Human Cell (2011) 24:65–73
DOI 10.1007/s13577-010-0005-9

RESEARCH ARTICLE

Effects of tamoxifen on the endometrium and its mechanism of carcinogenicity

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Received: 7 October 2010 / Accepted: 22 October 2010 / Published online: 22 January 2011

Abstract This study was conducted to clarify the clinicopathological characteristics of tamoxifen-associated endometrial carcinomas and its mechanisms of carcinogenesis. Seven patients with tamoxifen-associated endometrial carcinomas (TAM group) and 28 with sporadic endometrioid adenocarcinomas (EMC group) were included in the study. The clinicopathological factors, such as FIGO stage, histological type, grade, lymph node metastases, vascular invasion and the coexistence of hyperplasia, were investigated in both groups. The protein expression of p53, PTEN, *h*MLH1 and *h*MSH2 was investigated by immunohistochemistry. Microsatellite instability (MSI), *k-ras* and *p53* mutation were also examined. In the TAM group, the histological types included five endometrioid, one endometrioid combined with serous and one clear cell type. The rates of coexistence with hyperplasia (five of seven cases) and vascular invasion (four cases) were significantly higher in the TAM group. The rates of stage III/IV (four cases) and lymph node metastasis (three cases) tended to be higher in the TAM group. Although there were no significant differences in PTEN, *h*MLH1 and *h*MSH2 expression between the two groups, *p53* mutation was more frequent in three out of five cases (60%) in the TAM group compared with 2 of 15 cases in the EMC group (13.3%). No significant differences were observed concerning MSI and *k-ras* mutation in either group. These results suggested that TAM-associated endometrial carcinomas have overlapping biological characteristics of type I and type II endometrial carcinomas. This might explain the somewhat worse prognosis of these tumors than sporadic endometrioid carcinomas.

Keywords Tamoxifen · Endometrial carcinoma · *p53* · *k-ras* · *PTEN* · MSI

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Introduction

Many reports suggest that tamoxifen (TAM) is associated with carcinogenesis, and it is one of the risk factors for the development of endometrial carcinomas. TAM is a type of selective estrogen receptor modulator (SERM). It is still widely used for adjuvant therapy of breast cancers [1]. Although TAM is an anti-estrogen, it also has a weak estrogenic effect on the endometrium, and its long-term administration may induce endometrial carcinomas [2]. However, the exact mechanisms of the carcinogenicity of TAM on endometrial cells still remain under debate.

Endometrial carcinomas may be divided into two groups, types I and II [3, 4], based on clinicopathological characteristics. Type I is estrogen dependent, is associated with endometrial hyperplasia and has a relatively good prognosis. More specifically, excessive estrogen stimulation results in an increase in endometrial hyperplastic changes, and a process leading to a highly differentiated endometrioid adenocarcinoma is initiated. In contrast, type II is estrogen independent and includes subtypes such as poorly differentiated endometrioid type or serous and clear cell type, all of which may develop de novo into cancer without concomitant endometrial hyperplasia. Type II carcinomas generally carry a poor prognosis.

Thus far, molecular genetic studies have identified various genes that are involved in the endometrial carcinogenesis. Among them, it is believed that the changes in *PTEN* and *Ras* genes occur as a relatively early event, and the changes in the *p53* gene occur as a relatively late event [5]. Moreover, it has been reported that abnormalities of the *PTEN* or *Ras* genes are more commonly associated with the development of types I carcinomas, whereas abnormalities of the *p53* gene are associated with type II carcinomas [4–6].

Lynch syndrome (hereditary nonpolyposis colorectal cancer, HNPCC) has also been associated with a high incidence of endometrial carcinomas [7]. DNA mismatch repair genes *hMLH1*, *hMSH2*, *hMSH6*, *hPMS1*, and *hPMS2* have been isolated as causative genes of Lynch syndrome [8]. Abnormalities in the DNA mismatch repair result in repetitive sequential errors when DNA is replicated. These repetitive sequences are detectable by microsatellite markers in tumor tissue, thereby designating them as microsatellite instability (MSI) positive. The Lynch syndrome-associated endometrial carcinomas that occur concomitantly with other carcinomas (double cancer) are known to be associated with abnormalities of these DNA mismatch repair genes, which are identified by screening for MSI.

