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The *PTEN*, *BAX*, and *IGFIIR* Genes Are Mutated in Endometrial Atypical Hyperplasia

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To pursue the pathogenesis of endometrial carcinogenesis, we investigated microsatellite instability, mutations in the *PTEN*, *TGFβRII*, *IGFIIR*, and *BAX* genes, and LOHs on 10q in 18 putative endometrial premalignant lesions (11 endometrial atypical hyperplasias (ATHs), 2 complex hyperplasias, and 5 simple hyperplasias) as well as 8 endometrial cancers (ECs). In the ATH cases, MSIs as well as LOHs at 10q were observed at frequencies similar to those in ECs. Mutations in *PTEN*, *BAX*, and *IGFIIR* were observed only in ATHs and ECs. These results suggest that (1) *PTEN*, *BAX*, and *IGFIIR* are already mutated in ATHs, and (2) ATH is one of the precursor lesions which could lead to EC.

Key words: Endometrial hyperplasia — PTEN — IGFIIR — BAX — MSI

EH, including ATH, SH, and CH, are thought to be premalignant lesions that can lead to EC. It was reported that nearly 20% of EHs develop to ECs.[1, 2] Proliferation of the normal endometrium is regulated by estrogens and overgrowth can be caused by excessive estrogen exposure. Some of the hyperproliferative foci are reversible, and some are not; the latter form a group of high-risk lesions. It is clinically very important to discriminate the high-risk lesions from the others. However, the genetic pathways relevant to ECs are not yet well understood. To date, frequent microsatellite instability has been reported in ECs, and mutations in specific genes such as *IGFIIR* and *BAX* have been reported in endometrioid ECs.[3, 4] Frequent LOH was also observed in chromosome 10q in ECs.[5] *PTEN* was cloned and mapped to 10q23.3,[6, 7] and frequent somatic mutations were reported in ECs.[8–10] However, detailed analysis of the LOH studies suggested that there exists a 100-kb region of frequent allelic loss between *D10S587* and *D10S1723* on chromosome 10q25.3-q26.1 in ECs.[11, 12] We have now extended our study to premalignant lesions of the endometrium.

We first analyzed five microsatellite markers including two mononucleotide repeats (*BAT25* and *BAT26*)[13] and three dinucleotide repeats in 10q (*D10S587* and *D10S1723* at the critical region in 10q25.3-q26.1[11, 12] and *D10S2492* at the *PTEN* locus).[14] The study was conducted with twenty-one paired tumors and corresponding normal tissues (11 ATHs, 5 SHs, 2 CHs), as well as 8 ECs (five of which were accompanied by ATHs) obtained at endometrial curettage from Japanese patients with endometrial tumors at the Clinical Laboratory of Sendai City Medical Association and NTT Tohoku Hospital (Sendai). These samples were fixed in formalin and embedded in paraffin. DNAs were extracted according to methods described previously.[15] Histopathological diagnosis and clinical staging were done according to the criteria of WHO[16] and FIGO,[17] respectively.

Microsatellite analyses were performed according to methods described previously.[5] Examples are shown in Fig. 1. LOH was obvious in ATH case 1638; the intensity of the lower band, indicated by a thin arrow, was significantly reduced at *D10S1723*. ATH case 6013 had a lesion of ATH as well as EC in the same specimen. As indicated by an arrow in Fig. 1, LOH was evident at *D10S1723* only in cancer. In case 2993, however, LOHs were evident at *D10S587* in both ATH and EC. In each case, an independent experiment labeling the antisense strand was performed to confirm the result.

For the LOH study of *PTEN*, the 5-bp insertion/deletion polymorphism in intron 4 of the *PTEN* gene was also analyzed according to methods previously described.[18] We found that only one case of EC had a deletion at this locus (case 8694). Interestingly, the accompanying ATH lesion in this case retained both *PTEN* alleles. All of these results are summarized in Tables I through III; the incidences of LOHs at the *PTEN* and *D10S587*/*D10S1723* loci in ATH were quite similar to those in ECs.

