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MGMT promoter hypermethylation and K-RAS, PTEN and TP53 mutations in tamoxifen-exposed and non-exposed endometrial cancer cases

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Background: Tamoxifen has anti-oestrogenic and anti-tumour activity in the breast, but is oestrogenic and carcinogenic in the endometrium. It can induce experimental tumours by both hormonal and DNA-damaging mechanisms, but its carcinogenic mode of action in human endometrium remains unclear.

Methods: We investigated whether an epigenetic mechanism, involving promoter hypermethylation of the gene for the DNA repair enzyme MGMT (O6-methylguanine DNA methyltransferase), was associated with K-RAS, TP53 and PTEN mutations in endometrial tumours from women treated with tamoxifen (TAM, $n = 30$) or unexposed to the drug (EC, $n = 38$).

Results: There were significant ($P < 0.05$) differences in tumour grade between the TAM and EC groups, with more favourable morphology in the latter. K-RAS mutations, predominantly G > A, occurred in small numbers in both groups. TP53 mutations were of mainly A > G, C > T and indel modifications in both groups, but more frequent in TAM cases. PTEN mutations dominated in EC tumours and were of the type that has large impact on protein function, such as indel or nonsense mutations. These observations alongside the mutational spectrum in PTEN suggest that the malignancies arise from different backgrounds, hence pointing to an effect of tamoxifen. Both groups displayed MGMT promoter hypermethylation. This coincided with mutations more frequently in the TAM (78%) than in the EC (50%) group, even though there were significantly ($P < 0.05$) fewer mutations and methylations in TAM cases.

Conclusions: Although the difference in coincidence did not reach significance with the current sample size, the findings suggest that epigenetic processes may play a role in the way tamoxifen induces endometrial cancer.

Endometrial cancer is the fourth most common cancer in women in industrialised countries, with many risk factors including the use of exogenous oestrogens and certain drugs, such as tamoxifen (Fisher *et al*, 1998). Tamoxifen is a selective oestrogen receptor modulator, which acts antagonistically in oestrogen-dependent breast

cancer by competing for the oestrogen receptor (ER), thereby forming a nuclear complex that recruits co-repressors to stop oestrogen-driven transcription. This decreases DNA synthesis and causes G₀/G₁ phase arrest of the cell cycle (Shang *et al*, 2000). In human endometrium, however, tamoxifen displays oestrogenic activity.

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Tamoxifen gives rise to DNA adducts and liver cancer in both male and female rats (Davis *et al*, 2000). In rats, the minor metabolite alpha-OH-tamoxifen is a substrate for hydroxysteroid sulphotransferase *a*, which leads to bioactivation in the liver, whereas in humans the major metabolite 4-OH-tamoxifen is conjugated by oestrogen sulphotransferases leading to detoxification (Chen *et al*, 2002). This interspecies difference in tamoxifen metabolism may partly explain why the compound is more toxic for rats compared to humans. Nonetheless, the potential neoplastic effects of tamoxifen in human endometrium have been largely unexplored at the molecular level.

In humans, a major circulating inactive form of plasma oestrogen is oestrogen sulphate (E_1S and E_2S) that can be activated by intra-tumoural steroid sulphatases, thereby exerting additional *in situ* hormonal activity in oestrogen-dependent breast cancers (Chetrite *et al*, 2000). Similar observations have been made in endometrial tumours displaying increased steroid sulphatase and lowered oestrogen sulphotransferase activity (Utsunomiya *et al*, 2004), and in endometrial cancer cells where tamoxifen was shown to perturb the balance of oestrogen-metabolising enzymes and disposition of oestrogen metabolites (Williams-Brown *et al*, 2011).

Metabolism of tamoxifen also involves formation of quinone metabolites, which can oxidise and alkylate cellular macromolecules *in vivo* (Zhang *et al*, 2000). O6-methylguanine DNA methyltransferase (MGMT) is responsible for repairing alkylation DNA damage and also has a possible role in inhibiting ER-mediated cell proliferation (Teo *et al*, 2001). In addition, certain polymorphic variants of MGMT may influence endometrial cancer risk (Han *et al*, 2006). Expression of MGMT is in part controlled through epigenetic processes such as hypermethylation of the promoter region of the gene, which can lead to inactivation of the repair pathway (Esteller *et al*, 1999), but evidence for the role of MGMT in endometrial cancer is conflicting (Furlan *et al*, 2006; Rimel *et al*, 2009).