Therefore, in this study, we examined the mechanisms of carcinogenesis of TAM-associated endometrial carcinomas based on the following proposed mechanisms: [1] mechanisms by which type I carcinoma is formed as a result of the continuous estrogenic effect of TAM [2], mechanisms by which type II carcinoma is formed as a result of *p53* gene mutations due to TAM-induced genotoxic DNA adduct formation, and [3] mechanisms, independent of TAM, in the form of double cancers of breast and endometrial carcinomas cause MSI via abnormalities in the DNA mismatch repair gene. Focusing on the involvement of these three mechanisms, we investigated the mechanisms of carcinogenesis of TAM-associated endometrial carcinomas.

This study was conducted to clarify the clinicopathological characteristics of TAM-associated endometrial carcinomas and its mechanisms of carcinogenesis.

Materials and methods

Subjects

Of the 158 patients who were diagnosed with endometrial carcinomas at Fujita Health University hospital from 1987 to 2006, seven patients with TAM-associated endometrial carcinomas (TAM group; 4.4%) were included in this study. For comparisons with the TAM group, each case was matched, based on age and the date of surgery; a set of 28 cases of endometrioid adenocarcinoma (EMC group) who did not have concurrent breast cancers was recruited as a control group in this study.

All patients in the TAM and EMC groups were interviewed for their family history, taken to include second-degree relatives, to clarify whether they fulfilled the Revised ICG-HNPCC Criteria (Amsterdam Criteria II) [9].

The current study was approved by the institutional review board of Fujita Health University hospital.

Clinicopathological examination

We performed a comparative study on clinicopathological factors and the incidence of the coexistence of hyperplasia in the TAM and EMC groups. FIGO stage, grade, lymph node metastases, myometrial invasion, and vascular invasion were set as clinicopathological factors. We also performed a comparative study of disease-free survival in both groups.

Immunohistochemical examination

We performed an immunohistochemical examination of the protein expression of p53, PTEN, *h*MLH1, *h*MSH2, and *h*MSH6 for formalin-fixed, paraffin-embedded surgical specimens in the TAM and EMC groups. Antibodies used and dilution rates are listed below.

p53 (DO-7, DAKO, UK): 100×-fold dilution
PTEN (28H6, Novocastra, UK): 50×-fold dilution
*h*MLH1 (G168-728; PharMingen, CA, USA): 100×-fold dilution
*h*MLH2 (G219-1129, PharMingen, CA, USA): 200×-fold dilution
*h*MSH6 (Q-20, SantaCruz, CA, USA): 60×-fold dilution

For p53, PTEN, and *h*MSH6 proteins, the streptoavidin-biotin-peroxidase method was used. Briefly, these sections were deparaffinized and treated with 3% H_2O_2 in methanol to block endogenous peroxidase activity. After antigen retrieval in a microwave oven using citrate buffer (pH 7.0), sections were incubated with antibody at each dilution rate at 4°C overnight followed by incubation with a biotinylated secondary antibody. Staining was visualized using a streptoavidin-biotin-peroxidase complex solution (Histofine, Nichirei Biosciences, Inc., Japan) and 3, 3-diaminobenzidine-H_2O_2, with counterstaining by Mayer's hematoxylin. *h*MLH1 and *h*MSH2 proteins were stained using the amino acid polymer technique. Briefly, autoclavation in 1 mM EDTA buffer solution (pH 8.0) was performed. The primary antibody of each dilution rate was added in each specimen, and then a peroxidase-labeled secondary antibody was applied.

For p53 protein, the ratio of positive cells among tumor cells was calculated, and the mean value was set as p53 protein labeling index (LI). For PTEN protein, the tumor cells that stained equally to peripheral interstitial and vascular endothelial cells, were only weakly stained or were not stained were defined as retained, reduced or absent, respectively [10]. As for the expression of *h*MLH1, *h*MSH2 and *h*MSH6 proteins, when the nuclei of tumor cells were less stained than the deeply stained nuclei of the cells in the periphery, expression was considered to have disappeared [11].

