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ORIGINAL ARTICLE

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# Endometrial K-ras mutations in postmenopausal breast cancer patients treated with adjuvant tamoxifen or toremifene

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**Abstract** *Purpose*: Long-term use of tamoxifen is associated with a two- to threefold increased risk of endometrial cancer in postmenopausal women. Toremifene is another triphenylethylene antiestrogen, which is as effective as tamoxifen in postmenopausal breast cancer. Thus far, its use has not been associated with an increased risk of endometrial cancer. K-*ras* codon 12 mutations seem to be important in endometrial carcinogenesis, and these mutations have been found in endometrial samples of patients on tamoxifen. The present study was undertaken to investigate if there is any difference in the frequency of endometrial K-*ras* mutations among patients treated with tamoxifen or toremifene. *Methods*: Endometrial samples were taken from 23 postmenopausal breast cancer patients (tamoxifen, $n=11$; toremifene, $n=12$) before and after 36 months of treatment. DNA was isolated from formalin-fixed paraffin-embedded samples using a routine proteinase K digestion protocol. K-*ras* mutations in codon 12 were screened using real-time PCR and melting curve analysis in LightCycler equipment. Wild-type PNA oligomer was used to increase the sensitivity of the assay. *Results*: All baseline samples contained wild-type K-*ras*, while 10/23 (43%) of the follow-up samples carried a codon 12 mutation. Mutations were identified in 3 of the 11 in the tamoxifen group and in 7 of the 12 in the toremifene group. Seven were transitions (G → A), and three were transversions (two G → T, one G → C). One of the mutations in the toremifene group was associated with a polypoid endometrium. All the other mutations were found in an atrophic ($n=6$) or proliferative ($n=3$) endometrium. *Conclusions*: Both tamoxifen and toremifene induce endometrial K-*ras* codon 12 mutations. The significance of this finding to endometrial carcinogenesis remains to be elucidated.

**Keywords** K-*ras* codon 12 mutation · Antiestrogens · Tamoxifen · Toremifene · Endometrial cancer · Breast cancer

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## Introduction

Since 1989, the scientific community has acknowledged the carcinogenic potential of tamoxifen on the endometrium [7]. In a recent meta-analysis it was calculated that the relative risk of endometrial cancer is 2.70 in postmenopausal women on long-term tamoxifen [1]. Tamoxifen has partial estrogen-agonist properties on the endometrium [21] and unopposed exogenous estrogen is known to be carcinogenic on the endometrium [3], and the general opinion is that this is the main mechanism by which tamoxifen induces endometrial tumors. If it were the only mechanism, the endometrial cancers associated with the use of tamoxifen should be of low stage and grade, as are tumors induced by estrogen [22]. This, however, does not seem to be the case—tamoxifen-associated tumors tend to be more aggressive [2, 24].

Toremifene is a related triphenylethylene antiestrogen in which chlorine is substituted for one of the hydrogen atoms in the terminal carbon of the ethyl side chain [13]. Toremifene seems to be as effective as tamoxifen in

metastatic [9] and early breast cancer [11, 12]. Toremifene has tamoxifen-like effects in the postmenopausal endometrium [17, 23]. However, its genotoxic potential seems to be far less than that of tamoxifen in the preclinical setting, e.g. in inducing micronuclei in mcl-5a cells in vitro and aneuploidy in rat liver in vivo [10]. For the time being, an excess number of endometrial cancers has not emerged among patients treated with toremifene [5], although the number of patients treated with toremifene may still be too small for a carcinogenic effect to become evident [19].

Mutations in the K-*ras* oncogene seem to be important in endometrial carcinogenesis [21]. At least in human bronchial epithelial cells, codons 12 and 14 of the gene have been identified as hotspots for the formation of carcinogenic DNA adducts [6]. Nicolo et al. found an excess of endometrial K-*ras* codon 12 mutations in tamoxifen-treated patients as compared to untreated controls [18]. In a preliminary study, we found mutations of K-*ras* in the endometria of patients on toremifene and of those on tamoxifen [16]. The aim of the present study was to determine whether there are any differences in the number of endometrial K-*ras* codon 12 mutations in patients treated with adjuvant toremifene or tamoxifen, and to correlate the results with histological findings.

## Materials and methods

The population in this prospective study consisted of 26 postmenopausal women who participated in the Finnish Breast Cancer Group Adjuvant Trial in which tamoxifen 20 mg/day for 3 years was compared to toremifene 40 mg/day for 3 years in node-positive breast cancer [11, 12]. No patient was given adjuvant chemotherapy. The patients gave their informed consent to the study, and the study was approved by the Ethics Committee of Tampere University Hospital.

