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# Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma

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**Summary.** Epigenetic models for tumor formation assume that oncogenic transformation results from changes in the activity of otherwise normal genes. Since gene activity can be inhibited by DNA methylation, and inactivation of tumor suppressor genes is a fundamental process in oncogenesis, we investigated the methylation status of the retinoblastoma suppressor gene (*RB* gene) on chromosome 13, in blood and tumor cells from 21 retinoblastoma patients. Using methylation-sensitive restriction enzymes and a cloned DNA probe for the unmethylated CpG island at the 5′ end of *RB* gene, we obtained evidence of hypermethylation of this gene in a sporadic unilateral retinoblastoma tumor. The closely linked esterase D gene and a CpG-rich island on chromosome 15 were not affected. We suggest that changes in the methylation pattern of the *RB* gene play a role in the development and spontaneous regression of some retinoblastoma tumors.

## Introduction

DNA methylation is a key process in the epigenetic control of gene expression (Holliday 1987). Although methylation has been correlated with gene activity, it is not clear whether different methylation patterns cause, maintain, or merely reflect different activity states of a gene. Whereas constitutively expressed genes generally contain CpG islands that are unmethylated in all tissues (Bird 1986), tissue-specific genes are extensively methylated in germ cells and non-expressing tissues (Jahner and Jaenisch 1984). Is has been proposed that loss of methylation may lead to the reactivation of silent genes, and that these changes in gene expression may be important in oncogenesis (Holliday 1987). Although hypomethylation of DNA has been detected in tumor cells (Feinberg and Vogelstein 1983; Goelz et al. 1985) and shown to activate genes (Jones 1985), activation of oncogenes by mechanisms other than point mutations or translocations has not yet been observed.

In contrast, Kautiainen and Jones (1986) reported increased levels of DNA methyltransferase in tumor cells, and De Bustros et al. (1988) detected a hot spot for hypermethylation on the short arm of chromosome 11. Interestingly, the affected chromosome region is known to harbor tumor suppressor genes (Human Gene Mapping 9 1987). Inactivation of tumor suppressor genes underlies the formation and/or progression of several types of cancer (Klein 1987). It is possible therefore that hypermethylation of tumor suppressor genes plays a role in oncogenesis. Since the retinoblastoma suppressor gene (*RB* gene) on chromosome 13 is a paradigm for tumor suppressor genes, we investigated its methylation status in lymphocytes and retinoblastoma tumor cells to test this hypothesis.

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## Materials and methods

### *Patients*

Patients with retinoblastoma were ascertained through the Retionoblastoma Clinic of the Department of Ophthalmology. The diagnosis of retinoblastoma had been established by current ophthalmological and histological criteria. Fresh tumor specimens were obtained from 21 patients who hat not been treated by radiotherapy or chemotherapy prior to enucleation. Tumor G0288 was obtained from a 21-month-old male patient who was unilaterally affected and did not have any family history of retinoblastoma.

### *DNA analysis*

Total genomic DNA was isolated from whole blood and from retinoblastomas obtained after enucleation as described by Kunkel et al. (1977). DNA samples (1 μg each) were digested with the restriction enzymes indicated in the text. DNA fragments were separated by gel electrophoresis, transferred to DURALON-UV nylon membranes (Stratagene, La Jolla, Calif., USA) and hybridized with the radioactively labeled DNA probes as described previously (Horsthemke et al. 1987). Filters were exposed at −70°C to Fuji RX film with one Dupont Lightning Plus intensifying screen for 1 day.

## Results and discussion

Loss of both copies of the *RB* gene results in retinoblastoma, an intraocular tumor of early childhood (Knudson 1971; Benedict et al. 1983). The gene has recently been cloned (Friend et al. 1986; Lee et al. 1987; Fung et al. 1987) and shown to be expressed in fetal retina cells, adult retina cells, and other tissues (Lee et al. 1987). Using the restriction enzymes

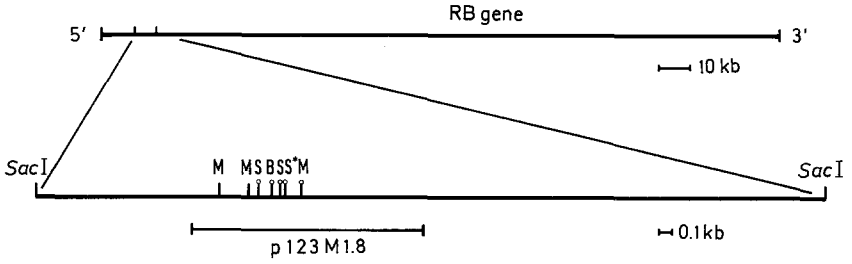


**Fig. 1.** Restriction site map of the CpG-rich island at the 5′ end of the *RB* gene. The map was deduced from single and double digests of clone p123M1.8 and genomic DNA with *Sac*II (*S*), *Sma*I (*M*), and *Bss*HII (*B*). The *asterisk* denotes the *Sac*II site present at the 5′end of the cDNA as identified by hybridization of the p123M1.8 fragments with the cDNA probe. The sites marked with a *circle* are methylated in tumor G0288 (see text and Fig. 3)