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Abbreviations: ATH, atypical hyperplasia; BAX, BCL2-associated X protein; CH, complex hyperplasia; EC, endometrial carcinoma; EH, endometrial hyperplasia; IGFIIR, insulin-like growth factor II receptor; LOH, loss of heterozygosity; MSI, microsatellite instability; PCR, polymerase chain reaction; PTEN, phosphatase and tensin homolog deleted on chromosome ten; SH, simple hyperplasia; TGFβRII, transforming growth factor β receptor type II.

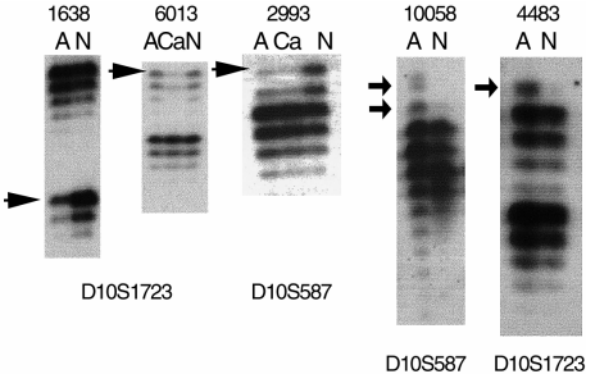

Fig. 1. Examples of allelotype study by the use of microsatellite markers in chromosome 10q. A, Ca, and N denote endometrial atypical hyperplasia, endometrial cancer, and normal tissues, respectively. Note that these two markers *D10S587* and *D10S1723* are within a 200-kb region on chromosome 10q25.3-q26.1.[12] In cases 1638, 6013, and 2993, LOHs (indicated by thin arrows) are clearly observed. In cases 10058 and 4483, MSIs (indicated by thick arrows) are clearly seen.

Table I. Summary of Microsatellite Analyses

Case	Histological diagnosis	MSI[a]	*BAT25*	*BAT26*	*PTEN*		*D10S587*	*D10S1723*
					Intron 4[b]	*D10S2492*		
7649	SH	–	H	H	ND	○	○	○
6012	SH	–	ND	ND	○	○	ND	○
7233	SH	–	H	H	ND	○	○	○
7480	SH	–	H	H	H	○	○	○
7257	SH	–	H	H	○	○	○	○
7083	CH	–	H	H	H	○	○	H
7806	CH	–	H	H	H	○	○	○
7830	ATH	–	H	H	○	○	○	○
4483	ATH	+/–	H	H	H	○	○	MSI
9193	ATH	+	MSI	MSI	H	MSI	MSI	MSI
10058	ATH	+	MSI	MSI	H	MSI	MSI	MSI
7626	ATH	–	ND	ND	H	○	●	●
1638	ATH	–	H	H	H	H	○	●
*8694	ATH	+	H	MSI	○	H	MSI	●
*6013	ATH	–	H	H	○	H	●	○
*2993	ATH	–	H	H	H	H	●	●
*1465	ATH	–	H	H	H	H	○	○
*241	ATH	–	H	H	○	○	○	○
*8694	EC	+	H	MSI	●	H	MSI	●
*6013	EC	–	H	H	○	H	●	●
*2993	EC	–	H	H	H	H	●	●
*1465	EC	–	H	H	H	H	○	○
*241	EC	–	H	H	○	○	○	○
10087	EC	–	H	H	H	H	●	H
5854	EC	+	H	MSI	○	○	MSI	MSI
3698	EC	–	H	H	○	H	○	●

SH, simple hyperplasia; CH, complex hyperplasia; ATH, atypical hyperplasia; EC, endometrial cancer. Asterisks denote samples of ATHs accompanied by ECs. Closed and open circles denote loss and retention of heterozygosity, respectively. H, homozygosity; ND, not determined due to the poor yield of PCR products; MSI, microsatellite instability.

a) Tumors in which altered sized bands were observed at two or more of the five microsatellite loci were defined as MSI+, and that at only one locus, as MSI+/–.

b) Previously reported 5-bp insertion/deletion polymorphism in intron 4 of *PTEN*.[18]

Table II. Results of Analyses of the *PTEN*, *BAX*, *IGFIIR*, and *TGFβRII* Genes

