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Loss of PTEN Expression in Breast Cancers

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Background : PTEN, located on chromosome 10q23.31, is a novel tumor suppressor gene. In the sporadic breast cancers, the incidence of the loss of heterozygosity of PTEN is approximately 10% to 40%, but the incidence of intragenic mutation of PTEN is less than 1%. To assess the role of the PTEN in the invasive ductal breast cancer, we studied the frequency of the loss of PTEN expression, its correlation with the commonly used prognostic factors of the breast cancer and with PTEN promoter hypermethylation status. **Methods :** Immunohistochemical staining with an anti-PTEN protein antibody was performed on the paraffin-embedded breast tissues from 129 women with a diagnosis of invasive ductal carcinoma. Methylation specific PCR was performed to detect hypermethylation in the PTEN gene on the 28 cases with the loss of PTEN expression. **Results :** Sixty-two (48%) of 129 breast tumors had the loss of PTEN expression. The loss of PTEN expression was correlated with lymph node metastasis and stage, and there was a near-significant correlation with the tumor size. PTEN promoter hypermethylation was found in five (18%) out of 28 patients. **Conclusion :** These results suggest that the loss of PTEN expression might play a role in the progression of the breast cancer and that the aberrant promoter methylation is one of the silencing mechanisms of PTEN.

Key Words : PTEN; Invasive ductal carcinoma; Breast; Promoter hypermethylation

PTEN (phosphatase and tensin homologue deleted in chromosome ten), is located on chromosome 10q23.31, and it is a novel tumor suppressor gene.[1] PTEN encodes a dual specificity phosphatase that has recently been reported to dephosphorylate focal adhesion kinase and to inhibit cell migration, spreading, and focal adhesion formation.[2] Moreover, PTEN plays an important role in modulating the 1-phosphatidylinositol 3-kinase pathway, and this pathway is involved in cell proliferation and survival.[3] Germ-line PTEN mutations have been identified in Cowden's disease, an autosomal dominant disorder associated with multiple mucocutaneous hamartomas and it has a predisposition to thyroid and breast tumors.[4] Somatic mutations in PTEN have been identified at a high frequency in many malignant tumors, including advanced glial tumors, prostate, endometrial, thyroid, ovarian and bladder carcinomas, and malignant melanoma.[1,5-7]

In sporadic breast cancers, although the incidence of the loss of heterozygosity (LOH) of PTEN is approximately 10 to 40%, intragenic mutations were observed at a frequency of less than 1%.[8-10] Therefore, PTEN promoter hypermethylation has been suggested to be one of the mechanisms of PTEN gene inactivation. Promoter hypermethylation of the tumor suppressor genes has been observed in a wide range of sporadic cancers, and this appears to be an alternative mechanism of gene inactivation in addition to mutation and deletion.[11] Methylation specific PCR (MSP) has been used to identify promoter hypermethylation because of the ease of detecting the methylation status in paraffin embedded tissue, and also because of the procedure's high sensitivity and the absence of false positivities resulting from incomplete restriction enzyme digestion.[12]

To assess the role of the PTEN gene in invasive ductal breast cancers, we studied the frequency of the loss of PTEN expression, its correlation with the commonly used prognostic factors of breast cancer and with PTEN promoter hypermethylation status.

MATERIALS AND METHODS

Tumor samples

Paraffin blocks from 129 cases of invasive ductal carcinoma were retrieved from the pathology fields at the Ewha Womans University Medical Center between 1995 and 2001. The tumor stages (AJCC/UICC TNM Classification and Stage grouping) were determined by reviewing the medical records.[13]

Clinicopathologic factors that were analyzed

The immunohistochemical and methylation results were correlated with age, tumor size, status of the lymph node, stage, nuclear grade and histologic grade, and the expression of estrogen receptor (ER), progesterone receptor (PR), c-erbB-2, and p53.

