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SHORT COMMUNICATION

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The human caspase-activated DNase gene (*hCAD*): genomic structure, exonic single-nucleotide polymorphisms, and a highly polymorphic dinucleotide repeat at the *hCAD* locus

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Abstract Caspase-activated DNase (CAD) cleaves chromosomal DNA during apoptosis. We determined its genomic structure and identified single-nucleotide polymorphisms (SNPs) within exons 5 and 7, as well as a highly polymorphic dinucleotide repeat of (CT)m(CA)n within the 5′ region of the human CAD gene (*hCAD*). The genomic structure of *hCAD* presented here, together with information concerning SNPs within the gene, as well as a highly polymorphic (CT)m(CA)n repeat fragment at the *hCAD* locus, may assist in the construction of genetic maps for exploring gene(s) that play pivotal roles in carcinogenesis.

Key words CAD (caspase-activated-DNase) · Dinucleotide repeat · SNPs

Introduction

Caspase-activated DNase (CAD) cleaves chromosomal DNA during apoptosis (Enari et al. 1998). Recently the human CAD gene (*hCAD*) was isolated and localized at chromosome 1p36.3 (Mukae et al. 1998), a T-band region rich in GC nucleotides that contains a number of important housekeeping genes (Gregory et al. 1998). Chromosome 1p36 is frequently involved in hemizygous deletions in several types of neoplasms, in particular in neuroblastoma, indicating the presence of one or more tumor suppressor genes in the region (White et al. 1995). Since *hCAD* acts as a key enzyme for the cleavage of DNA during apoptotic processes, it is a likely candidate for one of these tumor suppressors, because its inactivation may cause failure of cells to undergo apoptotic DNA degradation (Sakahira et al. 1998). As a step toward exploring somatic mutations of the *hCAD* in various types of malignant tumors, we determined its genomic structure and identified single-nucleotide polymorphisms (SNPs) within two exons, as well as a highly polymorphic dinucleotide repeat at the *hCAD* locus.

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Results and discussion

We used a full-length cDNA clone (4.2 kb), designated pKT-hCAD (Mukae et al. 1998), to screen a cosmid library prepared from human peripheral lymphocytes. Two cosmids containing genomic fragments of the *hCAD* were isolated. Hybridization of these cosmids with a $(GT)_{10}$ probe on Southern blots, after digestion by *Rsa*I, detected a CA-repeat in one of the fragments. The subcloned fragment was sequenced, to reveal a (CT)m(CA)n microsatellite within the 5′ region of *hCAD*.

The polymerase chain reaction (PCR) primers designed to flank this repeat sequence for the analysis of polymorphism were: forward, 5′-CAAGCTAACTCAGTTGCATG; reverse, 5′-GCATGGACTGTGTCCTTGAC. PCR experiments were performed in volumes of 10 μl containing 10 ng genomic DNA, 0.25 units of EX-Taq DNA polymerase (Takara, Tokyo, Japan), 1× PCR buffer (67 mM Tris [pH 8.8], 16.6 mM NH_2SO_4, 6.7 μM ethylenediaminetetraacetic acid (EDTA), 10 mM β-mercaptoethanol), 250 μM dNTPs, and 1 pmol each of α[^{32}P] end-labeled forward primer and non-labeled reverse primer. Cycle conditions were 94°C for 2 min, then 35 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s, with final extension for 5 min at 72°C. The PCR products were electrophoresed in 0.3-mm-thick denaturing 5% polyacrylamide gels containing 30% formamide and 7.7 M urea, at 2,200 V for 2–4 h. The

gels were blotted onto filter papers, which were dried at 80°C and autoradiographed. Allelic status at this microsatellite marker was examined in 43 unrelated Japanese individuals, of whom 34 (79%) showed heterozygosity, indicating that the frequency of heterozygosity at this locus was more than 0.79.

