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Alterations of the K-*ras* and *p53* genes in Tamoxifen-associated endometrial carcinoma

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Abstract. To better understand the molecular mechanisms of carcinogenesis induced in uterine endometrium by therapeutic anti-estrogenic Tamoxifen (TAM) exposure, 27 uterine tumors (4 benign endometrial polyps and 23 carcinomas) associated with TAM exposure were analyzed for the presence and spectrum of *p53* and K-*ras* mutations. Although there was no significant difference between TAM-associated endometrial carcinomas and sporadic endometrial tumors in the frequency of these mutations, the spectrum of *p53* mutations was characteristically unique to the TAM-associated tumors. The median duration of TAM exposure was significantly longer in patients with *p53* mutations than those without *p53* mutations (62 vs. 30 months, p=0.028). Our observation suggests that prolonged TAM exposure may directly inactivate the *p53* gene by acting as a mutagen in a significant fraction of TAM-associated endometrial carcinomas.

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Abbreviations: TAM, Tamoxifen; HPLC, high-performance liquid chromatography; dG-N^2-TAM, α-(N^2-deoxyguanosinyl)tamoxifen; CpG, 5'-CpG-3'

Key words: Tamoxifen, K-ras, p53, endometrial carcinoma, mutation

Introduction

Endometrial carcinoma represents 6% of all newly diagnosed malignancies yearly in women in the United States. It is the second most common malignancy of the female urogenital tract in Japan, where its incidence is increasing.

Endometrial carcinoma has been classified into two types according to the tumor's biological and clinical features (1). The Type I carcinoma includes low grade endometrioid carcinoma and represents approximately 70-80% of sporadic endometrial carcinomas. Type I is preceded by complex hyperplasia with atypia and it is often associated with excessive estrogenic stimulation. It occurs predominantly in premenopausal or perimenopausal women and is associated with obesity, hyperlipidemia, anovulation, infertility, and late menopause, and it has a favorable prognosis. In contrast, Type II carcinoma consists of high grade endometrioid and non-endometrioid tumors and represents 10-20% of sporadic endometrial carcinomas. The Type II tumor is usually not associated with estrogen stimulation or hyperplasia. It frequently occurs in postmenopausal women and it carries a higher mortality rate.

Tamoxifen (TAM) is a non-steroidal selective-estrogen-receptor-modulator (SERM); it has potent anti-estrogenic activity in the breast while displaying a contrasting weak pro-estrogenic effect in the endometrium. TAM has long been used as an agent for adjuvant chemotherapy against recurrent breast carcinoma. However, an disturbing increased incidence of endometrial carcinoma has been reported among post-menopausal women who had breast carcinoma followed by treatment with TAM (2). In large epidemiologic studies, the relative risk of a iatrogenic primary endometrial carcinoma has been estimated to double, or even triple, with the risk increasing commensurate with the duration and cumulative dosage of TAM treatments (3).

There are two plausible mechanisms for how TAM induces uterine endometrial cancer. TAM may work as a mild tumor promoter by way of its weak estrogenic activity on the endometrium (4). Alternatively, TAM may play an additional role as a tumor initiator via its weak genotoxicant metabolites (5).

TAM is reported to induce liver tumors in rats, where its detectable TAM-DNA adducts are correlated to its carcinogenesis (6,7). Divi *et al* have reported on the dose-response formation of TAM-DNA adducts in liver, as measured by chemiluminescence immunoassay and ^{32}P-postlabeling in female Fischer rats given TAM (8). Shibutani *et al* (9) and Schild *et al* (10) observed similar TAM-DNA adducts in the brain, ovary, and uterus, in addition to the liver, in monkeys treated with TAM. Further, Shibutani *et al* have reported finding TAM-DNA adducts in human endometrial samples, using a combination of ^{32}P-postlabeling and HPLC analysis (11), and they have suggested that the organ-specific formation of TAM-DNA adducts may be species-specific, referring to other reports (9,12). This may suggest that the tissue-specific repair efficiency of TAM-DNA adducts may be a key factor in TAM-induced carcinogenicity.

