Loss of PTEN Expression Is Associated With IGFBP2 Expression, Younger Age, and Late Stage in Triple-Negative Breast Cancer

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ABSTRACT

Objectives: To investigate the association between PTEN loss and IGFBP2 expression in a series of triple-negative breast cancers and to relate this expression to basal cytokeratin expression and clinicopathologic features.

Methods: One hundred and one formalin-fixed and paraffin-processed triple-negative breast cancer cases from the University of Malaya Medical Centre were tested immunohistochemically for cytokeratins 5/6 and 14, PTEN, and IGFBP2. The resulting slides were scored for proportion and intensity of staining.

Results: Loss of tumor nuclear and cytoplasmic staining for PTEN occurred in 48.3% of cases and was significantly associated with younger age at diagnosis (47 years compared with 57 years in those without PTEN loss; $P = .005$). Independent predictors of PTEN loss were late stage at presentation ($P = .026$),

*cytokeratin 5/6 positivity (*P *= .028), and IGFBP2 expression (*P *= .042). High levels of IGFBP2*

expression were seen in 32% of cases; independent predictors of high levels were cytokeratin 14

*negativity (*P *= .005). PTEN loss and high levels of IGFBP2 expression were associated with poorer*

survival but neither of these trends was significant.

Conclusions: *PTEN loss is a frequent event in triple-negative breast cancers and is significantly*

associated with younger age at onset of breast cancer, late stage, and IGFBP2 expression.

Triple-negative (TN) breast cancers are defined by their lack of expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), and account for 10% to 24% of all breast cancers.[1-4] Notably, TN breast cancers share many overlapping characteristics with basal-like breast cancers, in that the majority of TN breast cancers have elevated expression of high-molecular-weight cytokeratins and both have similar gene expression signatures.[5-9] TN tumors are predominantly high-grade and aggressive cancers with poor prognosis, and unlike hormone receptor– and HER2-positive breast carcinomas, no targeted therapeutic regimens have been shown to significantly improve survival.[10] The treatment of these tumors is therefore challenging and biomarker studies are required to better characterize these tumors with the aim of identifying improved therapeutic interventions.

Phosphatase and tensin homolog (*PTEN*) is a tumor suppressor gene that is lost or mutated in many types of cancer, including breast, prostate, and lung cancer.[11] PTEN dephosphorylates PIP_3, a product of the PI3K pathway, thereby inactivating the Akt signaling pathway, inhibiting cell growth and promoting apoptosis.[11] More recently, PTEN has been postulated to have an important role in DNA repair, because mutation or loss of PTEN results in a deficiency to repair DNA double strand breaks.[12]

Although one previous study reported PTEN loss in 48% of unselected breast cancer cases,[13] other studies have reported lower incidence of PTEN loss (8%, 15%, 28%).[13-15] However, these differences may reflect methodologic differences in testing and reporting PTEN loss. In contrast, a recent study suggests that up to 66% of basal-like breast cancers have loss of PTEN, which may occur more frequently in this phenotype than in other subtypes of breast cancer.[15] Insulin-like growth factor–binding protein 2 (IGFBP2) is a member of six binding proteins that modulate the action of insulin-like growth factors (IGF-I, IGF-II) that principally signal via the type 1 IGF receptor (IGF-IR).[16] This axis plays a critical role in the development and progression of many epithelial cancers, including

