

Evaluation of endometrial changes and p53 expression in tamoxifen treated women: Comparison of various methods

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Abstract

Objective: To compare transvaginal sonography (TVS), sonohysterography (SHG), hysteroscopy and endometrial aspiration (EA) and p53 expression in assessing endometrial abnormalities in women on tamoxifen.

Methods: In a cross sectional study of 50 pre- and post-menopausal women receiving tamoxifen for > 2 years, all participants underwent TVS and EA. Those with endometrial thickness > 4 mm on TVS underwent hysteroscopy and SHG. Serum p53 antibody and p53 immunohistochemistry were tested in all women.

Results: The sensitivity and specificity when compared with histopathology as the reference standard were as follows: TVS 100% and 33.3%, SHG 85.7% and 50%, hysteroscopy 92.8% and 80.8%, serum p53 50% and 83.3%, and p53 immunohistochemistry 57.1% and 61.1%. Prevalence of endometrial abnormalities was not significantly different in asymptomatic and symptomatic women.

Conclusion: Tamoxifen-users require routine testing for endometrial evaluation. TVS followed by hysteroscopy and biopsy is an effective option. p53 expression correlates with histological abnormalities.

Key words: Tamoxifen, Sonography, Sonohysterography, Hysteroscopy, Endometrium, p53

Breast cancer is the commonest malignancy in women worldwide and the second most common cause of cancer-related deaths in females¹. In India, it ranks second after cancer of the uterine cervix. The incidence has increased from 19 new cases per 100,000 population in 1995 to 23 new cases per 100,000 population in 1998². Tamoxifen, a non-steroidal anti-estrogen, has been widely used as an adjuvant cancer chemotherapy drug in the management of patients with breast cancer since the 1970s. Long-term tamoxifen at a dose of 20 mg/day has been associated with a six-fold higher risk of developing endometrial cancer³. In a recent study, the incidence of other endometrial abnormalities in 700 tamoxifen-treated women were as follows: polyps 23.1%; glandular hyperplasia 8%; and atypical hyperplasia 3%⁴. It appears, therefore, that women on long-term tamoxifen therapy may benefit from routine evaluation for early detection of endometrial abnormalities. Several modalities have been used for this purpose. Transvaginal ultrasonography (TVS) is superior to transabdominal scanning in the evaluation of endometrial abnormalities⁵. However, it is not sensitive in discriminating endometrial pathology when the endometrial thickness is more than 4 mm. Sonohysterography (SHG) or saline infusion sonography can offer additional information regarding the presence of endometrial polyps and other endometrial abnormalities like hyperplasia or

malignancy⁶. Hysteroscopy enables visualization of the entire cavity directly but is more invasive than TVS and SHG⁷. Histopathological evaluation of endometrium is the gold standard for diagnosis, but endometrial sampling is often inadequate in tamoxifen-treated women⁸.

Tamoxifen-induced endometrial carcinogenesis could be due to its capacity to induce DNA adducts in the endometrium and is independent of its action as an estrogen agonist^{9,10}. Studies on molecular alterations involved in pathogenesis of endometrial cancer have revealed mutations in the p53 tumour suppressor gene and accumulation of p53 protein has been detected in approximately 90% serous carcinoma and endometrial intraepithelial carcinomas^{11,12}.

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In response to p53 protein accumulation in cells, presence of circulating p53 antibodies has been observed in various cancers including gynaecological malignancies and they appear even before the clinical detection of disease¹³. The present study was conducted to determine the prevalence of endometrial abnormalities in women with breast cancer who had received tamoxifen for 2 or more years, and to compare TVS, SHG and hysteroscopy for the diagnosis of endometrial abnormalities, taking histopathology as the gold standard. The expression of p53 protein in endometrial aspirates and serum p53 antibodies were also studied in relation to the above-mentioned tests, to determine whether these tests can be used to predict the occurrence of early endometrial abnormalities in tamoxifen-treated women.

Materials and methods

This hospital based cross-sectional study was conducted in the Department of Obstetrics and Gynaecology of the All India Institute of Medical Sciences, New Delhi from April 1999 through April 2001. Fifty breast cancer patients who had received tamoxifen for 2 or more years were invited to participate in the study. Exclusion criteria were as follows: patients with previously diagnosed endometrial cancer and hyperplasia; evidence of significant haematological, renal or hepatic impairment; hormone replacement therapy (HRT) or any chemotherapy other than tamoxifen during the study period. Informed written consent was taken from all patients. Detailed history and general physical examination including bimanual pelvic examination was carried out. All patients underwent TVS using colour Doppler ultrasound machine with vaginal probe of high frequency 5 MHz (Siemens SI 400). The thickness of the endometrium was measured with electronic callipers in the longitudinal plane between the two basal layers of the anterior and posterior uterine walls (double layer thickness). Pre-menopausal patients underwent TVS in the postmenstrual phase. Patients with endometrial thickness of > 4 mm underwent SHG, hysteroscopy and endometrial aspiration. Where the endometrial thickness was ≤ 4mm, endometrial aspiration was carried out directly.