Microsatellite instability (MSI) analysis

MSI analysis was performed using five kinds of satellite markers (BAT25, BAT26, D2S123, D5S346 and D17S250) recommended by the National Cancer Institute [12]. The sets of primers of satellite markers used were described elsewhere [12].

For MSI analysis, DNA was extracted by the method described below. Briefly, formalin-fixed, paraffin-embedded tissues were incubated at 65°C for 15 min with 300 µl of cell lysis solution, and then homogenized. The cell suspension was incubated with 1.5 µl of proteinase K solution (4 mg/ml). RNase A solution (1.5 µl of 4 mg/ml) was added to the cell lysate and incubated at 37°C for 60 min. Protein precipitation solution (100 µl) was added, and after centrifugation, the DNA-containing supernatant was added to a 1.5-ml tube containing 300 µl of 100% isopropanol. After centrifugation, the supernatant was discarded and the pellets air dried. Three hundred microliters of 70% ethanol was added, and the DNA pellet was washed. After centrifugation and air drying, 25 µl of DNA hydration solution was added to dissolve the DNA.

MSI analysis was performed by the PCR-SSCP method. Briefly, PCR was performed using the five satellite marker primers, and 5 µl formamide dye solution (95% deionized formamide, 10 ml EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) were added to the 2 µl of the PCR product obtained. Then, the mixture was heat denaturated at 95°C for 5 min, rapidly cooled and analyzed by polyacrylamide gel electrophoresis. The electrophoresis bands within the gel were stained using silver stain kit (Daiichi Pure Chemicals, Japan). For assessing MSIs, sections were defined as positive when abnormalities were seen in at least one of the five satellite markers [13].

Analysis of *k-ras* gene mutations

After obtaining informed consent for analysis of the frozen tumor tissues, specimens from 5 of 7 and 15 of 28 cases of the TAM and EMC groups, respectively, were analyzed for *k-ras* (codon 12 of exon 1) mutations using PCR-PHFA (preferential homoduplex formation assay). The specimens were examined using the K-ras codon 12 mutation detection kit (Roche Diagnostic, Switzerland). This method allows semi-quantitative identification of *k-ras* gene mutations in the specimens by reacting in-tube solid-phased labeled DNA of six types of mutated genes (AGT, CGT, TGT, GAT, GCT, GTT), wild-type gene (GGT). The PCR reaction and the detection of mutated genes using AP-labeled anti-DNP antibody were performed according to the instruction manual.

Analysis of *p53* gene mutations

Specimens from 5 of 7 and 15 of 28 cases of the TAM and EMC groups, respectively, were also subjected to *p53* gene analysis. The specimens in which abnormalities were detected by the PCR-SSCP method were subjected to direct sequencing. DNA (100 ng) extracted from each tissue was prepared, and exons 5–8 were amplified by PCR using primers that were labeled with fluorescent dye Cy5 (Amersham Pharmacia Biotech, Sweden) at their 5′-end. The amplified product was heat-denaturated at 80°C for 5 min and electrophoresed on 5% acrylamide gel at 20°C. Specimen material with abnormal peaks in the SSCP analysis was amplified using the PCR system (Roche Diagnostics, Switzerland) using exons for which abnormal peaks had been found as primers. A cycle sequencing reaction was performed using each primer that had been used for the PCR-SSCP analysis, and the Thermo Sequence Fluorescent Labeled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Sweden). Reaction termination solution was added followed by denaturation at 80°C for 5 min. A 7-M urea/6% Long Range gel was prepared, 2 µl of reaction liquid was applied, and electrophoresis was performed. After analyzing the data obtained using Analysis Evaluation Software (Amersham Pharmacia Biotech, Sweden), the mutations were analyzed by comparing normal control and specimens.

Statistical analysis

Statistical analysis of the results of the clinicopathological examination and gene analysis were performed using chi-square test. All measured values are represented as mean value ± standard deviation, and a P value of <0.05 was considered statistically significant. Disease-free survival was examined by the Kaplan-Meier method, and statistical significance was determined using log-rank test.