breast cancer.[17] At the cellular level, the IGF-I receptor appears to play a fundamental role in maintaining the transformed phenotype.[18] Recent prospective epidemiologic studies have consistently shown strong associations between circulating IGF-I levels and the subsequent risk of developing a number of epithelial cancers, including breast cancer.[19] Although IGFBPs can act to either inhibit or enhance IGF-induced cell signaling, they can also exert effects in an IGF-I–independent manner, indicating that IGFBPs can intrinsically modulate aspects of cell growth and survival.[20] Busund et al[21] reported that IGFBP2 abundance was markedly higher in invasive breast carcinoma and carcinoma in situ compared with normal breast tissue or benign hyperplastic lesions that had very little IGFBP2 expression. Wang et al[22] reported that tumor expression of IGFBP2 could predict tumors most likely to metastasize. IGFBP2 clearly appears to play a role in breast cancer progression,[21,23-26] and interestingly, high levels of IGFBP2 expression have also been identified in a number of additional cancers, including prostate,[27] ovary,[28] stomach,[29] adrenal gland,[30] and bladder,[31] suggesting that IGFBP2 generally plays an important role in tumorigenesis.

An unbiased screen of human prostate and glioblastoma samples, using microarray-based expression profiling, identified IGFBP2 as the most significant marker of PTEN loss.[32] Although the mechanism of loss of expression of PTEN in breast cancer has yet to be fully elucidated in cell lines, PTEN activity is downregulated by the interaction of IGFBP2 with the β1 integrin receptor.[16]

The purpose of the current study was to investigate for the first time the association between PTEN loss and IGFBP2 expression in a relatively large series of TN breast cancers and to relate this expression to basal cytokeratin expression and the clinicopathologic features of stage, histologic grade, patient age, and overall survival.

Materials and Methods

Tissue blocks of all accessible TN breast carcinomas diagnosed between 2004 and 2009 at the University of Malaya Medical Centre (Kuala Lumpur, Malaysia) were used in this study, with a total of 101 identified as having adequate invasive tumor tissue for evaluation. All tissues had been fixed in 10% neutral-buffered formalin for 6 to 72 hours, and processed to paraffin wax blocks, from which sections were cut at 3-μm thickness on a rotary microtome and mounted onto Tissue Tek Plus glass slides (Sakura, Alphen aan den Rijn, The Netherlands) to ensure maximum adhesion.

Assessment of Tumor Grade and Stage:

Grading was performed according to the modified Bloom and Richardson criteria[33] and all slides were reviewed and regraded for this study by the histopathologists on the team. **[Au3: Pls include initials]** Clinical data on patient age, ethnicity, and stage (American Joint Commission on Cancer, 2003) were extracted from the database for this series of cases. Patients were staged based on criteria described in the 6th edition of the American Joint Commission on Cancer (AJCC) guidelines.[34]

Immunohistochemistry

Expression of ER, PR, HER2, IGFBP2, PTEN, cytokeratins 5/6, and cytokeratin 14 was tested using standard immunohistochemical methods. Briefly, slides were deparaffinized, treated with 0.3% hydrogen peroxide to block endogenous peroxidase, and incubated for 30 minutes in a 750-W microwave oven in the appropriate antigen retrieval buffer to expose tissue antigens. The slides were then incubated in the respective primary antibodies overnight at 4°C ∎Table 1∎. A horseradish peroxidase–conjugated avidin-biotin–based system was used for antibody detection and visualization (Vector Elite, Vector Laboratories, Peterborough, England). Nuclei were counterstained with Harris hematoxylin. The specificity of the IGFBP2 antibody was tested by adding a specific IGFBP2 blocking peptide (Santa Cruz Biotechnology, Dallas, TX) to the primary antibody mix before application. Positive cell line-block controls for ER, PR, and HER2 were used as described previously.[35]

Assessment of Immunohistochemistry

Immunohistochemical staining for ER and PR was assessed with the Allred scoring system described in the most recent American Society of Clinical Oncology/College of American Pathologists guidelines.[36] Briefly, nuclear staining of the invasive tumor cells was designated an intensity score (0 [no staining], 1 [weak staining], 2 [moderate staining], 3 [strong staining]) and a proportion score (0 [no staining], 1 [less than 1%], 2 [1%-10%], 3 [11%-33%], 4 [34%-66%], 5 [67%-100%]). The intensity and proportion scores were then summed to give a total score ranging from 0 through 8, with a score of more than 2 defined as positive for ER or PR. The same scoring system was used to assess the cytoplasmic staining of IGFBP2 in the invasive tumor compartment, with a total score of 0, 2 to 5, and 6 to 8 defined as no expression, low expression, and high expression of IGFBP2, respectively. For the purposes of comparing IGFBP2 expression with PTEN loss and other clinicopathologic variables, a cutoff of more than 5 was used to define IGFBP2 positivity. This cutoff was chosen based on the results of So et al (2008), **[Au4: Pls**

