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Advances in Brief

Genomic Structure of the Transforming Growth Factor β Type II Receptor Gene and Its Mutations in Hereditary Nonpolyposis Colorectal Cancers[1]

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Abstract

To characterize the tumorigenetic role of the transforming growth factor β type II receptor (*RII*) gene, we defined its genomic structure, which consists of seven exons. The sequences of exon-intron junctions were determined to facilitate mutation analysis of each exon. Twenty-five carcinomas and five adenomas from hereditary nonpolyposis colorectal cancer patients were analyzed for mutations in the entire coding region. Four missense mutations (two in adenomas and two in carcinomas) were found in the 10 cases carrying the polyadenine deletions in one allele. These results indicate that *RII* shares the two-hit inactivation mechanism with tumor suppressor genes and that mutations of it may occur in the early stage of tumorigenesis.

Introduction

TGF-β[3] is a potent inhibitor of epithelial cell growth. Loss of responsiveness to TGF-β is common in human cancers and is thought to be an important step in tumorigenesis. The growth inhibitory effect is primarily mediated by a heteromeric complex of two distantly related transmembrane serine/threonine kinases called receptors I and II, and inactivation of either receptor subtype can result in TGF-β resistance (1). Reports have shown that genetic alterations of *RII* occurred in gastric carcinoma cell lines (2) and head and neck squamous carcinoma cells (3). Recently, deletions within an $(A)_{10}$ repeat of *RII* were found in colonic and gastric cancer cell lines (4, 5), primary colorectal adenocarcinomas (6, 7), and endometrial cancers (5, 7), all of which showed microsatellite instability. Moreover, the cancer cells showed reduced malignancy when the wild-type *RII* was transfected (8). Based on this evidence, it was suggested that the *RII* gene may behave as a tumor suppressor in tumorigenesis.

We reported (7) that a deletion in the $(A)_{10}$ repeat occurred in 71% of the microsatellite instability-positive tumors in a major cancer susceptibility syndrome, called HNPCC, in which germline mutations of a group of mismatch repair genes, *i.e.*, *hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2*, were responsible for the cancer susceptibility (9). Among them, an $(A)_{10}$ deletion affecting both alleles occurred in more than one half of the cases, suggesting a similar inactivation mechanism for *RII* and tumor suppressor genes. However, the problem remained of the cases with only one or no allele affected in the $(A)_{10}$ repeat of *RII*. Is there any mutation that affects the wild-type allele(s) outside the repeat? To further characterize the role of the *RII* gene in tumorigenesis, we describe here the whole exon-intron organization of the *RII* gene and methods for analyzing individual exons. Moreover, we report the results of screening for mutations of *RII* in HNPCC patients.

Materials and Methods

Subjects. A total of 25 carcinomas and 5 colorectal adenomas from 16 Japanese HNPCC patients were collected. In the case of carcinomas, there were 22 in the colorectum, 2 in the endometrium, and 1 in the stomach. Among the 30 tumors, 10 had a homozygous deletion, 10 had a heterozygous one, and 10 had a normal sequence in the *RII* $(A)_{10}$ repeat, which we determined previously (7). Genomic DNA was extracted from surgically resected tumor tissues and corresponding normal tissues and from EBV-transformed lymphoblastoid cell lines or peripheral blood karyocytes as described previously (10).

Determination of the Exon-Intron Organization. The genomic fragments encoding the human *RII* gene were obtained by two methods. First, LA-PCR was performed for 35 cycles at 98°C (1 min), 55–60°C (1 min), and 72°C (8 min), with a final 20-min elongation at 72°C, using LA-*Taq* polymerase (Takara, Kyoto, Japan). Second, a newly developed method termed "suppression PCR" (11) was applied when LA-PCR failed to work. For this method, five so-called genomic libraries were created by digestion of human genomic DNA with *Eco*RV, *Sca*I, *Dra*I, *Pvu*II, and *Ssp*I, separately, and by ligation of a special adaptor to the ends of each DNA fragment. Two runs of suppression PCR were carried out using a PromotorFinder DNA Walking kit (Clontech, Palo Alto, CA). For the first run, using each of the five DNA libraries as a template, PCR was performed with one adaptor primer (AP1) and a specific primer of *RII* based on its cDNA sequence (12) for 35 cycles at 95°C (30 s) and 68°C (5 min), with a final elongation at 68°C for 8 min. For the second run, a nested PCR was performed with another inner adaptor primer (AP2) and an inner *RII*-specific primer. The conditions were the same as for the first run except for 24 cycles.