Next we determined the exon-intron boundary sequences of *hCAD*, using the oligonucleotide primers originally employed for sequencing the cDNA (Mukae et al. 1998) to amplify introns. The PCR primer-pairs were designed to flank cDNA fragments 200–300 bp long that overlapped at the ends, to ensure that the entire gene was screened. Both cosmids containing genomic fragments of *hCAD* were amplified by standard PCR protocols, and sequenced. A gap between them was filled by means of "long PCR", using 0.2 mM of each primer and 100 ng of total genomic DNA. Comparison of the published cDNA sequence of *hCAD* (Mukae et al. 1998; GeneBank accession no. AB013918) with the genomic sequences indicated that the *hCAD* consists of seven exons, and that all sequences at exon-intron boundaries conformed to the AG-GT consensus sequence, except for the intron 3/exon 4 boundary (Table 1). This structure is similar to that of murine *CAD* (Kawane et al. 1999).

We used the PCR-single-strand conformation polymorphism (SSCP) technique with 92 chromosomes of unrelated Japanese individuals to ascertain whether polymorphic sites were present within the coding region of *hCAD*. The coding sequence was divided into nine parts; primers for amplifying each segment (Table 2) were designed so that an end portion of each amplified fragment overlapped the 5′ end of the next one. Ten-nanogram aliquots of genomic DNA from 46 unrelated Japanese individuals were amplified by each pair of primers under the PCR conditions described above. Variant bands were detected by SSCP in exons 5 and 7, and the sequences of the corresponding PCR products were determined (Fig. 1A). To examine for polymorphism in exons 5 and 7, we carried out PCR experiments, using 61 normal DNA samples in volumes of 20 μl containing 20 ng genomic DNA, 67 mM Tris-Hcl (pH 8.4), 17 mM $(NH_4)_2SO_4$, 24 mM $MgCl_2$, 0.07% 2-mercaptoethanol, 6.7 μM EDTA (pH 8.8), 250 μM of each dNTP, 10 pmol of each primer (ex5F and ex5R or ex7-2F and ex7-2R), and 0.5 units of Taq polymerase. Cycle conditions were as described above. Each of the PCR products revealed a single-nucleotide polymorphism: in exon 5, a G-to-A transversion at the second nucleotide of codon 196 created a restriction site of *Alu*I and changed the amino acid from Arg to Lys; and an A-to-G transversion at the third nucleotide of codon 318 in exon 7 created an *Ava*I site but did not result in an amino-acid substitution (Fig. 1B). To clearly show fragments digested with *Alu*I, the PCR products (200-bp-long) containing exon 5 were amplified using primers of ex5F′: 5′-GGAGTGAGATGGATCGAGAG and ex5R′: 5′-CTCCTCTGTCGAAGTAGCTG. Three fragments (99-, 84-, and 17-bp) were generated from the A1 allele and four fragments (84-, 73-, 26-, and 17-bp) from the A2 allele (Arg196Lys), along with an additional *Alu*I site. The PCR

Table 1. Exon-intron boundary sequences of the *hCAD* coding region

Exon number	Exon length (bp)	cDNA position	Splice acceptor	Splice donor	Intron number
1	114	1–114		CCGCTTCCAG gtgcccgctg	1
2	127	115–241	gtccctgcag CTCCCTGAGC	TGGCAGGGCT gtgagtggca	2
3	189	242–430	ttggtggcag ATGTGAGCGA	TGGTTTGAAG gtgcgtgggg	3
4	80	431–510	gttttccttg GCTTGGAGTC	CCTGAGGGAG gtgagcctga	4
5	171	511–681	gttcctccag GTGAGCTCCT	CTCCTGCCAG gtgagctgtg	5
6	101	682–782	tggcccccag GGTCCCTTTG	TGGATCACAT gtaagctcac	6
7	235	783–1,017	tactttgcag AATAGAAAAG		