Procedure of SHG: An 8 or 12 French Foley's catheter was introduced into the uterine cavity under aseptic precautions. The balloon was inflated with 2-3 ml of sterile saline and withdrawn to the level of the internal os. The TVS probe was inserted into the vagina, and up to 50 ml of sterile saline solution was slowly instilled under direct sonographic visualization. Findings were considered 'normal'

when, after saline instillation, the cavity was seen distended, the endometrial lining regular and smooth without any bulge or protrusion into the cavity, and no subendometrial echolucency.

Procedure of hysteroscopy: Hysteroscopy was carried out in an operation theatre under sedation and paracervical block with 1% lignocaine solution. The cavity was distended with normal saline. Findings were considered 'normal' when the endometrium was smooth, both ostia seen and no areas of abnormal vascular, or growth.

Endometrial aspiration: Endometrial aspiration was done in all cases. Tissue obtained by endometrial aspirate was sent for histopathologic examination and immunohistochemical analysis in 10% buffered formalin. Dilatation and curettage (D&C) was done in cases where the endometrial sampling was inadequate.

p53 analysis: Five ml venous blood was collected from each patient for p53 ELISA test. Serum was separated and stored at -20°C until it was processed.

p53 immunohistochemical analysis: 5-7 µm thick paraffin embedded sections were fixed in acetone. Endogenous peroxidase activity was blocked with 0.3% (w/v) hydrogen peroxide for 20 minutes. Non-specific binding sites were blocked by incubating the sections with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hour. The sections were then incubated with primary antibody overnight at 4°C. Mouse monoclonal antibody (1µg/ml) DO1 was used for detecting p53 protein. The primary antibody was detected using biotinylated secondary anti-mouse IgG antibody, avidin-biotin complex and 3,3' diaminobenzidine tetrachloride as a chromogen. The intensity of immunohistochemical staining was evaluated in five areas of the section for correlation and confirmation of the tissue analysis. The p53 positive cases were evaluated semiquantitatively on a four-point scale based on the proportion of cells showing p53 staining: negative = <10%, +1 = 10-30%, +2 = 30-50%, +3 = ≥ 50%.

Circulating p53 antibodies: Recombinant baculovirus expressed p53 protein in SF9 insect cells was purified and used as antigen for detection of anti-p53 antibodies (p53-Abs). Briefly, immunopurified human p53 antigen (100 ng/well) or BSA (100ng/well) was coated in wells of 96-well microtitre plates. The plates were incubated for 16-18 hours. Non-specific binding was blocked by incubation with 3% (w/v) BSA for 1 hour, followed by incubation for 1 hour with patient's serum/normal serum (1:100 dilution in PBS). Antihuman IgG conjugated to Horse Radish Peroxidase (Dako A/C,

Copenhagen, Denmark) (1:5000 dilution in PBS) was subsequently added to the wells and allowed to incubate for 45 min. The colour was developed using the substrate ortho-phenylene diamine (0.5 mg/ml) in citrate phosphate buffer (0.1 M, pH 5.0). The reaction was stopped after adding 5N sulphuric acid and absorbance was measured at 492 nm. Appropriate controls for antigen, antibodies and horse radish peroxidase conjugate were used to exclude any non-specific background in patient samples. A known serum sample was used as an internal control in each batch of assay to take into account interassay variations. Each serum sample was assayed twice in triplicates using p53 antigen as well as BSA. Mean absorbance of all six observations (wells) was calculated. BSA was added as the irrelevant antigen to obtain a clear distinction between p53- specific binding and possible background binding to each of the tested serum samples. Sera were assayed a minimum of twice. A positive assay required a mean p53: BSA ratio of ≥ 1.5 .