Case	Histological diagnosis	*PTEN*[a)]	*BAX*[a)]	*IGFIIR*[a)]	*TGFβRII*[a)]
7649	SH	—	—	—	—
6012	SH	—	—	—	—
7233	SH	—	—	—	—
7480	SH	—	—	—	—
7257	SH	—	—	—	—
7083	CH	—	—	—	—
7806	CH	—	—	—	—
7830	ATH	—	2-bp del.	—	—
4483	ATH	—	—	—	—
9193	ATH	—	—	—	—
10058	ATH	1-bp del. (exon 7) 4-bp del. (exon 8)	—	1-bp del.	—
7626	ATH	—	—	—	—
1638	ATH	—	—	—	—
*8694	ATH	—	—	—	—
*6013	ATH	—	—	—	—
*2993	ATH	—	—	—	—
*1465	ATH	—	—	—	—
*241	ATH	—	—	—	—
*8694	EC	—	—	—	—
*6013	EC	—	—	—	—
*2993	EC	—	—	—	—
1465	EC	—	—	—	—
*241	EC	—	—	—	—
10087	EC	—	—	—	—
5854	EC	1-bp del. (exon 7)	—	—	—
3698	EC	—	—	—	—

SH, simple hyperplasia; CH, complex hyperplasia; ATH, atypical hyperplasia; EC, endometrial cancer. Asterisks denote samples of ATHs accompanied by ECs.
a) Repetitive sequences at codons 265–267 and 317–320 in *PTEN*, at codons 38–41 in *BAX*, at codons 1314–1317 in *IGFIIR*, and at codons 125–128 in *TGFβRII* were analyzed. Tumors in which no alterations were observed are indicated as (—).

Table III. Results of LOH Analyses

	PTEN	*D10S587*/*D10S1723*
SH	0/5	0/5
CH	0/2	0/2
ATH	0/4	5/9 (56%)
EC	1/5 (20%)	5/7 (71%)

MSIs were also observed in this experiment. ATH case 10058 gained one or two CA dinucleotide repeats, as indicated by thick arrows on the left (see Fig. 1). Similarly, in case 4483, gain of one CA repeat was clearly observed. Our results are summarized in Table I; MSI was observed in four (36%) of 11 ATHs. Among these four, two tumors showed MSIs at all five microsatellite loci examined. We

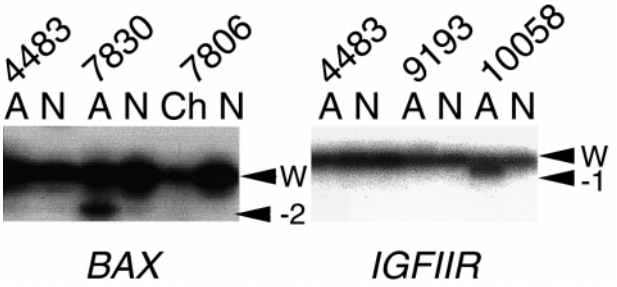

Fig. 2. Results of the screening for mutations of the poly$(G)_8$ tract in the *BAX* and *IGFIIR* genes. Ch, A, and N denote complex hyperplasia, atypical hyperplasia, and normal tissues, respectively. A 2-bp deletion in *BAX* in case 7830 (MSI–) and a 1-bp deletion in *IGFIIR* in case 10058 (MSI+) were observed. W, –1, and –2 denote the wild type and 1- and 2-bp deletions, respectively.