include So et al study in reference list] who showed that intermediate and strong staining of IGFBP2 (which equates to an Allred score of >5) was associated with worse breast cancer–specific survival in hormone receptor–negative disease. Positivity for HER2 was defined as intense and complete membrane staining (chicken wire pattern) of at least 30% of invasive tumor cells.[37] Most tumors were large and heterogeneous in nature with respect to PTEN expression. Consequently, PTEN expression was considered lost in cases of complete absence of staining (both cytoplasmic and nuclear) in at least two thirds of the invasive tumor compartment, with PTEN staining in the adjacent normal stromal tissue being used as the internal positive control. Cytokeratin 5/6 and cytokeratin 14 immunostaining was assessed on the basis of 10% or more invasive tumor cells showing cytoplasmic positivity, using appropriate localized staining of normal glands as positive internal controls. The basal-like phenotype was defined as positivity for cytokeratin 5/6 and/or cytokeratin 14.[8] Cases that were not possible to interpret or did not have sufficient positive control staining were omitted from analysis.

Follow-up Data

At the University of Malaya Medical Centre, all patients were followed-up via scheduled appointments in the specialist breast cancer clinics. Data on mortality were obtained from the hospitals' medical records, as well as through active follow-up. In addition, vital status was verified through direct linkage with the National Registration Department in Malaysia. Follow-up time was calculated as the interval between date of diagnosis and date of death, or date of last contact, whichever came first.

Statistical Analysis

Continuous variables (age) were described using medians and compared using the Mann-Whitney U test; categorical variables (ethnicity, stage, grade, IGFBP2 expression, PTEN loss, cytokeratin 5/6 expression, and cytokeratin 14 expression) were expressed as proportions and compared using either the χ^2 test or Fisher exact test. Variables significantly associated with PTEN loss and IGFBP-2 expression were simultaneously entered into a multivariate logistic regression model, with PTEN loss and IGFBP-2 as the outcome variables to determine the independent predictors of PTEN loss and IGFBP2 expression. Because information on cause of death was not available for most patients, we calculated relative survival rates (RSRs) to estimate the high mortality rate associated with breast cancer among the patient population.[36] Population mortality data for Malaysia were used to compute these estimates. The

relative survival rate adjusts for the general survival of the Malaysian population for the given sex, age, and year, and thus is a measure of net survival attributed to breast cancer independent of other causes of death. *P* values of less than .05 were considered statistically significant, as was the 95% confidence interval (CI) for odds ratio (OR) that did not include 1.0. All statistical analyses were carried out using IBM Statistics software (version 20; IBM SPSS, Armonk, NY) and Stata MP (version 14) software (StataCorp, College Station, TX).

Results

Following retesting of ER, PR, and HER2 and confirmation of the TN status of the breast cancers, 89 cases were available for analysis of PTEN loss and basal cytokeratin expression and 100 cases for analysis of IGFBP2 and basal cytokeratin expression. Patient age at onset of breast cancer ranged from 23 to 83 years, with a median of 53 years. Most cases (93%) were invasive ductal carcinomas, with the remaining being either medullary or metaplastic cancers. Sixty-two percent of the tumors were of basal-like phenotype based on their positive expression of cytokeratin 5/6 and/or cytokeratin 14.