K-RAS, PTEN and TP53 are often mutated in endometrial cancer, at frequencies that vary with tumour grade (Liu, 2007; Bansal *et al*, 2009). The aim of this project was to investigate associations between tamoxifen exposure, MGMT promoter hypermethylation and mutations in TP53, PTEN and K-RAS in endometrial tumours and tissue, in order to shed light on the carcinogenic mode of action of tamoxifen in humans.

MATERIALS AND METHODS

Patients. All samples were from cancer cases and subdivided according to the following criteria:

TAM ($n=30$): endometrial tumours from women who had taken tamoxifen; average age 69.4 ± 11.2 years; 10 with matching blood samples.

EC ($n=38$): endometrial tumours from patients not treated with tamoxifen; average age 67.0 ± 9.4 years; 24 with matching blood samples.

Treatment with tamoxifen ranged from 4 months to 20 years at 20 mg per day. In the majority of cases the tamoxifen use was restricted to approximately 60 months duration. Extended usage of tamoxifen was only in two cases with breast cancer recurrences.

The samples were from patients undergoing gynaecological surgery in the Royal Preston Hospital of Lancashire Teaching Hospitals NHS Foundation Trust in Preston between January 2006 and August 2012. Ethical committee approvals were obtained (Local Research Ethical Committee (LREC) approval no. 05/Q1302/83 and the Research and Ethics Committee (REC) approval no. 10/H0308/75). The consenting patients were between the ages of 37 and 90 years. Samples were formalin-fixed and paraffin-embedded (FFPE) and/or flash frozen (FF). Histopathology was

performed to determine the tumour type and grade. Shavings of the FFPE blocks and/or small pieces of the FF tissues, as well as frozen blood, were sent to King's College London for DNA isolation.

One patient in each group was found to have a silent germline mutation in TP53. These patients were excluded from the data compilation (Figures and Tables) and statistical calculation, although still included in Supplementary data.

Methylation specific-PCR analysis. DNA was extracted from triplicate shavings (10- μ m thickness) from tumour samples containing less than 10% normal tissue using a QIAamp DNA FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The extracted DNA was measured spectrophotometrically for purity and concentration, and 1 μ g (where possible) was bisulphite-treated using the Epitect Bisulfite Kit (Qiagen GmbH, Hilden, Germany). The bisulphite-converted DNA was amplified by PCR for the selected promoter region with the following primers synthesised in 25 nmol scale by Invitrogen (Carlsbad, CA, USA):

MGMT-MF: TTTCGACGTTTCGTAGGTTTTCG

MGMT-MR: GCACTCTTCCGAAAACGAAACG

MGMT-UF: TTTGTGTTTTGATGTTTGTAGGTTTTTGT

MGMT-UR: AACTCCACACTCTTCCAAAAACAAAACA

Primers were diluted in distilled water to 300 ng μ l⁻¹ and the following PCR mixture was prepared; 5 μ l of 10 \times PCR reaction buffer, 5 μ l of 50 mM MgCl₂ and 0.2 μ l Platinum Taq (1U; all from Invitrogen) was mixed with 5 μ l 10 mM dNTP-mix (Qiagen), 2 \times 1 μ l primer (forward and reverse), 10 μ l DNA template and 22.8 μ l of distilled water to a total volume of 50 μ l. Bisulphite-converted methylated and unmethylated human DNA, as well as non-converted unmethylated DNA that was converted along with the samples, and pure water, were included with each PCR analysis as positive or negative controls.

The thermocycler was programmed for a 5-min hot start at 95 °C, followed by 40 cycles of 94 °C for 40 s, 59 °C for 40 s and 72 °C for 40 s, and finally a 10-min elongation step at 72 °C. The PCR products were separated on 3% agarose at 140 V and visualised with EtBr. The bisulphite treatment and PCR amplification were carried out in triplicate; if a methylated band was observed at least two out of three times for a given sample, then it was deemed to be positive for methylation.