*Bss*HII, *Sac*II, and *Sma*I (recognition sequence GCGCGC, CCGCGG, and CCCGGG, respectively), we identified an unmethylated CpG island characteristic of constitutively expressed genes within a 6.1 kb *Sac*I fragment at the 5′ end of the *RB* gene (Fig. 1). In order to investigate the methylation status of the *RB* gene in lymphocyte and tumor DNA, we chose the restriction enzyme isoschizomers *Msp*I and *Hpa*II. Both enzymes recognize the tetranucleotide sequence CCGG, but *Hpa*II does not cleave DNA when the internal deoxycytosine residue is methylated (Bird and Southern 1978).

Lymphocyte and tumor DNA samples for 14 unilaterally and 7 bilaterally affected retinoblastoma patients were studied. Two-thirds of the tumors had lost one copy of the *RB* gene through mitotic non-disjunction or recombination (Cavenee et al. 1983). Gross structural alteration of the *RB* gene was detected in one tumor. Since in vitro culture is known to affect methylation (Shmookler-Reis and Goldstein 1982), only fresh blood and tumor samples were used. DNA samples were subjected to *Sac*I/*Msp*I and *Sac*I/*Hpa*II double digests and probed with clone p123M1.8 spanning the CpG island (Fig. 1). Except for one sporadic unilateral tumor (G0288), which had retained constitutional heterozygosity at chromosome 13 loci, the *Hpa*II patterns of lymphocyte and tumor DNA were the same; they were also identical with normal controls.

Figure 2A shows that in both cell types, some *Msp*I/*Hpa*II sites are methylated and not cleaved by *Hpa*II. In tumor G0288, an extra *Hpa*II fragment of 4.1 kb is present, whereas the 3.4 kb band has only half the normal intensity. Since *Msp*I and other methylation-insensitive enzymes did not reveal any gross structural alterations of the *RB* gene in this tumor, we conclude that the 4.1 kb band has arisen as a result of hypermethylation. The closely linked esterase D gene (Sparkes et al. 1980) and a CpG island on chromosome 15 (Buiting et al. 1988) are not affected (Fig. 2B, C). This result suggests that hypermethylation in G0288 is restricted to localized hot spots including the *RB* gene or to the *RB* gene alone. Because of the small amount of tumor material, the methylation of further loci could not be tested.

Since the large number of *Msp*I/*Hpa*II sites prohibited fine mapping of the hypermethylated sites, we measured methylation of the recognition sites for the methylation-sensitive enzymes *Bss*HII, *Sac*II, and *Sma*I. In contrast to lymphocyte DNA, the 6.1 kb *Sac*I fragment of the tumor DNA is refractory to complete cleavage by these enzymes (Fig. 3). The result points to methylation of the *Bss*HII site, the three *Sac*II sites, and the 3′ *Sma*I site of the CpG island within these *Sac*I fragments (Fig. 1).

The methylation pattern of the *RB* gene in tumor G0288 can be explained by three models: (1) methylation of both gene copies in 50% of the cells, (2) random methylation of one gene copy per cell, and (3) allele-specific methylation. Since the patient is homozygous for the *Bam*HI restriction

