Mutational Analysis of the Transforming Growth Factor β Receptor Type II Gene in Hereditary Nonpolyposis Colorectal Cancer and Early-onset Colorectal Cancer Patients[1]

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ABSTRACT

Somatic mutations in the transforming growth factor β receptor type II (*TGF-βRII*) gene have been observed in various human cancers showing microsatellite instability. Most of the mutations observed were additions or deletions of the mononucleotide repeat sequence present in *TGF-βRII* coding region, suggesting that the *TGF-βRII* may be a target gene of genomic instability in tumorigenesis. Recently, we reported germ-line frameshift mutations in the mononucleotide repeat sequence of the *hMSH6* gene, which is believed to be one of the target genes of genomic instability in tumorigenesis, suggesting the possibility of germ-line mutation in mononucleotide repeat sequences. Moreover, one case of germ-line mutation in the *TGF-βRII* gene was identified in a hereditary nonpolyposis colorectal cancer (HNPCC) kindred, indicating the involvement of *TGF-βRII* inactivation in tumorigenesis of HNPCC. However, germ-line mutation analysis of all of the coding sequences and the mononucleotide repeat sequence of the *TGF-βRII* in HNPCC patients has not yet been fully elucidated. Therefore, to further investigate the presence of germ-line mutations, we screened all of the coding region sequences and mononucleotide repeat sequence of *TGF-βRII* from 35 HNPCC, 44 suspected HNPCC, and 45 sporadic early-onset colorectal cancer patients. However, no pathogenic mutations other than silent mutations, introgenic mutation, and polymorphisms were identified. Two silent mutations at codons 309 (ACG to ACA) and 340 (CAT to CAC) in the kinase domain located in exon 4 were detected. A 1-bp cytidine deletion was observed 6 bases from the 3′ end of intron. Two polymorphisms were identified at codon 389 (AAC to AAT) and at the fourth-to-last base in intron 3. The polymorphism at codon 389 was more frequent in HNPCC (20%; 7 of 35) and suspected HNPCC patients (18%; 8 of 44) than in nonmalignant control group (10%; 5 of 50). Moreover, the frequency was significantly higher in early-onset colorectal cancer patients (31%; 14 of 45). This is the first report of a different frequency of polymorphism in HNPCC, suspected HNPCC, early-onset colorectal cancer patients, and healthy normal individuals. This result suggests that: (*a*) germ-line mutation of the *TGF-βRII* gene may be a rare event during tumorigenesis in HNPCC and sporadic early-onset colorectal cancer; (*b*) the mononucleotide repeat sequence of the *TGF-βRII* gene is an apparent target of genomic instability but not of germ-line mutation; and (*c*) the polymorphism of codon 389 (AAC to AAT) is frequent, especially in early-onset colorectal cancer patients, in which it is more frequent than in control group.

INTRODUCTION

HNPCC[3] is the most common hereditary condition predisposing patients to the development of colorectal cancer. Germline mutations in genes of the mismatch repair system, namely *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2*, and *hMSH6*, have been identified in patients with HNPCC (1–8). Cells with defective mismatch repair genes display an elevated instability of microsatellite sequences, indicating genomic instability.

Somatic mutations in the *TGF-βRII* gene have been identified in various human cancers, including HNPCC, sporadic colorectal, gastric, and ovarian carcinomas (9–13). Most of the mutations found were additions or deletions of the mononucleotide repeat sequence present in the *TGF-βRII* coding region (9, 14–16), suggesting that *TGF-βRII* may be a target gene of genomic instability in tumorigenesis. However, the recent observation of a germ-line mutation of the *TGF-βRII* gene in an HNPCC patient indicated a involvement of *TGF-βRII* inactivation in the tumorigenesis of HNPCC (17).

Therefore, to clarify whether or not germ-line mutation in the entire coding region of *TGF-βRII* is implicated in tumorigenesis, we screened 35 HNPCC patients satisfying the ICG-HNPCC criteria and 44 patients suspected of having HNPCC who did not fulfill the criteria of the ICG-HNPCC (4, 18). Forty-five early-onset colorectal cancer patients (who developed

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[3] The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; ICG, International Collaborative Group; MSI, microsatellite instability; SSCP, single-strand conformation polymorphism; TGF, transforming growth factor; TGF-βRII, TGF-β receptor type II.

