

Human Pituitary Adenomas Infrequently Contain Inactivation of Retinoblastoma 1 Gene and Activation of Cyclin Dependent Kinase 4 Gene

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Abstract. Components of cyclinD1/cyclin-dependent kinase 4 (CDK4) /p16^{INK4a}/pRb pathway are the frequent target of many tumor types. We examined the role of retinoblastoma susceptibility gene (*RB1*) and the *CDK4* gene in human pituitary tumorigenesis. For the *RB1* gene, pRb expression and loss of heterozygosity (LOH) on 13q in pituitary adenomas were analysed. Immunostaining of pRb revealed lack of expression in 1 of 29 pituitary adenomas. In 4 of 31 pituitary adenomas, allelic imbalances including LOH of *RB1* on 13q14 were detected. Three of 4 pituitary adenomas, in which one adenoma lacked pRb expression, had a common LOH region at least from *D13S219* on 13q12.3-q13 to *D13S265* on 13q31-32. Interphase fluorescence *in situ* hybridization with a probe of *RB1* showed 2 copies of *RB1* gene suggesting that mitotic recombination events, not deletion or chromosome loss, led to LOH in the 3 pituitary adenomas analyzed. All 27 exons, intron-exon boundaries, and essential promoter region of *RB1* gene were then sequenced in genomic DNA from 4 pituitary adenomas with allelic imbalance on 13q14 including one adenoma without pRb expression and 3 adenomas with pRb expression. Any somatic mutations, insertions, or microdeletions in the *RB1* gene were not detected in 4 pituitary adenomas. Methylation sensitive (MS)-polymerase chain reaction (PCR) and bisulfite sequencing analysis revealed hypomethylated status of CpG islands in the promoter region of the *RB1* genes of 4 pituitary adenomas. In addition, activating mutations of *CDK4* gene, which is a component of cyclinD1/CDK4/p16^{INK4a}/pRb pathway, were not detected in 31 pituitary adenomas. Based on these results, it is concluded that somatic mutations of the *RB1* gene or *CDK4* gene do not appear to play a major role in pituitary tumorigenesis. This supports the presence of potential tumor suppressor gene(s) on 13q12.3-q13 to 13q31-32 in pituitary adenomas.

Key words: Pituitary adenomas, *RB1*, *CDK4*, Mutations, Loss of heterozygosity

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THE *RB1* gene on 13q14 encodes a pRb that is phosphorylated and dephosphorylated synchronously with the cell cycle [1]. The phosphorylation of pRb by the

cyclin D1/CDK4/6 complex in late G1 results in the release of nuclear proteins and transcription factors, including the E2F family, thereby initiating the expression of genes critical for transition into the S phase of the cell cycle. Because both cyclin D1 and p16^{INK4a} have the potential to complex with CDK4, they function as positive and negative regulators of pRb phosphorylation, respectively. Recently, Hibberts *et al.* reported frequent overexpression of cyclin D1 in pitu-

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itary adenomas [2]. Hypermethylation of CpG island in the *p16^{INK4a}* gene resulted in loss of *p16^{INK4a}* protein expression in pituitary adenomas [3]. Thus, a component of the cyclin D1/CDK4/*p16^{INK4a}*/pRb pathway appears to be mutated or deregulated in pituitary adenomas.

Mutations and deletions of the *RB1* gene have been found in retinoblastomas and many other types of tumors, suggesting the importance of *RB1* in human oncogenesis [4]. Ikeda *et al.* reported that a somatic *RB1* mutation was detected in 1 of 25 pituitary adenomas using PCR-single strand conformation polymorphism screening [5]. However, the tumor having a mutation of *RB1* did not show LOH at the *RB1* locus. Furthermore, LOH at *RB1* intragenic markers was not identified in human pituitary adenomas [6–8]. Instead, other data showed LOH at sites telomeric or centromeric to the *RB1* locus in some aggressive pituitary adenomas [9, 10]. These data argue for independent tumor suppressor gene(s) on 13q that are closely linked with, but distinct from, *RB1*.

To assess the role of *RB1* gene in tumorigenesis of the pituitary gland, 31 human pituitary adenomas were examined for pRb expression by immunohistochemistry, LOH on 13q, somatic mutations in the *RB1* gene, and methylation status in the promoter of *RB1* gene. In addition, a somatic mutation of Arg-to-Cys in codon 24 (R24C) in the *CDK4* gene, which generates a dominant oncogene with resistance to normal physiologic inhibition by *p16^{INK4a}* [11, 12], in pituitary adenomas was analyzed.