Table 2. Primer sequences for amplifying *hCAD* coding region

Name of primer	Nucleotide sequences	Product size (bp)
ex1F	5′-AGAGGGCTTGAGGACATCTG-3′	196
ex1R	5′-ACCTCCTATTCTCCCCACAC-3′	
ex2F	5′-TCCTCCTCCTGTTGCTTCTC-3′	179
ex2R	5′-CCAAAGTCCTTGCCACTCAC-3′	
ex3-1F	5′-GTTTGTCCCATTGGTGGCAG-3′	157
ex3-1R	5′-GACGTTGTGCAGGAGGTCAG-3′	
ex3-2F	5′-CACAGAGGCAGAGGCAGAGGCTGCTG-3′	144
ex3-2R	5′-CCTCAGACTCCAAAGAAGCC-3′	
ex4F	5′-GTGGACTTGGGGGTCTTCTC-3′	141
ex4R	5′-AGCATACCCGGTCTTCACTC-3′	
ex5F	5′-CCGACGTTCCTTGGTTCCTC-3′	216
ex5R	5′-GGATAAAGGGCACACAGCTC-3′	
ex6F	5′-TCTGGCCTTCCCTCATTGTC-3′	161
ex6R	5′-GGTCTGAACCTCGCTCTGTG-3′	
ex7-1F	5′-TGTGACCACAGAAAATGATGTC-3′	181
ex7-1R	5′-GGGGTTTGTAGATTCTGCTC-3′	
ex7-2F	5′-CACCCACAAGCTCAACTGTG-3′	145
ex7-2R	5′-GTTAAAATGATGCCCACGTC-3′	