Results

Clinicopathological examination

By interviewing the TAM and EMC groups for details regarding their family history, no subjects who fulfilled the revised ICG-HNPCC criteria were found.

The cases in the TAM group had a history of TAM exposure for 24–144 months (median 69 months). As for the histological type of seven patients in the TAM group, five had endometrioid adenocarcinoma, one had mixed carcinoma of endometrioid and serous type, and one had clear cell type. The age of the patients in the TAM and EMC groups ranged from 51 to 75 years (median 61 years) and from 49 to 74 years (median 60 years), respectively. Regarding FIGO stage, in the TAM group, 3 patients were stage I, 3 were stage III, and 1 was stage IV, and in the EMC group, 22 and 6 cases were stages I and III, respectively; no differences were observed ($P = 0.11$). Regarding the grade and myometrial invasion, no differences were observed in the two groups, respectively. The number of cases of lymph node metastases in the TAM group was significantly higher than that in the EMC group, 3 of 7 cases (42.9%) compared to 3 of 28 cases (10.7%) ($P = 0.043$). More cases in the TAM group than in the EMC group were positive for vascular invasion, 4 of 7 cases (57.1%) compared to 3 of 28 cases (10.7%) ($P = 0.006$). Furthermore, a significantly high incidence of coexisting hyperplasia was observed in the TAM group compared to the EMC group, 5 of 7 cases (71.4%) compared to 6 of 28 cases (21.4%) ($P = 0.01$) (Table 1). Moreover, although there was no significant difference in disease-free survival between the two groups, with observation periods of 21–104 months (median 50 months) and 5–116 months (median 65 months) in the TAM and EMC groups, respectively, the disease-free survival in the TAM group was slightly lower than that in the EMC group ($P = 0.19$) (Fig. 1).

Table 1 Clinicopathological characteristics of the TAM and EMC groups

	TAM group ($n = 7$)	EMC group ($n = 28$)	P value
FIGO stage			
I	3	22	0.11
II	0	0	
III	3	6	
IV	1	0	
Grade			
G1	3	12	0.54
G2	3	15	
G3	1	1	
Lymph node metastases			
Negative	4	25	0.043
Positive	3	3	
Myometrial invasion			
1/2≥	4	21	0.34
1/2<	3	7	
Vasucular invasion			
Negative	3	25	0.006
Positive	4	3	
Coexistence of hyperplasia			
Negative	2	22	0.01
Positive	5	6	

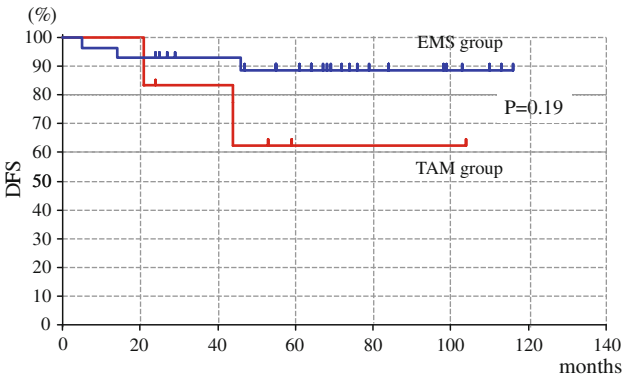

Fig. 1 Disease-free survival of the TAM and EMS groups. There was no significant difference in disease-free survival between the two groups; however, the disease-free survival in the TAM group was slightly lower than that in the EMC group ($P = 0.19$)

Immunohistochemical examination

Examples of cases that stained positive for p53 protein are shown in Fig. 2. The LI of p53 protein in the TAM and EMC groups was 42.9 ± 31.3 and 34.8 ± 28.9, respectively. Although there were no significant differences between the two groups, the LI of the TAM group appeared to be slightly higher than that of the EMC group ($P = 0.17$) (Table 2). The expression pattern of PTEN protein is shown in Fig. 2, and the number of cases assessed as retained, reduced or absent was comparable in the two groups. *h*MLH1 protein-positive and -negative examples are shown in Fig. 3. The frequency of *h*MLH1 protein negative cases was comparable in the two groups. Furthermore, regarding *h*MSH2 and *h*MSH6 protein expression-negative cases, no significant differences were observed between the two groups (Table 2).