PTEN

PTEN staining occurred in both the nucleus and cytoplasm of cancer cells. Loss of nuclear-cytoplasmic immunostaining for PTEN occurred in 48.3% of TN cases; the remaining cases had weak PTEN staining or stronger staining of PTEN equal to that of the surrounding stromal tissue ▮Image 1 ▮ Univariate **[Au5: ok? Is this what you mean?]** analysis showed that loss of PTEN immunostaining was associated with younger age at onset; the median age of patients at diagnosis with tumors showing PTEN loss was 47 years, compared with 57 years in those without PTEN loss (*P* = .005). PTEN loss was also associated with expression of cytokeratin 5/6 and IGFBP2, but these trends were not statistically significant on univariate analysis (*P* = .097 and *P* = .073, respectively). In multivariate analysis, independent predictors of PTEN loss were younger age at onset (*P* = .041), late stage (*P* = .026), cytokeratin5/6 positivity (*P* = .028), and IGFBP2 expression (*P* = .042) ▮Table 3 ▮ **[Au6: Table 2 not cited. Pls cite before Table 3]** ▮Image 2 ▮, and ▮Image 3 ▮.

IGFBP2

IGFBP2 staining was predominantly confined to the cytoplasmic compartment of the invasive cancer cells with little stromal staining or expression in normal glands. Addition of the IGFBP2 blocking peptide resulted in complete absence of staining in cases shown to be strongly positive for IGFBP2 ▐Image 4▐. Of 100 TN cases that were assessable for IGFBP2 staining, 32% showed high levels of IGFBP2 expression (Allred score >5). Univariate analysis revealed no significant differences in high IGFBP2 expression and age, stage, or cytokeratin 5/6 but IGFBP2 expression was associated with lack of staining for cytokeratin 14 (P = .004). Multivariate analysis showed that independent predictors of high levels of IGFBP2 expression were lack of positivity for cytokeratin 14 (P = .005) and PTEN loss (P = .047) ▐Image 5▐. There was also a trend for lymphovascular invasive cases to have high levels of IGFBP2 expression but this trend was not significant (P = .064).

Survival Analysis

TN cases with PTEN loss seem to have a poorer survival than cases without PTEN loss; the 4-year RSR for patients with PTEN loss was 65.0% (95% CI, 45.2%-79.7%) compared with 77.8% (95% CI, 60.8%-89.0%) in those without PTEN loss. Similarly, cases with high levels of IGFBP2 experienced marginally lower survival than their counterparts with low or negative IGFBP2; the 4-year RSR was 68.4% (95% C,: 44.5%-84.5%) for high levels of IGFBP2 compared with 74.9% (95% CI, 60.8%-85.1%) for cases with low or no IGFBP2 expression ▐Figure 1▐ and ▐Figure 2▐.

Discussion

In the current study, PTEN expression in tumor cells is lost in nearly half of all TN breast cancers when tested using the antibody clone 6H2.1. Notably, the extent of PTEN loss in TN breast cancer is significantly higher than that reported using the same PTEN antibody in an unselected breast cancer cohort.[15] Clone 6H2.1 is the recommended marker for immunohistochemical analysis of PTEN loss because it is the only antibody that exhibits a correlation with molecular alterations in PTEN[38] and shows a correlation between western blot analysis and PTEN mutational and allelic status.[14] Loss of PTEN staining in the invasive tumor compartment is readily assessable; strong positive staining of the adjacent nontumor stroma serves as an excellent internal positive control, as previously reported.[14]

Interestingly, loss of PTEN was significantly associated with a younger age at diagnosis. This reflects the findings by Anders et al[39] that PTEN expression and genes involved in related signaling pathways were altered in breast cancers that occurred in younger patients (≤45 years).

Although not significant, PTEN loss was associated with breast cancer survival; this is in agreement with the significant findings of Depowski et al[40] in a cohort of breast cancers not selected on the basis of TN status. TN cohorts generally tend to have poorer survival than unselected cohorts of breast cancer cases. Consequently, it is probably necessary to study larger TN cohorts with PTEN loss to establish whether the trend observed in the current study becomes significant when larger numbers of cases are included.