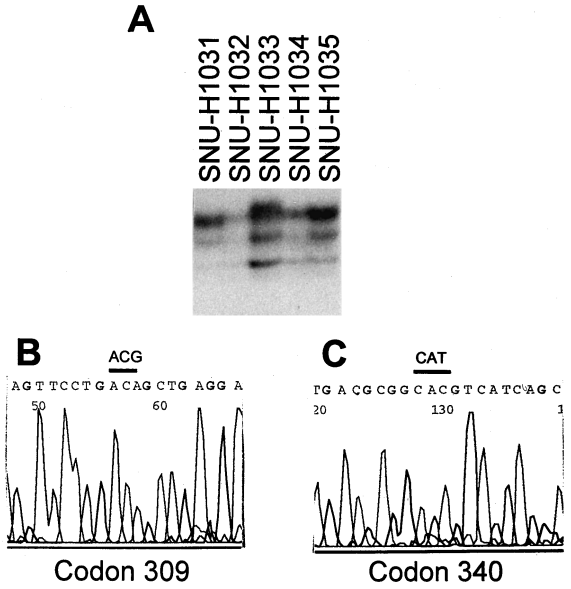

Fig. 1 Silent mutations of the *TGF*-β*RII* gene in suspected HNPCC patient (SNU-H1033) *A*, PCR-SSCP analysis of exon 4-3 of *TGF*-β*RII*. An abnormal band pattern was detected in patient SNU-H1033. *B*, a silent mutation at codon 309 from ACG (Thr) to ACA (Thr). *C*, a silent mutation at codon 340 from CAT (His) to CAC (His).

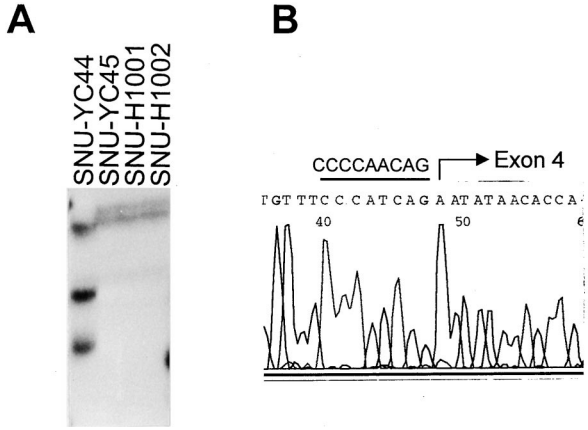

Fig. 2 Genetic alterations in intron 3 of the *TGF*-β*RII* gene from a sporadic early-onset colorectal cancer patient (patient SNU-YC44) *A*, an abnormal band pattern was detected in patient SNU-YC44 by PCR-SSCP analysis. *B*, a 1-bp cytosine deletion at the sixth-to-last base and A/T polymorphism at the fourth-to-last base were identified.

colorectal cancer before the age of 40 years) without any family history of colorectal cancer were also examined.

MATERIALS AND METHODS

Subjects and DNA Isolation from Blood Samples. The previously reported samples, 35 HNPCC, 44 suspected HNPCC, and 45 sporadic early-onset colorectal cancer patients registered in the Korean Hereditary Colorectal Cancer Registry were used in this study (8). All patients enrolled in this study were Korean and were the proband case of (suspected) HNPCC family or patients of early-onset colorectal cancer, and thus all of them originated from unrelated families. Twenty ml of peripheral blood from each patient were used to prepare genomic DNA from WBCs, as described elsewhere (19).

Fifty blood samples for control were taken from patients with benign proctological diseases (48 patients with hemorrhoids and 2 patients with anal fissure). None of individuals in the control group had family history suggesting the HNPCC or development of colon cancer in earlier age. There were few differences in demographic findings between control and patient groups. The only significantly different finding was that patients with early-onset colorectal cancer were approximately 10 years younger than the other groups.

PCR and SSCP Analysis. The PCR-SSCP method was used to screen for mutations of all of the exons of the *TGF*-β*RII* gene. The sequences of the primers and the detailed reaction conditions for amplification have been described previously (10).

Analysis of the Mononucleotide Repeat Sequence in TGF-βRII. We analyzed frameshift mutations in the repeat sequence by modified PCR-SSCP. The PCR primers for the 10-bp polyadenine repeat in exon 3 of *TGF*-β*RII* were as follows: sense, 5′-TGA CTG ATA CTT CTA CCA GC-3′; antisense, 5′-AAC ATT TGT TCC TCA CCT GC-3′. The repeat sequences were amplified from 100 ng of genomic DNA using the sense primer of each gene labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase and the unlabeled antisense primer of each gene. The PCR conditions consisted of 35 cycles at 95°C for 30 s, 45°C for 1 min, and 70°C for 1 min. The PCR products were denatured, separated on 7% polyacrylamide gels at a constant 60 W, and visualized by autoradiography.