MSI analysis

One of seven cases (17%) of the TAM group was assessed as MSI positive since an allelic band shift in the two

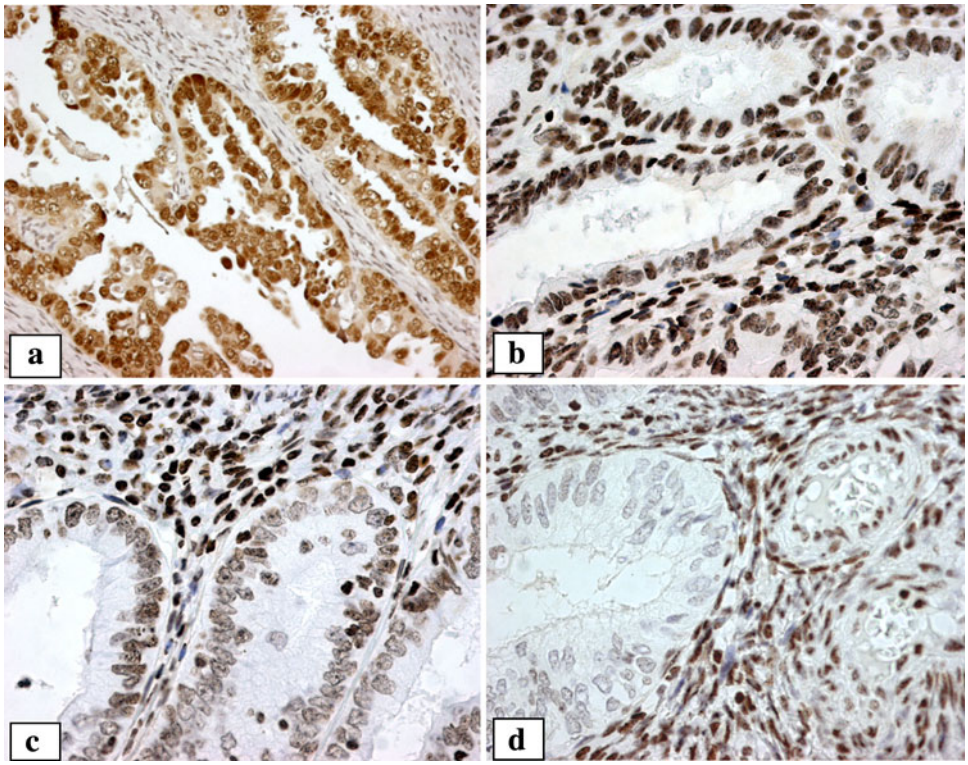

Fig. 2 Immunohistochemical staining of p53 and PTEN proteins. **a** p53 protein expression (×200), **b** PTEN protein-retained case (×400), **c** PTEN protein-reduced case (×400), **d** PTEN protein-absent case (×400)

Table 2 Immunohistochemical and molecular analysis of the TAM and EMC groups

	TAM group ($n = 7$)	EMC group ($n = 28$)	P value
p53 expression			
LI	42.9 ± 31.3	34.8 ± 28.9	0.17
PTEN expression			
Retained	3 (50%)	13 (46%)	0.96
Reduced	3 (50%)	12 (42%)	
Absent	1 (0%)	3 (12%)	
*h*MLH1 expression			
Positive	6 (85%)	18 (64%)	0.27
Negative	1 (15%)	10 (36%)	
*h*MSH2 expression			
Positive	7 (100%)	26 (92%)	0.46
Negative	0 (0%)	2 (8%)	
*h*MSH6 expression			
Positive	6 (85%)	26 (92%)	0.54
Negative	1 (15%)	2 (8%)	
MSI			
Positive	1 (17%)	7 (25%)	0.54
Negative	6 (83%)	21 (75%)	

regions of the loci D2S123 and D17S250 was observed (Tables 2, 3). Figure 4 shows an example of the electrophoresis of normal and malignant tumor tissue of this case. While no differences were observed at locus BAT25, this case was assessed as MSI positive because when compared to the normal tissue, an allelic band shift was observed in the malignant tumor tissue at D2S123. Furthermore, this case was *h*MLH1 protein expression negative. In contrast, 7 of 28 cases (25%) of the EMS group were MSI positive, and no significant differences with the TAM group were observed ($P = 0.54$) (Table 2).