Mutation Screening with PCR-SSCP. According to the exon-intron boundary sequences we determined, 10 sets of primers were designed to amplify the entire coding region, including each splicing site, of the *RII* gene (Table 1). PCR comprised 35 cycles at 94°C (1 min), 55–65°C (2 min), and 72°C (1 min) in standard solutions. Nonradioisotopic SSCP was performed according to the method described previously (13). Electrophoresis was carried out on 10–15% polyacrylamide gels supplemented with 10% glycerol in a Tris-glycine buffer, using a minislab gel apparatus (Atto Co., Ltd., Tokyo, Japan). The running conditions were 350 V for 1.5–2.5 h, and the gels were stained with silver (Silver Stain kit; Daiichi Co., Ltd., Tokyo, Japan).

DNA Sequencing. In the case of LA-PCR or suppression PCR, each fragment was purified from the agarose gel with a GENECLEAN II kit (Bio 101, Inc., La Jolla, CA). In the SSCP cases, the PCR products were purified using a QIA-quick spin PCR purification kit (Qiagen Inc., Chatsworth, CA). All of the fragments were directly sequenced with a cycle sequencing kit (TAKARA) using end-labeled primers and the conditions specified by the manufacturer. All of the primer sequences for LA-PCR, suppression PCR, and sequencing are available upon request.

Results

Structure of the *RII* Genomic Locus. The genomic structure of *RII* was determined using the following steps. According to the *RII* cDNA sequence (12), we designed eight sets of PCR primers, dividing the whole coding region averagely. These primers generate eight fragments overlapping each other with sizes from 280 to 300 bp on a

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[3] The abbreviations used are: TGF-β, transforming growth factor β; *RII*, TGF-β type II receptor; HNPCC, hereditary nonpolyposis colorectal cancer; $(A)_{10}$, ten adenines; SSCP, single-strand conformation polymorphism; LA, long and accurate; LOH, loss of heterozygosity.

Table 1 *List of primer sequences for amplification of individual exons of the TGF-β RII gene*

Exon	Sense primer sequence[a] (5′–3′)	Antisense primer sequence (5′–3′)	Size of PCR product (bp)
1	TATGACGAGCAGCGGGGTCTGCC[b]	cgactgtcaagcgcagcgtagag	182
2	gctgcctgcagttggataac	cactgactgtgtgtactatg	294
3	cctcgcttccaatgaatctc	ttggcacagatctcaggtcc	267
4-1	cctaccacatccaactccttc	ACGTGGAGCTGATGTCAGAGC	283
4-2	CGAGCACTGTGCCATCATCC	GAACTGGAGTATGTTCTCATGC	283
4-3	GAGAAGGACATCTTCTCAGAC	ATTGGAGCTCTTGAGGTCCC	285
4-4	CCTCCACAGTGATCACACTC	taaaggggatctagcactagc	205
5	aatgatggcctcactgtctg	ccactacacatatctggtcc	202
6	agtgaccctgtgtttgctgg	cctaagaggcaacttggttg	214
7	cctttggatctctttcccgc	AGAGGGGCAGCCTCTTTGG[c]	249

[a] Upper case letters correspond to exons, and lower case letters to introns.
[b] Designed according to untranslated sequences upstream of the initiating ATG.
[c] Designed according to untranslated sequences downstream of the stop codon.

cDNA template. When genomic DNA was used as a template, two overlapping fragments were obtained with the same size as the corresponding cDNA sequence, indicating that there is no intron in the two fragments and that they are in the same exon. Using LA-PCR, we obtained two more amplifiable genomic fragments. Sequencing showed that each fragment contained one intron, with a size of 2.0 kb or 3.0 kb, respectively. For the remaining four parts which could not be amplified by LA-PCR, suppression PCR was applied for the isolation of genomic fragments. A total of eight sets of suppression PCR was performed, and we could isolate the specific fragments in at least one of the five genomic libraries in all of the cases. The sequencing revealed the sites and boundary sequences of each exon-intron junction. As shown in Fig. 1*A*, the human *RII* gene consists of seven exons and six introns, the sizes of the exons ranging from 128 to 800 bp. The ATG start codon is included in exon 1 and the termination signal in exon 7 is followed by 51 bp of the 3′ untranslated region. The whole exon sequences were identical to those of the *RII* cDNA, and all exon-intron junctions followed the GT–AG rule. The boundary sequences of each exon-intron, which are useful for primer designing, are shown in Fig. 1*B*.