Terashima *et al*, while investigating the mutagenic specificities of the TAM adducts in simian kidney cells, found that a significant amount of guanine to thymine substitutions were induced by a dG-N^2-TAM-adduct, when using a shuttle vector consisting of an oligodeoxynucleotide containing dG-N^2-TAM (13). Davies *et al* reported that the major class of mutation induced by TAM was a G:C→T:A transversion in the liver of C*II* transgenic rats (16/28) (14).

These observations suggest that TAM has at least the potential to directly inactivate tumor suppressor genes and/or activate proto-oncogenes which may lead to endometrial tumorgenesis. However, there are only a few reports regarding actual gene alternations in TAM-related human endometrial lesions (15-17). The present study was designed to reveal whether there is any association between the frequency and spectrum of mutations in *p53* and K-*ras*, two widely used endogenous carcinogen-fingerprint *in vivo* reporter genes which are frequently mutated in endometrial carcinoma (18,19), and endometrial carcinomas having a history of exposure to TAM, which may help us to better understand the TAM mechanisms in carcinogenesis.

Materials and methods

Tissue specimens and DNA extraction. This study was conducted as an Ethics Committee-approved cooperative study of Gynecologic Oncology of the Obstetrical Gynecological Society of Kinki District Japan. From seven hospitals, a total of 27 patients with a primary breast cancer who had subsequent endometrial cancers were registered for this study. Informed consent was obtained from all patients. All had a history of TAM exposure for 6-120 months (median 36 months) as an adjuvant therapy for their breast cancer, and all subsequently developed endometrial lesions. None had a family history suggestive of hereditary non-polyposis colorectal cancer. The median age of the patients was 61 years (range 40-87 years). Twenty-three patients (85%) were post-menopausal.

Tissues obtained by hysterectomy were formalin-fixed and paraffin-embedded. The tissue block was cut at 4 μm and non-tumor tissue was trimmed away from the tumor tissue using parallel hematoxylin-eosin-stained sections as a guide. Normal tissue was also collected as an internal control. DNA was extracted using DNA Extraction Kit (Qiagen, CA, USA), according to the manufacturer's instructions.

PCR amplification and cloning of target exons of the p53 and K-ras genes. Exons 5-8 of the *p53* gene, and the sequence immediately surrounding codon 12 of the K-*ras* gene, were amplified individually from genomic DNA using previously described primers and PCR conditions (18,20). The PCR products were electrophoresed and purified using a QIAquick Gel Extraction Kit (Qiagen). The purified PCR products were sub-cloned using the pGEM-T Easy Vector System (Promega, WI, USA).

Detection of mutations by sequencing. At least 10 independent colonies per case were collected at random and sequenced at least twice in both directions. Sequencing was accomplished using the Big Dye Terminator (v3.1) Cycle Sequencing Kit (Applied Biosystems, CA, USA) with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) using the Sp6 reverse primer and the T7 forward primer, respectively.

Statistical analysis. The significance of differences in the frequency with which mutations occurred in different categories of lesions was estimated using the χ^2 test; the median duration of TAM administration was estimated using the Mann-Whitney U test. Differences were considered to be significant at p-value <0.05.

Results

Clinicopathology of the tumors. Of the 27 endometrial lesions considered, four were benign endometrial polyps, 18 were endometrioid adenocarcinomas, two were serous adenocarcinomas, and three were carcinosarcomas (Table I). Of the 18 endometrioid adenocarcinomas, eight were Grade 1 tumors, nine were Grade 2 tumors, and one was a Grade 3 tumor.

Simple hyperplasia with no cellular atypia (which may suggest the presence of a history of continuous estrogenic stimulation) was present adjacent to the neoplastic tissue in only 3 of the 8 G1 endometrioid adenocarcinomas. In contrast, only atrophic glands were present adjacent to the tumor tissue in the remaining 15 endometrioid adenocarcinomas

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Clinicopathological and molecular characters of TAM-induced endometrial lesions.

