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Association of Replication Error Positive Phenotype with Lymphocyte Infiltration in Endometrial Cancers

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Microsatellite instability (MI) has been detected in certain sporadic cancers as well as in hereditary non-polyposis colorectal cancer (HNPCC). In order to determine the precise clinicopathological characteristics of MI in endometrial cancer, we examined 90 sporadic endometrial cancers (83 endometrioid adenocarcinomas, 3 adenosquamous carcinomas, 3 papillary serous carcinomas, and 1 clear cell carcinoma) and eight lesions of endometrial hyperplasia for replication error (RER) using polymerase chain reaction amplification of CA repeated microsatellite sequences at 15 loci. RER was observed in 23 (28%) of the 83 endometrioid adenocarcinomas at at least one locus and in 19 (23%) at two or more loci (RER+ phenotype) in the seven most commonly observed loci, but not in carcinomas of other histological types or in endometrial hyperplasia. Lymphocyte infiltration around carcinoma cells, which is one of the histological features seen in tumors from HNPCC, was severer in RER+ phenotype tumors (79%, 11/14) than in the RER– tumors (25%, 11/44) (marked/moderate infiltration versus slight, $P<0.001$, χ^2 test), when 58 tumors with muscular invasion were examined. The RER+ phenotype was associated with a higher parity and gravidity ($P<0.05$, Wilcoxon test). However, RER+ phenotype was not associated with tumor stage, histological grade, muscular invasion, lymph node metastasis or patient survival. In conclusion, MI occurs in a subset of endometrial cancers, which often show marked infiltration of lymphocytes around the tumor.

Key words: Microsatellite instability — Replication error — Endometrial cancer — Clinicopathological characteristics — Lymphocyte accumulation

It is now generally understood that accumulation of genetic changes, both in proto-oncogenes and tumor suppressor genes, plays a critical role in the development of human cancer.[1)] In addition to the oncogene and suppressor pathway of carcinogenesis, a novel mechanism has recently been discovered in hereditary non-polyposis colorectal cancer (HNPCC) syndrome.[2)] A defect in the mismatch repair (MMR) system of aberrant genetic sequences during DNA replication causes development of this disease. Disruption of one of the MMR genes, such as *hMSH2*, *hMLH1*, *hPMS1*, *hPMS2* and/or *GTBP*, results in loss of the ability to repair or correct aberrant DNA segments.[2, 3)] Thus, a mutated MMR gene causes replication errors (RER) in repeated genetic sequences at several regions, including critical genes responsible for human tumorigenesis.[4)] These findings support the notion that alteration in either of the MMR pathways is likely to be associated with the development of human cancer.[3, 5)] Microsatellites are short repetitive DNA sequences located throughout the genome. When MMR is defective, expansion or contraction of repetitive sequences at microsatellite regions frequently occurs during DNA replication, and this is called microsatellite instability (MI) or a replication error positive (RER+) phenotype. This alteration has been found in sporadic colorectal cancer, endometrial cancer, and many other cancers as well as HNPCC.

Since endometrial cancer is the second most common tumor in women with HNPCC syndrome,[2)] it is important to verify the effect of MI on the clinicopathological features of this cancer. Although previous reports on MI in endometrial cancer have shown that its incidence was almost the same (17–23%)[6–10)] as that in sporadic colon cancer (17%),[11)] the association of MI with specific clinicopathological features of the tumor, which was analyzed well in colon cancer, is still not clear in endometrial cancer. Accordingly, we used polymerase chain reaction (PCR) microsatellite analysis to investigate MI in 90 patients with endometrial cancer, in whom we were able to obtain precise clinicopathological data including a long

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enough follow-up period for assessment of survival. The incidence of MI in our series was similar to that reported previously, but some specific clinicopathological associations were found.