Mutation Analysis of HNPCC Tumors. For SSCP analysis, PCR amplification of genomic DNA from primary tumors was performed using intron-based primers surrounding each of the seven exons, except that exon-based primers were created overlapping PCR products for large exon 4. A total of 10 sets of PCR generated fragments covering the whole coding region and splicing sites of the *RII* gene. The size of each PCR fragment was less than 300 bp, which is suitable for SSCP analysis. All of the primer sequences for PCR-SSCP are presented in Table 1.

The $(A)_{10}$ repeat is located in exon 3 of the *RII* genome. We detected 20 SSCP variants in this region. Sequencing of representative SSCP variants showed that 10 cases had a heterozygous and 10 had a homozygous 1-bp deletion in the $(A)_{10}$ repeat (data not shown). These results were in agreement with our previous results (7).

The serine/threonine kinase domain spanned from a part of exon 4 to exon 7. We detected four SSCP variants in this area. One occurred in exon 5 and the other three in exon 7 (Fig. 2*A*). All of these variants were only observed in the tumor DNA, indicating somatic mutations, and only in the 10 heterozygously deleted cases, which suggested that the mutations may have occurred on the wild-type allele.

Sequencing revealed that one mutation occurred in kinase subdomain VIII and three in XI (11). As shown in Table 2, a T to C transition was found at codon 441 in tumor 769, which resulted in a substitution of proline for serine. Since the mutation created a new

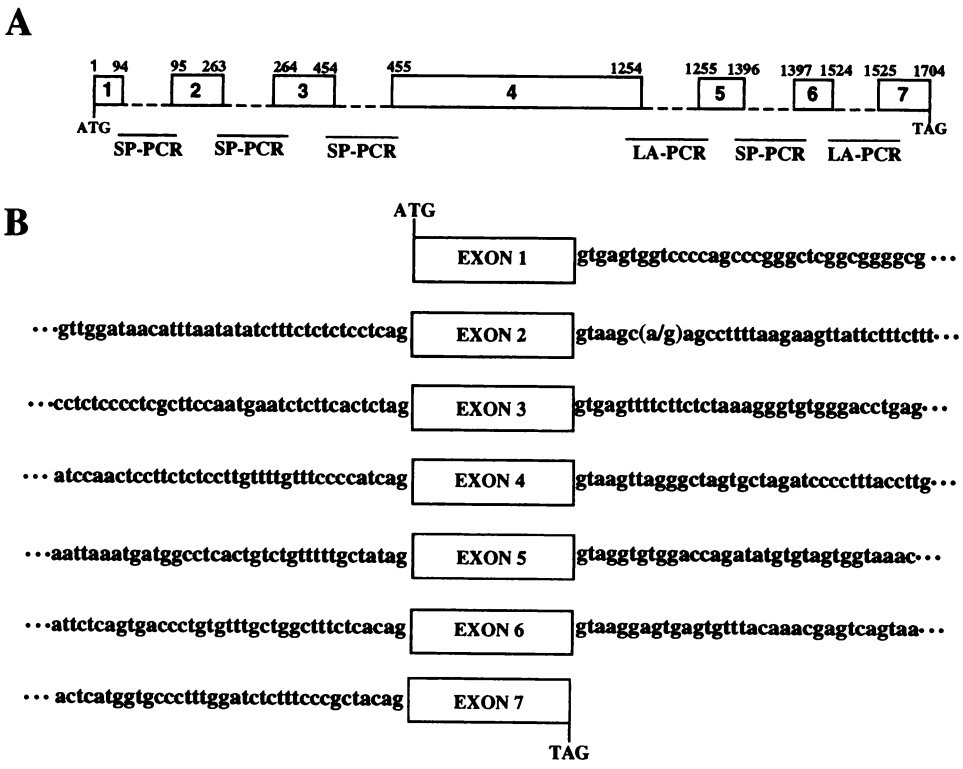

Fig. 1. Genomic structure of the *RII* gene. *A*, a schematic diagram of the organization of the *RII* locus and methods for isolation of genomic fragments. *Boxes*, exons; *numbers in boxes*, numbers of the exons. – – – introns; *numbers above the boxes*, nucleotide numbers corresponding to the sequence of *RII* cDNA (12). The 5′ boundary of exon 1 is defined by the ATG start codon, and the 3′ boundary of exon 7 is defined by the TAG termination codon. *SP-PCR*, suppression PCR. *B*, sequences for the intronic region surrounding each *RII* exon. An a/g polymorphism is shown at the seventh base of intron 2. The GenBank accession nos. for the *RII* sequences are U69146–U69152.