Materials and Methods

Patients

Thirty-one sporadic pituitary adenomas with matched blood samples were obtained from patients who had undergone hypophysectomy. This study was approved by the institutional review board of Toranomon Hospital and Okinaka Memorial Institute for Medical Research, and fully informed consent was obtained from all subjects. This study was also conducted in accordance with the WHO guidelines on ethical issues in medical genetics and genetic services.

DNA was extracted from frozen tumor sections and peripheral blood as previously described [13]. In frozen tumor sections, tumor and normal tissues were

separated under a microscope by cutting them into small pieces using a razor blade. We microscopically confirmed that tumor tissues were scarcely contaminated with normal tissues. The clinical and pathological features of adenomas in adenomas are shown in Table 1 [13–15].

pRb immunohistochemical study

Tissues were routinely formalin-fixed and paraffin-embedded. Monoclonal antibody, clone G3-245 (BD PharMingen, San Diego, CA) recognizing an epitope between amino acids 332–344 of the human pRb, was used in 1:100 dilution. Biotinylated secondary antibody and peroxidase conjugated avidin-biotin complex were applied at the dilution and the time recommended by the vendor (ABC kit, Vector Lab., Burlingame, CA). Tissue sections on glass slides were pretreated with microwaving before immunostaining. The reactions were visualized with 3, 3' diaminobenzidine. Negative controls included omission of the primary antibody and tissue from a retinoblastoma tissue with negative staining. Only nuclear positivity was assessed; cytoplasmic staining was regarded as non-specific [16]. Tumors were scored positive if all cells were stained or the staining pattern was heterogeneous with a portion of the cells showing immunopositivity. Tumors were scored pRb negative only if all of the malignant cells showed no pRb staining in the presence of nuclear immunoreactivity in surrounding or infiltrating normal cells.

Detection of LOH by microsatellite analysis

Because the *RB1* gene is located at 13q14, LOH in pituitary adenomas were examined in regard to 3 microsatellite markers located on 13q12-q31: *D13S267*, *D13S153*, and *D13S170* (Table 2). With regard to adenomas showing decreased allelic ratios in the 3 markers, 21 additional markers on chromosome 13 were analyzed for better characterization of the pattern and extent of LOH for the chromosomal region (Table 2). The primer sequences and the linear ordering of these markers are based on UniSTS [17].

Microsatellite markers were PCR-amplified from 31 paired normal and tumor DNA samples. One of each primer pair was labeled with 6-FAM fluorescent dye (Applied Biosystems, Foster City, CA). PCR was performed as reported previously [15]. Gel electrophore-

Table 1. Clinical features and pRb expression in pituitary adenomas

Sample number	Age/Sex	Clinicopathological diagnosis	Hardy's classification	pRb expression in adenomas
1.	44/F	Somatotroph adenoma	II-A*	+
2.	65/F	Somatotroph adenoma	I-0	+
3.	34/F	Somatotroph adenoma	III-C	ni**
4.	25/M	Somatotroph adenoma	II-A	+
5.	47/M	Somatotroph adenoma	III-A*	+
6.	49/M	Somatotroph adenoma	III-0	+
7.	38/M	Somatotroph adenoma	III-0	ni**
8.	59/M	Somatotroph adenoma	II-0	+
9.	35/F	Somatotroph adenoma	II-0	+
10.	43/M	Somatotroph adenoma	III-A	+
11.	46/F	Mixed GH/PRL tumor	III-B	+
12.	45/F	Mixed GH/PRL tumor	I-0	+
13.	44/M	Prolactinoma	II-A	+
14.	42/M	Prolactinoma	III-B	-
15.	27/F	Prolactinoma	II-0	+
16.	56/M	Prolactinoma	III-A*	+
17.	70/F	Prolactinoma	II-A*	+
18.	47/F	Prolactinoma	I-0	+
19.	41/M	Thyrotroph adenoma	IV-0	+
20.	67/F	Thyrotroph adenoma	IV-0*	+
21.	15/M	Corticotroph adenoma	II-A	+
22.	55/M	Endocrine inactive	II-A	+
23.	72/F	Endocrine inactive	II-B	+
24.	74/F	Endocrine inactive	II-B	+
25.	67/M	Endocrine inactive	II-B	+
26.	54/M	Endocrine inactive	III-B	+
27.	63/F	Endocrine inactive	II-A	+
28.	55/M	Endocrine inactive	IV-0	+
29.	65/F	Endocrine inactive	II-B*	+
30.	60/F	Endocrine inactive	II-B	+
31.	70/M	Endocrine inactive	III-C	+

*denotes cavernous sinus invasion.

