Apoptosis as a Possible Candidate Mechanism for Removal of Tamoxifen-related Endometrial Cells with KRAS Mutations

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Abstract. Background: Endometrial cell KRAS mutations are frequent in tamoxifen (TAM)-treated breast cancer patients. We previously demonstrated that most KRAS mutations disappeared after TAM cessation, suggesting the existence of a removal mechanism for endometrial cells with KRAS mutation. Here, the role of apoptosis in this mechanism was investigated. Patients and Methods: DNA was extracted from frozen endometrial polyps of 31 TAM-treated breast cancer patients. Codon 12 mutations in KRAS were detected by enriched polymerase chain reaction enzyme-linked minisequence assay. Apoptosis was detected by the TdT-mediated dUTP-biotin nick end-labeling (TUNEL) method and Ki-67 expression by immunohistochemistry. Relationships between KRAS mutations, the apoptosis index, and the Ki-67 index were determined. Results: KRAS mutations were observed in 9 of these patients. There was no significant relationship between the Ki-67 index and KRAS mutation. However, the apoptosis index was significantly higher in polyps with KRAS mutation (p=0.002). Conclusion: Apoptosis may play an important role in removing TAM treatment-related endometrial cells with KRAS mutations.

Tamoxifen (TAM) is a nonsteroidal triphenylethylene derivative that is widely used for adjuvant treatment and chemoprevention in breast cancer patients. TAM is a selective estrogen receptor modulator and possesses estrogen-agonistic and estrogen-antagonistic effects in various types of tissue (1). It is well known that the incidence of endometrial cancer will increase among breast cancer patients receiving TAM. Several large epidemiological studies have confirmed that the relative risk of endometrial cancer is estimated to be two- to three-fold that of controls, while the risk increases with both the duration and cumulative dose of TAM treatment (2-4).

Endometrial polyps and hyperplasia are among the most common pathological changes in women treated with TAM (5). The frequency of mutations in codon 12 of the KRAS gene ranged from 27% to 76% in endometria of TAM-treated patients (6-9). These frequencies were greater than those seen in cases of sporadic atypical endometrial hyperplasia (4.5% to 23%) (10, 11) and cases of sporadic endometrial cancer (11% to 26%) (12, 13). Prasad et al. reported that 5 (17%) out of 29 TAM-related endometrial tumors had KRAS mutations and the frequency was similar to that in sporadic endometrial cancer (14). The frequency of KRAS mutations in TAM-related endometrial cancer is lower than that of TAM-treated patients overall.

We reported detection of endometrial cell KRAS mutations in 13 (46%) out of 28 TAM-treated patients at initial examinations and the subsequent examinations performed more than two years after cessation of TAM treatment did not detect endometrial cell KRAS mutations in any of these patients (9). No endometrial cells with KRAS mutation were detected in premenopausal patients and the 13 patients with KRAS mutations were of postmenopausal amenorrheic status. These results suggest that menstruation probably plays an important role in the removal of endometrial cells with KRAS mutations but that a mechanism for removal of cells with mutations other than by menstruation exists. The purpose of the present study was to investigate the role of apoptosis as a possible candidate for removal of endometrial cells with KRAS mutations in postmenopausal and amenorrheic patients.
Patients and Methods

Patients. Since 1993, gynecological examinations of patients who received adjuvant treatment after undergoing surgery for breast cancer have been performed at the Outpatients' Gynecologic Clinic of Fukuoka University Hospital. Until 2006, hysteroscopy was performed on 59 patients presenting with vaginal bleeding who exhibited an endometrial polyp on ultrasound and/or were thought to have either premalignant or malignant lesions based on endometrial cytological studies. Forty-five patients were of postmenopausal or amenorrheic status. Hysteroscopic findings of these 45 patients included endometrial polyps (n=31), cystically atrophic changes in the endometrium (n=9), and endometrial carcinoma (n=5). Thirty-one frozen endometrial polypectomy specimens without malignancy were available for analysis.

