Methylation of CpG island promoters in uveal melanoma

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ABSTRACT

Background: Inactivation of tumour-related genes by promoter hypermethylation is a common epigenetic event in the development of a variety of tumours.

Aim: To investigate in primary uveal melanoma the status of promoter methylation of genes thought to be involved in tumour development: p16, TIMP3, RASSF1, RARB, FHIT, hTERT and APC.

Methods: Gene promoter methylation was studied by methylation-sensitive single-strand conformation analysis and dot-blot assay in a series of 23 primary uveal melanomas. All DNA samples were obtained from paraffin-embedded formalin-fixed tissue blocks.

Results: hTERT promoter methylation was found with a relatively high frequency (52%). Promoter methylation of p16, TIMP3, RASSF1, RARB, FHIT and APC was a rare event. For none of these genes did promoter methylation exceed 15% of tumour samples, and, for some genes (FHIT and APC), no methylation was found at all. Furthermore, promoter methylation was absent in 39% (9/23) of cases. In only 22% (5/23) of cases was hypermethylation of at least two promoters observed.

Conclusions: Promoter methylation of hTERT is a regular event in uveal melanoma. Hypermethylation of the other genes studied does not seem to be an essential element in the development of this tumour. As promoter methylation of APC, RASSF1 and RARB is often observed in cutaneous melanoma, these results suggest that different epigenetic events occur in the development of cutaneous and uveal melanoma.

Uveal melanoma is the most common primary intraocular tumour in adults, with a stable incidence of 4.3 new cases per million per year over the last 25 years.¹ Despite new treatment modalities, mortality has not decreased,² mainly because of liver metastases. Although numerous studies have addressed the genetic events involved in the development of uveal melanoma, only a few have focused on the epigenetic events that may occur during tumorigenesis.

CpG islands promoter methylation, associated with transcriptional gene silencing, has emerged as one of the most important epigenetic alterations in the development of human malignancies.³ Promoter methylation has been observed in many tumour types,^{4–5} but there are few reports on uveal melanoma. p16 promoter has been found to be methylated in up to 32% of primary tumours and 50% of cell lines.^{6–8} In derived cell lines, TIMP3 expression was found to be lower in liver metastatic cells than primary uveal tumour, and it was suggested that TIMP3 promoter methylation may be the cause of TIMP3 downregulation.⁹ More recently, RASSF1 promoter methylation was identified in 50% of primary uveal melanoma, and a correlation was

noticed between RASSF1 promoter methylation status and the development of metastasis.¹⁰

Although cutaneous and uveal melanoma share common morphological features, they differ substantially in their behaviour, metastatic spread and response to chemotherapy. There is increasing evidence that this is related to differences in their molecular phenotype. cDNA analysis has revealed that uveal and cutaneous melanoma cell lines have different expression profiles.¹¹ BRAF mutations, often observed in cutaneous melanoma, were not found in uveal melanomas.^{12–13} Hypermethylation of RASSF1, RARB and APC has been identified in cutaneous melanoma.^{14–16} However, except for RASSF1 in one recent study,¹⁰ these epigenetic events have not been investigated in uveal melanoma.

In this study, we investigated methylation profiles of several genes commonly involved in cancer development (p16, TIMP3, RASSF1, RARB, FHIT, hTERT and APC) in a series of 23 uveal melanomas. We specifically examined key promoter genes that have previously been shown to be methylated in cutaneous melanoma and other cancers. As loss of one copy of chromosome 3 was found in 50% of uveal melanomas and associated with metastatic disease,¹⁷ we were particularly interested in the CpG island methylation status of genes located on chromosome 3, namely RASSF1, RARB and FHIT.

MATERIALS AND METHODS

Tissue samples

Uveal melanoma samples were obtained from consecutive patients recruited in the Jules Gonin Eye Hospital of Lausanne. Cases in which there had been previous irradiation were excluded from the study. Twenty-three patients (12 female, 11 male) were selected; their mean age was 58.4 years (range 25–84). Table 1 summarises the histopathological data. Nine uveal melanomas had involvement of the ciliary body. There were 12 medium and 11 large uveal melanomas according to Collaborative Ocular Melanoma Study (COMS) criteria. Most tumours were mixed (n = 14), and the others were spindle cell (n = 5) and epithelioid cell (n = 4) types. Vascular patterns were assessed as previously described.¹⁸ The local ethics committee authorised the use of human tissues for this study. The study adhered to the tenets of the Helsinki Declaration.