**ni, not informative due to inadequate samples for pRb immunohistochemistry.

sis, data collection, and analysis were performed on a Model 377 DNA sequencer with GENESCAN 672 software (Applied Biosystems). Each fluorescent peak was assessed by peak height. The ratio of alleles 1 and 2 was calculated for leukocyte DNA as the representative normal tissue and the adenoma DNA [15]. The ratio in the adenoma sample was then divided by the ratio in the normal tissue. Calculation was made as

$T1:T2/N1:N2$, where T1 and N1 were the peak height values of the shorter allele product peak for the pituitary adenoma DNA and the normal tissue DNA, respectively, and T2 and N2 were the peak height values of the taller allele product for the tumor DNA and the normal tissue DNA, respectively.

Table 2. Allelic ratios of markers on chromosome 13 in pituitary adenomas

Locus	Sample number				
	28	4	9	14	17
centromere					
D13S175 (13q11)	ni	50	ni	73	ni
D13S1304 (13q)	92	20	95	55	ni
D13S217 (13q12)	ni	20	85	ni	42
D13S1246 (13q)	97	56	ni	ni	60
D13S289 (13q12.1)	90	ni	95	ni	58
D13S260 (13q12.3)	89	21	95	76	75
D13S171 (13q12.3)(BRCA2)	ni	21	97	ni	60
D13S267 (13q12.3)	91	20	92	51	54
D13S220 (13q12.3-q13)	97	ni	ni	ni	ni
D13S219 (13q12.3-q13)	89	24	10	17	ni
D13S1253 (13q)	96	45	11	ni	51
D13S263 (13q14.1-q14.2)	96	24	6	16	87
D13S1312 (13q)	94	21	ni	12	ni
D13S164 (13q14.3)	93	28	14	ni	55
D13S153 (13q14.3) (RB1)	76	18	15	18	58
Rb 1.20 (13q14.3)	84	17	8	12	44
D13S284 (13q14.3)	93	14	10	ni	55
D13S1303 (13q)	87	20	11	ni	70
D13S162 (13q21.2-q31)	91	25	12	11	49
D13S170 (13q31)	95	26	19	11	54
D13S1283 (13q)	90	ni	10	ni	ni
D13S265 (13q31-q32)	96	ni	13	38	54
D13S159 (13q32)	ni	22	9	ni	ni
D13S1265 (13q32)	81	22	23	ni	60
qter					
mean \pm SD	91 \pm 6			58 \pm 11	

ni, not informative

Interphase fluorescence in situ hybridization (FISH) analysis

Interphase FISH was performed as described previously [15]. Briefly, tissue sections of 6 μ m in thickness from paraffin blocks were deparaffinized. After protein digestion, the biotin-labeled probe for *RB1* (RB1, Oncor, Gaithersburg, MD) was directly added to the tissue sections, hybridized, and washed. After the *in situ* hybridization, tissue sections were counterstained with propidium iodide. The number of hybridized probes per nucleus detected with fluorescein-

conjugated avidin under confocal fluorescent microscopy was counted in at least 100 nuclei in each sample.

Sequence analysis of exons 1–27 and the promoter region of RB1

To investigate the possibility of inactivating mutations or microdeletion of *RB1*, 4 adenomas that showed allelic imbalance in 13q were subjected to sequence analysis of whole exons (exons 1–27) and the promoter. Specific oligonucleotides were designed to encompass exons and the surrounding intronic sequences. Primer pairs for the promoter, exons 1 and 2, and exons 3–27 were synthesized as reported by Simpson *et al.* [18], Ikeda *et al.* [5], and Sachse *et al.* [19], respectively.