Histology	Grade	Age	Stage	MI	Meno-pause	Duration of TAM exposure (months)	Coexisting simple hyperplasia	*p53* mutation			K-*ras* mutation	
								Exon	Codon	Sequence	Codon	Sequence
Benign												
Endometrial polyp		68			Yes	36	Yes		wt			wt
Endometrial polyp		71			Yes	47	No		wt			wt
Endometrial polyp		59			Yes	65	No		wt			wt
Endometrial polyp		54			Yes	68	No		wt			wt
Malignant												
Endometrioid	2	40	Ib	<1/2	No	6	No		wt			wt
Endometrioid	2	70	Ib	<1/2	Yes	9	No		wt			wt
Endometrioid	2	63	Ib	<1/2	Yes	12	No		wt			wt
Endometrioid	1	50	Ia	None	No	19	Yes		wt			wt
Endometrioid	1	47	Ia	None	No	20	Yes		wt			wt
Endometrioid	1	52	Ia	None	Yes	24	Yes		wt		12	GGT→GAT
Endometrioid	1	64	Ib	<1/2	Yes	24	No		wt			wt
Endometrioid	2	51	IIIa	None	Yes	24	No		wt			wt
Endometrioid	2	69	Ia	None	Yes	30	No		wt			wt
Endometrioid	1	57	Ib	<1/2	Yes	36	No		wt			wt
Serous		61	Ib	<1/2	Yes	48	No	7	238	TGT→TGTT		wt
Carcinosarcoma		77	Ib	<1/2	Yes	60	No	6	214	CAT→CGT		wt
Carcinosarcoma		74	Ib	<1/2	Yes	60	No		wt			wt
Endometrioid	2	65	Ib	<1/2	Yes	60	No		wt			wt
Endometrioid	1	71	Ia	None	Yes	61	No	7	238	TGT→TGTT	12	GGT→GTT
Endometrioid	1	58	IIa	<1/2	Yes	62	No		wt			wt
Endometrioid	2	63	Ia	None	Yes	62	No		wt			wt
Endometrioid	2	70	Ia	None	Yes	63	No	5	163	TAC→GAC		wt
Endometrioid	3	52	Ia	None	Yes	63	No		wt			wt
Endometrioid	1	47	Ib	<1/2	No	72	No		wt			wt
Carcinosarcoma		55	Ib	<1/2	Yes	84	No	5	173	GTG→ATG		wt
Endometrioid	2	53	IIIc	<1/2	Yes	84	No		wt			wt
Serous		87	IIb	<1/2	Yes	120	No	5	136	CAA→TAA		wt

MI, myometrial invasion. Wt, wild-type.

(5/8 G1 tumors, 9/9 G2 tumors and 1/1 G3 tumors), and also in the two serous adenocarcinomas and three carcinosarcomas.

The association between tumor histopathology and the duration of TAM exposure was evaluated. All three of the carcinomas with adjacent hyperplasia had been exposed to TAM for a relatively shorter time than the remaining non-hyperplasia containing lesions, but the difference was not statistically significant (p=0.067 by Mann-Whitney's U test) (Fig. 1A). All three carcinomas with adjacent hyperplasia were from perimenopausal women. When the tumors were histologically classified into those with a favorable prognosis (G1 and G2 endometrioid adenocarcinomas) and those with a poor prognosis (G3 endometrioid adenocarcinoma, serous adenocarcinoma and carcinosarcoma), those with a poorer prognosis were exposed to TAM significantly longer than those with a good prognosis ($p<0.05$ by Mann-Whitney's U test) (Fig. 1B).