IGFBP2 positivity was associated with lymphovascular invasion and lower breast cancer survival rates. However, neither of these reached statistical significance, possibly because of the small number of cases in the current study. These data are consistent with those of So et al,[26] who showed that of 3,117 breast tumors that were assessable for both ERα status and IGFBP2 expression, IGFBP2 was not prognostic among the ERα-positive tumors, but a trend showed lower breast cancer disease-specific survival rates in the ER-negative tumors. Furthermore, in vitro studies have demonstrated that overexpression of IGFBP2 in ER-negative breast cancer cell lines and cell lines of other cancer types that include those of prostate, glioma, and bladder conferred a growth advantage, enhanced invasion and migration, and chemoresistance.[22,26,31,41] Interestingly IGFBP2 expression in tumors was linked to lack of staining for the basal cytokeratin 14. TN breast cancers are enriched for characteristics of epithelial mesenchymal transition and we speculate that these tumors may be undergoing epithelial mesenchymal transition. Further studies would be required to confirm this. A recent study showed that IGFBP2 promotes angiogenesis in neuroblastoma cells via direct activation of the vascular endothelial growth factor (VEGF) promoter[42] and anti-VEGF therapy of gliomas; infiltrating tumor was associated with increased levels of IGFBP2.[43] Because VEGF is already considered a target in TN breast cancer, perhaps cotargeting IGFBP2 might be of benefit. In addition, overexpression of EGFR and IGFBP2 has been observed in high-grade astrocytomas and coexpression of these genes was strongly associated with high-grade gliomas and lower survival. This report suggested that coexpression of these genes had a more important clinical and biological impact than the expression of each individual gene alone.[44] EGFR is strongly associated with TN breast cancer and is a potential target[45,46]; there may now be a rationale for assessing EGFR status in relation to IGFBP2 expression in TN breast cancers because perhaps cotargeting both of these would provide a better outcome.

This is the first immunohistochemical study to show an association between IGFBP-2 expression and PTEN loss and supports the evidence from studies using in vitro cell lines that show IGFBP2 downregulates PTEN[16] and conversely that overexpression of PTEN has been shown to reduce IGFBP2 expression.[47] These studies indicate IGFBP2 plays a role in the PI3K signaling pathway that is known to be involved in promoting survival and growth.[48] Several pathways could be responsible for this inverse relationship, including increased IGFBP2 expression by breast cancer cells diminishing or ablating the expression of PTEN protein, potentially via integrin receptors.[16] Further work could also investigate how IGFBP2 affects the catalytic activity of PTEN. PTEN possesses a carboxy-terminal, noncatalytic regulatory domain with three phosphorylation sites (Ser380, Thr382, and Thr383) that regulate its biological activity.[49,50] Antibodies are available that recognize phosphorylation at these sites and may prove useful in further studies to investigate the relationship between IGFBP2 expression and PTEN phosphorylation and thus activity.

In summary, we have shown that loss of PTEN can be readily assessed using immunohistochemistry; that PTEN loss is a frequent event in TN breast cancers; and that this is significantly associated with a younger age at onset of breast cancer, late stage of presentation, and high levels of IGFBP2 expression. **[Au7: Tables 4 and 5 not cited anywhere. Pls cite in numerical order]**

References

1.   Bauer KR, Brown M, Cress RD, et al. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype. Cancer. 2007;109:1721-1728.

2.   Reis-Filho JS, Tutt AN. Triple negative tumours: a critical review. Histopathology. 2008;52:108-118.

3.   Tan GH, Taib NA, Choo WY, et al. Clinical characteristics of triple-negative breast cancer: experience in an asian developing country. Asian Pac J Cancer Prev. 2009;10:395-398.