MATERIALS AND METHODS

Patients and DNA extraction We examined 90 patients with endometrial cancer and 8 patients with endometrial adenomatous hyperplasia (4 with and 4 without atypia) who underwent surgery. The histological type of endometrial cancer included 83 endometrioid adenocarcinomas, 3 adenosquamous carcinomas, 3 papillary serous carcinomas, and 1 clear cell carcinoma. Of these 98 patients, 50 cancer patients and 8 hyperplasia patients underwent surgery at Ehime University Hospital (Ehime) from January 1984 to December 1991 and the remaining 40 patients were operated on at Kurashiki Central Hospital (Okayama) from October 1979 to December 1991. Paraffin-embedded tissue blocks were available in all cases, as well as data on stage, histologic subtype, grade of histological differentiation, lymph node metastasis, and peritumoral infiltration of lymphocytes. Paired tumor and corresponding normal tissue DNA was extracted from formalin-fixed, paraffin-embedded specimens by the method described elsewhere.[12] Before surgical resection, neither radiation therapy nor chemotherapy was performed. Tumor staging was done by the surgical staging method of the International Federation of Gynecology and Obstetrics.[13] Lymph node resection was performed in 76 patients. Histologic classification was performed with hematoxylin and eosin-stained sections adjacent to those used for DNA extraction, and was done according to the World Health Organization classification, except for papillary serous carcinoma.[14, 15] The infiltration of lymphocytes into the peritumoral stroma was examined at the interface of non-necrotic tumor and the adjacent stroma in 58 patients with uterine myometrial invasion, and was classified as marked, moderate, slight, or absent according to the abundance of lymphocytes (Fig. 1).

Analysis of microsatellite instability Fifteen dinucleotide microsatellite markers were used for detection of replication error. The loci examined were TP53 (17p13.1),[16] D17S31 (17p13.3), D17S579 (17q11-q21),[17] DCC (18q21),[18] D16S450 (16q22.2-q23.1), D16S389 (16q21), D16S318 (16q22.1), D16S285 (16q12), D16S390 (16q12.1-q13), D16S347 (16q22.1), D16S301 (16q22.1), D16S395 (16q23.1-q24.2), D16S289 (16q23.1-q24.2), D16S393 (16q23.2-q24.3), D16S305(16q24.3).[19] The initial seven loci were examined in all 98 specimens and the latter eight loci were examined in the 58 specimens resected at Ehime University Hospital. The procedure of PCR microsatellite analysis was described previously.[20] In brief, template DNA (50 ng) from tumor and corresponding normal tissue was amplified with each microsatellite primer for 27 to 35 cycles at the recommended annealing temperature. The PCR product was