The endometrial samples ($n = 52$) were taken at the Outpatient Clinic of the Department of Obstetrics and Gynecology of Tampere University Hospital during 1995–2001. Two samples were taken from each patient, one at baseline and the other after 36 months of treatment. Thus, 26 samples were taken from patients on tamoxifen and 26 from patients on toremifene. The endometrial samples were taken by the Pistolet or Pipelle method and fixed in formalin. One baseline sample was insufficient in the tamoxifen group, while one follow-up sample each was insufficient in the tamoxifen and toremifene groups. This left 12 and 11 evaluable patients in the toremifene and tamoxifen groups, respectively. The median age of the tamoxifen-treated patients was 61 years (range 51–72 years) and of the toremifene-treated patients was 59.5 years (51–76 years). One patient in the tamoxifen group had postmenopausal bleeding during the study period. All the others were asymptomatic. All except one of the patients remained free of breast cancer recurrence during the 36-month study period. Skeletal metastases were found in one patient in the tamoxifen group at 12 months.

One patient in the tamoxifen group had been and continued throughout the study period to be on low-dose methotrexate (2.5 mg daily 4 days/week) for rheumatoid arthritis. Hormonal replacement therapy (HRT) had been used prior to the diagnosis of breast cancer by 17 of the patients. All of them had discontinued the therapy before the study began.

DNA was isolated from formalin-fixed, paraffin-embedded endometrial samples using routine proteinase K digestion protocol. DNA from the cancer cell lines NCI-H23 and MDA-MB-134, both of which contain a mutation in codon 12 of K-*ras*, was used as a positive control. DNA from normal human lymphocytes was used as a negative control.

The samples were first screened for K-*ras* mutations using real-time PCR and melting curve analysis in a LightCycler (Roche). A modification of the Clamped Probe Assay (CPA), a sensitive new method for the detection of point mutations in cell populations in which only a fraction of the cells contain the mutation [14], was utilized. Briefly, the method involves the use of a wild-type peptide nucleic acid (PNA) oligomer, which competes with the hybridization probes for template binding. PNA inhibits amplification of the wild-type allele, and blocks the fluorescent signal from the wild-type allele during the melting curve analysis. The assay is able to detect one mutated cell in a background of $10^5$ wild-type cells.

**Table 1** Properties of primers, probes, and PNA. Numbering refers to human lung adenocarcinoma (PR310) K-*ras* oncogene sequence with Genbank accession number K01519. The K-*ras* wild-type sequence in the probe region is presented in the last row (*p* phosphate, *640* LightCycler Red 640, *705* LightCycler Red 705, *F* fluorescein)

| Name | DNA sequence listing 5′ → 3′,<br>PNA sequence listing $NH_2$ → $CONH_2$ | Sense/antisense | K01519 |
|---|---|---|---|
| K-*ras* F | AAGGCCTGCTGAAAATGACTG | Sense | 1–20 |
| K-*ras* R | GGTCCTGCACCAGTAATATGCA | Antisense | 164–143 |
| Anchor-12 | CGTCCACAAAATGATTCTGAATTAGCTGTATCGTCAAGGCACT-F | Antisense | 283–241 |
| 12Gly wt | 640-TGCCTACGCCACCAGCTCCAA-p | Antisense | 59–39 |
| 12Cys sensor | 705-TTGCCTACGCCACAAGCTCCAA-p | Antisense | 60–39 |
| PNA-15 | TACGCCACCAGCTCC | Antisense | 55–41 |
| Wild-type sequence | GTTGGAGCTGTTGGCGTAGGCAAG | Sense | 38–61 |

**Table 2** K-*ras* mutations and endometrial histopathological findings in 23 postmenopausal breast cancer patients before and after 36 months of treatment with tamoxifen ($n = 11$) or toremifene ($n = 12$)

| Treatment | Patient | Histology | | Genotype | |
|---|---|---|---|---|---|
| | | Baseline | 36 months | Baseline | 36 months |
| Tamoxifen | 1 | Atrophy | Atrophy | Wild-type | Wild-type |
| | 2 | Atrophy | Atrophy | Wild-type | Wild-type |
| | 3 | Atrophy | Atrophy | Wild-type | Wild-type |
| | 4 | Atrophy | Atrophy | Wild-type | Wild-type |
| | 5 | Proliferation | Atrophy | Wild-type | Wild-type |
| | 6 | Atrophy | Proliferation | Wild-type | Wild-type |
| | 7 | Atrophy | Proliferation | Wild-type | Wild-type |
| | 8 | Atrophy | Proliferation | Wild-type | Wild-type |
| | 9 | Atrophy | Atrophy | Wild-type | GAT/Asp |
| | 10 | Atrophy | Atrophy | Wild-type | GTT/Val |
| | 11 | Atrophy | Atrophy | Wild-type | GAT/Asp |
| Toremifene | 12 | Atrophy | Atrophy | Wild-type | Wild-type |
| | 13 | Atrophy | Atrophy | Wild-type | Wild-type |
| | 14 | Atrophy | Atrophy | Wild-type | Wild-type |
| | 15 | Atrophy | Atrophy | Wild-type | Wild-type |
| | 16 | Proliferation | Atrophy | Wild-type | Wild-type |
| | 17 | Atrophy | Atrophy | Wild-type | GAT/Asp |
| | 18 | Atrophy | Atrophy | Wild-type | GAT/Asp |
| | 19 | Atrophy | Atrophy | Wild-type | GAT/Asp |
| | 20 | Atrophy | Proliferation | Wild-type | GAT/Asp |
| | 21 | Atrophy | Proliferation | Wild-type | GCT/Ala |
| | 22 | Atrophy | Proliferation | Wild-type | TGT/Cys |
| | 23 | Proliferation | Polypoid | Wild-type | GAT/Asp |