k-ras gene mutations

Among the frozen tumor specimens for which we obtained informed consent for analysis and for which analysis was possible, no *k-ras* mutations in codon 12 were observed in either group (Table 3).

p53 gene mutations

p53 gene mutations were detected in 3 of 5 cases (60%) in the TAM group and 2 of 15 cases (13.3%) in the EMC group. Although no significant difference was observed, a greater trend of mutations was found in the TAM group than in the EMC group ($P = 0.072$) (Table 3). In two of three cases of the TAM group in which *p53* gene mutations were detected, a CGG → TGG point mutation was found in exon 7, codon 248 (Fig. 5), and in one case, a CAT → CCT point mutation was found in exon 5, codon 179. These mutations have previously been reported in endometrial carcinomas and are not specific to TAM usage.

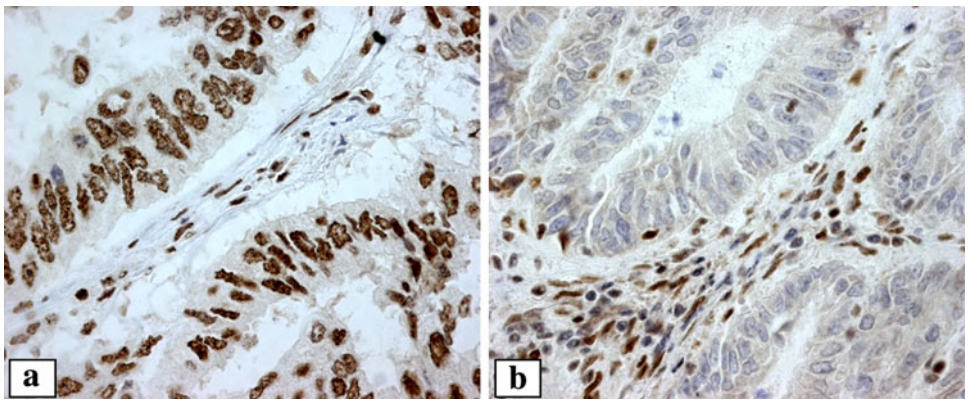

Fig. 3 Immunohistochemical staining of *h*MLH1 protein. **a** Immune-positive example (×400), **b** immune-negative example (×400)

Table 3 Immunohistochemical and molecular characteristics of TAM-associated endometrial carcinomas

Case	Immunohistochemistry					*p*53 mutation	*k-ras* mutation	MSI/microsatellite markers				
	*h*MLH1	*h*MSH2	*h*MSH6	PTEN	p53 (LI)			BAT25	BAT26	D2S123	D5S346	D17S250
1	+	+	+	Reduced	40	NE	NE	−	−	−	−	−
2	−	+	+	Retained	70	Exon 7	−	−	−	+	−	+
3	+	+	+	Reduced	100	NE	NE	−	−	−	−	−
4	+	+	+	Reduced	80	Exon 7	−	−	−	−	−	−
5	+	+	+	Retained	5	−	−	−	−	−	−	−
6	+	+	+	Retained	30	Exon 5	−	−	−	−	−	−
7	+	+	+	Retained	30	−	−	−	−	−	−	−

NE not evaluated

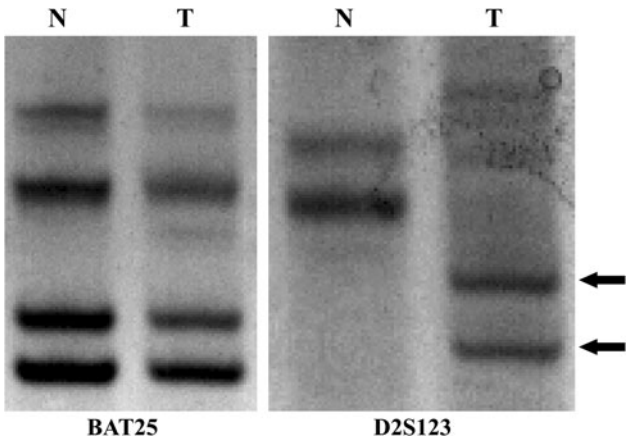

Fig. 4 MSI analysis in one case of the TAM group. Paired normal and malignant tumor DNA displayed an allelic band shift at a microsatellite locus, D2S123 (*arrows*). *N* DNA from normal tissue, *T* DNA from malignant tumor tissue