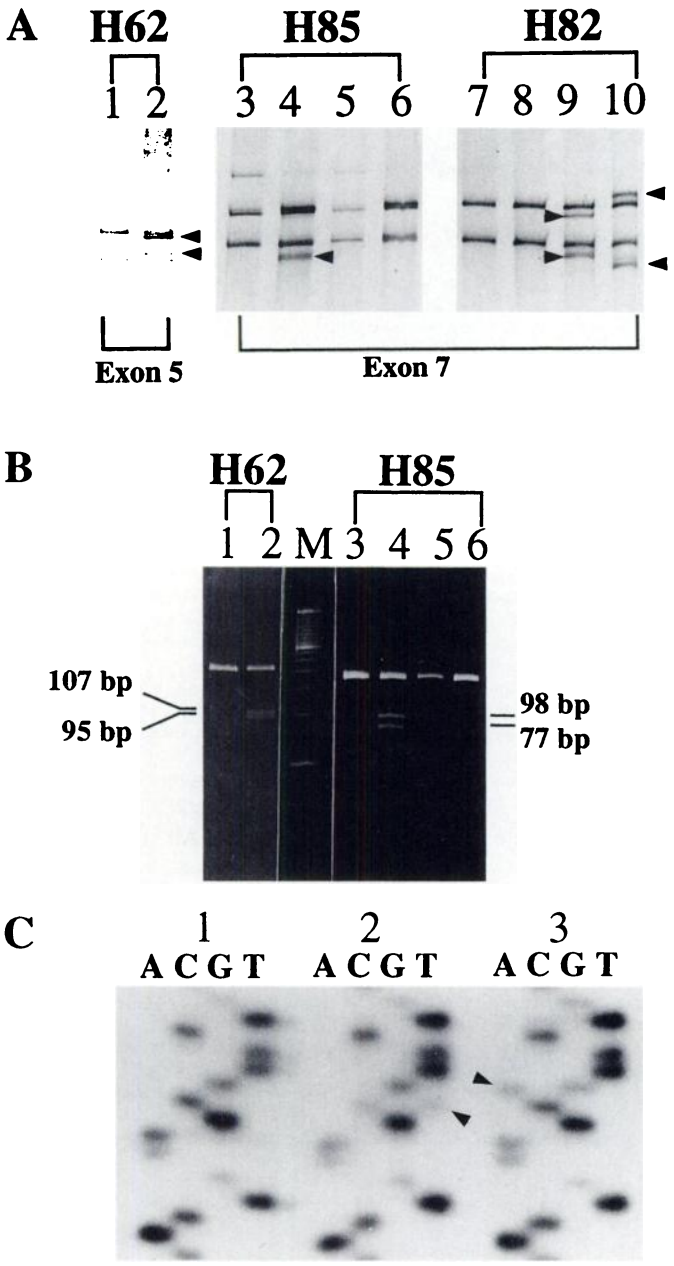

Fig. 2. Mutation analysis of the *RII* gene in HNPCC tumors. *A*, SSCP analysis of exon 5 in patient H62 (*Lanes 1* and *2*) and exon 7 in H85 (*Lanes 3–6*) and H82 (*Lanes 7–10*). Mobility shifts were detected in endometrial cancer T769 (*Lane 2*), adenomas T781 (*Lane 4*) and T797 (*Lane 9*), and rectal cancer T798 (*Lane 10*), as shown by *arrowheads*. Corresponding normal tissues (*Lanes 1, 3,* and *7*), colonic (*Lane 5*) and endometrial cancers (*Lane 6*), and adenoma T796 (*Lane 8*) showed a normal SSCP pattern. *B*, *BAN*II digestion of PCR products of *RII* exon 5 (202 bp) from patient H62 (*Lanes 1* and *2*). Mutant bands (107 bp + 95 bp) caused by the mutation were detected for cancer T769 (*Lane 2*), but not for the corresponding normal tissue (*Lane 1*). *Stu*I digestion of PCR products containing a part of exon 7 (175 bp) from patient H85 (*Lanes 3–6*). Adenoma T781 (*Lane 4*) showed mutant bands (98 bp + 77 bp) due to a new enzyme site created by the mutation. Normal tissue (*Lane 3*) and colonic (*Lane 5*) and endometrial cancers (*Lane 6*) were not digested by *Stu*I. *M*, 50-bp DNA ladder marker. *C*, sequence analysis in patient H82 DNA. The sequence should be read in the antisense way. G to A (*arrowhead*) and C to T (*arrowhead*) transitions were detected in T797 (*row 2*) and T798 (*row 3*), respectively, compared with in normal tissue (*row 1*).

*BAN*II restriction enzyme site, we confirmed it with this enzyme (Fig. 2*B*, *Lanes 1* and *2*). In tumor 781, a C to T transition was detected at codon 528, which changed arginine to cysteine. We also detected this mutation with the *Stu*I enzyme created by this mutation (Fig. 2*B*, *Lanes 3–6*). The other two mutations occurred in the same codon, *i.e.*, codon 537. One was G to A and the other C to T transitions in tumors 797 and 798, resulting in substitution of histidine and cysteine for the same arginine, respectively (Fig. 2*C*).

LOH Analysis of HNPCC Tumors. Four polymorphisms were found in the *RII* genome. Two were used for LOH analysis because of their relatively high rates of heterozygosity. One is at the seventh base of intron 2 (Fig. 1*B*), an a/g polymorphism resulting in an obvious heterozygous SSCP pattern. Informative cases due to this polymorphism comprised 12 of the 30 cases (40%). The other one is a silent mutation caused by a C to T transition at codon 389, as described previously (3). The frequency of informative cases caused by this polymorphism comprised about 47% (14/30). Taking the two polymorphisms together, 18 of the 30 tumors were informative for the LOH study. None of these 18 tumors showed LOH of the *RII* gene (data not shown). The other two sequence variants are C to T at codon 439 and a to c at nucleotide 1710 of the 3′ nontranslated region. In contrast to the polymorphism reported previously (6), both of the variants only showed a homozygous T at codon 439 and c at nucleotide 1710 in all of our cases.