Statistical analysis

Descriptive statistics were used to describe the prevalence of the abnormal findings. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for each test. Endometrial thickness of >4 mm on TVS, presence of polyps and subendometrial cysts on SHG and hysteroscopy were taken as the cut-off for defining abnormal findings. The gold standard was histopathology, and diagnoses of polyps or endometrial hyperplasia or cancer were taken as abnormal findings. Categorical data were compared using Fisher's exact test. P value < 0.05 was considered statistically significant. The data was entered in MS "Excel" and analyzed with STATA 8.0 software packages.

Results

Mean age (SD) of the study population was 51.3 (10.8) years, with a range of 28-70 years. Twelve (24%) women were pre-menopausal, and 38 (76%) were postmenopausal. Seventeen (34%) patients had a family history of cancer. Thirty-five (70%) women had Stage II A and 10 (20%) women stage II B breast cancer. All patients underwent primary surgery, i.e., modified radical mastectomy, after which 25 patients (50%) received adjuvant chemotherapy, radiotherapy or combined therapy.

After radical mastectomy all patients received oral tamoxifen, 20 mg daily, for a period varying from 24-

84 months (mean 49.2 months). The majority of patients (n=38, 76%) were asymptomatic at the time of recruitment. Among the symptomatic women (n=12), post-menopausal bleeding was present in 10 (20%) and polymenorrhagia in 2 (4%) women.

Transvaginal Sonography: Mean endometrial thickness was 10.3 mm (range 1.9-23.3 mm). Out of 12 pre-menopausal women, 7 (58.3%) had an endometrial thickness of >4 mm as compared to 31/38 (81.5%) post-menopausal women receiving tamoxifen.

Sonohysterography (SHG): As per protocol, SHG was performed in 38 patients whose endometrial thickness was > 4 mm on TVS. Table 1 shows the findings on SHG in relation to endometrial thickness on TVS. There were no post-procedure complications in these women.

Hysteroscopy: Diagnostic hysteroscopy was performed in 38 patients: 16 underwent the procedure on the same day as of the SHG; in 22 women the procedure was carried out after 1 week. Table 1 shows hysteroscopic findings in relation to varying endometrial thickness on TVS.

Histopathology: The distribution of histopathological findings in relation to menopausal status and symptoms of the patient is presented in Table 2. All three cases of endometrial hyperplasia had simple hyperplasia without atypia. There was significant difference in the histopathology findings between pre-menopausal (n=12) and post-menopausal women (n=38) women, as all 3 cases each of endometrial hyperplasia and endocervical polyp and seven of the eight endometrial polyps were seen in post-menopausal women. Amongst the 12 symptomatic women, 7 (58.5%) had abnormal endometrial findings. However, 7 out of 38 asymptomatic women (20%) too had abnormal findings on histopathology (Table 2).

p53 estimation: On immunohistochemistry p53 protein was found to be over-expressed in 22/50 cases. The semi-quantitative assessment was as follows: Negative-28 (56%); + - 17 (34%); ++ - 4 (8%); +++ - 1 (2%). Out of these 22 cases, 13 were positive for circulating anti-p53 antibodies. Test characteristics of TVS, SHG and hysteroscopy, serum p53 and p53 immunohistochemistry in relation to histopathology are shown in Table 3.

Table 1: Findings on Hysteroscopy and Sonohysterography in relation to endometrial thickness on Transvaginal Sonography (TVS)

Test Results	Endometrial thickness on TVS			Total % (n=38)
	5-8 mm	9-12 mm	>12 mm	
Hysteroscopy				
Normal	6	5	4	15 (39.4%)
Atrophic	1	2	3	6 (16%)
Polyp	1	2	10	13 (34.2%)
SEC*	-	1	2	3 (8%)
Endocervical Polyp	-	1	-	1 (2.7%)
Sonohysterography				
Normal	4	3	1	8 (20.6%)
Polyp	1	2	5	8 (20.6%)
SEC	4	3	7	14 (37%)
Polyp + SEC	-	1	6	7 (18.5%)
Endocervical Polyp	-	1	-	1 (2.7%)

Table 2: Endometrial abnormalities in relation to symptoms and menstrual status

Histopathology	Asym* (n=38)	Sym* (n=12)	p-value	Premens (n=12)	Postmen (n=38)	p-value
Atrophic	24	2	0.007	2	24	0.007
Proliferative	4	-	0.560	4	-	0.002
Secretory	3	3	0.141	5	-	0.0001
Endomet. Polyp**	5	3	0.397	1	7	0.661
EH**	1	2	0.139	-	3	1.00
Ecp**	1	2	0.139	-	3	1.00

* Asym=Asymptomatic; Sym=Symptomatic;

