Allelic Loss on Chromosome 17p and p53 Mutations in Human Endometrial Carcinoma of the Uterus

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ABSTRACT

To understand the involvement of allelic losses and inactivation of tumor suppressor genes for the development of endometrial carcinoma of the uterus (EC), 24 cases of EC were examined for loss of heterozygosity (LOH) using a total of 57 polymorphic DNA markers covering all 23 pairs of chromosomes. LOH was observed at 27 loci on 10 different chromosomes, i.e., chromosomes 1, 3, 6, 11, 13, 15, 17, 18, 20, and 21, but was not detected at loci on chromosomes 4, 5, 7, 9, 10, 12, 14, 16, and X. It was observed only in seven of 24 cases, and the other 19 cases did not show LOH at any loci examined, including five cases of tumors with a high proportion of adenomatous hyperplasia. Among seven tumors with LOH at one or more loci, five tumors showed LOH at loci on the short arm of chromosome 17. Furthermore, mutations of the p53 gene, which is located on the short arm of chromosome 17, were detected in three of these 24 tumors by a polymerase chain reaction-single strand conformation polymorphism analysis and subsequent DNA sequencing. In two of these three tumors, p53 mutations were accompanied by the loss of wild-type p53 alleles. These results suggest that inactivation of the p53 gene is involved in the development of EC as in the case of several other types of human cancers.

INTRODUCTION

Although EC is the most common female pelvic malignancy (1), little is known about the molecular genetic events which contribute to the development of EC. Recent molecular genetic studies have shown that either activation of oncogenes or inactivation of tumor suppressor genes is involved in the development of a wide variety of human tumors (2). RFLP analysis is a powerful method to determine candidate loci of tumor suppressor genes inactivated in tumors, and it has been shown, using this method, that allelic losses are common chromosomal events in human tumors (3). Subsequently, several tumor suppressor genes have been isolated from the chromosomal regions showing frequent LOH in tumors (4). We examined 24 fresh tumors of EC for LOH by a RFLP analysis using 57 polymorphic DNA markers that identify RFLPs determined by loci on all 23 different chromosomes. LOH was detected most frequently at loci on the short arm of chromosome 17, suggesting the presence of tumor suppressor gene(s) for EC on this chromosome. Since the p53 gene is located on chromosome 17p and considered to be a tumor suppressor gene, we further searched for p53 mutations by a PCR-SSCP analysis, which is a rapid and sensitive method to detect sequence mutations (5). Mutations of the p53 gene were detected in three of 24 tumors by this method, and it was revealed by subsequent sequencing that these three tumors carry point mutations in either intron 5, exon 8, or exon 10 of the p53 gene. These results indicated that inactivation of the p53 gene is involved in the development of EC.

MATERIALS AND METHODS

Human Tissue Samples. Twenty-four primary tumors together with adjacent noncancerous tissue or peripheral blood were obtained at the time of surgery. The tumors were histologically reviewed and classified according to the Histological Typing of Female Genital Tract Tumours by the World Health Organization (6) and staged according to the International Federation of Gynecology and Obstetrics staging systems. Twenty-three cases were adenocarcinomas, one of which complicated a clear cell carcinoma, and the other one was an adenosquamous carcinoma. Eleven cases were classified as being grade 1 (well-differentiated type), five as grade 2 (moderately differentiated type), two as grade 3 (poorly differentiated type), three as a mixture of grades 1 and 2, one as a mixture of grades 2 and 3, and two as a mixture of grades 1, 2, and 3. There were 18 with stage Ia, three with stage Ib, two with stage II, and one with stage III. Although atypical hyperplasia and adenomatous hyperplasia of the endometrium are considered to be precursors of EC and often present in tumor masses of EC (7), these tumors were also histologically classified into two groups: group I, tumor without atypical hyperplasia or adenomatous hyperplasia; and group II, tumor with atypical hyperplasia or adenomatous hyperplasia which occupies obviously greater space than the carcinoma nest. Nineteen tumors were classified into group I and the other five into group II. None of the 24 cases had received any chemotherapy or radiotherapy before the surgical removal of the tumors.

RFLP Analysis. High molecular weight DNA was prepared by proteinase K digestion and phenol/chloroform extraction as described previously (8). DNA was digested with appropriate restriction endonuclease, electrophoresed on 0.8% agarose gel, transferred to nylon filters, and hybridized to 32P-labeled DNA probes prepared by random primer labeling (Molecular Biology Boehringer Mannheim). The 57 polymorphic DNA markers used in this study are listed in Table 1. These probes detected RFLPs at 57 loci on all 22 autosomal chromosomes and the X chromosome. The allele lengths observed were identical with those published previously (9, 10). Allelic loss was considered to have occurred if the signal intensity of one of the allelic fragments in the tumor was <50% of that in the corresponding normal tissue.