Immunohistochemistry

PTEN (monoclonal mouse anti-human PTEN [A2B1], Santa Cruz Biotech, Santa Cruz, CA) immunohistochemical staining was performed with an indirect biotin avidin system on the 4 μm formalin-fixed, paraffin-embedded sections that were made from a representative block for each case.[13] The slides were deparaffinized with using xylene and graded ethyl alcohol, and then they were rinsed in water. Antigen retrieval was performed by boiling the slides in 0.01 mol/L sodium citrate buffer, pH 6.0 in a microwave oven at 98℃ for 15 min. The sections were incubated in 0.3% hydrogen peroxide for 30 min to block the endogenous peroxidase activity. The slides were incubated with anti-PTEN antibody (dilution 1:40) overnight in a humidified chamber at 4℃. The slides were subsequently incubated with a biotinylated universal secondary antibody and with an avidin horseradish peroxidase label. After color development was done with 3-amino-9-ethylcarbazole, the slides were counterstained with hematoxylin. The adjacent benign tissue and the fibrocystic disease tissue were used as positive controls. The immunostaining was graded as either negative (-) or positive (+). The group showing less than 5% tumor staining was assessed as negative and this was considered to indicate PTEN protein loss.

Table 1. PCR primers used for methylation specific PCR

Primer set	Primer
PTEN-M	5′-TTCGTTCGTCGTCGTCGTATTT-3′ 5′-GCCGCTTAACTCTAAACCGCAACCG-3′
PTEN-U	5′-GTGTTGGTGGAGGTAGTTGTTT-3′ 5′-ACCACTTAACTCTAAACCACAACCA-3′

Primers are the same as reported by Salvesen *et al.*[21]

Methylation specific PCR

We performed methylation specific PCR on 28 paraffin-embedded tumor samples that had PTEN protein loss and adequate conservation of the DNA. The DNA was isolated by digestion from paraffin-embedded tumors with proteinase K in sodium chloride TrisEDTA and 0.5% SDS and this was followed by a standard phenol-chloroform extraction and ethanol precipitation. The methylation status of the PTEN gene in the tumors was determined by methylation-specific PCR, as described by Herman *et al.*[12] Briefly, the genomic tumor DNA was modified by treatment with sodium bisulfite. Two primer sets were used to amplify the promoter region of the PTEN gene that incorporated a number of CpG sites specific for the unmethylated sequence (PTEN-U) and the other for the methylated sequence (PTEN-M, Table 1). The positive control for the unmethylated primer set was sodium bisulfite treated lymphocyte DNA from individuals who were without cancer. The positive control for the methylated primer set was genomic tumor DNA that was treated with excess SssI methyltransferease (New England Biolabs, Beverly, MA), and this generated DNA completely methylated DNA at all the CpG sites. This was then followed by treatment with sodium bisulfite. Reactions without DNA were included as negative controls for all the primer sets.

Statistical analysis

Statistical comparisons were carried out with SPSS 11.0 software (Chicago, Illinois, USA) using the Mantel-Haenszel χ^2 test to determine the significance of the association between the different variables. Statistical differences were considered significant at a p value of less than 0.05.

RESULTS

The one hundred twenty nine patients ranged in age from 26 to 76 years (median age 45). Lymph node metastasis was present in 66 (51%) cases, whereas 63 (49%) cases were node negative. Twenty-nine (23%) cases were stage I, 74 (57%) cases were stage II, and 26 (20%) cases were stage III. The tumors were histologically grade 1, grade 2, and grade 3 in 25, 66, and 38 cases, respectively. Nuclear grades were 1, 2, and 3 in 22, 73, and 34 cases,