DNA extraction and bisulfite modification

Samples were retrieved from formalin-fixed paraffin-embedded tissue blocks. After deparaffinisation, selected areas in tumour tissue sections were microdissected, and the DNA extracted as previously described.¹⁹ DNA was modified with sodium bisulfite as previously described.¹⁹

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Table 1 Uveal melanoma histology

Case	Location	Base (mm)	Height (mm)	Cell type	Vascular pattern
1	ch	8	8	M	Back to back loops
2	ch	11	10	E	Arcs with branching
3	cb	4	4	M	Normal
4	ch	10	7	M	Normal
5	cb	10	8	S	Back to back loops
6	cb	15	11	M	Back to back loops
7	cb	12	18	M	Arcs with branching
8	ch	9	8	E	Back to back loops
9	cc	18	8	E	Back to back loops
10	cc	10	8	M	Back to back loops
11	ch	11	12	M	Back to back loops
12	ch	20	8	M	Normal
13	ch	18	10	E	Arcs with branching
14	cb	7	4	M	Parallel without cross-linking
15	cc	16	8.5	S	Silent
16	ch	9	12	M	Arcs with branching
17	ch	13	12	M	Arcs with branching
18	ch	10	8	S	Normal
19	ch	11	11	S	Normal
20	cc	12	13	M	Back to back loops
21	ch	9	9	S	Arcs with branching
22	ch	9	4	M	Arcs with branching
23	ch	9	10	M	Back to back loops

ch, choroidal; cb, ciliary body; cc, ciliochoroidal; M, mixed cell type; S, spindle cell type; E, epithelioid cell type.

Methylation-sensitive single-strand conformation analysis (MS-SSCA)

Nested PCR was performed for the seven selected gene promoters with the following amplification profile: 94°C for 30 s, 45 s at T_m, and 72°C for 75 s.²⁰ For the outer PCR, 2 µl modified DNA was used in a total volume of 20 µl; the inner PCR was performed with 1 µl diluted first-PCR product in a total volume of 20 µl. For the outer and inner PCR, 40 and 20 cycles, respectively, were performed. Table 2 lists conditions and primer sequences. Amplification was confirmed by analysis on a 2% agarose gel. Single-strand conformation analysis was performed as previously described.¹⁹ For each gene promoter, the percentage of methylated alleles was semiquantitatively estimated by comparing the intensity of the methylated and unmethylated bands with an external standard.

Methylation-sensitive dot-blot assay (MS-DBA)

All amplified PCR samples were submitted to MS-DBA, as previously described.^{20, 21} Briefly, NaOH-denatured nested PCR products and positive controls were immobilised in duplicate on two NytranN membranes (Schleicher and Schuell, Dassel, Germany). The probes were labelled using a DIG oligonucleotide 3'-End Labeling Kit (Roche, Rotkreuz, Switzerland) following the manufacturer's instructions. Specific probes were developed to detect the amplified DNA. One probe was designed to hybridise to methylated DNA containing two CG dinucleotides, and the other one contained two TG dinucleotides in order to recognise the unmethylated DNA. Table 3 describes the conditions of the dot-blot and probe sequences. The results were obtained by comparing the intensity of the spots on both membranes.

Positive and negative controls

SssI methylase (New England Biolabs, Beverly, Massachusetts, USA) was used to methylate 15–20 µg normal colon DNA obtained from frozen colon mucosa. Full methylation was

confirmed by digestion with MspI (Promega, Madison, Wisconsin, USA) and HpaII (Amersham Biosciences, High Wycombe, Buckinghamshire, UK). SssI-methylated and unmethylated DNA were mixed at different ratios to obtain a methylation scale (0%, 50% and 100% of methylation), which was used as positive control. For each ratio, 2 µg DNA was modified with sodium bisulfite and amplified by nested PCR as described above.

RESULTS

Uveal melanoma samples from 23 patients were analysed for the methylation status of seven CpG island promoter genes, including p16, TIMP3, RASSF1, RARB, FHIT, hTERT and APC. Methylation patterns of the different promoter genes were determined by MS-SSCA and MS-DBA. Different patterns of methylation were found by MS-SSCA: no methylation, full hypermethylation, or a mixture of unmethylated and fully hypermethylated alleles in varying ratios (fig 1A). The results obtained by MS-SSCA were confirmed by MS-DBA (fig 1B). Figure 2 gives detailed results for the DNA methylation analysis of the seven genes in uveal melanoma samples.

Of the uveal melanomas evaluated, 39% (9/23) did not contain a significant level of promoter methylation for any of the genes studied. Of the 14 cases with at least one methylated promoter, only five were methylated for two of the seven analysed gene promoters. Only hTERT showed a relatively high frequency of promoter methylation (52%; 12/23 cases). A low frequency of methylation was observed for RASSF1 (13%), RARB (13%), TIMP3 (9%) and p16 (4%). No methylation was found for APC and FHIT.

No correlation was established between promoter methylation status and either the histopathological characteristics of the tumours or the age of the patients.