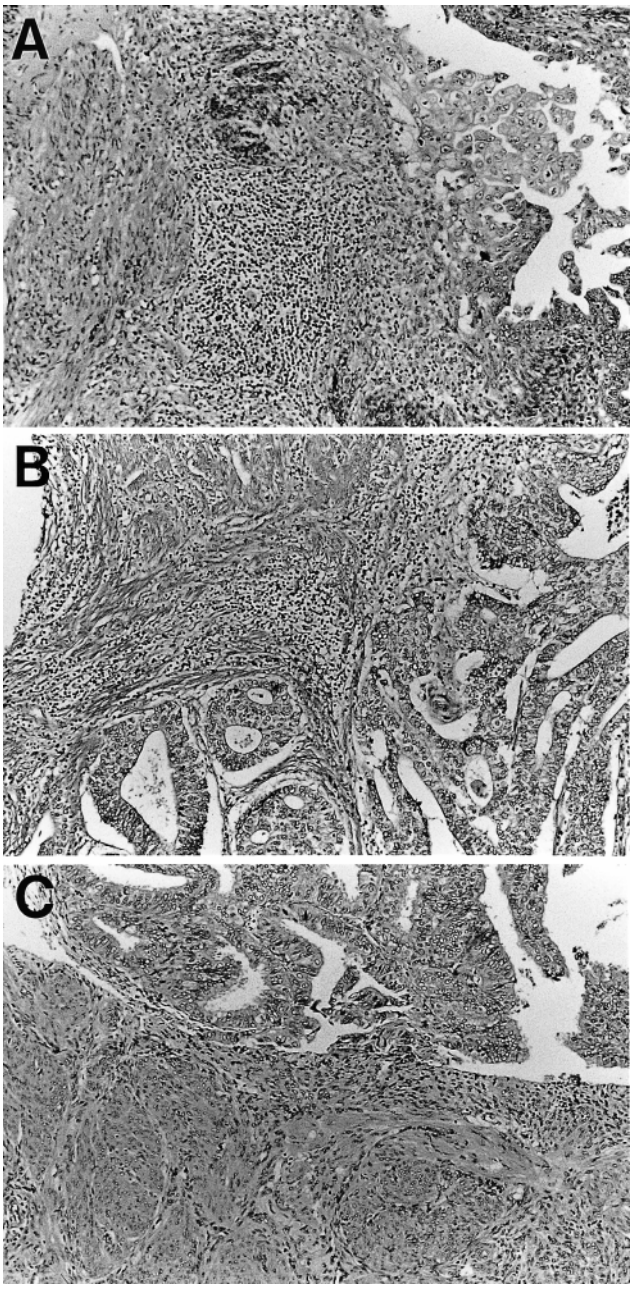

Fig. 1. Hematoxylin-eosin-stained sections showing lymphocyte infiltration. A: Marked lymphocyte infiltration in the peritumoral stroma of the cancer E26. B: Moderate lymphocyte infiltration around tumor E18. C: Slight lymphocyte infiltration around tumor K26. E26 and E18 showed replication error at 12 and 10 of the 15 loci examined, respectively, whereas K26 showed no alteration of any of the microsatellite markers examined.

labeled with ^{32}P during amplification in a 20-μl reaction mixture, consisting of 10 m*M* Tris-hydrochloride (pH 8.3), 1.0–1.5 m*M* magnesium chloride, 50 m*M* potassium chloride, 0.01% gelatin, 200 μM each of dATP, dGTP, dTTP, 50μM dCTP, 10 pmol of each pair of primers, 1μCi of [α-^{32}P]dCTP, and 0.5 units of *Taq* polymerase. Subsequently, the product was diluted three-fold with formamide loading buffer and denatured at 90°C for 5 min. Then, electrophoresis was done on 6% polyacrylamide gel containing 8 *M* urea at a constant 80 W, followed by autoradiography using X-OMAT AR film (Kodak, New York, NY). RER was evaluated as positive at a particular locus when different bands were seen in the tumor PCR product compared with those observed in normal DNA. All of the specimens and loci showing RER were re-examined. The tumor was defined as being RER+ phenotype when tumor DNA showed RER at two or more loci in the seven most commonly observed loci among 15 loci tested.

Analysis of *TGF-β RII* gene mutation In 90 endometrial cancers, we examined slippage or addition of the base in a 73-bp region (nucleotides 665 to 737) of exon 3 of the *TGF-β RII* gene encompassing the polyadenine repeat sequence.[4, 21] The PCR mixture was the same as that for microsatellite analysis, except for the primer pair (TA10-F1:5′-CTT TAT TCT GGA AGA TGC TGC-3′ and TA10-R1:5′-GAA GAA AGT CTC ACC AGG-3′). Thirty PCR cycles, consisting of 94°C for 30 s, 55°C for 1 min, and 70°C for 1 min, were performed. Gel electrophoresis and autoradiography were done in the same way as for microsatellite analysis. Mutation was defined as the presence of bands in tumor DNA that were not present in the corresponding normal DNA.