Sequencing. DNA was isolated from FFPE tissues as described above, and from FF tissues and blood using a DNeasy Blood & Tissue kit, (Qiagen). The purified DNA was measured for concentration and aliquots were portioned into 96-well plates and immediately frozen at -80 °C. Sequencing was performed by Beckman Coulter Genomics (United States) on K-RAS exon 1-4, PTEN exons 1-9 and TP53 exons 4-11.

Statistical analysis. Statistical analysis was performed using a two-tailed Z-test at the 95% confidence level by evaluating the proportions of given occurrences in the different patient groups (TAM and EC). Differences were statistically significant when $P \leq 0.05$.

RESULTS

Tumour histology and grading. Patients with tamoxifen-associated endometrial cancer (TAM) had significantly lower occurrence ($P \leq 0.05$) of type I tumours than those in the non-exposed (EC) group with 5 out of 29 (17%) and 17 out of 37 (46%), respectively (Table 1). TAM cases had more type II tumours of predominantly serous, carcinosarcoma and mixed carcinoma, whereas in the EC group these were mainly grade 3 endometrioid carcinomas followed by clear cell and carcinosarcoma (Table 1).

Table 1. Tumour histology and grading of morphologies found in the patient groups of tamoxifen and unexposed, categorised into Type I, Type II or others

Endometrial tumours	N	TAM 29	EC 37
TYPE I			
G1 endometrioid	15	3	12
G2 endometrioid	7	2	5
TYPE II			
G3 endometrioid	7	1	6
Uterine papillary serous carcinoma	7	5	2
Clear-cell endometrioid	4	1	3
Carcinosarcoma	14	8	6
Mixed carcinoma	8	5	3
OTHERS			
Simple hyperplasia	1	1	0
Complex atypical hyperplasia	1	1	0
Endometrial polyp	0	0	0
Endometrial polyp with stromal hyperplasia	1	1	0
Inactive endometrium	0	0	0
Menstrual endometrium (progestagen effect)	0	0	0
Proliferative	1	1	0
Secretory	0	0	0

Grouping together the endometrioid tumours of all grades (G1–G3), a significant ($P \leq 0.05$) overrepresentation of 23 out of 27 (62%) was found among the EC cases. There was also a significant difference ($P \leq 0.05$) between these two groups with respect to other diverse lesions, including hyperplasia, polyps, inactive, menstrual, proliferative and secretory endometrium, which were completely absent from the EC group, but represented about 4 out of 29 (14%) of the malignancies among the TAM cases.

MGMT promoter hypermethylation. Patients scored positive for promoter hypermethylation in both groups (Table 2), with TAM 9 out of 29 (31%) and EC 14 out of 37 (38%). There was no statistical difference between any of the groups and no correlation between the type or grade of tumours and *MGMT* promoter hypermethylation (Supplementary data).

Mutation analysis

Overall mutations. Counting the number of patients with mutations, regardless of type of gene or multiple mutations in the same gene, revealed no difference between the two tumour groups. There was a trend in higher occurrence of methylations or mutations in the EC as compared to the TAM group, which was significant ($P \leq 0.05$) when adding up patients with either of the modifications present. The reverse was observed in patients who exhibited both a mutation and methylation (Table 2). Thus, although not statistically verified at the 95th percentile, a closer look at how many patients with methylation also exhibited mutations showed that this occurrence was higher in the TAM compared with the EC group, with 7 of 9 (78%) and 7 of 14 (50%), respectively (Table 2).

Specific mutations. K-RAS mutations were found in both groups and were mainly of the missense type and distributed over codons 11–13 in exon 2; four in TAM and two in EC (Table 3). The mutations in the TAM group consisted of one G>T transversion and three G>A transitions. In the EC group, the K-RAS mutations were one G>A transition at codon 13 and a T>C transversion (Supplementary data).