PCR was carried out in a volume of 20 μ l containing 100 ng of genomic DNA, 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 2 mM MgCl₂, 0.2 mM each dNTP, 10 pmole of each primer, and 0.5 units of AmpliTaq Gold (Applied Biosystems). PCR samples were denatured at 94°C for 10 min, then subjected to 35 cycles of 94°C for 30 sec, 55–58°C, 30 sec, 72°C for 1 min and 72°C for 8 min. The PCR products were electrophoresed in an 8% polyacrylamide gel to confirm amplification. After treatment of PCR products with exonuclease I and shrimp alkaline phosphatase (Amersham Bioscience, Tokyo), samples were subjected to direct sequencing in sense and antisense directions using an ABI PRISM BigDye™ terminator v3.0 cycle sequencing kit (Applied Biosystems), and analyzed on an ABI PRISM 3700 or 3100 analyzer (Applied Biosystems).

In regard to exons 15 and 16, PCR samples were subcloned into plasmids. After PCR, bands detected by polyacrylamide gel analysis were excised from a polyacrylamide gel. PCR products were eluted in distilled water at 55°C for more than 30 min and cloned into the pCR II vector with a TA cloning kit (Invitrogen, San Diego, CA). DNA sequences of at least 10 clones that were amplified in more than two separate experiments were determined in sense and antisense directions.

Bisulfite treatment of DNA samples

DNA was treated with sodium bisulfite, using the CpGenome DNA Modification Kit (Intergen Inc., Gaithersburg, MD), according to the manufacturer's

instruction.

Methylation status by methylation-sensitive PCR

To discriminate between methylation and unmethylation of the CpG island in the *RB1* promoter region, we used two primer sets of RB1M and RB1U (18). PCR reactions contained primer sets specific for the methylated (RB1M) or unmethylated (RB1U) promoter region (10 pmole of each primer), 1.5 mM MgCl₂, and 100 ng of bisulfite-treated template DNA. PCR was carried out for 30 cycles (55°C for 30 s, 72°C for 30 s, and 94°C for 1 min). PCR products were run on 8% nondenaturing polyacrylamide gels, and stained with ethidium bromide.

To ensure PCR amplification of the methylated *RB1* promoter sequence after modification, we methylated genomic DNA from lymphocytes *in vitro* with the CpG methylase enzyme *SssI*. This DNA was then subjected to sodium bisulfite treatment as described above and served as a positive control. A water blank was used as a negative control.

Methylation status by sequencing analysis

A fragment of the CpG island region of the *RB1* gene was amplified by PCR using the pair of primers (RB1U). PCR of bisulfite-treated template DNA was carried out for 30 cycles (55°C for 30 s, 72°C for 30 s, and 94°C for 1 min). PCR products were subjected to direct sequencing in sense and antisense directions. In addition, gel-purified PCR fragments were cloned into the pCR II plasmid and 10 clones for each sample were sequenced.

Analysis of mutations of codon 24 in the CDK4 gene

An activating mutation resulting from an Arg to Cys amino acid substitution at codon 24 (R24C) in the *CDK4* gene was analyzed. DNA from adenomas was subjected to PCR amplification of codon 24 and the surrounding region of the *CDK4* gene using a pair of primers (sense, 5'-GCTACCTCTCGATATGAGC-3'; antisense, 5'-ACTCTTGAGGGCCACAAAG-3'). PCR was carried out with annealing temperatures of 60°C for 3 cycles, 58°C for 12 cycles, 56°C for 12 cycles, and 54°C for 13 cycles. PCR products were then subjected to direct sequencing as described above.

Results

pRb expression

Out of 31 adenomas, 2 samples (samples 3 and 7) were inadequate for pRb immunostaining, because pRb in the normal endothelial cells were not stained. Out of the remaining 29 tumors, all tumors except for one adenoma (sample 14, prolactinoma) showed positive staining of pRb (Table 1). Figure 1 showed that sample 14 had no tumor cells with nuclear staining of pRb, but there was nuclear staining of pRb in normal endothelial cells.