DNA Sequencing Analysis. When abnormal patterns were detected by PCR-SSCP analysis, the PCR products were purified with the QIAquick PCR purification kit (Qiagen, Inc., Chatsworth, CA) and then sequenced directly with a Taq dideoxy terminator cycle sequencing kit on an ABI 377 automatic DNA sequencer (Perkin-Elmer, Foster City, CA).

Statistical Analysis. To compare the differences in the portion of cases harboring mutation of specific type of *TGF*-β*RII* gene polymorphism, statistical analysis was performed using Fisher's exact test.

RESULTS AND DISCUSSION

We examined all of the exons of the *TGF*-β*RII* gene by PCR-SSCP analysis. An abnormal band pattern was revealed in exon 4 of a suspected HNPCC patient (patient SNU-H1033), in which germ-line mutations of *hMSH2* and *hMLH1* were not detected (Fig. 1*A*). Subsequent sequencing analysis of the *TGF*-β*RII* gene revealed that SNU-H1033 has two silent mutations at codon 309 (ACG to ACA) and codon 340 (CAT to CAC) that reside on different alleles (Fig. 1, *B* and *C*). In a sporadic early-onset colorectal cancer patient (patient SNU-YC44), a 1-bp cytosine deletion 6 bases from the 3′ end of intron 3 was found (Fig. 2).

Furthermore, we found a previously reported polymorphism at the third nucleotide of codon 389 (AAC to AAT) in exon 4 (13). Three types of PCR-SSCP patterns were identified

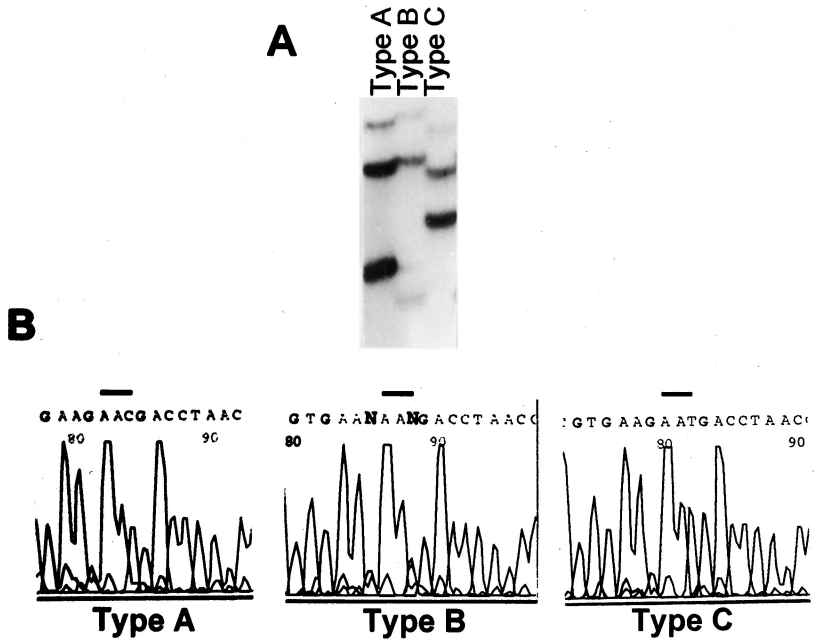

Fig. 3 Polymorphism analysis of the *TGF*-β*RII* gene at codon 389 *A*, PCR-SSCP analysis of exon 4-4 shows three types of band pattern. *B*, sequencing analysis reveals that type A is AAC/AAC, type B is AAC/AAT, and type C is AAT/AAT.