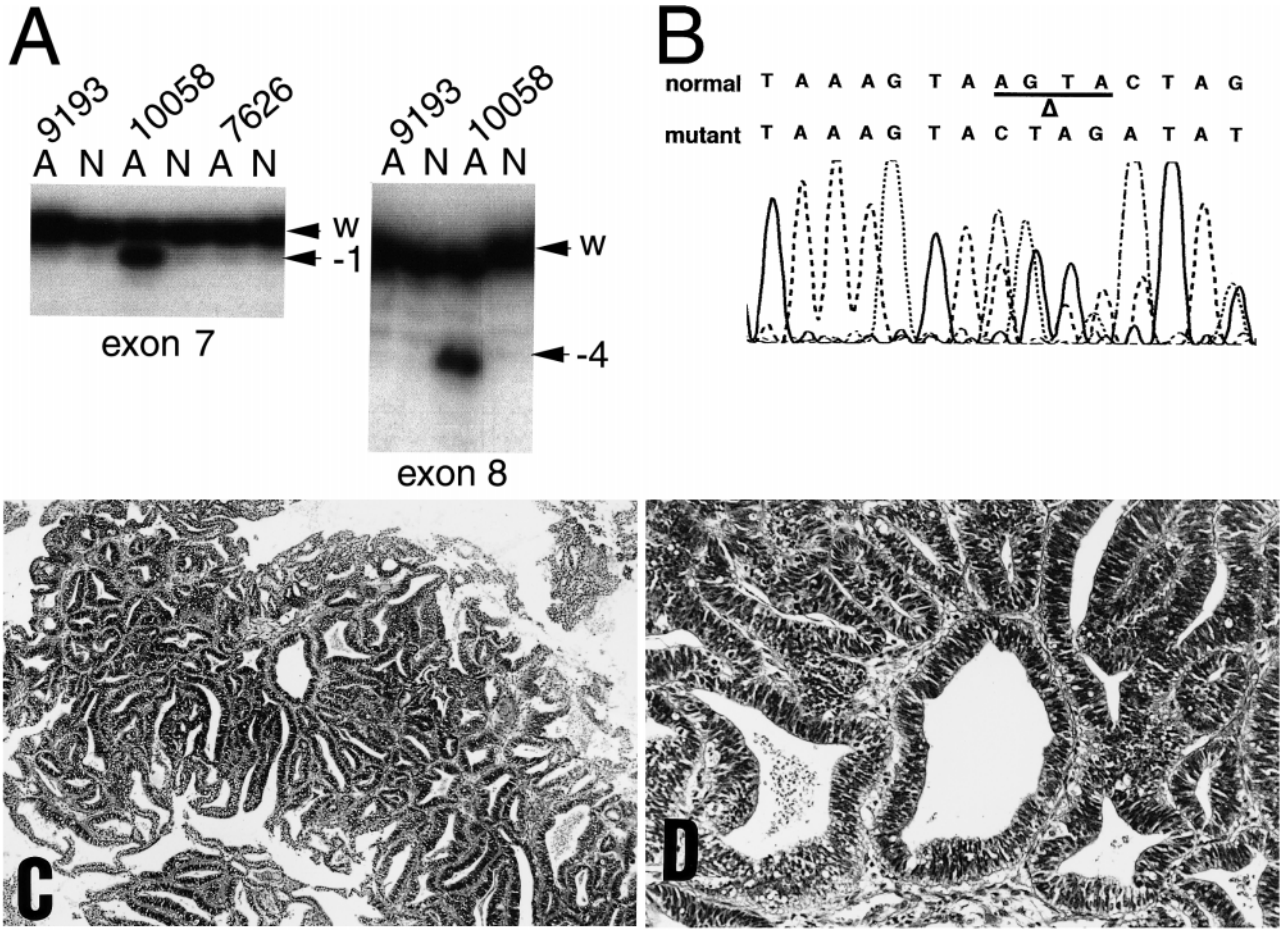

Fig. 3. (A) Screening for mutation of exons 7 and 8 in *PTEN*. In ATH case 10058 (MSI+), a 1-bp deletion in exon 7 and a 4-bp deletion in exon 8 were clearly seen. A and N denote endometrial atypical hyperplasia and normal tissues, respectively. W, –1, and –4 denote the wild type and 1- and 4-bp deletions, respectively. (B) Nucleotide sequencing analysis around codons 317–320 in exon 8. As indicated by underlining and △, a 4-bp deletion (see Fig. 3A right side) in case 10058 at the direct repeat in this region was observed. The nucleotide sequence of the antisense strand is shown. (C), (D) Light microscopic features of ATH case 10058 (69-year-old Japanese female). Marked glandular crowding, resulting in the compression of endometrial stroma, was observed. The glands are lined by stratified cells showing cytological atypia (hematoxylin and eosin stain, original magnification for (C) is ×45 and for (D) is ×175).