Allelic ratios of 13q in pituitary adenomas

The first screening analysis of LOH at the *RB1* locus showed that samples 4 (somatotroph adenoma), 9 (somatotroph adenoma), and 14 (prolactinoma) out of 31 adenomas revealed LOH for an intragenic marker of *RB1*, *D13S153*. Sample 17 (prolactinoma) showed the allelic ratio of 58% at *D13S153*. With regard to adenomas showing decreased allelic ratios at *D13S153*, a total of 24 microsatellite markers were analyzed for better characterization of the extent of LOH for the chromosomal region (Table 2). The reproducibility of the allelic ratios for these samples was confirmed by 2–4 independent PCR amplifications for each sample. In sample 17, the decreased mean allelic ratio of 58% with regard to multiple microsatellite markers on the entire 13q (Table 2) suggested the possibility of trisomy 13. Three samples of 4, 9, 14 showed decreased allelic ratios at 2 *RB1* loci of *D13S153* and Rb1.20. *D13S153* and Rb1.20 were localized in introns 2 and 20 of the *RB1* gene, respectively. In sample 4, LOH seemed to be located entirely on 13q. In sample 9, the decreased allelic ratio extended from *D13S219* (13q12.3-q13) to *D13S1265* (13q32). In sample 14 which showed absence of pRb expression, a decreased allelic ratio was observed from *D13S219* (13q12.3-q13) to *D13S265* (13q31-q32). LOH on the *BRC A2* locus of *D13S171* was limited in sample 4 (Table 2). With regard to *D13S170* at 13q31, 27 samples (excepting samples 4, 9, 14, and 17) showed no allelic imbalance (data not shown).

FISH

The decreased mean allelic ratio of 18%, 12%, and

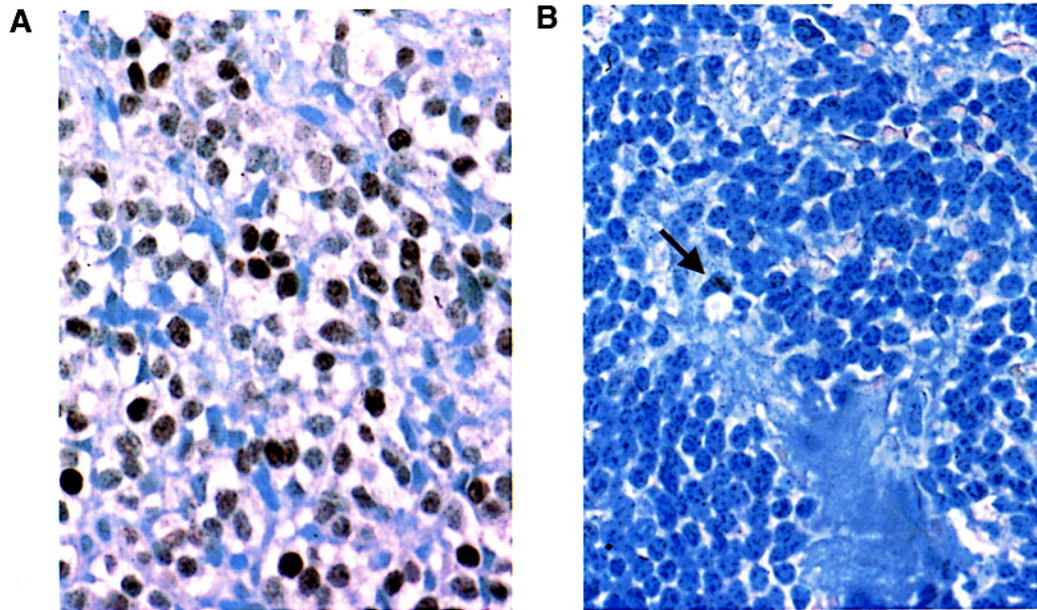


Fig. 1. Immunohistochemical analysis of pRb expression in pituitary adenomas. Only nuclear positivity was assessed; cytoplasmic staining was regarded as nonspecific. **A.** Nuclear reactivity for pRb throughout the section in pituitary adenoma (no. 28) (original magnification, x 200). **B.** Absence of nuclear pRb immunoreactivity throughout the section in a pituitary adenoma (no. 14) (original magnification, x 200). Arrow shows nuclear staining of pRb in normal endothelial cells.