Sequencing analysis was conducted for evaluation of the alteration within the polyadenine tract of the *TGF-β RII* gene. Forty cycles of PCR were performed in 100-μl volume using the TA10-F1 and TA10-R1 primer pair. The amplified product was purified on a Microcon-10 (Amicon) column and concentrated to 7 μl. Then, the product was analyzed with a BigDye Terminator Cycle Sequencing FS kit (Applied Biosystems) using protocols and kit obtained from the supplier. The product was analyzed on an ABI PRISM 310 automatched DNA sequencer (Applied Biosystems). When the *TGF-β RII* gene mutation was unable to be detected in tumors owing to contami-

Table I. Microsatellite Loci and *TGF-β RII* Mutation in 23 Tumors Showing Replication Error

Case number	Microsatellite locus showing replication error	Altered loci / Tested loci (%)	*TGF-β RII* mutation
E26T	D17S579, D17S31, TP53, D16S389, D16S389, D16S305, D16S285, D16S347, D16S393, D16S301, D16S289, D16S395	12/15 (80)	yes
E18T	D17S31, TP53, D16S450, D16S389, D16S318, D16S305, D16S285, D16S347, D16S393, D16S289	10/15 (67)	—
E30T	D17S579, D17S31, DCC, D16S450, D16S389, D16S305, D16S285, D16S347, D16S393, D16S301	10/15 (67)	—
E24T	D17S579, D17S31, TP53, DCC, D16S389, D16S390	6/15 (40)	—
E07T	D17S31, TP53, DCC, D16S389, D16S305	5/15 (33)	—
E08T	D17S31, TP53, DCC, D16S389	4/15 (27)	—
K24T	D17S579, D17S31, TP53, DCC	4/7 (57)	—
K48T	D17S579, D17S31, DCC, D16S450	4/7 (57)	yes
E38T	D17S579, D17S31, D16S318	3/15 (20)	—
K50T	D17S579, TP53, DCC	3/7 (43)	—
K28T	D17S579, D16S450	2/7 (29)	—
E29T	D17S579, D16S318	2/15 (13)	yes
K25T	DCC, D16S389	2/7 (29)	—
K38T	D17S31, D16S389	2/7 (29)	yes
K31T	D17S579, TP53	2/7 (29)	—
K37T	D17S579, D17S31	2/7 (29)	—
K39T	D17S579, TP53	2/7 (29)	—
K41T	D17S31, D16S450	2/7 (29)	—
K51T	D17S31, D16S450	2/7 (29)	—
E09T	D17S31	1/15 (7)	—
E22T	TP53	1/15 (7)	—
K13T	TP53	1/7 (14)	—
K19T	D17S579	1/7 (14)	—

nation with normal cells, tumor DNA was carefully re-extracted from paraffin-embedded tissue rich in the tumor cells under microscopic examination or the mutated DNA band of the *TGF-β RII* gene in the polyacrylamide gel was excised and extracted.[22] The DNA was then subjected to the sequencing analysis.

Statistical analyses The association of RER+ phenotype with various clinicopathological features and *TGF-β RII* gene mutation was examined by use of the χ^2 test or Fisher's exact test. The Wilcoxon test was used to assess differences of gravidity and parity between RER+ phenotype and RER− phenotype groups. Survival curves were drawn by the Kaplan-Meier method and differences in survival were calculated by use of the log-rank test. The mean follow-up period was 68.2 months (range: 2 to 192 months).

Table II. Microsatellite Instability and Clinicopathological Features of Endometrial Cancer