4.   Viale G, Rotmensz N, Maisonneuve P, et al. Invasive ductal carcinoma of the breast with the "triple-negative" phenotype: prognostic implications of EGFR immunoreactivity. Breast Cancer Res Treat. 2009;116:317-328.

5. Cheang MC, Voduc D, Bajdik C, et al. Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. Clin Cancer Res. 2008;14:1368-1376.

6. Dent R, Trudeau M, Pritchard KI, et al. Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res. 2007;13:4429-4434.

7. Kreike B, van Kouwenhove M, Horlings H, et al. Gene expression profiling and histopathological characterization of triple-negative/basal-like breast carcinomas. Breast Cancer Res. 2007;9:R65.

8. Rakha EA, El-Sayed ME, Green AR, et al. Prognostic markers in triple-negative breast cancer. Cancer. 2007;109:25-32.

9. Tan DS, Marchio C, Jones RL, et al. Triple negative breast cancer: Molecular profiling and prognostic impact in adjuvant anthracycline-treated patients. Breast Cancer Res Treat. 2008;111:27-44.

10. Carey L, Winer E, Viale G, et al. Triple-negative breast cancer: disease entity or title of convenience? Nat Rev Clin Oncol. 2010;7:683-692.

11. Salmena L, Carracedo A, Pandolfi PP. Tenets of PTEN tumor suppression. Cell. 2008;133:403-414.

12. Shen WH, Balajee AS, Wang J, et al. Essential role for nuclear PTEN in maintaining chromosomal integrity. Cell. 2007;128:157-170.

13. Panigrahi AR, Pinder SE, Chan SY, et al. The role of PTEN and its signaling pathways, including AKT, in breast cancer: an assessment of relationships with other prognostic factors and with outcome J Pathol. 2004;204:93-100.

14. Perren A, Weng LP, Boag AH, et al. Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. Am J Pathol. 1999;155:1253-1260.

15. Lopez-Knowles E, O'Toole SA, McNeil CM, et al. PI3K pathway activation in breast cancer is associated with the basal-like phenotype and cancer-specific mortality. Int J Cancer. 2009;126:1121-1131.

16. Perks CM, Vernon EG, Rosendahl AH, et al. IGF-II and IGFBP-2 differentially regulate PTEN in human breast cancer cells. Oncogene. 2007;26:5966-5972.

17. Meinbach DS, Lokeshwar BL. Insulin-like growth factors and their binding proteins in prostate cancer: cause or consequence? Urol Oncol. 2006;24:294-306.

18. Baserga R, Peruzzi F, Reiss K. The IGF-1 receptor in cancer biology. Int J Cancer. 2003;107:873-877.

19. Roddam AW, Allen NE, Appleby P, et al. Insulin-like growth factors, their binding proteins, and prostate cancer risk: analysis of individual patient data from 12 prospective studies. Ann Intern Med. 2008;149:461-471, W83-8.

20. Holly J, Perks C. The role of insulin-like growth factor binding proteins Neuroendocrinology. 2006;83:154-60.

21.	Busund LT, Richardsen E, Busund R, et al. Significant expression of IGFBP2 in breast cancer compared with benign lesions. J Clin Pathol. 2005;58:361-366.

22.	Wang H, Arun BK, Wang H, et al. IGFBP2 and IGFBP5 overexpression correlates with the lymph node metastasis in T1 breast carcinomas. Breast J. 2008;14:261-267.

23.	Wang M, Liu YE, Ni J, et al. Induction of mammary differentiation by mammary-derived growth inhibitor-related gene that interacts with an omega-3 fatty acid on growth inhibition of breast cancer cells. Cancer Res. 2000;60:6482-6487.

24.	Gebauer G, Jager W, Lang N. mRNA expression of components of the insulin-like growth factor system in breast cancer cell lines, tissues, and metastatic breast cancer cells. Anticancer Res. 1998;18:1191-1195.