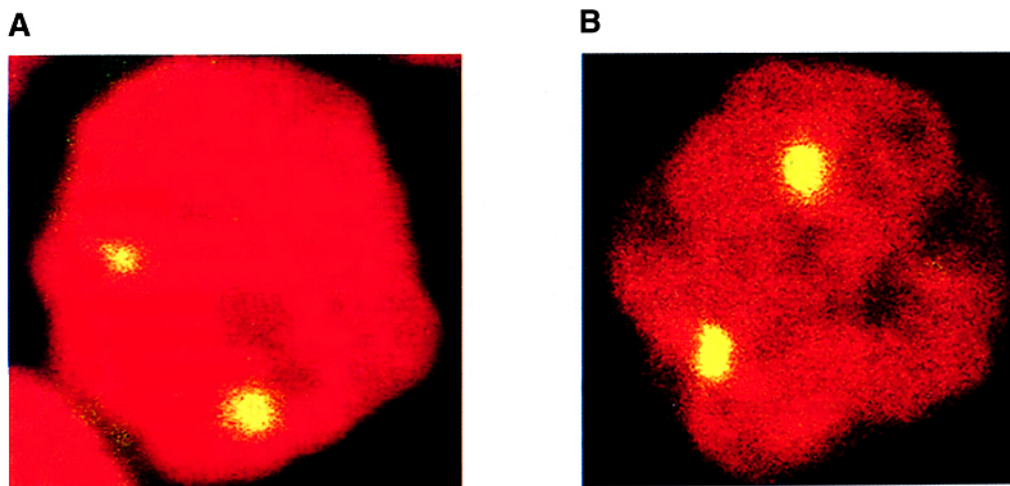


Fig. 2. Interphase FISH with the *RBI* probe. **A.** One typical interphase nucleus from sample 28 (retention of *RBI*) showed two nuclear hybridization signals. **B.** One typical interphase nucleus from sample 14 (LOH at *RBI*) showed two nuclear hybridization signals.

15% in samples 4, 9, and 14 (Table 2), respectively, with regard to intragenic markers of *D13S153* and *Rb1.20*, suggested the possibility of deletion of the *RBI* locus. Interphase FISH with the *RBI* probe showed that intact and independent nuclei were examined with confocal fluorescent microscopy, and 2 sig-

nals of the *RBI* were detected in 66.0%, 67.5%, and 60.7% of examined nuclei in samples 4, 9, and 14, respectively (Fig. 2). In addition, we detected 2 signals at a frequency of 61.1% in control sample 28 (Fig. 2), which retained both alleles of *RBI* (Table 2). The mean allelic ratio of 58% in sample 17 (Table 2) on the

entire 13q suggested 3 copies of chromosome 13. Because paraffin-embedded tissue of sample 17 for FISH was not available, the confirmative result of trisomy 13 was not obtained.

DNA sequence of whole RB1 exons and the promoter region

The sample 14 without pRb expression and 3 samples with pRb expression were subjected to PCR amplification of whole exons and the essential promoter in the *RB1* gene using gene-specific oligonucleotide primers. Any somatic mutations, insertions, or microdeletions in the *RB1* gene were not detected in one adenoma without pRb expression as well as 3 adenomas with pRb expression. A single nucleotide change in intron 25 was detected: heterozygous T to A change in the exon 26 intervening sequence (IVS)-10 was detected in samples 9 and 17. The nucleotide changes were detected in their leukocytes DNA. A single nucleotide polymorphism (SNP) in the exon 26 IVS-10 was already reported (5, 19).

Methylation status of RB1 promoter region

To determine the methylation status of the promoter region of *RB1* in sample 14 showing lack of pRb expression, we used methylation sensitive PCR analysis. Genomic DNA samples from the 4 pituitary adenomas including sample 14 were treated with sodi-

um bisulfite and tested for the presence of methylated and unmethylated CpG dinucleotides in the promoter region of the *RB1*. Sample 14 contained unmethylated promoter sequences as well as samples 4, 9 and 17 (Fig. 3). Furthermore, we determined the CpG methylation status in the promoter region of *RB1* by sequencing sodium bisulfite-treated DNA from 4 pituitary adenomas. In these 4 pituitary adenomas, no methylation was found in CpG sites in the promoter (data not shown).

CDK4 mutations in pituitary adenomas

We analysed for the R24C mutation in the *CDK4* gene in 31 pituitary adenomas. Direct sequencing of PCR products did not show any mutations within the codon 24 or surrounding nucleotide sequences in pituitary adenomas (data not shown).