Discussion

Recent studies have suggested that the *RII* gene may behave as a tumor suppressor in several cancers (6, 7). To further define the role of the *RII* gene in tumorigenesis, we have determined the exon-intron organization of the *RII* gene. This information will lead to DNA-based diagnostic methods for detecting *RII* mutations in many types of tumors using a variety of clinical samples. Such methods have considerable advantage over other methods such as reverse transcription-PCR-based methods. This is because DNA samples are more widely available than RNA samples, particularly in the case of archival samples that have been stored for long periods, and because DNA samples do not suffer from the problem of underrepresentation of mutant species that can occur with RNA samples.

In this study, unlike the common cloning method from a genomic library, we mainly used suppression PCR to determine the *RII* genomic structure. This method was useful for cloning the promoter region and also could be applied for walking from a known region to an unknown region even in uncloned genomic DNA (11). Using this method, we successfully obtained genomic fragments when LA-PCR failed to work. Therefore, suppression PCR is a simple, rapid, and powerful means of determining the exon-intron boundaries of genes.

Previously, three and two missense mutations of *RII* have been reported in colon cancer cell lines (6) and head and neck cancer cell lines (3), respectively, whereas no reports concerning mutations other than the $(A)_{10}$ repeat in primary tumors have appeared. This may be due to the lack of genomic information on the *RII* gene. Using the DNA-based screening method, we found four new missense mutations in primary tumors from HNPCC patients. These mutations, together with the previous results (3, 6), were all in the conservative kinase domain, especially in subdomain XI, indicating the important role of this region. The sites of mutations we detected may be critical in the TGF-β signaling pathway. The serine at codon 441 is a consensus phosphorylation site in protein kinases (12), which might also be a potential site for phosphorylation in *RII*. The arginine at codon 528 is conserved in all protein kinases (14), and the ion pair formed by this arginine is essential for serine/threonine kinase functions observed on X-ray crystallography (15). The arginine at codon 537 is also a conservative site and seems to be a hotspot for mutations, since two of our mutations and one mutation reported previously (3) occurred in this site. In three cases, *i.e.*, T769, T781, and T798, a charged residue was replaced by an uncharged one, which may affect protein folding significantly and result in changes in catalytic activity or substrate recognition (16). Additional studies are in progress to define the biochemical and biological properties of the four mutations.