and revealed AAC/AAC (type A), AAC/AAT (type B), and AAT/AAT (type C; Fig. 3). Among the 35 HNPCC patients, 28 were of the AAC/AAC type (80%), and 7 were of the AAC/AAT type (20%). Thirty-six of the AAC/AAC type (82%) and 8 of the AAC/AAT type (18%) were identified in the 44 suspected HNPCC patients. Among the 45 sporadic early-onset colorectal cancer patients, 31 were of the AAC/AAC type (69%), 10 were of the AAC/AAT type (22%), and 4 were of the AAT/AAT type (9%). Furthermore, to determine the existence of any association between the polymorphism and an altered risk for colorectal cancer, 50 samples from individuals with nonmalignant tumors were genotyped by means of PCR-SSCP. As shown in Table 1, this type of polymorphism was especially frequent at sporadic early-onset colorectal cancer patients (31%; $P = 0.02$). The polymorphism was also more frequent in the ICG-HNPCC (20%; $P = 0.22$) and suspected HNPCC (18%; $P = 0.37$) groups than in the control group (10%), although it was not statistically significant. The polymorphism, however, was not associated with the age of patients, tumor site, or differentiation of tumor, as shown Table 2. Although the biological significance of the polymorphism is still unclear, we report the first description of the prevalence and genotype of the AAC/AAT polymorphism in HNPCC, suspected HNPCC, sporadic early-onset colorectal cancer patients, and normal controls. A previously reported introgenic polymorphism was also found at the fourth-to-last base in intron 3 (Ref. 20; Fig. 2).

Compelling evidence has indicated that *TGF*-β*RII* is a tumor suppressor gene and is mutationally inactivated in several types of human cancers exhibiting MSI (10–15). The predominant region of mutations of the *TGF*-β*RII* gene is within a 10-bp polyadenine repeat at exon 3. MSI frequently occurs when the DNA mismatch repair genes *hMSH2* and *hMLH1* are defective. Although MSI has been detected in a variety human cancers, the role of MSI in tumorigenesis remains unclear.

Table 1 Frequencies of polymorphism at codon 389 (AAC to AAT) in patients with ICG-HNPCC, suspected HNPCC, and sporadic early onset colorectal cancer

Group	Frequency (%)	Probability (*P*)[a]
Control group	5/50 (10)	
ICG-HNPCC[b]	7/35 (20)	0.22
Suspected HNPCC	8/44 (18)	0.37
Early-onset colorectal cancer	14/45 (31)	0.02

[a] Probability of difference between control group and disease groups.
[b] International Collaborative Group on HNPCC.

Several studies have shown a frequent frameshift mutation in simple repeat sequences within the open reading frame of the *TGF*-β*RII*, *BAX*, *hMSH3*, or insulin-like growth factor II receptor (*IGFIIR*) genes in cancer tissues and cell lines exhibiting MSI, indicating that they may be the target genes of genomic instability in tumorigenesis (14, 21–23). Recently, we reported germ-line frameshift mutations in a polycytidine repeat sequence of *hMSH6*, which is also one of the target genes of genomic instability in tumorigenesis, suggesting a possibility of germ-line mutation in a mononucleotide repeat sequence (8). In this study, one of our aims was to investigate whether a germ-line frameshift mutation exists in the 10-bp polyadenine repeat of the *TGF*-β*RII* gene. However, no such alteration was observed in any of the tested HNPCC, suspected HNPCC, and sporadic early-onset colorectal cancer patients.

In summary, our results suggest that germ-line mutation of the *TGF*-β*RII* gene may be a rare event in the tumorigenesis of HNPCC and sporadic early-onset colorectal cancer, and the 10-bp polyadenine repeat sequence of the *TGF*-β*RII* gene is an apparent target of genomic instability but not of germ-line

Table 2 Clinical characteristics of colorectal cancer patients with or without polymorphism at codon 389 (AAC to AAT)
No parameters had statistical significance according to the presence or absence of the AAT type polymorphism (Student's *t* test).

	AAT type polymorphism at codon 389 (no.)	Mean age[a] (yr)	Right colon (%)	Poorly differentiated or mucinous type (%)
ICG-HNPCC	Yes (7)	52.1	2 (29)	2 (29)
	No (28)	44.5	11 (39)	7 (25)
Suspected HNPCC	Yes (8)	53.2	3 (38)	3 (38)
	No (36)	47.2	12 (33)	8 (22)
Early-onset sporadic cancer	Yes (14)	32.6	2 (14)	3 (21)
	No (31)	33.2	8 (26)	5 (16)
Total	Yes (29)	43.0	7 (24)	8 (28)
	No (95)	41.8	31 (33)	20 (21)

[a] Mean age of the control group, 43 years.

mutation. Although the biological significance of the polymorphism at codon 398 (AAC to AAT) remains unknown, the frequency of polymorphism is higher in patients with HNPCC and suspected HNPCC and especially in sporadic early-onset colorectal cancer patients than in normal, healthy individuals. Therefore, study of the biological significance of the polymorphism and further rigorous statistical investigation are warranted.

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