         | 990–1020      | 64             |
| CTNNB3’      | CAAACACCTCAAAAAAACAACACTCCCTCC         | X89448         | 1142–1170     | 64             |
| ALCAM5’      | ATTATTTAAGTTTTTTAATGGAATAGG            | AC078806       | 73938–73968   | 64             |
| ALCAM3’      | TTCCATATTTCCCTCTTCTTCTTAAATACCC        | AC078806       | 74217–74247   | 64             |

All primer sequences are given in the 5’ to 3’ orientation. The primers were designed based on the sequence information (accession) provided by GenBank database at NCBI. The primer positions (position) according to the GenBank data file and annealing temperatures (annealing) used for PCR are also given. C, T and were added in equal concentration during primer synthesis.
RARB–MSP; however, the primer concentrations were adjusted to 0.8 µM each. After the initial denaturation step (at 94 °C for 5 min), 35 cycles of denaturation (at 95 °C for 15 s), annealing (at 60 °C for the FHIT PCR and 64 °C for the ALCAM and CTNNB PCR for 15 s) and extension (at 72 °C for 15 s) were performed, followed by a final extension step (at 72 °C for 5 min). PCR products were separated on 2.5% agarose gels and visualized by ethidium bromide staining. Agarose gel slices containing PCR products were excised from the gels and purified using the MiniElute Extraction Kit (Qiagen, Hilden, Germany).

DNA sequencing

ALCAM, FHIT and CTNNB PCR products were sequenced using an ABI 3100 automated capillary genetic analyser (Applied Biosystems, Foster City, CA), using the corresponding 3′ primers as sequencing primers. To enable the SequenceAnalyse software for automated analysis, 1 µl Matrix Standard Set DS-01 (Applied Biosystems, Foster City, CA) was added to each sample before loading it onto the machine. As the sequence reactions were started with the 3′ primer, formerly methylated cytosines were indicated by a G-peak in the electropherogram.

Results

Established MSP protocols were used to analyse the methylation status of the VHL, CDH1, CDKN2A, RARB, and RB1 promoter/exon 1 regions as well as the imprinted SNRPN and D15S63 loci (Herman et al., 1996; Zeschnigk et al., 1997, 1999; Runte et al., 2001). The accuracy of MSP was controlled by the use of methylated control DNA as a template. To determine the methylation patterns of the FHIT, CTNNB1 and ALCAM genes, we generated PCR primers (Table 1) that hybridize to bisulphite-treated DNA in the corresponding promoter/exon 1 regions. After performing PCR on bisulphite-modified genomic tumour DNA and methylated control templates, the PCR products were purified and sequenced. The use of premethylated DNA as a control confirmed that all of the assays were capable of detecting methylated alleles.

The methylation status of all 10 loci was tested in 40 tumour samples, 50% of which had monosomy 3. Detection of any methylation in non-imprinted CpG islands is described as methylated. As summarized in Table 2, none of the tumours showed methylation in the promoter/exon 1 regions of the FHIT, CTNNB1, VHL, ALCAM, CDH1 and RB1 genes. Two tumours with disomy 3 (T21 and T22) and one tumour with monosomy 3 (T15) showed methylation of the RARB gene on chromosome 3 (Figure 1a). In addition to a methylated allele, the MSP displayed the presence of an unmethylated allele in these tumours. The observation of two RARB alleles appears to be in conflict with the presence of monosomy 3 in tumour T15, but could be explained by methylation mosaicism.

The CDKN2A promoter region was methylated in three different tumours, one tumour with monosomy 3 (T16) and two tumours with disomy 3 (T25, T36) (Figure 1b). In all three tumours, in addition to the methylated allele, a PCR product specific for an unmethylated allele was present.

The imprinted loci SNRPN and D15S63 are known to be methylated on the maternal chromosome, and unmethylated on the paternal chromosome, in normal individuals. Surprisingly, the PCR product specific for the unmethylated D15S63 alleles (143 bp) was absent in two tumours (T27 and T35), and a third tumour (T24) showed only a weak signal. In one of these tumours (T27), the PCR product specific for the unmethylated SNRPN allele (221 bp) was also reduced (Figure 2). These results are compatible with the assumption of de novo methylation of the paternal D15S63 alleles in the tumours. Alternatively, they may be explained by loss of the paternal D15S63 allele. However, LOH is unlikely for tumours T24 and T35, because both show two differentially methylated alleles at the closely linked SNRPN locus. It is noteworthy that the altered methylation of the SNRPN/D15S63 region was only observed in tumours with disomy 3.