Table 2 *Somatic mutations of the TGF-β RII gene in HNPCC tumors carrying heterozygous mutations in the $(A)_{10}$ repeat*

Patient[a]	Sex	Age (yr)	Tumor	Ad[b]/carcinoma	Site	Exon	Nucleotide[c]	Codon	Genomic DNA	Amino acid
H62	F	52	769	Carcinoma	En	5	1321	441	TCC→CCC	Ser→Pro
H85	F	35	781	Ad	A	7	1582	528	CGT→TGT	Arg→Cys
H82	M	62	797	Ad	D	7	1610	537	CGC→CAC	Arg→His
			798	Carcinoma	R	7	1609	537	CGC→TGC	Arg→Cys

[a] The germline mutations of HNPCC patients are: a nonsense mutation at codon 419 of *hMSH2* in H62 (21), and a mutation at the splicing site of intron 16 of *hMLH1* in H85 (unpublished data). A germline mutation in the whole coding region of either *hMSH2* or *hMLH1* was not found in H82.

[b] Ad, adenoma; F, female; M, male; En, endometrium; A, ascending colon; D, descending colon; R, rectum.

[c] Nucleotide and codon numberings correspond to the sequence published in Ref. 11 (GenBank accession no. M85079).

Five adenomas were included in our study, which may be helpful for determining the stage of *RII* gene inactivation. Two adenomas, T781 and T797, had heterozygous deletions in the $(A)_{10}$ repeat, and additional missense mutations were found in both cases. In contrast, no mutations were detected in the remaining three cases with the normal $(A)_{10}$ repeat. These data indicate that two-hit inactivation of the *RII* gene may occur in the early stage of HNPCC colon cancer development.

As for the extracolonic cancers of HNPCC, we found a missense mutation in one endometrial cancer (T769) with a heterozygous deletion in the $(A)_{10}$ repeat. We also detected one gastric cancer with a homozygous deletion in the $(A)_{10}$ repeat previously (7). These results suggest similar mechanisms of tumorigenesis in the two extracolonic cases with some colorectal cancers.

The *RII* gene has been mapped to chromosome 3p22 (17), where delections commonly occur in several tumors (18, 19) but are rare in colon cancers (20). Using the polymorphisms inside the *RII* gene, we did not observe LOH in the 18 informative cases, suggesting that loss of the wild-type allele in *RII* may be rare in HNPCC. Considering loss in this region was common in several cancers, such as head and neck cancers (18) and bladder cancers (19), these polymorphisms may be helpful for defining the nature of deletions in these tumors.

In the cases of the remaining six tumors with a heterozygous deletion in the $(A)_{10}$ repeat of *RII*, we could find no mutation outside the repeat. LOH was not found either in the four informative cases. These cases might be explained by: (*a*) the SSCP method for screening may not be sensitive enough to detect all mutations and (*b*) mutations may occur outside the coding region of *RII*, such as in the promoter or introns.

We previously showed that more than one half of HNPCC tumors carried a homozygous deletion in the $(A)_{10}$ repeat (7). Parsons *et al.* (6) reported three additional mutations in cancer cell lines heterozygous for the $(A)_{10}$ deletion. In this study, four missense mutations in highly conservative sites were found in the 10 heterozygous cases. It is unknown whether these mutations occurred on the wild-type or mutant-type allele. However, no mutations were detected in the tumors with a homozygous deletion or with a normal sequence in the $(A)_{10}$ repeat. The difference was statistically significant when we compared the heterozygous cases with either the homozygous or normal ones by means of Fisher's exact probability test ($P < 0.05$), showing that these mutations outside the $(A)_{10}$ repeat may affect the wild-type allele. Combining the 10 cases with heterozygous $(A)_{10}$ deletions and 10 homozygous ones, 14 of the 20 *RII*-mutated cases (70%) have mutations affecting both alleles. These data constitute evidence that *RII* is a tumor suppressor gene in HNPCC and maybe in several other cancers.

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