Discussion

Functional inactivation of *RB1* is a pathogenic factor for several forms of human malignancy [4]. Although earlier studies [6–8] reported infrequent LOH of *RB1* intragenic markers in human pituitary tumors, more recent reports [9, 10] described an increased frequency of LOH within the 13q14 region in invasive pituitary tumors compared with their noninvasive counterparts. In addition, these studies [9, 10] de-

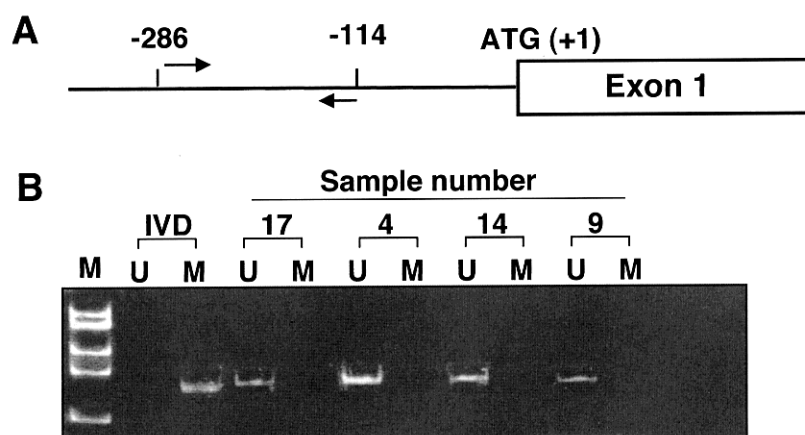


Fig. 3. MS-PCR analysis of the *RB1* promoter region in pituitary adenomas. A. Schematic representation of the promoter region and exon 1 of the *RB1* gene. The positions of primers are shown as arrows relative to the translation start site (+1). B. Bisulfite-treated DNA samples were subjected to PCR with a pair of primers specific for unmethylated (U) or methylated (M) DNA. PCR products were run on an 8% polyacrylamide gel, and stained with ethidium bromide. IVD, *in vitro* methylated genomic DNA with *SssI*; M, ϕ X174 *HaeIII*-digested DNA fragments used as molecular markers.

scribed pRb expression as being unassociated with loss of an *RB1* intragenic marker. Pei *et al.* [9] demonstrated frequent LOH in an intragenic marker of Rb1.20 on 13q14.3 in 13 (93%) out of 14 aggressive pituitary tumors. In contrast, LOH in the other 3 markers on 13q14.3 was rare. The Rb protein, however, was identified by immunohistochemistry in all aggressive pituitary tumors with chromosome 13 allelic loss, suggesting another suppressor gene located on 13q14.3 adjacent to the *RB1* locus. Bates *et al.* [10] reported that 11 of 44 invasive pituitary tumors had LOH at *D13S155*, a more centromeric marker than *RB1*. However, 5 among these 11 tumors retained *D13S153*, an intragenic marker of *RB1*. These results support the view of a tumor suppressor gene(s) on 13q14.3 other than *RB1*.

To shed light on the role of *RB1* and other possible tumor suppressor genes on 13q in pituitary tumorigenesis, we examined pRb expression and LOH on chromosome 13 in pituitary adenomas. We detected pRb expression and allelic imbalance on chromosome 13 in 28 of 29 and 4 of 31 pituitary adenomas, respectively. In addition, 3 of 4 pituitary adenomas had a common LOH region at least from *D13S219* located on 13q12.3-q13 to 13q31-32. This observation was partly supported by Fan *et al.* [20]. They reported that loss of 13q was frequently observed in pituitary adenomas and the minimum overlapping region of losses in 13q was 13q21-q31, outside the region of *RB1*.

Allelic imbalance in chromosome 13 in samples 4, 9, and 14 suggested loss of a part of chromosome 13 or gene conversion by recombination as the most likely causes [21]. To distinguish between these, we performed interphase FISH using a probe of *RB1* in pituitary adenomas. Interphase FISH showed the presence of both *RB1* alleles in each sample. The results suggested that mitotic recombination events, not deletion or chromosome loss, led to a reduction to homozygosity in 3 pituitary adenomas. Sample 17 with a mean allelic ratio of 58% through chromosome 13 was suggested to have trisomy 13.

Tumors showing LOH at *RB1* by mitotic recombination events were expected to have point mutations and microdeletions of *RB1* gene. We examined the nucleotide sequence of exons 1–27 of the *RB1* gene for the presence of inactivating mutations or microdeletions. To our knowledge, this study is the first report to analyze the entire exons and promoter region of *RB1* gene in pituitary adenomas. No mutations except for SNP

were detected in any exons or exon-intron boundaries in sample 14 without pRb expression as well as other 3 adenomas with allelic imbalance.