Table 2. Percentage of *MGMT* promoter hypermethylation, mutation, both, either or neither in the TAM and EC groups

Groups	Total (n)	Methyl.	Mutation	Both	Either ^a	Neither ^a
TAM	29	31% (9 of 29)	52% (15 of 29)	24% (7 of 29)	62% (17 of 29)	41% (12 of 29)
EC	37	38% (14 of 37)	68% (25 of 37)	19% (7 of 37)	86% (32 of 37)	14% (5 of 37)

^aIndicates that there is a significant ($P < 0.05$) difference between the tamoxifen (TAM) and unexposed to the drug (EC) groups.

Table 3. Specific types and numbers of mutations found in the different groups

Groups	Gene	Spec mut.	Type of mut.
TAM	K-RAS	14% (4 of 29)	G>A (3 of 4) G>T (1 of 4)
	P53	34% (10 of 29)	A>G (2 of 10) A>T (1 of 10) C>T (2 of 10) G>A (1 of 10) G>T (1 of 10) Indel (3 of 10)
	PTEN	21% (6 of 29)	C>T (1 of 6) Indel (5 of 6)
EC	K-RAS	5% (2 of 37)	G>A (1 of 2) T>C (1 of 2)
	P53	38% (14 of 37)	A>G (3 of 14) C>T (6 of 14) G>A (1 of 14) T>A (1 of 14) Indel (3 of 14)
	PTEN	59% (22 of 37)	A>C (1 of 22) A>G (1 of 22) A>T (2 of 22) C>A (2 of 22) C>G (2 of 22) C>T (4 of 22) G>T (2 of 22) T>A (1 of 22) T>G (1 of 22) T>C (1 of 22) Indel (5 of 22)

Abbreviations: EC = unexposed to the drug; TAM = tamoxifen. The number of mutations is based on each individual mutation found in the patients, which includes multiple mutations in the same gene.

TP53 mutations occurred in both groups to a similar extent (34% in TAM and 38% in EC) and were of diverse types (Table 3). The tumour groups displayed mainly missense or indel alterations, with the addition of a few nonsense mutations in EC, and predominantly clustering within the central DNA-binding core domain within residues 102–292. Some of the missense mutations designated with SNP ascension numbers were found to be associated to Li-Fraumeni syndrome (see Discussion). Two patients, one in each group, had a germline silent mutation in *TP53* (Arg213Arg rs1800372), which was confirmed in corresponding blood samples (Supplementary data).

PTEN was extensively mutated in the EC group (Table 3) and found within varying grades and types of tumours and of the sort that has great impact on protein function; i.e., truncated via a stop-codon, or frameshift through either insertion or deletion. In fact, out of the 22 *PTEN* mutations observed in the EC group, 11 were either stop or indel (supplementary data). Only six patients from the TAM group displayed *PTEN* mutations, out of which five were indel and just one was a silent mutation (Table 3). Most alterations occurred in exon 5 (phosphatase domain and core motif), 7–8 and one mutation each in exons 3, 4, 6 and 9 (C-terminal region). No correlation to methylation status was found in any of the cases.

The mutation spectrum of the two tumour groups showed that the most commonly mutated gene following tamoxifen treatment was *TP53*, with A>G (27%), C>T (20%) and indel (30%) modifications (Figure 2). In the *PTEN* gene, indel was by far the most common alteration with 83%. The EC group displayed high degree of mutation in both *TP53* and *PTEN*. In *TP53*, the most prominent base-change was C>T (43%), followed by A>G (22%) and indel (21%), and occurred mainly in tumours of non-endometrioid character. In *PTEN*, C>T (18%) and indel (23%) dominated, and over half of all mutations were found in endometrioid tumours (Supplementary data).

Interpretation. Epidemiological studies have shown an increased risk of endometrial cancer with tamoxifen exposure, correlating risks ranging from 2.53 to 7.5 with length of exposure/treatment (van Leeuwen *et al*, 1994; Fisher *et al*, 1998). The mechanisms of action are yet to be fully evaluated; despite the demonstration of the DNA adduct forming capacity of metabolically activated tamoxifen in animals and in some experimental systems, there is conflicting evidence on the genotoxicity of tamoxifen in humans (Phillips, 2001; Kim *et al*, 2004; Singh *et al*, 2007, 2008). Here we aimed to investigate if there was a possible connection between tamoxifen treatment, promoter hypermethylation of *MGMT* and mutations in genes known to play a role in endometrial cancer.