Mutations of the p53 gene. Mutations of *p53* were found in six of 23 (26%) TAM-associated endometrial carcinomas, three were in exon 5, one in exon 6, and two in exon 7. Of the six cases with a *p53* mutation, one was a Grade 1 (G1)

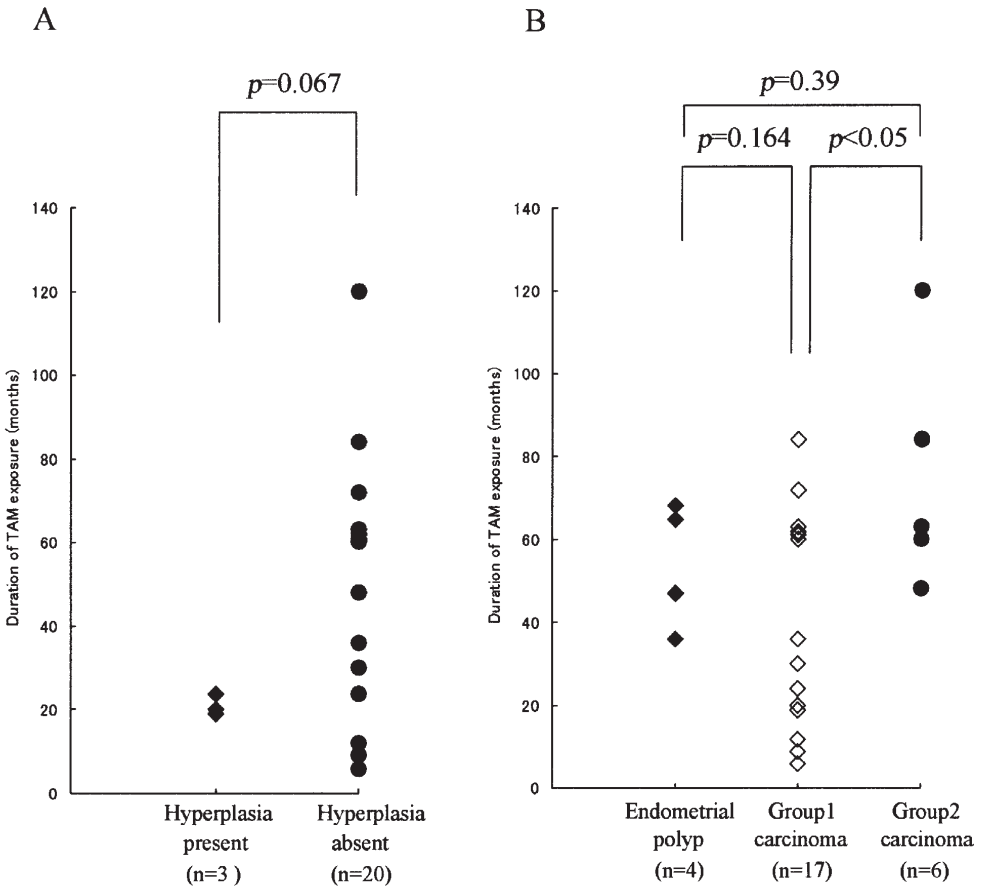

Figure 1. Association of histology and duration of Tamoxifen exposure. Endometrial carcinomas were divided into those with adjacent hyperplasia present and with no adjacent hyperplasic lesions. No association was observed between duration of Tamoxifen exposure and presence of hyperplasic lesion (median 46 months vs. 60 months, p=0.067 by Mann-Whitney's test) (A). Group 1 carcinoma includes G1 and G2 endometrioid adenocarcinoma. Group 2 carcinoma includes G3 endometrioid adenocarcinoma and serous adenocarcinoma, carcinosarcoma. Group 2 carcinoma was exposed to TAM significantly longer than Group 1 ($p<0.05$ by Mann-Whitney's U test) (B).

endometrioid adenocarcinoma, one was a G2 endometrioid adenocarcinoma, two were serous adenocarcinomas, and the other two were carcinosarcomas. No *p53* mutation was found in the four endometrial polyps. Mutations of *p53* were found significantly more frequently in advanced G3 endometrioid adenocarcinomas, serous adenocarcinomas and carcinosarcomas than in early G1 and G2 endometrioid adenocarcinomas (4/6 vs. 2/17; $p<0.036$ by the χ^2 test) (Table I).

Of the six cases with *p53* mutations, four were missense mutations resulting in an amino acid substitution (two G:C→A:T, one A:T→C:G, one A:T→G:C), and two were insertion mutations (both TGT→TGTT) (Table I). There was an association between the presence of *p53* mutation and duration of TAM exposure. Patients with *p53* mutation had a significantly longer exposure to TAM than patients with wild-type *p53* (median; 62 vs. 30 months, p=0.035 by Mann-Whitney's U test) (Fig. 2A).