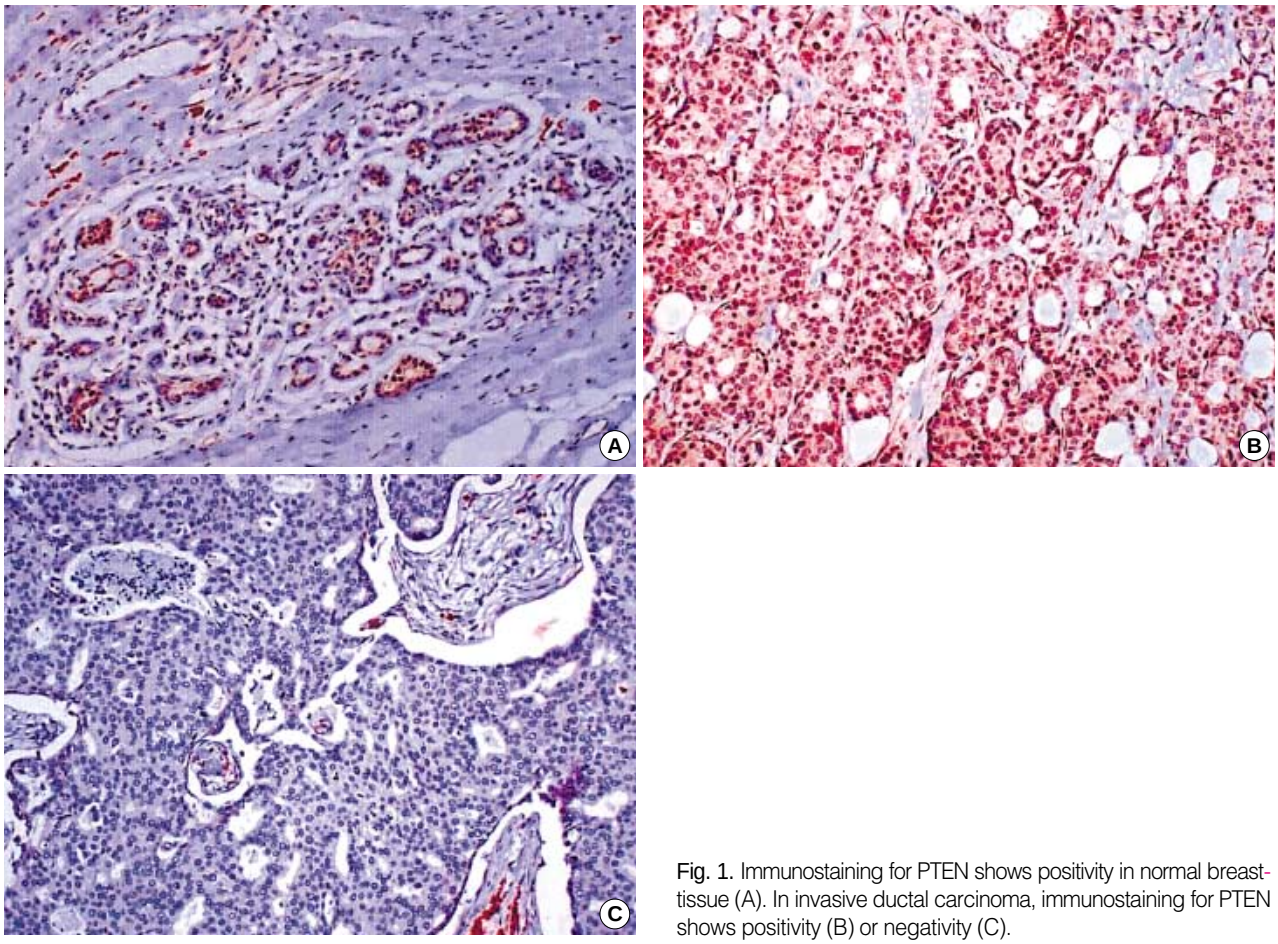

Fig. 1. Immunostaining for PTEN shows positivity in normal breast tissue (A). In invasive ductal carcinoma, immunostaining for PTEN shows positivity (B) or negativity (C).

respectively. Seventy percent (64/92 cases) were positive for ER and 72% (66/92 cases) were positive for PR. C-erb-B2 overexpression was observed in 43% (24/56 cases). Forty-seven samples were immunostained for p53 and 29 (62%) cases showed positivity for p53.