Tumour histology and grading. High-grade malignancies were more frequent in the TAM group compared to EC, which consisted mainly of type I category malignancies of endometrioid character and G1–G2 morphology (Table 1), corroborating previous observations that women who underwent tamoxifen treatment for breast cancer and subsequently developed uterine cancers had higher-grade tumours with less favourable prognosis (Magriples *et al*, 1993; van Leeuwen *et al*, 1994). A possible bias towards high-grade tumours at medical centres that are better equipped for treating more severe cases is disputed based on the scarcity of the tamoxifen-exposed cases even with influx of large patient numbers, the endeavours of peripheral centres to report and collect all available samples, and that none of the patients opted out of the study despite the length of time. In addition, the medical histories of the patients were taken into consideration, where previous/adjuvant treatments in patients with breast cancer in the TAM group involved either radiotherapy or a combination of 5FU/epirubicin/cyclophosphamide. These therapies and this combination of drugs have not been reported in the literature to be associated with increased risk of endometrial cancer.

When assessing the occurrence of type I tumours, there was a statistically significant difference ($P \leq 0.05$) between the TAM and EC groups, which also held true when evaluating the occurrence of all endometrioid morphologies as a whole (G1–G3; Table 1). It is reported that the majority of endometrial cancers (70–80%), designated as type I carcinomas, follow the oestrogen-related pathway and seemingly arise in the background of hyperplastic endometrium with endometrioid differentiation (Liu, 2007). However, many endometrial carcinomas are overlapping in their clinical, morphologic, immunohistochemical and molecular features between types I and II. Lesions with various histologies including polyps, hyperplasia, late/mid-secretory and proliferative

endometrium, and progesterone effect were only observed in the TAM group. Many of these are normal features and are universal components of the menstrual cycle, although some forms of hyperplasia (especially complex atypical) can be premalignant. Polyps are, for instance, not precancerous, but still occur more commonly in women on tamoxifen (Biron-Shental *et al*, 2003).

MGMT promoter hypermethylation. There was no statistical difference between groups with regard to *MGMT* promoter hypermethylation, which was seen in 9 out of 29 (31%) and 14 out of 37 (38%) cases, in TAM and EC groups, respectively (Table 2). There is some support for this in the literature, but also some studies that contradict our finding. Rimel *et al* (2009) found an absence of *MGMT* promoter hypermethylation in 120 endometrial cancer cases, which was also supported by previous findings in a small number ($n=17$) of endometrial cancers (Jacinto and Esteller, 2007). On the other hand, in a study involving synchronous ovarian and endometrial primary tumours there was a similar rate of *MGMT* promoter hypermethylation to what we report here (Furlan *et al*, 2006). One aspect to consider is methodology where it can be argued that studies finding positive methylation were done with MS-PCR, which may increase sensitivity and methylation-positive cases with increasing cycles of amplification (Shen *et al*, 2005). However, different methodologies (MS-PCR and/or combined bisulphite restriction analysis) have been used on various DNA sources, often in parallel, and results were found to be consistent (Xiong and Laird, 1997; Shen *et al*, 2005; Cheng *et al*, 2010). In addition, primer design needs to meet certain criteria (Herman *et al*, 1996; Brandes *et al*, 2007) and proper controls need to be added in the analysis to avoid problems of false-positives. Hence, we optimised the melting temperature with gradient PCR, using both converted and non-converted DNA and water to minimise false-positives and these controls were included in every amplification step from then on (Figure 1B). The quality of the DNA does not explain discordance with the literature, as methylation was also observed in both groups and no difference in the extent of methylation was seen between FFPE or flash-frozen tissues. When comparing methylation to tumour type there were no significant correlations.