Point mutations in the K-ras gene. Of twenty-seven TAM-associated endometrial tumors, two point mutations of K-*ras* were found, both in G1 endometrioid adenocarcinomas (7.4%). Both mutations were missense mutations at codon 12 resulting in an amino acid substitution (one G:C→A:T, the other G:C→T:A) (Table I).

Discussion

The prevalence of K-*ras* and *p53* mutations in the present study was compared with our data on sporadic (non-TAM associated) endometrial carcinoma. In the current study, the frequency of K-*ras* mutation in TAM-associated endometrial cancer was lower than we found in sporadic carcinomas but the difference was not significantly different (2/23 vs. 34/114, p=0.066 by the χ^2 test) (18,21). The frequency of *p53* mutation in TAM-associated endometrial carcinoma was 26% and the frequency was comparable to that of endometrial carcinomas with no prior history of TAM exposure (18,21). These observations are consistent with the report by Prasad *et al*, who found no difference in K-*ras*, *PTEN*, or *p53* mutations between TAM-associated and non-TAM-associated endometrial cancer (15). Mutations of *p53* were found significantly more frequently in G3 carcinomas and serous carcinomas than in G1 and G2 endometrioid carcinomas (4/6 vs. 2/17, p=0.035 by χ^2 test). This tendency was similar to that of sporadic endometrial carcinomas (18,21).

Concerning the pattern and the position of *p53* mutations, Enomoto *et al* previously reported that G:C→A:T mutations were found in 15 of 33 cases of sporadic endometrial carcinomas (45%) and all of them were found at methylatable CpG sites (22). Wada *et al* reported G:C→A:T mutations were