Approximately 100 ng of each sample was used for analysis. Sequences of the primers, hybridization probes, and PNA are given in Table 1. The reactions contained 0.5 $\mu M$ of each primer, 0.3 $\mu M$ anchor probe, 0.15 $\mu M$ sensor probe, 0.15 $\mu M$ or no PNA, and 4 m $M$ $MgCl_2$ and the LightCycler–FastStart DNA Master Hybridization Probe reaction mix (Roche Diagnostics) in a final volume of 20 µl. The amplification was performed in a LightCycler (Roche) starting with 10 min denaturation at 95°C, followed by 45 cycles of 10 s denaturation at 95°C, 15 s annealing at 60°C and 5 s extension at 72°C. Melting curve analysis was performed after 20 s denaturation at 95°C and hybridization for 20 s at 40°C by increasing the temperature from 40°C to 85°C with a slope of 0.1°C/min. Detection was in channel F2, gain 15, for the LightCycler-Red 640 labeled probe, and in channel F3, gain 30, for the LC-Red 705 labeled probe.

Cycle sequencing with a BigDye Terminators v3.0 cycle sequencing kit was performed from both strands of the samples amplified in the presence of PNA using an ABI PRISM 3100 genetic analyzer. For histology, the formalin-fixed and paraffin-embedded samples were stained using the routine hematoxylin-eosin method.

### Statistical analysis

The data from the two groups were compared using Fisher's exact test.

## Results

Sequencing of the follow-up samples amplified in the presence of 0.15 µl PNA showed ten of them to have mutations. All of them were missense mutations in codon 12 of K-*ras*. Seven were transitions (G → A), and three were transversions (two G → T, one G → C) (Table 2). Two of the baseline samples showed variations in the CPA. Sequencing, however, showed them to be wild-type. Altogether, 43% of the follow-up samples carried a codon 12 mutation, whereas none of the baseline samples showed the mutation. The frequency of the mutations was 3 of 11 in the tamoxifen group and 7 of 12 in the toremifene group. The difference was not statistically significant (Fisher's exact test). The patient with breast cancer recurrence had wild-type K-*ras* in both samples.

The patient who was on methotrexate treatment had wild-type codon 12 of k-*ras* both at baseline and at 36 months. Of the 17 previous HRT users, 8 had a mutation at 36 months and 9 did not.

None of the patients had malignant lesions on endometrial histology (Table 2). One patient in the toremifene group, who had a proliferative endometrium at baseline, developed polypoid changes during the treatment. She had a mutation. The rest of the mutations were associated with a proliferative ($n = 3$) or atrophic endometrium ($n = 6$).

## Discussion

Because only wild-type K-*ras* was seen in the baseline endometrial samples, the mutations found after 3 years exposure to toremifene and tamoxifen were likely to have been caused by the medication. Although toremifene has been shown clearly in preclinical studies to be less genotoxic than tamoxifen [10], and in the clinical setting thus far no excess of endometrial cancers related to the use of toremifene has been noted [5], there were no fewer K-*ras* mutations in the group treated with toremifene than in the one treated with tamoxifen. Since there was no correlation of mutations with any estrogenic effect as evaluated by endometrial histology, the mutations were probably not related to the estrogen-agonist properties of the drugs. This interpretation is in accordance with the findings of earlier studies, which have implied that toremifene is somewhat less estrogenic than tamoxifen [4, 17].

It is likely that K-*ras* mutations are related to endometrial carcinogenesis, because premalignant lesions such as endometrial hyperplasias and polyps, as well as overt carcinoma harbor K-*ras* mutations [21]. Hachisuga et al. have demonstrated that tamoxifen-induced endometrial polyps also contain K-*ras* mutations [8]. In the present study, none of the mutations in the tamoxifen group was found in a polypoid endometrium, but all three were associated with endometrial atrophy. In the toremifene group, one of the seven mutations had occurred in a polypoid endometrium.

Nicolo et al. found endometrial K-*ras* codon 12 mutations in 25 of 33 patients who had been on tamoxifen 20 mg/day for a median of 36 months [18]. Of the mutations, 15 were G → T transversions and the rest G → A transitions. At least in pancreatic tumors, G → T mutations seem to be more important than G → A mutations [15]. In the present study, 7 of 10 mutations were G → A transitions, which may explain why no correlation with premalignant or malignant lesions was found. Interestingly, the only mutation-polypoid combination found was a G → A transition in a polypoid endometrium.

In conclusion, both toremifene and tamoxifen are able to induce K-*ras* mutations in postmenopausal endometria after 3 years of use. However, the present study failed to demonstrate any correlation between K-*ras* mutations and endometrial carcinogenesis.

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