Detection of a mutation in codon 12 of the KRAS gene. DNA was extracted from the available frozen specimens. Each sample was placed in liquid nitrogen and pulverized in a blender. The resultant powder was dissolved in lysis buffer (0.1% sodium dodecyl sulfate with 200 mg/ml proteinase K). DNA was isolated after phenol/chloroform extraction and precipitation with ethanol. Mutations in codon 12 of the KRAS gene were analyzed by an enriched polymerase chain reaction (PCR)-enzyme-linked minisequence assay (Sumitomo Metal Industry Inc, Tokyo, Japan) (15). This assay was based on the enrichment of the mutant KRAS gene as previously described (16), followed by the incorporation of a biotin-labeled nucleotide specific for the mutant gene, which was then quantified enzymatically using a chromogenic substrate. The KRAS gene amplified by PCR was captured by probes designed to detect the wild-type KRAS codon 12 (GGT) and 6 mutants (GAT, GCT, GTT, AGT, CGT, and TGT), which were subsequently measured by a microtiter plate reader to detect their presence and quantity. The results of a semiquantitative analysis were scored according to the percentage of mutant KRAS genes: 3+, >20%; 2+, 2%−20%; 1+, 0.2%−2%; ±, <0.2%; −, 0% (not detected). The oligonucleotide primers used were:

 upstream for the first and second PCR, 5'-CAAATGATCTGAATTAGCTG-3'.
 downstream for the first PCR, 5'-GTTGGATCATATTCGTACAC-3'.
 upstream for the second PCR, 5'-TAAACTTGTGGTAGTTGGAACT-3'.
 downstream for the second PCR, 5'-GTTGGATCATATTCGTACAC-3'.

Detection of apoptotic cells. The 4 μm sections from paraffin-embedded blocks were deparaffinized, immersed in 3% H2O2 in methanol to block endogenous peroxidase activity. After briefly being rinsed in Tris-buffered saline, sections (in 0.01 M citrate buffer) were irradiated in a microwave oven (800 W). After the sections were cooled and rinsed in Tris-buffered saline, they were incubated for 6 hours at room temperature with monoclonal antibody directed against Ki-67 (clone MIB-1 [1:100 dilution]; Dako, Glostrup, Denmark). The thoroughly washed sections were subjected to immunostaining for 30 minutes using an alkaline phosphatase-mediated system (EnvisionAP; Dako). Sections were counterstained with hematoxylin. For each specimen, the Ki-67 labeling index (%) was determined by counting 1,000 cells in the most active area. The primary antibody was omitted in negative control specimens.

Statistical analysis. Statistical analysis was performed using Dr. SPSS II 11.0.1 J (SPSS Japan, Tokyo, Japan). The Mann-Whitney U-test was used to assess the association between categorical variables. Statistical significance was set at a value of p<0.05.
Results

Clinical features are summarized in Table I. All patients were either of postmenopausal or amenorrheic status. The age of the patients at polypectomy ranged from 48 to 82 years with a mean of 62.7 years. Patients were treated with 20 mg of TAM daily for 6 to 98 months, with a mean of 31.7 months. The total dose of TAM ranged from 3.6 to 59.6 g, with a mean of 19.2 g. Mutations in codon 12 of the KRAS gene were observed in 9 (29%) out of 31 patients. Six patients underwent chemotherapy and one patient underwent adjuvant radiotherapy. No patients underwent combination therapy with gonadotropin-releasing hormone agonist.

There was no statistically significant association between age, duration of TAM use, total TAM dose, and maximum diameter of polyp and presence of KRAS mutations (Table II). Immunohistochemically, there was no statistically significant association between Ki-67 index and presence of KRAS mutations (Table II). However, as shown in Figure 1, apoptotic bodies were found more frequently in endometrial polyps with KRAS mutations (Figure 1 A, B, and C) than in polyps without KRAS mutations (Figure 1 D). Statistically, the apoptosis index was significantly higher in polyps with KRAS mutations (Table II).