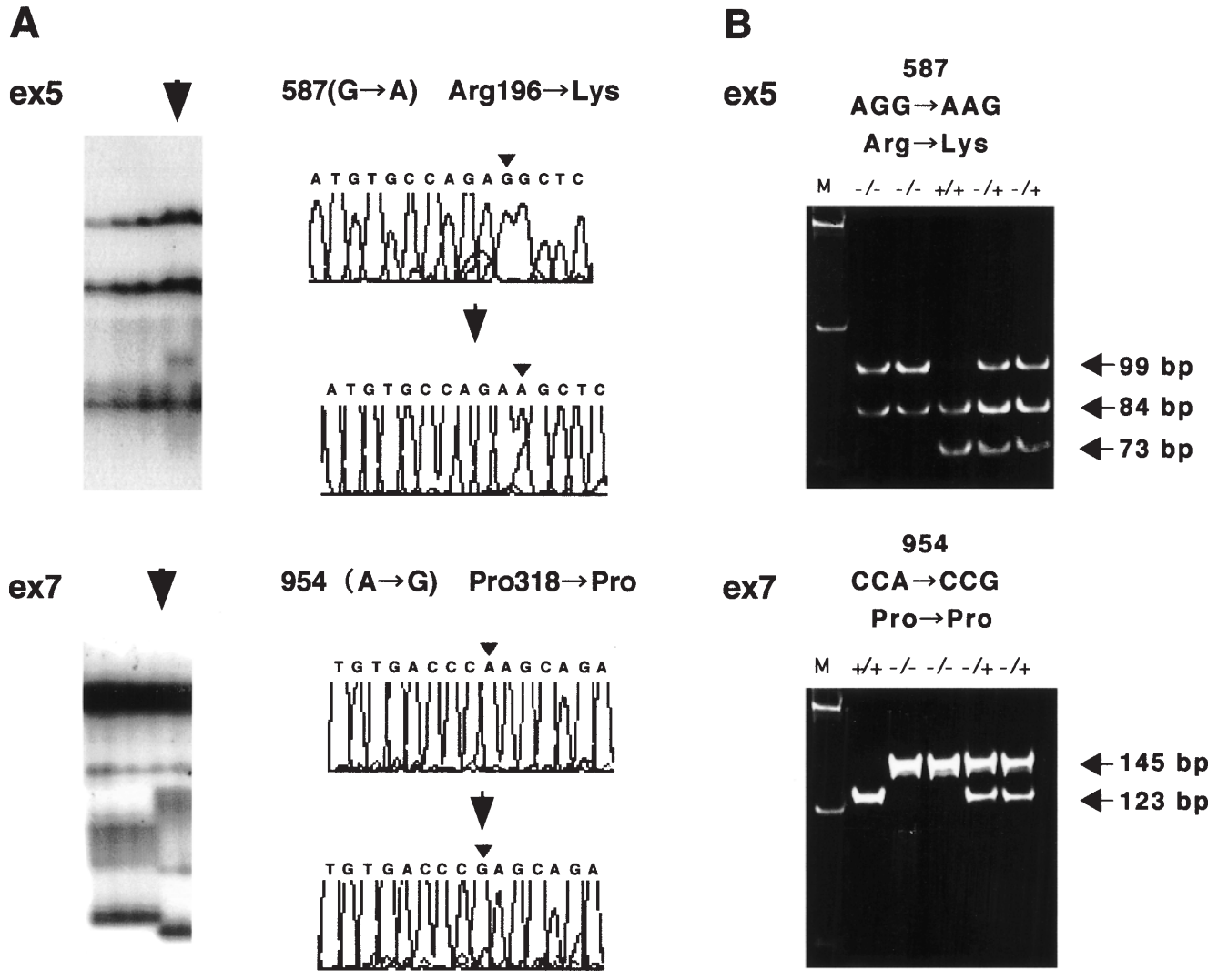

Fig. 1. **A** Single-strand conformation polymorphism (*left panels*) and sequence analysis of exons 5 and 7 of the *hCAD*. *Arrows* indicate variant band patterns. In exon 5, a G-to-A transversion was detected at the second nucleotide of codon 196, resulting in an amino acid change of Arg → Lys. An A-to-G transversion at the third nucleotide of codon 318 was detected in exon 7. **B** Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis of exons 5 and 7. *Alu*I digestion generated three fragments from exon 5 (99-, 84- and 17-bp) from the A1 (AGG) allele, and four fragments (84-, 73-, 26-, and 17-bp) from the A2 (AAG) allele (Arg196Lys) with an additional *Alu*I site. *Ava*I digestion of the PCR products from exon 7 produced a 145-bp fragment (B1 allele, CCA) lacking the *Ava*I site, and 22-bp and 123-bp fragments from the B2 (CCG) allele that retained the recognition sequence

products obtained with primers ex7-2F and ex7-2R and digested with *Ava*I produced a 145-bp fragment in the B1 allele that lacked the *Ava*I site, and 22-bp and 123-bp fragments in the B2 allele that retained the recognition sequence. The allelic frequencies of these single-nucleotide polymorphisms (SNPs) were estimated in 122 chromosomes from unrelated Japanese individuals; the results are listed in Table 3.

Chromosomal band 1p36 is frequently involved in nonrandom translocations in hematological malignancies (Mitelman et al. 1997); moreover, loss of heterozygosity (LOH) and chromosomal deletions in this region have been observed in carcinomas arising in a variety of tissues. The *hCAD* is likely to be a candidate for a tumor suppressor gene. In our additional experiment, allelic imbalances (AI) were observed in only 2 of 31 neuroblastomas, but none of the mutations were detected in any of 7 exons of *hCAD* in these two patients (data not shown). The genomic structure of the *hCAD* presented here, together with information concerning SNPs within the gene, as well as a highly polymorphic (CT)m(CA)n repeat fragment at the *hCAD* locus, may assist in the construction of genetic maps for exploring gene(s) that play pivotal roles in carcinogenesis.

Table 3. Allelic frequencies of single nucleotide polymorphism among 61 unrelated human subjects

		Genotypes			Allelic frequencies	
	n	A1/A1	A1/A2	A2/A2	A1	A2
ex5	61	40	20	1	0.82	0.18
	n	B1/B1	B1/B2	B2/B2	B1	B2
ex7	61	19	27	15	0.53	0.47

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