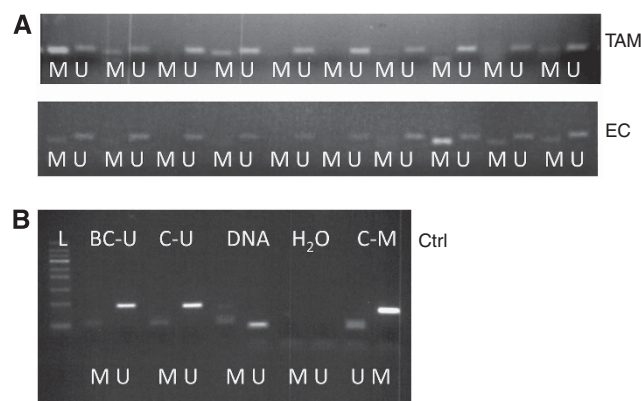


Figure 1. Examples of MS-PCR analysis (A; showing the first lane of gels, including 10 patient samples of each group). 'M' represents methylated bands and 'U' unmethylated. Controls (B) were used at each analysis with BC-U being unmethylated human DNA that was bisulphite converted during sample workup procedures to assess the success of the conversion step. 'C-U' and 'C-M' were commercially available bisulphite converted unmethylated and methylated human DNA, respectively. 'DNA' represents non-converted human DNA. The H₂O samples were lacking DNA of any sort in the PCR mixture. Note that close to the bottom line of the ladder corresponding to about 50 bp, bands can be seen. These are primer dimers. The product bands for both 'M' and 'U' are around 100 bp with only minor differences.

In a study by Turbinder *et al* (2008), *K-RAS* mutations appeared to be more common among endometrial carcinomas in patients with history of tamoxifen exposure (21%) compared with informative endometrial carcinomas (11%), and were comparably low in both tumour-adjacent groups, although the difference was not statistically significant. Previous reports also found that endometrial polyps in patients treated with tamoxifen had a significantly higher frequency of *K-RAS* mutations compared with sporadic endometrial hyperplasia (Hachisuga *et al*, 2003) and treatment with tamoxifen or toremifene gave rise to *de novo* *K-RAS* mutation in a seemingly normal endometrial tissue (Wallen *et al*, 2005). Accordingly, our study showed *K-RAS* mutations in 4 out of 20 (20%) mutations among TAM patients, and 2 out of 38 (5%) in the EC cases.

Only six patients with *PTEN* alterations were from the TAM group with five indel and a silent mutation (Table 3). The difference in the number of patients with *PTEN* mutation between the TAM and EC groups was statistically significant with $P \leq 0.05$ at the 95% confidence level. This is in agreement with a similar observation in the literature, where *PTEN* mutations occurred less frequently in the tamoxifen-treated patients, although not statistically significant due to that cohort size (Turbiner *et al*, 2008).

According to the literature *TP53* mutations in endometrial cancer are more abundant in high-grade tumours with poor prognosis that are often oestrogen independent (Lax *et al*, 2000; Bansal *et al*, 2009). In our study, most of the *TP53* mutations occur in the serous, clear-cell or serous/clear-cell tumours in the TAM group. In exon 7, two missense mutations (Arg273His rs28934576 and Arg282Trp rs28934574) were found associated with Li-Fraumeni syndrome (Malkin *et al*, 1992; Toguchida *et al*, 1992). Only three tumours were of other morphology; i.e., adenocarcinoma, carcinosarcoma and sarcoma with high-grade FIGO 1B. In the EC group, a similar pattern emerged, where the majority of *TP53* mutations clustered in serous, clear-cell or serous/clear-cell tumours and only three were in either carcinosarcoma or endometrioid