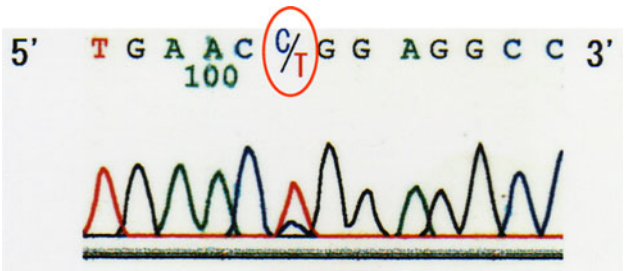

Fig. 5 Mutational analysis of the *p53*. In two cases of the TAM group, a CGG → TGG point mutation was found in exon 7, codon 248

Discussion

In 1985, Killackey et al. [14] first reported an association between TAM and endometrial carcinomas in three patients. Since then, many more have been reported, and the association between TAM and endometrial carcinomas has been addressed. The relative risk of endometrial carcinoma as a result TAM administration has been reported by the American National Surgical Adjuvant Breast and Bowel Project (NSABP) [1] and the British Early Breast Cancer Trials' Collaborative Group (EBCTCG) [15].

There are several reports of clinicopathological studies on TAM-associated endometrial carcinomas. While TAM-associated endometrial carcinomas are generally considered to have a good prognosis compared to sporadic endometrial carcinomas, subtypes such as serous and clear cell type carry a poor prognosis [16, 17]. Two studies did not find differences among the TAM-associated and

sporadic endometrial carcinomas in terms of FIGO stage and histological differentiation [18, 19]. In the present study, the incidences of lymph node metastases, vascular invasion and coexistence of hyperplasia in TAM-associated endometrial carcinomas were significantly higher as compared with sporadic endometrial carcinomas. While there were no significant differences between the two groups, FIGO stages III and IV were more frequent in the TAM group, and the disease-free survival also showed a slightly worse trend in the TAM group. This observation suggests that TAM-associated carcinomas may not carry a favorable prognosis as previously thought. This is consistent with the observation of Hachisuga et al. [20] who found a significantly high frequency of subtypes such as serous or clear cell type, and carcinosarcoma in patients aged 60 years or older. Additionally, cases with lymph node metastases, vascular invasion and myometrial invasion of 50% or more were also more frequent.

The findings of concurrent hyperplasia with a type II carcinoma was an interesting finding. It is possible that TAM-associated carcinomas are a distinct entity possessing attributes of type I and type II tumors. This may explain the somewhat worse prognosis of these tumors.

The frequency of cases of hereditary endometrial carcinomas that fulfill the revised ICG-HNPCC criteria is approximately 1–3.5% [21–23]. However, no patients in the two groups fulfilled this criteria in the present study. MSI is a genetic instability that is found at a rate of 75% in Lynch syndrome pedigrees [24, 25] and at a rate of 25–30% in sporadic endometrial carcinomas with no family history of Lynch syndrome [4]. In the present study, the frequency of MSI-positive cases in the TAM and EMC groups was 17 and 25%, respectively, and thus, no significant differences were observed between the two groups. Therefore, the association between TAM-associated endometrial carcinomas and MSI was considered to be low. Regarding the expression of *h*MLH1, *h*MSH2 and *h*MSH6 proteins, no significant differences between the two groups were observed. In the TAM group, no case had a family history suggestive of a hereditary cancer syndrome, and there were no instances of synchronous cancers of different types in the setting of genetic instability. This observation is consistent with that of Takano et al. [26] whose study of cancer patients with concurrent breast and endometrial carcinomas found no association between genetic instability and TAM-associated endometrial carcinomas.