PCR-SSCP Analysis. The oligonucleotide primers for exons 2–3, 4, 5–6, 7–8, 9, 10, and 11 of the p53 gene were prepared according to the published sequence (11), and all primers had additional nucleotides to create EcoRI sites at their 5' ends. Genomic DNA, 100 ng, was amplified in a total volume of 20 μl in a buffer containing 1.0 μl of [32P]dCTP (3000 Ci/mm, 10 Ci/ml). Thirty cycles consisting of 40 s at 94°C (denaturation), 40 s at 55°C (annealing), and 90 s at 72°C (extension) were performed. The PCR product, 2 μl, was diluted 100-fold by a buffer consisting of 20 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.05% bromphenol blue, and 0.05% xylene cyanol. The diluted samples, 40 μl, were heated at 80°C for 2 min, and 1 μl of the samples was applied.
to a 6% neutral polyacrylamide gel (5). Five or 10% glycerol was added to gels for the analysis of exons 4, 5–6, 7–8, 9, and 10. Electrophoresis was performed at 30 W for 1.5–2 h with cooling by fans. The gels were dried and exposed to Kodak XAR films at −80°C for 10–24 h with an intensifying screen.

Cloning and Sequencing. PCR products were purified, digested with EcoRI, and ligated into the EcoRI site of pGEMI plasmid. Sequencing was performed by the dideoxy termination method using Sequanase kit (U.S. Biochemical, Version 2.0). Either 50–100 pooled plasmid clones or one of the plasmid clones was used as a template for sequencing.

RESULTS

Loss of Heterozygosity in Endometrial Carcinoma. We surveyed a total of 57 loci on 23 different chromosomes for LOH in 24 cases of EC by a RFLP analysis. LOH was observed at 27 loci on 10 different chromosomes: chromosomes 1, 3, 6, 11, 13, 15, 17, 18, 20, and 21. No LOH was detected at any loci on chromosomes 4, 5, 7, 9, 10, 12, 14, 16, and X. Information was not available for chromosomes 2, 8, 19, and 22 because of constitutional homozygosity at these chromosomal loci in all cases examined (Table 1). The frequency of LOH at each locus ranged from 7% (1 of 15) at the GH2 locus on 17q to 44% (4 of 9) at the D17S5 locus on 17p. Only seven of 24 cases showed LOH at one or more loci on several chromosomes, and no LOH was detected in the remaining 17 cases at any loci examined (Table 2).

Among 10 different chromosomes, the short arm of chromosome 17 was lost most frequently in the seven tumors carrying allelic loss of some chromosomal loci; five of seven tumors showed LOH on the short arm of chromosome 17 (Fig. 1). LOH was also observed on several other chromosomal loci, including chromosome 3p in two of three tumors and chromosomes 1p, 11p, and 18 in two of seven tumors, respectively (Table 2).

Since LOH was detected only in seven of 24 tumors examined, these 24 cases were classified according to clinical and pathological characteristics with regard to the presence or absence of allelic losses in tumors. LOH at one or more loci was observed in four of 18 with stage Ia, one of three with stage Ib, one of two with stage II, and one of one with stage III. It was detected in four of 16 cases with grade 1, four of 11 with grade 2, and three of five with grade 3. Seventeen of 19 tumors with group I (without atypical hyperplasia or adenomatous hyperplasia) show LOH, while none of 5 tumors with group II (with atypical hyperplasia or adenomatous hyperplasia) carries LOH.

Mutations of the p53 Gene in Endometrial Carcinoma. Since chromosome 17p was lost most frequently in EC, we further examined for mutations of the p53 gene in these tumors by a PCR-SSCP analysis. Seven different portions of the p53 gene covering all the coding exons, exon 2–11, were amplified by PCR, denatured, and electrophoresed on nondenaturing acrylamide gels. After electrophoresis and autoradiography, altered mobility of the PCR products was observed in three of the 24 tumors; mobility of the DNA fragments corresponding to exon 5–6 was shifted in case 29, exon 7–8 in case 24, and exon 10 in case 2 (Fig. 2). When DNA fragments for exon 5–6 and exon 10 were electrophoresed, two bands representing the two complementary single strands of DNA were detected in normal tissue samples, while one or two additional bands corresponding to the two strands of the mutated allele were detected in tumors (Fig. 2, A and C). DNA polymorphisms were observed in the region corresponding to the exons 2–3, 4, and 7–8. When DNA fragments corresponding to exon 7–8 were amplified, four bands were seen in the normal tissue DNA from case 24 because of heterozygosity as shown in Fig. 2B, and the mutated allele was observed in the tumor as the additional two bands with altered mobility.