Although Simpson *et al.* [18] detected LOH at intragenic marker *D13S153* in 2 of 30 pituitary adenomas, 10/45 (22%) tumors showed no staining in pRb immunohistochemistry. In addition, they detected homozygous deletion of exons 20–24 and exon 24 in 2/30 and 1/30 pituitary adenomas, respectively. Furthermore, methylation specific PCR data showed that 4/45 tumors had methylated promoters. Our fine analysis of the allelic ratio showing LOH in 13q was observed in 3/31 tumors. However, somatic mutations of whole exons and exon-intron boundaries in the *RB1* gene were not detected in 3 adenomas including sample 14 without pRb expression. Compared to their results, our data showed a relatively low percentage of Rb immunonegativity (one prolactinoma out of 29 adenomas, 3%) in Japanese pituitary adenomas. This difference in frequency might be due to a racial difference or the small population of our pituitary adenomas.

Mutation [22] and microdeletion [23] of the *RB1* promoter region are frequently associated with an absence of pRb expression in hereditary retinoblastomas and prostate carcinomas, respectively. However, nucleotide changes in the promoter of the *RB1* were not detected in the pituitary adenomas examined. Recently, methylation errors resulting in the *de novo* methylation of CpG islands, which were not methylated in normal DNA, have been shown to contribute to progressive inactivation of tumor suppressor gene [24]. The *RB1* gene has a small CpG island of size 600 bp encompassing the essential promoter region, which remains unmethylated in all tissues during development. Aberrant methylation of the CpG island within the *RB1* promoter region was described in unilateral retinoblastoma [25, 26], which is associated with a loss of pRb expression. To clarify the cause of lack of pRb expression in sample 14, we examined the methylation status of the CpG island contained within the promoter region of the *RB1* gene. MS-PCR and bisulfite sequencing analysis revealed hypomethylated status of CpG islands in the promoter region of *RB1* gene of 4 pituitary adenomas including sample 14.

Our sample 14 showed no tumor cells with nuclear staining of pRb. However, neither mutation in any exon and the promoter of *RB1* or hypermethylation status of *RB1* promoter was detected. In addition, multiple PCR analysis of exons 20–24 of the *RB1* gene

with the *CYP2C19* gene at 10q24 or the *carbamoyl-phosphate synthetase/aspartate transcarbamoylase/dihydroorotase* gene at 2p21 in the sample 14 showed no homozygous deletion (data not shown). The molecular mechanism of undetectable pRb in the sample 14 remains to be explained.

A mutation in *CDK4* was implicated in the genesis and progression of familial human melanoma [12]. The importance of the *CDK4* locus in human cancer first became evident following the identification of a germ line *CDK4*-Arg24Cys (R24C) mutation, which abolishes the ability of *CDK4* to bind to p16^{INK4a}. To determine the role of *CDK4* (R24C) germ line mutation in the genesis of other cancer types, the R24C mutation in the *Cdk4* locus of mice by using Cre-loxP-mediated "knock-in" technology was introduced [27, 28]. Expression of deregulated *Cdk4*R24C resulted in hyperphosphorylation of the Rb family proteins, suggesting inactivation of Rb protein family function. Homozygous *Cdk4*^{R24C/R24C} mice developed tumors within 8 to 10 months of life. The majority of these tumors were found in the pancreatic islets, pituitary gland, brain, mammary gland, and skin. Furthermore, pituitary adenomas or carcinoma were derived from the adenohypophysis origin [27, 28], although mutants of Rb^{+/-} mice develop pituitary tumors with inter-

mediate lobe origin [29]. The *CDK4* gene was thus supposed to be a candidate oncogene in human pituitary tumorigenesis. However, no activating mutations were detected in 31 human pituitary adenomas.

In conclusion, we found that somatic mutations of the *RB1* gene or *CDK4* gene did not appear to play a major role in the pituitary tumorigenesis. Losses in 13q12.3-q13 to 13q31-32 were commonly detected in 3 pituitary adenomas, suggesting the presence of potential tumor suppressor gene(s) other than *RB1* on 13q12.3-q13 to 13q31-32 in pituitary tumorigenesis.

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