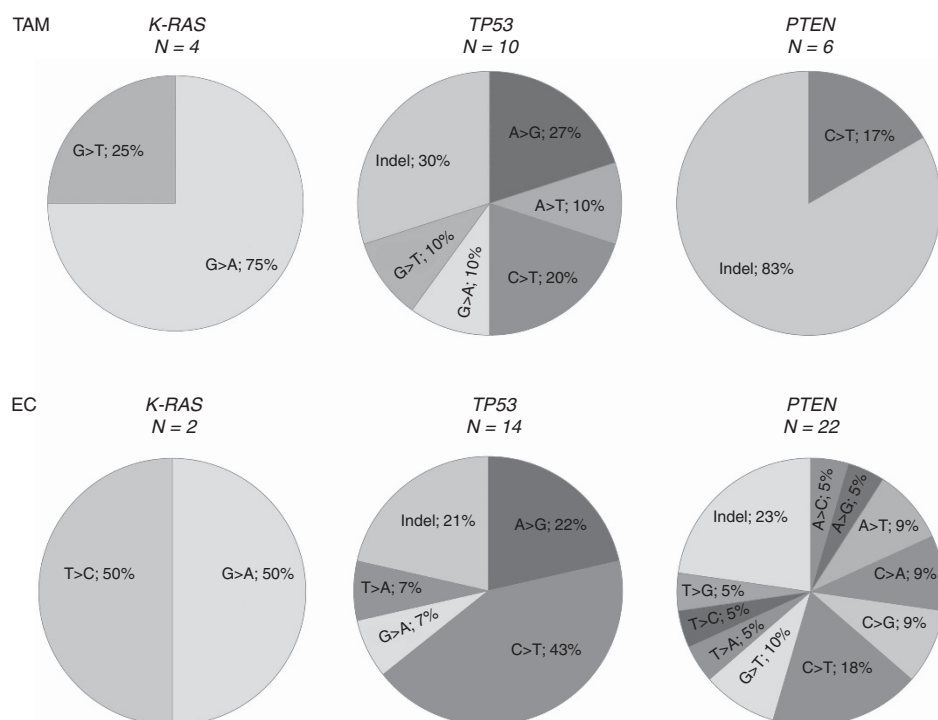


Figure 2. Mutational spectrum of *K-RAS*, *TP53* and *PTEN* in tumour tissues from TAM and EC patients.

tumours. In the EC group 38% of the mutations were found in *TP53*, with a mix of missense, homindel and stop mutations. In exon 6 a missense mutation was found with SNP accession number rs28934575, also present in Li-Fraumeni syndrome (Toguchida *et al*, 1992).

It has been reported that *TP53* mutations do not concur with either *PTEN* (Koul *et al*, 2002) or *K-RAS* (Lax *et al*, 2000) mutations in endometrial cancer, but rather with loss of p16. This is in part what has been observed in this study, as virtually none of the mutations in either of the genes coincided. However, it has been shown that *TP53* gene mutations occur in some endometrioid endometrial cancers in the presence of *PTEN* mutations, suggesting that both these genes participate in the development of these tumours (Janiec-Jankowska *et al*, 2010).

Our study supports in part some observations made by Prasad *et al* (2005), that endometrial tumours from these two groups contain similar genetic alterations with regard to the type of gene mutations. However, the mutational spectrum within some of the genes is clearly different (Figure 2). Although not much can be said for *K-RAS* due to the low occurrence, and the similarities in *TP53*, *PTEN* mutations are quite different between tamoxifen-exposed and non-exposed cases, suggesting the malignancies arise from different backgrounds, hence pointing to an effect of tamoxifen.

We have also observed an interesting coincidence between methylation and mutation, which was regardless of tumour morphology or genes affected (Table 2). This coincidence was between 7 of 9 (78%) and 7 of 14 (50%) in TAM and EC, respectively. The higher occurrence of this in TAM, despite the significantly lower levels of either alterations in this group, suggests that there may be an effect of tamoxifen exposure, although a larger number of cases would be needed to assess this fully. This suggests that tamoxifen exposure, apart from hormonal effects, possible adduct forming capacity, may also be driving malignancy through epigenetic processes.

CONCLUSION

Patients receiving tamoxifen developed more high-grade tumours with less favourable prognosis. Genes commonly mutated were *TP53* and *PTEN*, with *TP53* being the predominant one in the TAM cases and *PTEN* in the EC group. We also confirmed the presence of *MGMT* promoter hypermethylation and, in addition, a coincidence between methylation and mutation in both groups. The occurrence of this, however, was higher in the TAM group, although not significantly. Our findings suggest that hypermethylation of *MGMT* promoter may play an epigenetic role in the induction of endometrial cancer by tamoxifen.

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