Factor	Microsatellite instability		*P*-value
	Present	Absent	
	Number of patients (%)		
(1) Age			
≤52 years	9 (39)	14 (61)	<0.05[a]
>52 years	10 (15)	57 (85)	
(2) Parity (mean±SD)	2.1±2.0	2.3±1.7	<0.01[b]
(3) Gravidity (mean±SD)	3.1±2.4	3.5±2.4	<0.01[b]
(4) Histology			
endometrioid adenocarcinoma	19 (23)	64 (77)	
grade 1	10 (19)	43 (81)	
grade 2	5 (28)	13 (72)	NS[a, c]
grade 3	4 (33)	8 (67)	
other histology[d]	0 (0)	7 (100)	
(5) Stage (FIGO,[e] 1988)			
stage I	18 (25)	54 (75)	
Ia	5 (16)	27 (84)	
Ib	6 (32)	15 (68)	
Ic	4 (21)	15 (79)	
II	0 (0)	3 (100)	NS[a]
III	4 (31)	9 (69)	
IV	0 (0)	2 (100)	
(6) Depth of invasion			
limited to endometrium	5 (16)	27 (84)	
<50% of the myometrium	7 (28)	18 (72)	NS[a]
>50% of the myometrium	5 (19)	21 (81)	
invasion of serosa	2 (29)	5 (71)	
(7) Lymph node metastasis			
positive	0 (0)	8 (100)	NS[a]
negative	8 (16)	43 (86)	
not examined	11 (35)	20 (65)	
(8) Lymphocyte infiltration[f]			
marked	6 (75)	2 (25)	
moderate	5 (36)	9 (64)	<0.001[a]
slight/absent	3 (8)	33 (92)	

a) *P*-value by the χ^2 test.
b) Wilcoxon test.
c) Not significant.
d) Three adenosquamous cell carcinomas, 3 serous papillary cancers, 1 clear cell adenocarcinoma.
e) International Federation of Gynecology and Obstetrics.
f) Fifty-eight tumors with myometrial invasion were examined for lymphocyte infiltration.

RESULTS

Ninety-eight endometrial tumor specimens, consisting of 90 carcinomas and 8 endometrial hyperplasia lesions, were examined for RER by PCR microsatellite analysis. Among the 90 endometrial cancers, 23 (26%) showed RER at at least one locus, and 19 tumors (21%) showed RER at two or more loci of the seven most commonly observed loci (D17S579, D17S31, TP53, DCC, D16S450, D16S389, D16S318), which was defined as RER+ phenotype, or MI positive (Table I). All 23 tumors showing RER were endometrioid adenocarcinomas, which is the most common histological type of endometrial cancer, so the incidence of RER+ phenotype was 23% (19/83) in endometrioid adenocarcinoma. We could not detect RER in seven other histological types of tumors (three adenosquamous carcinomas, three papillary serous carcinomas and one clear cell carcinoma) and eight hyperplastic lesions at any of the loci tested. Representative autoradiograms showing RER are displayed in Fig. 2. The clinicopathological differences between the 19 RER+ phenotype carcinomas and the remaining 71 carcinomas (RER−) are summarized in Table II. An association of RER+ phenotype with the degree of the lymphocytic infiltration around the tumor cells was observed ($P<0.001$, χ^2 test) (Table II, Fig. 1). RER+ phenotype was more frequent in the tumors of patients aged 52 years or less than in those of patients over 52 ($P<0.05$, χ^2 test). Comparison of the obstetrics history revealed that patients with RER+ phenotype tumor had a higher gravidity and parity than patients with RER− phenotype tumor (both $P<0.01$, Wilcoxon test). The frequency of RER+ phenotype in endometrial cancer increased as the histology became less differentiated, although there was no statistically significant difference. There was also no significant difference between RER+ phenotype and RER− phenotype tumors with regard to other clinicopathological factors including stage, myometrial invasion, and lymph node metastasis. There was also no difference between the prognoses of the patients with tumors of RER+ phenotype and RER− phenotype in regard to Kaplan-Meier survival curves ($P=0.072$, log-rank test) (Fig. 3). The presence of MI in endometrial