PTEN, *k-ras* and *p53* gene alterations are also frequently observed in endometrial carcinomas. An association between the *PTEN* gene and endometrial carcinomas has been reported, and in sporadic endometrial carcinomas, *PTEN* gene mutations are observed at a rate of 30–50% [27]. There are only a few reports examining TAM-associated endometrial carcinomas and *PTEN* gene mutations. Holtz et al. [28] conducted a comparative study on the expression of PTEN protein in TAM-associated endometrial and sporadic endometrial carcinomas, but found no differences between the two groups; their results were similar to those of our study.

Regarding the *k-ras* gene, generally the frequency of *k-ras* mutations in sporadic endometrial carcinoma and atypical endometrial hyperplasia has been reported to be between 10–30 and 15%, respectively. The *k-ras* gene is thought to be involved in the early events from hyperplasia to the development of carcinoma [29, 30]. Regarding the role of *k-ras* in TAM-associated endometrial carcinomas, Barakat et al. [31] have reported a high frequency of *k-ras* mutations in TAM-associated endometrial carcinomas. Furthermore, Hachisuga et al. [32] found *k-ras* mutations in codon 12 in 64% of cases with TAM-associated endometrial polyps and reported the possibility of a polyp-cancer sequence. Kim et al. reported that a G → C translocation in the *k-ras* codon 12 is produced in the endometrium from the TAM-DNA adduct [33]. Although *k-ras* mutations are suggested to be involved in TAM-associated endometrial carcinomas as in sporadic endometrial carcinomas, we did not find *k-ras* mutations in any of the cases in the present study. Further studies using other methods besides PCR-PHFA with a large number of cases may be needed to clarify the relationship between *k-ras* mutations and TAM-associated endometrial carcinomas.

Regarding the *p53* gene, despite reports in which no association between the *p53* gene and TAM-associated endometrial carcinomas has been found [34, 35], in a study by Bergman et al., high expression levels of p53 protein were observed in TAM-associated endometrial carcinomas compared to non-TAM administered endometrial carcinomas, alluding to the importance of *p53* gene alterations in TAM-associated endometrial carcinomas [36]. Fujiwara et al. [37] also reported that prolonged TAM may act as a mutagen and directly inactivate the *p53* gene in a significant fraction of TAM-associated endometrial carcinomas. Although there were no significant differences in p53 protein overexpression and *p53* gene mutations between the two groups, a higher trend of p53 protein expression was observed in the TAM group in the present study. This suggests that the *p53* gene is involved in the carcinogenesis of TAM-associated endometrial carcinomas. Sherman et al. [38] considered endometrial intraepithelial carcinoma (EIC) as a premalignant lesion of serous type, noting that the *p53* gene was involved in serous type. In their study, p53 protein overexpression in atypical endometrial hyperplasia, a precancerous lesion of endometrioid adenocarcinoma, was less than 1%, but was 86% in EIC. It has also been reported that in serous type, the frequency of MSI is also high compared to endometrioid adenocarcinomas [39]. The TAM-associated endometrial carcinomas of the

present study included one case with mixed carcinoma of the endometrioid and serous type. Consistent with previous observations, this case was positive for a *p53* gene mutation (exon 7) and MSI. Regarding clear cell type, a trend of significantly high p53 protein expression has been reported. In one case with TAM-associated clear cell type in the present study, a *p53* gene mutation in exon 5 was found. It is plausible that TAM is involved in the development of serous and clear cell type via *p53* gene mutations.

Assessment of the carcinogenesis mechanism of TAM-associated endometrial carcinomas suggests the involvement of at least two mechanisms. Type I carcinomas are generated through the continuous estrogenic effect of TAM, and type II carcinomas arise following *p53* gene mutations generated by TAM-induced genotoxic DNA adduct formation. We hypothesize that TAM-associated endometrial carcinomas have overlapping biological characteristics of type I and type II endometrial carcinomas. However, the numbers of cases including TAM-associated and sporadic endometrial carcinomas recruited in this study were relatively small. Therefore, larger studies are needed to confirm our preliminary findings regarding the molecular biological features of TAM-associated endometrial carcinomas.

Conflict of interest No conflicts of interest to declare.

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