previously observed MSIs in 26% (26/100) of endometrial cancers.[3, 4] In this study, we also analyzed ECs and found that two (25%) of eight cases showed MSIs. The overall frequencies of MSI+ in ATHs and ECs were almost equivalent. Our results suggested that MSI is one of the early events in endometrial carcinogenesis, in good agreement with the ideas of Mutter *et al*.[19]

We then searched for mutations in the *TGFβRII*, *IGFIIR*, and *BAX* genes using a PCR-based assay.[3] The poly$(A)_{10}$ tract in the *TGFβRII* gene and the poly$(G)_8$ tract in *IGFIIR* and *BAX* within the coding regions were analyzed. Primers used for mutational analyses were described previously.[3, 4] Typical results are shown in Fig. 2. ATH case 7830 (MSI–) had a 2-bp deletion in the *BAX* gene, and ATH case 10058 (MSI+) had a 1-bp deletion in the *IGFIIR* gene. These mutations were confirmed by nucleotide sequencing (data not shown).

In our previous study,[3, 4] *IGFIIR* and *BAX* were mutated in MSI+ ECs at the frequencies of 15.4% (4/26) and 11.5% (3/26), respectively. Therefore, these two are thought to be the target gatekeeper genes in ECs. In the present study, although the number of tumors analyzed was not large, 25% (1/4) of the MSI+ ATHs harbored mutations in IGFIIR (see Tables I and II). Mutation in the *BAX* gene was also observed in one ATH case that did not show the MSI phenotype. On the other hand, no mutations in *TGFβRII* was observed, in good agreement with previous observations in ECs.[20] These results suggest that

the *BAX* and *IGFIIR* genes, but not the *TGFβRII* gene, are the gatekeepers at the early stage of endometrial carcinogenesis.

Mutation of the *PTEN* gene has frequently been observed in endometrial cancer, especially in MSI+ cases.[8–10] Since the amount of DNA was limited and the samples were formalin-fixed and paraffin-embedded tissues, it was not possible to survey the entire coding region of the gene; we only surveyed the mutational hot spots of the *PTEN* gene in ECs[8] using a PCR-based assay. Thus, we analyzed a poly$(A)_6$ stretch at codon 265–267 in exon 7 and a poly$(A)_6$ stretch with two direct repeats as well as a palindromic structure spanning codons 316–323 in exon 8. Nucleotide sequences of the primers for the target region in exon 7 are 5′-ATCAAAGTAGAGTTCTTCCA-3′ and 5′-TCCCAATGAAAGTAAAGTACA-3′ and those in exon 8 are 5′-CGTGCAGATAATGACAAGGAA-3′ and 5′-CGGTTGGCTTTGTCTTTATTTGC-3′. Typical examples of the results are shown in Fig. 3A. Two somatic mutations were observed in ATH case 10058: a 1-bp deletion in exon 7 and a 4-bp deletion in exon 8. The nucleotide sequence of the antisense strand of exon 8 is shown in Fig. 3B: a 4-bp deletion at the direct repeat spanning codons 317–320 was clearly seen. The nucleotide sequence in exon 7 was also analyzed, and confirmed a 1-bp deletion in the poly$(A)_6$ stretch (data not shown). We could not determine whether a two-hit mutation had occurred in this ATH tumor. Histopathological features of this tumor are also shown in Fig. 3C and 3D. Atypical hyperplasia with no sign of cancer was observed. Recently, mutations of *PTEN* in endometrial hyperplasias were reported by Maxwell *et al.*,[21] in good agreement with the results of our *PTEN* mutation analysis.

In this study, we analyzed the "premalignant lesions" of the endometrium and found mutations in the *BAX*, *IGFIIR*, and *PTEN* genes as well as frequent LOH at 10q25.3-q26.1 and MSI in ATHs, but not in SHs or CHs. Genetic alterations observed in ATHs were similar to those observed in ECs.[3–5, 8–11, 20] Although we could not examine a large number of the "premalignant lesions," our results support the idea that ATHs are at high risk for cancer development, but SHs and CHs are not. Further studies are needed to understand the multistep carcinogenesis of the endometrium.

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