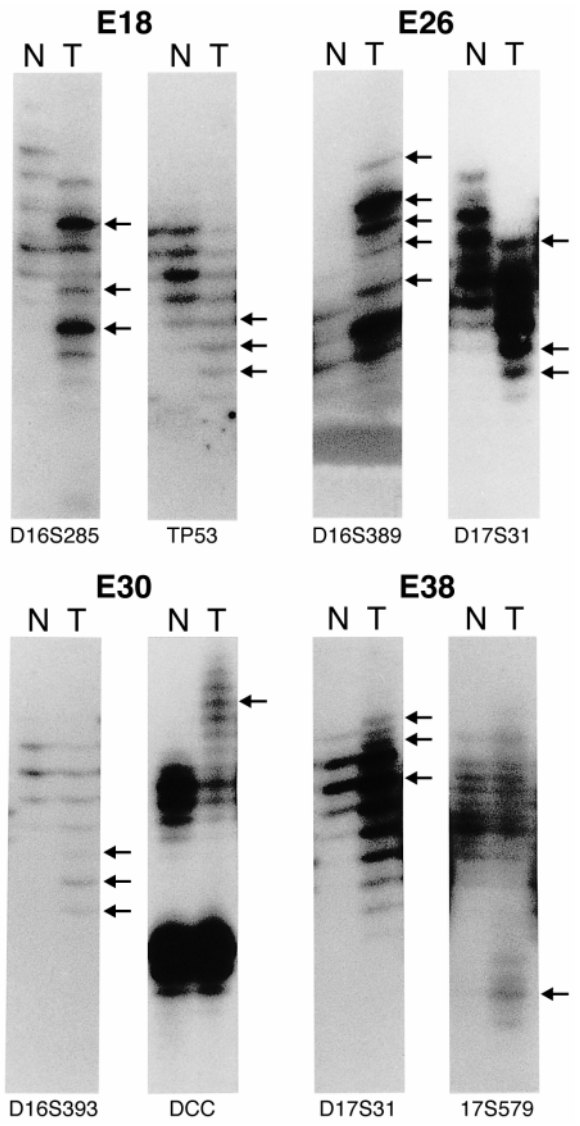

Fig. 2. Representative examples of replication error in 4 endometrial adenocarcinomas (E18, E26, E30, and E38). Additional bands are observed in tumor DNA (T). N, corresponding normal DNA; arrows, replication error.

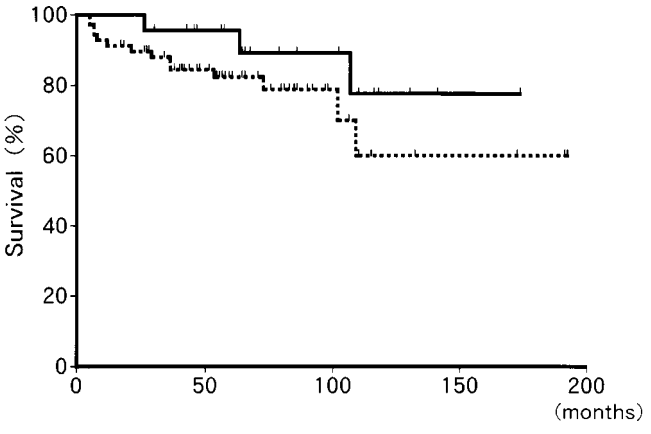

Fig. 3. Kaplan-Meier survival curves for 90 patients with endometrial carcinoma. Survival of 19 patients with RER+ phenotype tumor was compared with that of the remaining 71 patients (RER−). There was no significant difference in survival between patients with and without RER+ ($P=0.072$, log-rank test).
—— RER+ phenotype, ····· RER− phenotype.