Discussion

Although KRAS mutations are the most common oncogenic mutations in human malignancies, their incidence varies widely among carcinomas. TAM-related endometrial cancer has an incidence of 17% of KRAS mutations, which is similar to the proportion found in cases of sporadic endometrial cancer (17%) (14). However, the incidence of KRAS mutations in TAM-related endometrial polyps (64%) was greater than that in sporadic endometrial hyperplasia (4.5% to 23%) (17). These results suggest that KRAS mutation is a key molecular event of carcinogenesis for both sporadic and TAM-related endometrial cancer but that the signaling pathway might differ between these two malignancies.

The exact mechanism of endometrial carcinogenesis by TAM is still unknown. Historically, endometrial carcinogenesis was explained by the estrogen-agonistic effect that TAM exerts in the endometrium. However, Wu et al. reported that genes targeted by TAM are clearly different from those targeted by estrogen (18). Another hypothesis stipulates a genotoxic effect of TAM with formation of DNA adducts. The formation of DNA adducts is a key event in the multistage process of carcinogenesis. Unrepaired or inefficiently repaired DNA adducts may cause mispairing during DNA replication, resulting in mutations (19). Specific mutations in crucial genes encoding proteins for cell cycle control and growth might trigger tumorigenesis. Although some investigators have failed to find evidence of TAM–DNA adducts in endometrium (20, 21), Shibutani et al. (22) detected TAM–DNA adducts in 50% of endometrial samples obtained from TAM-treated women.

Site-specific N2-deoxyguanosinyl tamoxifen (dG-N2-TAM) adducts display a high miscoding and mutagenic potential, and primarily generate G-to-T transversions in mammalian cells (23). The presence of alpha-acetoxytamoxifen-induced DNA damage causes a dose-related increase in mutation frequency (24). Codons 12 and 14 of the KRAS gene are reported to be hotspots for carcinogen–DNA adduct formation in human bronchial epithelial cells (25). The DNA adducts that formed at codon 12 of the KRAS gene were poorly repaired in comparison to those at other codons, including codon 14 (25). A high incidence of mutations in codon 12 of the KRAS gene was also found in TAM-related endometrial polyps (17).

Mammalian cells have developed numerous repair systems to deal with various types of DNA damage and maintain genomic integrity. Bulky DNA adducts, such as those formed by TAM, are typically removed by the nucleotide excision repair system (NER) (26). One study comparing cells proficient and deficient in NER showed that this system plays a significant role in the removal of TAM–DNA adducts (24). The reported absence of endometrial cells with KRAS mutations in more than half of all patients who receive TAM treatment (20-22) indicates that the level of TAM–DNA adducts in uterine samples depends on individual variability in either the extent of metabolic activation or capacity of NER. In our previous study (9), no endometrial KRAS mutations were detected in premenopausal patients, suggesting that menstruation probably plays an important role in the removal of endometrial cells with KRAS mutations. We also revealed that endometrial cell KRAS mutations were detected initially in 13 (46.4%) out of 28 TAM-treated patients and no endometrial cell KRAS mutations had been detected in any of these patients more than two years after cessation of TAM treatment. The fact that these 13 patients were postmenopausal or amenorrheic suggested the existence of a mechanism for removal of KRAS mutations other than that by menstruation in humans. This event is supported by the presence of various repair systems (26) and/or checkpoint-mediated failsafe mechanisms, such as apoptosis or cellular senescence, in response to the acute induction of KRAS mutations in humans (27).

Several reports indicate that KRAS mutation participates in the induction of apoptosis. KRAS mutation subverts the antiapoptotic role of NRAS and cell sensitivity to apoptotic stimuli is increased by the presence of mutant of KRAS (28). KRAS downstream Raf/MEK/MAPK pathway is required for the induction of apoptosis in endometrial cells (29). In the present study, we clarified the possibility that apoptosis may play an important role in removal of TAM-related endometrial cell KRAS mutations in postmenopausal amenorrheic patients.
In summary, apoptotic bodies were found more frequently in endometrial polyps with KRAS mutations than in those without KRAS mutations, which suggests the possibility that apoptosis counteracts excessive mitogenic signaling from oncogenes as part of a cellular failsafe program.

### References