DNA sequencing was performed in these three samples to confirm the presence of mutated p53 genes and to determine the type of mutations. As described in "Materials and Methods," 50–100 pooled plasmid clones were used as templates. In case 2, a C to T substitution in the codon 342 (CGA→TGA) in exon 10, resulting in a new stop codon (TGA), was detected (Fig. 3A). A point mutation in codon 282 (CGG→TGG) in exon 5, resulting in a new stop codon (TGA), was detected in case 2, a C to T substitution in the codon 342 (CGA→TGA) in exon 10, resulting in a new stop codon (TGA), was detected (Fig. 3A). A point mutation in codon 282 (CGG→TGG) in exon 10, resulting in a new stop codon (TGA), was detected (Fig. 3A). A point mutation in codon 282 (CGG→TGG) in exon 10, resulting in a new stop codon (TGA), was detected (Fig. 3A). A point mutation in codon 282 (CGG→TGG) in exon 10, resulting in a new stop codon (TGA), was detected (Fig. 3A). A point mutation in codon 282 (CGG→TGG) in exon 10, resulting in a new stop codon (TGA), was detected (Fig. 3A). A point mutation in codon 282 (CGG→TGG) in exon 10, resulting in a new stop codon (TGA), was detected (Fig. 3A).

Since LOH on chromosome 17p was detected in cases 2 and 24 by a RFLP analysis (Table 2), it is obvious that one of the
Table 2 Clinical and pathological characteristics of seven endometrial carcinomas with allelic losses

<table>
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<td>+</td>
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<td>+</td>
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<td>+</td>
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* AD, adenocarcinoma; CC, clear cell adenocarcinoma; +, retention of heterozygosity; -, loss of heterozygosity; NI, not informative because of constitutional homozygosity.

DISCUSSION

A RFLP analysis was performed to determine the chromosomal loci of tumor suppressor gene(s) associated with the development of EC, and it was found that the short arm of chromosome 17 was the locus most frequently lost in EC. Therefore, we further examined for possible abnormalities of the p53 gene in these tumors. By a PCR-SSCP analysis, mutations of the p53 gene were detected in three cases, and two of them (cases 2 and 24) showed LOH on chromosome 17p by a RFLP analysis. Three tumors carrying mutated p53 genes were staged into Ia, Ib, and II and histologically classified into grades 1, 2, and 1-3. These results strongly suggest that alterations of the p53 gene are involved in the development of EC, as in the case of several other types of human cancers (4).

By DNA sequence analysis, two unique mutations were detected. One was a C to T substitution in the codon 342 in exon 10, resulting in a new stop codon (CGA-TGA). The other mutation was a single base pair mutation from GC to GT at position 9 from the splicing donor site of intron 5. At present, the significance of the intronic mutation in the latter case is unknown. It is possible that this mutation creates a new splice site at the mutated GT sequence since splicing abnormalities were reported to be due to several mutations within intron sequences (12-14). Although the majority of p53 mutations have been reported to occur in highly conserved regions, we show novel p53 mutations in two cases of EC. In our experience, mutations in p53 exon 4 or 10 lead to new stop codons rather than amino acid substitutions.

Although a total of 57 polymorphic DNA markers covering all 23 different chromosomes were used in this study, LOH was detected only in seven of 24 tumors examined by RFLP analysis, and mutations of the p53 gene were detected only in three of 24 tumors. There are two possible explanations for the low incidence of allelic losses and p53 mutations in EC in this study: (a) it is possible that there are other chromosomal loci showing frequent LOH specifically in this type of tumor; (b) the incidence might be underestimated because of the high percentage of noncancerous cells in tumor specimens. Since tumor speci-
mens of EC often contain a high proportion of noncancerous cells, including atypical hyperplasia and adenomatous hyperplasia, it is likely that genetic alterations in tumor cells are masked by these noncancerous cells. As expected, LOH was not detected in any of five cases with group II (with atypical hyperplasia or adenomatous hyperplasia), while it was detected in seven of 19 cases with group I (without atypical hyperplasia or adenomatous hyperplasia).

LOH was also observed on chromosomes 1, 3, 6, 11, 13, 15, 18, 20, and 21 in this study. There have been few reports showing specific cytogenetic or molecular genetic alterations in EC. Recently, Yamada et al. (15) reported that introduction of normal human chromosomes 1, 6, 9, and 11 individually suppressed tumorigenicity of the endometrial carcinoma cell line HHUA, suggesting the presence of tumor suppressor genes for EC on these chromosomes. Although LOH at the D13S1 locus on chromosome 9 was not detected in this study (0 of 6), LOH on chromosomes 1, 6, and 11 was detected with low frequency. It is possible that tumor suppressor genes on these chromosomes are also involved in the development of EC. Further studies using a number of RFLP markers for these chromosomes would be necessary to clarify the pathogenetic significance of LOH on these chromosomes for the development of EC.

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