** Endomet. Polyp = Endometrial polyp; EH = endometrial hyperplasia;
Ecp = Endocervical polyp

Table 3: Test characteristics of Transvaginal Sonography, Sonohysterography, hysteroscopy and p53 in relation to the reference standard (Endometrial histopathology)

	Histopathology								
	Pos	Neg	TP [†]	TN [†]	Sens [‡]	Spec [‡]	PPV [‡]	NPV [‡]	DA [‡]
Transvagial Sonography	14	36	14	11	100	33.3 (18.5 – 50.9)	36.8 (21.8 – 54.2)	100	52.0 (37.4 – 66.3)
Sonohystero-graphy	14	36	12	18	85.7 (57.1 – 98.2)	50.0 (32.9– 67.0)	40.0 (22.6– 59.3)	90.0 (68.3– 98.7)	60.0 (45.1 – 73.1)
Hysteroscopy	14	36	13	32	92.8 (66.1 – 99.8)	80.8 (73.9 – 96.9)	76.4 (50.1 – 93.2)	90.2 (84.2– 98.9)	90.0 (78.1 – 96.7)
p53 immuno-histochemistry	14	36	8	22	57.1 (28.9 – 82.3)	61.1 (43.4 – 76.8)	36.3 (17.2 – 59.3)	78.6 (59.0– 91.7)	60.0 (45.2 – 73.5)
p53 serum antibody	14	36	7	30	50.0 (66.1 – 99.8)	83.3 (73.9 – 96.9)	53.8 (50.1 – 93.2)	81.1 (84.2– 98.9)	74 (78.1 – 96.7)

* Values in brackets are 95% confidence intervals

[†] TP= true positive, TN = true negative, FP =false positive, FN= false negative

[‡] Sens (sensitivity)= (TP/ TP+FN) x 100

Spec (specificity)= (TN/TN+FP) x 100

PPV (positive predictive value) = (TP/ TP+FP) x 100

NPV (negative predictive value) = (TN/TN+FN) x 100

DA (diagnostic accuracy) = (TP+TN/ TP+FP+TN+FN) x 100

Discussion

Tamoxifen-induced abnormalities of the endometrium are of great concern in the light of the ever-increasing number of women taking this drug or contemplating its use. Overall endometrial pathologies have been histologically diagnosed in 21 to 39% of asymptomatic postmenopausal breast cancer patients who were treated with tamoxifen: simple hyperplasia in 2.1-11.6%; atypical hyperplasia in 2-16%; endometrial polyps in 4.1-8%; and endometrial cancer in 1.3-3.2%^{14,15,16,17}. In the present study, out of 50 women, 8 (16%) patients had tamoxifen associated endometrial polyps, 3 (6%) patients had endocervical polyp and 3 (6%) patients had endometrial hyperplasia without atypia. No cases of endometrial carcinoma were reported in this study although we have previously encountered cases of endometrial carcinoma following tamoxifen treatment. The small sample size could have been responsible for this, coupled with the fact that the majority (76%) of cases were asymptomatic. Mourtis et al reported that as many as 95% patients on tamoxifen may be asymptomatic¹⁸.

It has been generally observed that patients presenting with abnormal uterine bleeding more likely to have endometrial abnormalities than asymptomatic patients¹⁹. Although we did not find any case of endometrial cancer, there was significant difference between the distribution of endometrial polyp, endometrial hyperplasia and endocervical polyp in gynaecologically symptomatic and asymptomatic group (Table 2). The risk of endometrial abnormalities increases as treatment continues for longer than 2 years¹⁷. In the present study, out of 14 abnormal histopathological diagnoses, 6 were found with tamoxifen use of < 48 months and 8 after 48 months. However, 2 of the 3 endometrial hyperplasias occurred after 5 years of tamoxifen use.

Since the majority of patients on tamoxifen therapy are asymptomatic, the procedure for monitoring these patients has been the subject of debate. Measurement of endometrial thickness by TVS has been shown to be 100% sensitive in detection of endometrial pathology in these women using a cut-off of 4 mm and above^{18,19}. However, the drawback of TVS as a screening tool is its high false positivity rate, which has been reported to be as high as 46%⁸. Similar findings were found in the present study. If a higher cut-off of 8 mm had been used, as suggested by Cohen et al²⁰, one case of endometrial polyp would have been missed, in which the endometrial thickness was 4.4mm. The apparent endometrial thickness seems to be directly proportional to the duration of

tamoxifen treatment and does not necessarily indicate serious endometrial pathology²⁰. TVS frequently fails to differentiate between endometrial hyperplasia and polyps²⁰. In our study, all the cases of endometrial hyperplasia on histopathology had an endometrial thickness of >8mm, but there were 13 cases, where atrophic endometrium was found on histopathology despite the endometrial thickness being > 8 mm on TVS. This is a common feature in tamoxifen-users who develop thick, irregular endometrium containing cystic areas, which is the effect of tamoxifen on the myometrium rather than the endometrium²⁰. Interestingly, in the present study subendometrial cysts could be identified in 18 patients on TVS as well. There was a learning curve in this respect, and after doing a few cases of SHG, identification of these cysts on TVS also improved.