DISCUSSION

Most molecular genetic studies performed on uveal melanoma have focused on mutation or allelic loss of tumour suppressor genes leading to a loss of gene expression or function. DNA methylation resulting in epigenetic silencing of tumour suppressor genes is an alternative mechanism for loss of tumour suppressor gene function. Patterns with selective methylation of specific tumour suppressor gene promoters have been described in a wide range of tumours, creating a unique methylation profile characteristic of a particular tumour type.^{4, 22} We determined in 23 uveal melanomas the promoter methylation profile of seven genes commonly involved in tumour development. In our series, hTERT promoter methylation was found with relatively high frequency (52%). Promoter methylation of p16, TIMP3, RASSF1, RARB, FHIT and APC was a rare event. For none of these genes did promoter methylation exceed 15% of tumour samples, and, for some genes (FHIT and APC), no methylation was found at all. Furthermore, promoter methylation was absent in 39% (9/23) of cases.

Three gene promoters often methylated in cutaneous melanoma (APC, RARB and RAS1) were found to be either not or rarely methylated in uveal melanoma. The APC gene promoter has been found to be methylated in 60% of primary cutaneous melanomas and 90% of metastases,¹⁶ but promoter methylation of this gene was not identified in any of our series of uveal melanomas. Methylation of RARB has been observed in 70% of cutaneous melanoma,¹⁵ whereas this epigenetic alteration seems to be rare in uveal melanoma (13%). RASSF1 was methylated in only 13% of our uveal melanoma compared with 41–49% of cutaneous melanomas.^{14, 15} In a recent study, RASSF1 promoter methylation was identified in 50% of primary uveal melanoma,

Table 2 Amplicon location, number of CpG sites, primer sequences and PCR conditions

Gene	GenBank accession No	Amplicon location relative to transcription start site	PCR conditions			
			CpG sites	PCR	Primer sequences (5'–3')	T _m (°C)
p16	AF0022809	–46 to +126		Outer	GGGGGAGATTTAAATTTGGCAAC CCCTCCTCTTCTT	54
			10	Inner	GATTTAGGGGTGTATATTCAA CCCTCCTCTTCTT	54
TIMP3	AF001361	–189 to –36		Outer	GTATTATTTTATAGGATTG CCTAGCTACTACCTCTCTAC	50
			11	Inner	GGTTTAGTTTTTTTTGGAGCC TACCTACTACCTCTCTAC	50
RASSF1	AC002481	–2 to +167		Outer	CCCCACAATCGCTACACCCAAA TGTTTGGTAGTTTAAATGAGTT AGGTTTTT	55
			14	Inner	ACTCTCCTCAACTCCTCCGTTT TGGTAGTTTAAATGAGTTAGTT TTTT	54
RARB	X56849	–306 to –150		Outer	GTAAAGGGAGAGAGTTGGTGT CAACTCCCAAAATCTCACAA	55
			7	Inner	AAGGGAGAGAGTTGGTGTTA AAACAAACCAACCAAAAAACA AA	50
FHIT	AF399855/NM_002012	–126 to +42		Outer	GTTTTTGAATTAGGGTTATTGTT CCTAAAAATATACCCACTAAAT A	53
			11	Inner	GTATTATGGTTTTTAAATGGTT CAAAAAACAAAAAATCCTATAA C	50
hTERT	AF128893	–387 to –219		Outer	GGGTTATTTTATAGTTAGGTAA TCCCAATCCCT	53
			20	Inner	GGGTTATTTTATAGTTAGGTAA TCCACTAAAAACCC	48
APC	U02509	–210 to –52		Outer	GGGGTTAGGGTTAGGTAGGAAC TACACCAATACAACCCATA	53
			13	Inner	GGGTTAGGGTTAGGTAGGTGT CCCACACCCCAACCAATC	53

Table 3 Oligoprobe sequences and conditions for methylation-sensitive dot-blot assay

Gene	CG-probe sequences (5'–3')	TG-probe sequences (5'–3')	Tm Hyb (°C)	Tm washes (°C)
p16	TCCGAGGGGGTTTTTCGTT	GTTGGAGGGGGTTTTTTGTT	50	53
TIMP3	GTCGATGAGGTAATGCGGTT	GGTTGATGAGGTAATGTGGTT	50	52
RASSF1	CAAAACCAACGAAACACGAAC	CAAAACCAACAAACACAAACC	50	53
RARB	GGAAAGAAAACGTCGGTTTGT	GGAAAGAAAATGTTGGTTTGTG	50	53
hTERT	TAGTTGCGTTGTCGGGGTTA	GTAGTTGTGTTGTTGGGGTTA	50	53
FHIT	GGTTTCGTTTTATCGTGGG	GGGTTTTGTTTTATTGTGGG	48	52.5
APC	GATGCGGATTAGGCGTTTT	GGATGTGGATTAGGGTGTITT	50	53

and a correlation was found between RASSF1 promoter methylation status and the development of metastasis.¹⁰ As the same promoter region was evaluated in this study and ours, the discrepancy in the results may be explained by the selection of tumours of larger size and at a later stage of development in the Dutch study.