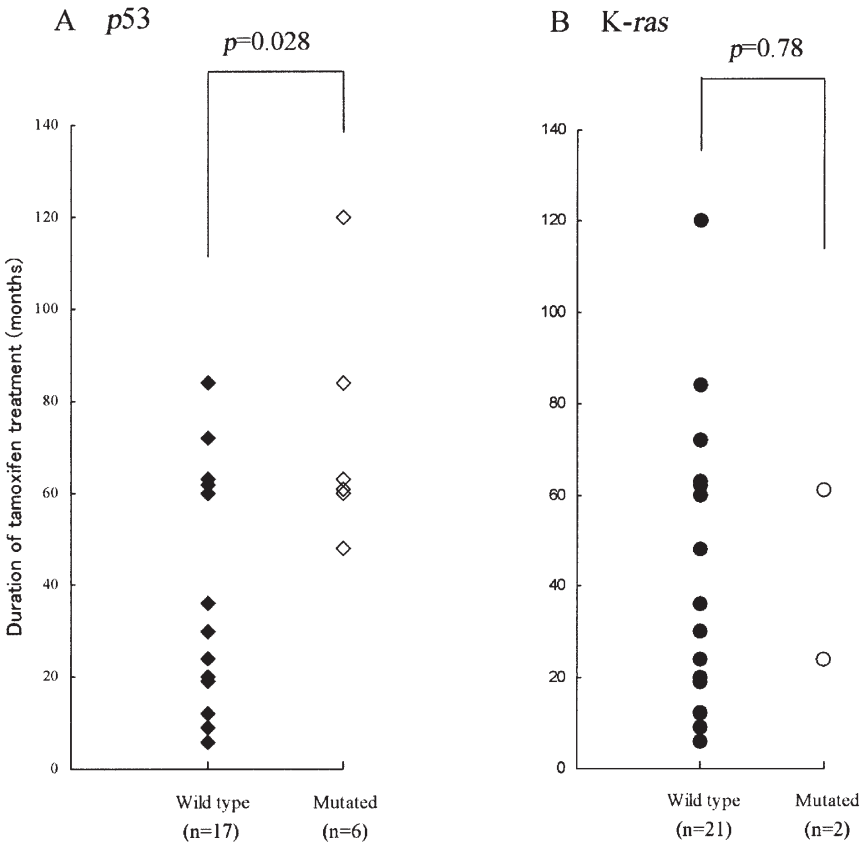

Figure 2. Association of K-*ras* and *p53* status and duration of Tamoxifen exposure in endometrial carcinoma. Endometrial carcinoma with mutated *p53* were exposed to TAM significantly longer than those with *p53* mutations (median duration 30 months (wild-type) vs. 62 months (mutated), p=0.028 by Mann-Whitney's test) (A). No association was observed between K-*ras* mutation and duration of TAM exposure (B).

found in six of eight carcinosarcoma cases (75%) and three of them were at a CpG site (21). In this study, a G:C→A:T transition was found in two of six tumors associated with TAM exposure but none of the mutations was at a CpG site. The IARC *p53* mutation database reports that the G:C→A:T mutation occurring at a CpG site is the most frequent *p53* mutation (5834/23544, 24.7%) occurring in human cancer (23). The G:C→A:T mutation occurring at a CpG site is known to arise through the spontaneous deamination of the methylated cytosine. In contrast, *p53* mutations at non-CpG sites are thought to be more associated with an exposure to specific environmental carcinogens. For example, dietary aflatoxin B_1 intake is correlated with a G:C→T:A transversion in hepatocellular carcinoma, and cigarette smoke is correlated with a G:C →T:A transversion in lung carcinoma (24).

The risk of endometrial carcinoma is reported to correlate with the duration and cumulative dose of TAM exposure (3). Our observations that none of the *p53* mutations in TAM-associated endometrial carcinoma were at CpG sites, and that a correlation was found between *p53* mutation and the period of TAM administration, may imply that long administration of TAM can inactivate *p53* directly, which may lead to more malignant changes of the endometrium.

The most frequent pathologic finding in the benign endometrial condition of the TAM-treated patients is that endometrial polyps are associated with TAM exposure. Such polyps contain markedly fibrotic, sometimes myxoid stroma and cystic glands lined by benign epithelium. Inactive and cystic glands in the TAM-associated polyps are sometimes found to be associated with hyperplasia and to coexist with hyperplastic glands. K-*ras* mutation is very frequently found (>60%) in TAM-associated benign endometrial polyps (16). Cohen *et al* showed neoplastic change of the endometrial polyps is more frequently found in TAM-exposure patients than in non-TAM-exposure patients (3% vs. 0.48%), and they proposed that such endometrial polyps would be the precursor of endometrial cancer induced by TAM (25). If such polyps are truly the precursor of TAM-associated endometrial carcinoma, K-*ras* mutation would be found in TAM-associated endometrial carcinoma as frequently as in benign endometrial polyps. However, our present data, together with the data by Prasad *et al* (15), show a much lower incidence of K-*ras* mutation in TAM-associated endometrial cancer (9% vs. 17%), suggesting that benign endometrial polyps may not necessarily be the precursor of TAM-associated endometrial carcinoma and there may be an alternative pathway for this tumor development.

Concerning the pathological type of TAM-associated endometrial carcinoma, some groups reported that these carcinomas are similar in grade and histological subtype to carcinomas arising in non-TAM users (26). However, resent reports suggest a higher frequency of unfavorable histology of endometrial cancer (serous and clear cell) in TAM-associated carcinomas (27,28). Analysis of over 300 cases of TAM-

associated endometrial carcinoma showed long-term TAM-users are more likely to develop endometrial carcinomas of advanced stage and malignant phenotype (carcinosarcoma and sarcoma) (29). Our finding that the duration of TAM exposure correlated with the malignant phenotype (G3 endometrioid adenocarcinoma, serous carcinoma and carcinosarcoma) supports the same observation made by Bergman *et al* (29).

In conclusion, although this study is limited by the small number of patients available for evaluations, our study suggests that TAM may act as a mutagen and directly inactivate the *p53* gene in at least a fraction of TAM-associated endometrial carcinoma. Further study to confirm these findings is required.

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