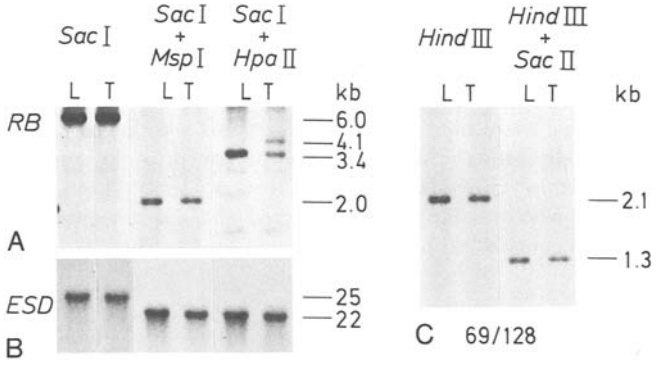


**Fig. 2A–C.** Methylation status of the *RB* gene, the esterase D gene (*ESD*), and a chromosome 15 CpG-rich island in lymphocytes (*L*) and tumor G0288 (*T*) from a patient with retinoblastoma. **A** DNA samples were digested with *Sac*I, *Sac*I+*Msp*I, and *Sac*I+*Hpa*II and analyzed by Southern blot hybridization with the *RB* gene probe p123M1.8. Under the conditions used (Horsthemke et al. 1987), weak unspecific cross-hybridization is visible on some blots. The size of the fragments was determined with the help of a lambda *Hin*dIII molecular weight standard. In tumor G0288, an extra 4.1 kb *Hpa*II fragment ist present, whereas the 3.4 kb band has only half the normal intensity. The same pattern was obtained with increasing concentrations of enzyme, demonstrating complete digestion of cleavable sites (not shown). **B** After removal of the *RB* probe, the Southern blot shown in **A** was rehybridized with the 250 bp *Pst*I-*Acc*I fragment from the 5′ end of the *ESD* cDNA (Squire et al. 1986). This fragment contains nine CpG doublets, one of which is part of an *Msp*I/*Hpa*II site (Young et al. 1988). No differences between lymphocyte and tumor DNA were observed. **C** DNA samples were digested with *Hin*dIII and *Hin*dIII+*Sac*II and analyzed by Southern blot hybridization with probe 69/128, which detects a CpG-rich island on chromosome 15 (Buiting et al. 1988). No differences between lymphocyte and tumor DNA were observed

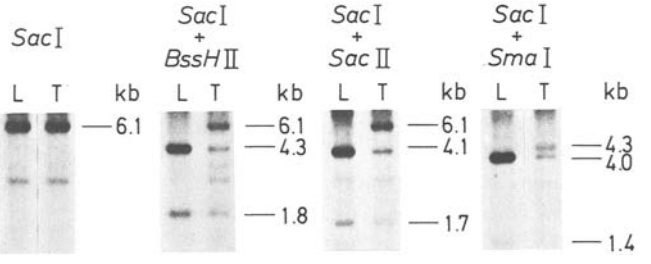


**Fig. 3.** Methylation of the CpG-rich island of the *RB* gene in tumor G0288. DNA samples were digested with *Sac*I, *Sac*I+*Bss*HII, *Sac*I+*Sac*II, and *Sac*I+*Sma*I and analyzed by Southern blot hybridization with the *RB* gene probe p123M1.8. In tumor G0288, the 6.1 kb *Sac*I fragment is refractory to complete cleavage by the CpG enzymes. The same patterns were observed with increasing concentrations of enzymes, demonstrating complete digestion of cleavable sites (not shown)

fragment length polymorphism (Bookstein et al. 1988), which is the only known DNA polymorphism in this part of the gene, it is not possible to test these models.

The presence of two cell populations in tumor G0288, as implicated in the first two models, suggests that hypermethylation occurred after the inactivation of the *RB* gene as a result of changes in the activity or specificity of a trans-acting factor involved in the maintenance of hypomethylation. The persistence of hypomethylation at other loci would suggest that there is a factor specific for the *RB* gene or a class of genes including the *RB* gene. We consider this to be unlikely.

Allele-specific methylation (third model) has been demonstrated in several systems (Chandler et al. 1987; Silva and White 1988), but its mechanism and function is not clear. Hypermethylation of one *RB* allele in tumor G0288 may be the consequence of an as yet unidentified structural gene defect or may represent the primary event in the inactivation of this allele. Hypermethylation of an otherwise normale *RB* allele, as implicated in the latter assumption, may be caused by erroneous methylation of the *RB* gene in the progenitor cell of the tumor and transmission of this epimutation (Holliday 1987) to the daughter cells. Alternatively, it is possible that the tumor results from clonal expansion of an early embryonic cell that has retained the methylation pattern of a specific parental allele (Reik and Surani 1989). Provided the methylated allele is inactive, one mutation at the homologous gene locus will be sufficient to trigger tumor development. Genomic imprinting has been invoked in explaining the preferential mutation of the paternally derived *RB* gene as the initial event in sporadic osteosarcoma, but the molecular basis remains unknown (Toguchida et al. 1989).

Epigenetic changes may also explain spontaneous tumor regression observed in some patients: the maintenance methylase does not replicate methylation patterns during DNA synthesis with absolute fidelity (Holliday 1987), and progressive loss of methylation may eventually lead to the reactivation of the *RB* gene and suppression of tumor growth. Interestingly, two *Sma*I sites of the hypermethylated CpG island are not methylated. It is tempting to speculate that this reflects the process of demethylation.

In conclusion, we would like to emphasize that so far there is no functional evidence for the assumption that the *RB* gene is inactivated by hypermethylation. Such evidence will be difficult to obtain; indeed, it has not yet been obtained for any other autosomal gene studied in vivo. Because of the small amount of tumor material and lack of identification of the other *RB* gene mutation in tumor G0288, it was not possible to perform Northern blot analysis and correlate hypermethylation and loss of expression of the *RB* gene. Nevertheless, our data represent the first tentative evidence for the shutting-off of endogenous genes by methylation in diploid somatic cells, apart from X-chromosome inactivation (Gartler et al. 1985; Lyon 1988). We suggest that hypermethylation of tumor suppressor genes is an infrequent but potential event in the development of human neoplasia, and that loss of hypermethylation may be involved in the spontaneous regression of some tumors.

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