PTEN expression by immunohistochemistry

The PTEN expression was involved in nucleus and cytoplasmic compartment of the tumor cells as well as normal ductal epithelial cells and myoepithelial cells. Endothelial cells and nerves showed strong PTEN expression and were useful as internal positive controls. Of the 129 invasive ductal breast cancers, the loss of PTEN expression was seen in 62 (48%). Thirty-seven of the 66 (56%) cases with lymph node metastasis showed the loss of PTEN expression. Nine of 29 (31%) cases in stage I, 35 of 74 cases (47%) in stage II, and 18 of 26 cases (69%) in stage III showed the loss of PTEN expression. The loss of PTEN expression was significantly correlated with lymph node metastasis (p=0.046) and the stage (p=0.005). There was a near significant association between the loss of PTEN expression and tumor size (p=0.072). There were no correlations between loss of PTEN expression and age (p=0.830), histologic grade (p=0.485), nuclear grade (p=0.950), ER status (p=0.937), PR status (p=0.996), overexpression of c-erbB-2 (p=0.444), or the expression of p53 (p=0.421) (Fig. 1, Table 2).

PTEN promoter hypermethylation by MSP

PTEN promoter hypermethylation was seen in 5 (18%) of the 28 invasive ductal breast cancers with PTEN protein loss (Fig. 2). Prognostic factors were not associated with methylation status, except for the stage which produced statistical significance, though the number of cases was too small (Table 3).

DISCUSSION

PTEN is a candidate tumor suppressor that appears to have a

Table 2. Relationship between PTEN expression and clinicopathologic factors in the breast cancer

Clinicopathologic features		No. of cases	PTEN expression		p value
			Negative (%)	Normal	
Age (years)	<50	82	40 (49)	42	0.830
	≥50	47	22 (47)	25	
Tumor size (pT)	pT1	50	20 (40)	30	0.072
	pT2	69	35 (51)	34	
	pT3	10	7 (70)	3	
Lymph node metastasis	absent	63	25 (40)	38	0.046
	present	66	37 (56)	29	
Stage	I	29	9 (31)	20	0.005
	II	74	35 (47)	39	
	III	26	18 (69)	8	
Nuclear grade	1	22	10 (46)	12	0.950
	2	73	36 (49)	37	
	3	34	16 (47)	18	
Histologic grade	1	25	12 (48)	13	0.485
	2	66	29 (44)	37	
	3	38	21 (55)	17	
ER	positive	64	28 (44)	36	0.937
	negative	28	12 (43)	16	
PR	positive	66	29 (45)	37	0.996
	negative	25	11 (44)	14	
c-erbB-2	positive	24	13 (54)	11	0.444
	negative	32	14 (44)	18	
p53	positive	29	11 (38)	18	0.421
	negative	18	9 (50)	9	

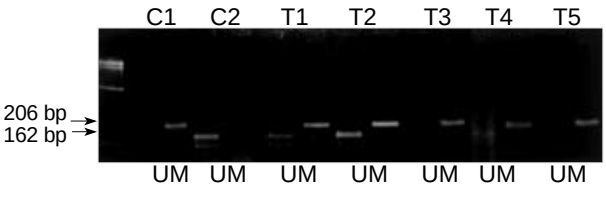

Fig. 2. Methylation specific polymerase chain reaction. The 162 bp product is indicative of an unmethylated PTEN allele (U), whereas the 206 bp product is indicative of a methylated PTEN allele (M). C1 is the positive control for the methylated primer set and C2 is the positive control for the unmethylated primer set. T denotes tumor sample.

multifunctional role, and it is involved in cell proliferation, migration and invasion.[2] Recent reports have shown a high rate of LOH in breast cancer.[8-10] Despite these findings, mutations have been infrequently observed and has been suggested that PTEN does not play a major role in pathogenesis or progression of breast cancers. Regarding the PTEN expression, we found significant PTEN protein loss (48%) in invasive ductal breast cancer by using immunohistochemical methods. This finding is similar to the results of previous immunohistochemical studies that reported weak or negative tumor staining in from 33% to 63% of the breast cancers that were analyzed.[14-18] Summarized findings indicate that PTEN protein loss is common in breast cancers.