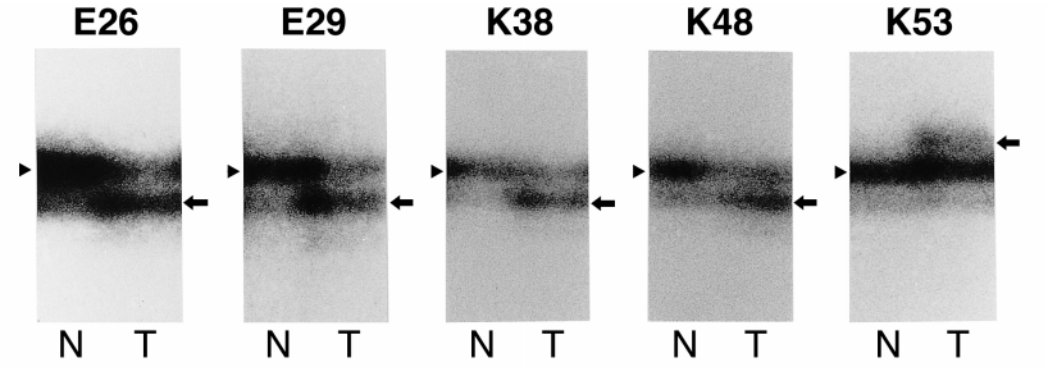

Fig. 4. Analysis of mutation in 73-bp fragment of the *TGF-β RII* gene encompassing the polyadenine repeat sequence. N, normal DNA; T, tumor DNA. Case numbers for each pair of normal and tumor DNAs are shown above the panel. Tumors E26, E29, K38, and K48 were classified as RER+ phenotype, and tumor K53 was RER–. Arrowheads show the 73-base-pair fragment of the wild-type PCR product. Arrows show a stronger band in tumor DNA than in normal DNA in cases E26, E29, K38, and K48, indicating one base deletion. A novel tumor DNA band in case K53 indicates a one-base insertion. All of these changes result in frameshift mutation.

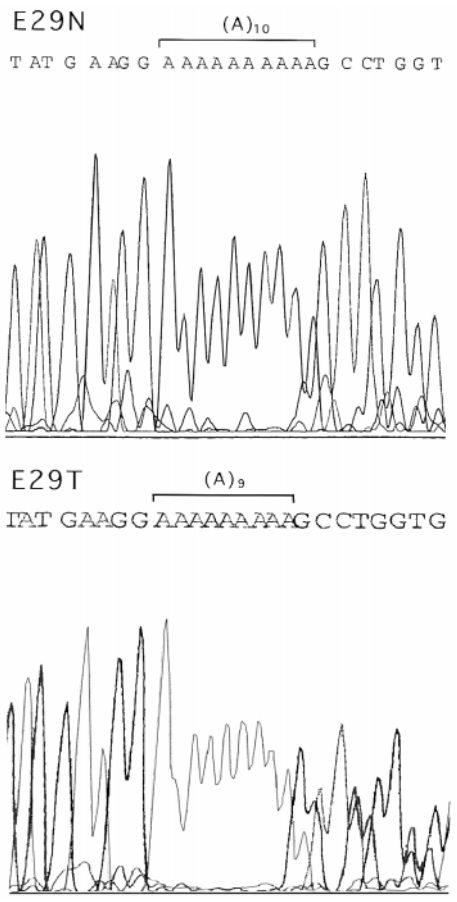

Fig. 5. Sequencing analysis of 10-bp polyadenine repeat in exon 3 of the *TGF-β RII* gene and the flanking regions. The tumor DNA (E29T) is revealed to have a 9-bp adenine repeat ($(A)_9$), and the corresponding normal DNA (E29N), a 10-bp adenine repeat ($(A)_{10}$).

carcinoma is summarized in Table I. The most frequently involved locus was D17S31 (61%, 14/23), and the least frequently involved loci were D16S390 and D16S395.

We examined mutation of the 73-base-pair region of the *TGF-β RII* gene encompassing the polyadenine repeat sequence, and detected the mutation in five out of 90 carcinomas (5.5%) (Fig. 4). Sequencing analysis revealed that the mutation was present within the 10-bp polyadenine repeat of the *TGF-β RII* gene. An example of sequencing analysis was shown in Fig. 5. The *TGF-β RII* gene mutation was more frequently observed in RER+ tumors (21%, 4/19) than in RER− tumors (1.4%, 1/71) ($P<0.01$, Fisher's exact test). The five patients with *TGF-β RII* mutation had no distinguishing clinicopathological characteristics.