In summary, aberrant methylation was detected in nine out of 40 primary uveal melanomas and was restricted to the TSGs RARB and CDKN2A and the imprinted region on chromosome 15. Altered methylation was less frequent in tumours with monosomy 3 (2/20) than in tumours with disomy 3 (7/20).
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Table 2. The methylation status of 10 genes/loci in 40 uveal melanomas

| Genes/loci | Uveal melanomas with disomy 3 | Uveal melanomas with monosomy 3 |
|------------|-------------------------------|---------------------------------|
|             | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 |
| FHIT       | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| CTNNB1     | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| VHL        | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| ALCAM      | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| CDH1       | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| RB1        | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| RARB       | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| CDKN2A     | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| SNRPN      | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| D15S63     | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − |

Discussion

To identify TSGs in uveal melanoma, we analysed the methylation status of the promoter/exon 1 regions of a set of TSGs in 40 tumour samples. As the loss of chromosome 3 in this tumour is associated with metastatic disease and poor survival, we focused our study on genes located on chromosome 3. No methylation was present in the CpG islands of the VHL, FHIT, CTNNB1 and ALCAM genes, which are located on chromosome 3. While methylation-mediated silencing of CTNNB1 and ALCAM have not previously been reported in any tumour, VHL and FHIT are known to be inactivated by promoter methylation in a wide range of other tumour entities, indicating that this alteration is a common inactivation mechanism of both TSGs. The absence of this epigenetic modification in 40 uveal melanomas makes a major role of VHL und FHIT in uveal melanoma tumourigenesis unlikely.

Three uveal melanomas showed methylation of RARB, which is thought to function as a TSG (Liu et al., 1996; Houle et al., 1993). This gene was shown to be inactivated by methylation in 38% of primary breast cancers Widschwendter et al. (2000) and 40% of primary non-small cell lung cancers (Zöchbauer-Müller et al., 2001). Although the frequency of RARB methylation is much lower in uveal melanomas (7.5%), our results suggest that RARB may be involved in uveal melanoma formation. However, it is unlikely that RARB is a major gene involved in metastatic disease in tumours with monosomy 3, because two of the three uveal melanomas with methylation of RARB have disomy 3, and methylation in the tumour with monosomy 3 is present in only some of the cells.

Among the 40 uveal melanomas investigated here, three (7.5%) showed methylation of CDKN2A. The frequency of CDKN2A methylation in our study corresponds well to the findings of Merbs et al. (1999), who identified methylated CDKN2A alleles in two out of 33 uveal melanomas (6%), but it is in contrast to the results of van der Velden et al. (2001), who investigated 22 tumours and found CDKN2A methylation in 34% of them. In all three studies, MSP was used to determine the methylation status, and we have no explanation for the discrepancy between these results. Moreover, van der Velden et al. found an association between
CDKN2A methylation and tumour location and presence of epithelioid cells, which are prognostic factors for metastatic disease. In our tumour set, however, CDKN2A methylation was not associated with monosomy 3, which is the best predictor of metastatic disease known so far.

Recently, LOH at a polymorphic locus within the RB1 gene was found in five (21%) out of 27 uveal melanomas, suggesting that this gene is involved in the pathogenesis of uveal melanoma (Scholes et al., 2001). In retinoblastoma, a tumour that invariably shows biallelic inactivation of the RB1 gene, methylation is observed in 10% of tumours. As we did not find methylated RB1 alleles in 40 analysed samples, this gene is most likely not the TSG targeted by the LOH at 13q14 in uveal melanoma. As discussed by Scholes et al. (2001), a locus closely linked to, but distinct from, RB1 may be involved.

In several tumour types, the loss of E-cadherin is associated with invasive disease and metastasis. In uveal melanomas, this gene was found to show varying levels of expression, at both the RNA and the protein level (Anastassiou et al., 2001). As we found no methylated CDH1 alleles among 40 tumours, the low expression of this gene in some uveal melanomas is most probably not caused by promoter methylation.
We found alterations in the DNA methylation of the imprinted region on chromosome 15 in three uveal melanomas, all with disomy 3. In one tumour (T27), both SNRPN and D15S63 appeared to be hypermethylated. In principle, the absence of an unmethylated allele in the tumours can be due to loss of heterozygosity; however, the presence of two differentially methylated alleles at the closely linked SNRPN locus in tumour T24 and T35 shows that altered D15S63 methylation is the likely cause of the observed results in these tumours. Interestingly, alterations of the SNRPN/D15S63 methylation pattern were restricted to tumours with disomy 3, which correlates with a good prognosis. In a previous study (Dittrich et al., 1993), we found various degrees of D15S63 hypomethylation in different tumours. As D15S63 and SNRPN are unlikely to play a role in tumour formation, it is likely that these changes reflect loss of imprint maintenance caused by tumour formation.

In summary, our data indicate that methylation of known tumour suppressor genes is infrequent in uveal melanoma. Specifically, several genes on chromosome 3 are not preferentially methylated in tumours with monosomy 3. However, we found methylation of the TSGs CDKN2A and RARB in 7.5% of primary tumours. While promoter methylation, as well as homozygous deletions, of CDKN2A have been reported previously in uveal melanomas (Merbs and Sidransky, 1999; van der Velden et al., 2001), this is the first report showing methylation alterations of the RARB gene in this tumour. Therefore, we suggest that this gene may play a role in uveal melanoma formation.

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