Methylation of the hTERT promoter was identified in 52% of the uveal melanomas. The first studies of the methylation status of the hTERT CpG island had led to a paradox. In normal somatic cells, this CpG island was unmethylated while the gene was transcriptionally silent. However, in most cancer cells, this region was hypermethylated, whereas telomerase activities and hTERT mRNA were unambiguously detected.^{23–26} These observations contrast with the general association between promoter methylation and gene silencing.²⁷ We recently solved this paradox. hTERT methylation does indeed prevent binding of negatively acting transcription factors, such as CTCF inhibitor,²⁸ and partial hypomethylation of the short region of the hTERT promoter can result in some level of transcriptional activity.²⁹ No previous studies have evaluated hTERT methylation in uveal melanoma, but moderate telomerase activity has been reported in a series of 14 tumours.³⁰ As telomerase activity and mRNA expression correlate with the level of its promoter methylation,²⁵ our data are in agreement with this report. However, we were not able to identify a correlation between hTERT promoter methylation status and tumour morphological characteristics (cell type, tumour size, tumour localisation, vascular pattern and pigmentation). Likewise, Heine *et al*³⁰ showed no correlation between telomerase activity and morphology or the growth fraction of the tumour.

In previous studies performed in uveal melanoma, much attention has been paid to cell cycle proteins such as p16. Various levels of p16 expression have been identified by immunohistochemistry, ranging from complete preservation of expression to loss of expression in 15–66% of cases.^{6–31–32} Loss of heterozygosity of 9p21 has been reported in 24–32% of cases.^{6–33} Sequence analysis failed to reveal any inactivating mutations.^{5–6} Inactivation of p16 can also occur through methylation of its promoter. In our study, only one case (6%) showed methylation of the p16 promoter. This result corroborates previous studies on primary uveal tumours.^{6–7} However, one group found a methylation rate of 32% in primary uveal melanomas and 50% in cell lines derived from them.⁸ The discrepancy between these data may be related to the sensitivity of the different techniques used for methylation analysis. The p16 promoter has often been found to be methylated in studies using methylation-specific PCR. This technique is the most widely used because of its high sensitivity and ease of execution; however, it can generate false-positive results through PCR overamplification. In contrast, p16 methylation has rarely been detected when MS-SSCA or sequencing has been used. Furthermore, analysis of promoter methylation in cell lines does not necessarily represent promoter methylation status in primary tumour specimens.³⁴

As monosomy of chromosome 3, an event identified in over 50% of uveal melanoma, correlates with the development of metastatic disease,¹⁷ much attention has been paid to the involvement of possible candidate genes located on chromosome 3 in the progression of the disease. We evaluated in this context the promoter methylation status of three genes located on chromosome 3, FHIT, RARB and RASSF1. On the basis of our data, CpG

Figure 1 hTERT promoter methylation by methylation-sensitive single-strand conformation analysis (MS-SSCA) and methylation-sensitive dot-blot assay (MS-DBA) in uveal melanomas. (A) hTERT promoter methylation by MS-SSCA. Three cases (13, 17 and 20) show full methylation, and another one (19) methylation of about 30% of the alleles. Enzymatically methylated DNA from placenta was used as control for the methylation scale. Bars indicate the methylated or unmethylated bands. Methylation level (%): 0, no methylation; 50, 50% of methylated alleles; 100, 100% of methylated alleles. (B) hTERT promoter methylation by MS-DBA. Dots hybridised with an oligoprobe specific for either the methylated DNA (Methyl. CpGs) or the unmethylated DNA (Unmeth. CpGs). The percentage of methylated alleles is indicated under each sample. In cases 13, 17, 19 and 20, dots are observed in the upper layer as a result of hybridisation with an oligoprobe specific for methylated DNA.

Figure 2 (A) Methylation analysis of p16, TIMP3, RASSF1, RARB, FHIT, hTERT and APC promoters in 23 uveal melanomas. The numbers identify the individual cases. The methylation analysis was performed using methylation-sensitive single-strand conformation analysis (MS-SSCA) and methylation-sensitive dot-blot assay (MS-DBA). (B) Percentage of methylated samples for each gene.

island methylation does not appear to be a common mechanism involved in the silencing of these genes in uveal melanoma.

In conclusion, our study indicates that epigenetic alterations of the hTERT gene is a significant event in uveal melanoma. Promoter methylation of p16, TIMP3, RASSF1, RARB, FHIT and APC is rare in primary uveal melanoma. Our findings do not, however, exclude silencing of these genes by promoter methylation during later tumour progression. They also show that different epigenetic events occur in the development of cutaneous and uveal melanoma.

Competing interests: None.

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