DISCUSSION

In the present study, RER+ phenotype was observed only in adenocarcinomas of endometrioid histology, not in those of other histological types. The highest frequency of MI has been reported in pancreatic cancer and poorly differentiated gastric cancer,[23] and it is less frequent in bladder cancer. The variation in the incidence of MI among cancer types[6, 23] and histological types suggests that the prevalence of MMR defects differs between tumor types or histological types. Although MI was not detected in endometrial hyperplasia in our study, it has been reported in atypical hyperplasia adjacent to well differentiated adenocarcinoma containing MI, indicating a direct link between atypical endometrial hyperplasia and endometrial adenocarcinoma but not between endometrial hyperplasia and atypical hyperplasia.[24] Thus, MI may occur at a relatively early step in endometrial carcinogenesis. It might also occur during any evolutionary step of endometrial

cancer, since RER+ phenotype was independent of the tumor stage and degree of tumor differentiation.

Marked infiltration of lymphocytes was more often observed in the peritumoral stroma of RER+ phenotype tumor than RER− tumors (Table II, Fig. 2, A, B, and C). Lymphocyte accumulation around the tumor has been histologically identified in HNPCC and in RER+ gastrointestinal cancers.[25, 26] The association of RER+ phenotype with stromal infiltration of lymphocytes may indicate that genetic instability induces the altered expression of tumor-associated or tumor-specific antigens, such as immunogenic determinants, which allows recognition by the immune surveillance system of the host.[5, 25] MMR-deficient tumor is expected to incur a number of mutations producing abnormal gene products that would lead to responses by the T-cell host immune system. When a repetitive DNA sequence in the coding region of an immunogenic stimulant is mutated under such a detrimental status of MMR deficiency, a host immune reaction would be expected to be generated. It is important to clarify what is the stimulant of the host reaction to induce lymphocytic infiltration around the RER+ tumors and to know whether or not such massive lymphocyte infiltration is universal among malignancies of RER+ phenotype. Bicknell *et al.* reported the presence of mutation in the 8-bp-repeat CT pairs in the β_2-microglobulin (β_2M) gene in colon cancer cell lines.[27] Since β_2M is one of the gene products in the chains of HLA class I molecules, altered β_2M is a possible candidate as a target of immunogenic response.

It has been reported that MI is associated with a favorable prognosis of sporadic colon and gastric cancers.[11, 26] Marked peritumoral inflammatory infiltrates due to host response represent a possible explanation for the good prognosis of RER+ gastrointestinal cancers.[2, 5] In our endometrial cancer series, the prognosis of patients with RER+ tumor, which is often seen with less differentiated histology,[10] was not worse than that of patients without it (Fig. 3). It has been documented that genetic alterations, such as *p53* gene mutation and loss of heterozygosity at certain chromosomal loci, are significantly associated with a poor prognosis of endometrial cancer.[20, 28] It is conceivable that accumulation of such genetic changes enhances tumor aggressiveness during the multistep progression of human cancer. Even if tumors with MI truly have MMR-deficient phenotype, which induces the alteration of repetitive sequences in multiple genomic regions, MI might not have much influence on the activation of proto-oncogenes or the inactivation of tumor suppressor genes, which influence tumor aggressiveness. Mutation of the *TGF-β RII* gene was demonstrated to be infrequent in this and previous[21] studies. However, knowledge of the presence or absence of RER+ phenotype in a tumor sample is clinically important, since RER+ phenotype is frequently seen in tumors of patients with multiple cancers.[29] Careful surveillance to detect second primary tumors that may develop in our patients with RER+ tumor is necessary. Testing for RER+ phenotype might be a convenient screening method to detect MMR deficiency in comparison with the direct examination of five or more MMR-related genes for mutation, because it is a laborious task to sequence all of these genes and it is possible that other known or unknown MMR-related genes also contribute to the development of this disease.

In conclusion, our data show that RER+ phenotype is a genetic event strongly associated with marked lymphocyte accumulation around the tumor.

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