Table 3. Relationship between PTEN promoter hypermethylation and clinicopathologic factors in breast cancers with loss of PTEN expression

Clinicopathologic features		No. of cases	PTEN expression		p value
			Negative (%)	Normal	
Age (years)	<50	17	3 (18)	14	0.830
	≥50	11	2 (18)	9	
Tumor size (pT)	pT1	10	1 (10)	9	0.072
	pT2	13	1 (8)	12	
	pT3	5	3 (60)	2	
Lymph node metastasis	absent	10	1 (10)	9	0.046
	present	18	4 (22)	14	
Stage	I	3	0 (0)	3	0.005
	II	16	1 (6)	15	
	III	9	4 (44)	5	
Nuclear grade	1	3	1 (33)	2	0.950
	2	15	2 (13)	13	
	3	10	2 (20)	8	
Histologic grade	1	4	0 (0)	4	0.485
	2	10	1 (10)	9	
	3	14	4 (29)	10	
ER	positive	13	2 (15)	11	0.937
	negative	8	1 (13)	7	
PR	positive	13	2 (15)	11	0.996
	negative	8	1 (13)	7	
c-erbB-2	positive	9	2 (22)	7	0.444
	negative	10	0 (0)	10	
p53	positive	10	2 (20)	8	0.421
	negative	6	0 (0)	6	

PTEN mutations are frequently associated with an advanced tumor stage of various cancers. PTEN mutations were detected at a greater frequency in metastatic prostate cancer than in localized cancers, though these mutations were found to occur with equal frequency at all stages of endometrial cancer.[7,19] Mutation analysis has confirmed that homozygotic inactivation of the PTEN gene occurs in a large fraction of glioblastomas but not in the low grade gliomas.[20] The correlation between PTEN protein loss and the prognostic factors for breast cancer has not been well characterized due to some discrepancies between different observations. Our study found that the loss of PTEN expression significantly correlated with lymph node metastasis and the stage. These findings are similar to those findings of some previous reports and they indicate that PTEN protein loss might contribute to the progression of breast cancer and is associated with a poor prognosis.[14,17,18]

An association between PTEN protein loss and the ER or PR status have been described,[14-16] however, we and Bose *et al*. found no association between PTEN loss and the ER or PR status.[18] A significant association between PTEN protein loss and c-erb-B2

expression or p53 expression has not been reported.[16,18]

PTEN promoter hypermethylation has been described in endometrial (19%), gastric carcinoma (39%), and nonsmall cell lung cancer (35%).[21-23] One study on advanced prostate cancer xenografts found the loss of PTEN expression to be frequent despite the lack of mutations.[24] The finding that the PTEN expression was restored in PTEN non-expressing prostate cancer cells by an in vitro treatment with the demethylating agent, 5-azadeoxycytidine, indicates that the mechanism of inactivation may occur through hypermethylation.[24] We found that 18% of the tumors were hypermethylated in the PTEN promoter region of the invasive ductal breast cancer with PTEN protein loss. This finding supports the suggestion that promoter hypermethylation may play a role in inactivating the PTEN gene in breast cancer. The discrepancy between the frequency of the PTEN protein loss and the frequency of PTEN promoter hypermethylation in our study suggest other epigenetic mechanisms, such as decreased protein synthesis or increased protein turnover, might contribute to PTEN protein loss as well.

PTEN promoter hypermethylation was reported to be associated with metastatic endometrial carcinoma despite the early inactivation of PTEN in endometrial tumorigenesis.[7,21] We found that PTEN promoter hypermethylation was more frequently observed in the higher tumor stages. These findings suggest that PTEN promoter hypermethylation might contribute to the progression of breast cancer.

In conclusion, PTEN protein loss was observed in 48% of the invasive ductal breast cancers that we tested and this loss correlated with lymph node metastasis and stage. PTEN promoter hypermethylation was found in five (18%) of 28 patients. These results suggest that PTEN protein loss might play a role in the progression of breast cancer and that the aberrant promoter methylation is one of the silencing mechanisms of PTEN.

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