Sonohysterography increases the sensitivity for detection of intraluminal masses such as polyps and enhances the differentiation between space-occupying lesions in the endometrial cavity such as endometrial polyps, subendometrial cysts (SECs) and other endometrial pathology²¹. However, as a primary screening modality in asymptomatic women, it has a limited role due to high false positive rate²². When compared with hysteroscopy, overall there was a good correlation but subendometrial cystic spaces were better seen on SHG. Only 6 out of the 13 polyps diagnosed by hysteroscopy could be picked up by SHG. On the other hand, out of 14 SECs diagnosed by SHG, 7 (50%) were normal and 5 (35.7%) were interpreted as atrophic on hysteroscopy.

Hysteroscopy allows direct visualization of the endometrial cavity. Hysteroscopic visual impressions showed a high concordance with the histological findings in our study. All women with atrophic endometrium (n=6/38) on hysteroscopy had atrophic endometrium on histology. Conversely, 12 out of 13 patients with a diagnosis of polyp on hysteroscopy had an abnormal histopathology report, one polyp being reported as atrophic endometrium on histopathology. Endometrial and endocervical polyps were seen more clearly on hysteroscopy than on TVS or SHG. However, it is an uncomfortable and relatively invasive technique that requires specialized equipment, making it an inappropriate screening tool^{7,23}.

Although histopathology is regarded as the gold standard in diagnosis of endometrial abnormalities there is often inadequacy in obtaining endometrial samples in tamoxifen users. Love et al reported that it is difficult to perform biopsy on tamoxifen-treated

endometrium even in the presence of apparent hysteroscopic abnormalities, and despite using various biopsy instruments, they were often unsuccessful in obtaining material for histology⁸. In the present study also, 15 out of 50 patients had to undergo D&C, due to very scanty or no tissue being obtained on endometrial aspiration. Even on D&C, scanty material was obtained, which was reported to be atrophic endometrium.

Positive immunostaining is usually indicative of abnormalities of the p53 gene and its product. Pisani et al reported p53 expression in 30% of invasive adenocarcinoma in contrast to 12% of cases of simple hyperplasia with atypia and in 14% cases of complex hyperplasia with atypia²⁴. In our study we found p53 protein over-expression in endometrial aspirates of 22 (42%) patients. However, due to a small sample size we did not find any correlation between histopathological findings and p53 protein expression and additional studies are necessary before this can be clearly established.

Circulating p53 antibodies in the sera can be yet another strategy for assessing p53 aberrations in several cancers including endometrial malignancies^{13,24}. However, it has not been studied in tamoxifen-induced endometrial pathology. We found significant correlation between histopathology and p53-Abs in the serum. Five of 13 patients showing increased levels of antibody titers had endometrial polyps and 3 had endometrial hyperplasia. Thus by comparing the clinicopathological features of a patient to p53-Ab sera analysis, early identification of high-risk groups undergoing tamoxifen therapy is possible.

The most important point of concern is whether asymptomatic women should be screened or not^{8,20,25}. In our study, 20% of asymptomatic women had abnormal endometrium on histology. Besides, 20 out of 30 abnormal SHG findings and 13 of 17 abnormal hysteroscopic findings were found in asymptomatic women. However, there was not a single case of endometrial cancer. Therefore, it appears reasonable to recommend that women on tamoxifen, both asymptomatic and symptomatic, should be screened for endometrial abnormalities. TVS should be the initial choice and women with increased endometrial thickness be subjected to further testing. The diagnostic accuracy of hysteroscopy and sonohysterography depends on the experience and skill of the operator. Any abnormalities in the above tests should prompt histopathological confirmation. Each centre should thus plan its own diagnostic

work-up according to the expertise and facilities available.

Conclusion

Asymptomatic women on tamoxifen require routine testing for endometrial evaluation. TVS followed by hysteroscopy and biopsy is an effective option. p53 expression correlates with histopathology

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