25.	McGuire SE, Hilsenbeck SG, Figueroa JA, et al. Detection of insulin-like growth factor binding proteins (IGFBPs) by ligand blotting in breast cancer tissues. Cancer Lett. 1994;77:25-32.

26.	So AI, Levitt RJ, Eigl B, et al. Insulin-like growth factor binding protein-2 is a novel therapeutic target associated with breast cancer. Clin Cancer Res. 2008;14:6944-6954.

27.	Degraff DJ, Aguiar AA, Sikes RA. Disease evidence for IGFBP-2 as a key player in prostate cancer progression and development of osteosclerotic lesions. Am J Transl Res. 2009;1:115-130.

28.	Yan XJ, Tian Y, Wang C, et al. The expressions and clinical significance of IGFBP-2, -3 in both serum and tumor tissues in patients with epithelial ovarian cancer. Sichuan Da Xue Xue Bao Yi Xue Ban. 2009;40:639-643.

29.	Zhang L, Huang W, Chen J, et al. Expression of IGFBP2 in gastric carcinoma and relationship with clinicopathologic parameters and cell proliferation. Dig Dis Sci. 2007;52:248-253.

30.	Shi Z, Henwood MJ, Bannerman P, et al. Primary pigmented nodular adrenocortical disease reveals insulin-like growth factor binding protein-2 regulation by protein kinase A. Growth Horm IGF Res. 2007;17:113-121.

31.	Miyake H, Hara I, Yamanaka K, et al. Introduction of insulin-like growth factor binding protein-2 gene into human bladder cancer cells enhances their metastatic potential. Oncol Rep. 2005;13:341-345.

32.	Mehrian-Shai R, Chen CD, Shi T, et al. Insulin growth factor-binding protein 2 is a candidate biomarker for PTEN status and PI3K/Akt pathway activation in glioblastoma and prostate cancer. Proc Natl Acad Sci U S A. 2007;104:5563-5568.

33.	Elston CW, Ellis IO. Pathological prognostic factors in breast cancer: I, the value of histological grade in breast cancer—experience from a large study with long-term follow-up. Histopathology. 1991;19:403-410.

34.	Singletary SE, Connolly JL. Breast cancer staging: working with the sixth edition of the AJCC cancer staging manual. CA Cancer J Clin. 2006;56:37-47.

35.   Rhodes A, Sarson J, Assam EE, et al. The reliability of rabbit monoclonal antibodies in the immunohistochemical assessment of estrogen receptors, progesterone receptors, and HER2 in human breast carcinomas. Am J Clin Pathol. 2010;134:621-632.

36.   Hammond ME, Hayes DF, Wolff AC, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer. J Oncol Pract. 2010;6:195.

37.   Wolff AC, Hammond ME, Schwartz JN, et al; American Society of Clinical Oncology, College of American Pathologists. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J Clin Oncol. 2007;25:118-145.

38.   Pallares J, Bussaglia E, Martinez-Guitarte JL, et al. Immunohistochemical analysis of PTEN in endometrial carcinoma: a tissue microarray study with a comparison of four commercial antibodies in correlation with molecular abnormalities. Mod Pathol. 2005;18:719-727.

39.   Anders CK, Hsu DS, Broadwater G, et al. Young age at diagnosis correlates with worse prognosis and defines a subset of breast cancers with shared patterns of gene expression. J Clin Oncol. 2008;26:3324-3330.

40.   Depowski PL, Rosenthal SI, Ross JS. Loss of expression of the PTEN gene protein product is associated with poor outcome in breast cancer. Mod Pathol. 2001;14:672-676.

41.   Kiyama S, Morrison K, Zellweger T, et al. Castration-induced increases in insulin-like growth factor-binding protein 2 promotes proliferation of androgen-independent human prostate LNCaP tumors. Cancer Res. 2003;63:3575-3584.

42.   Azar WJ, Azar SH, Higgins S, et al. IGFBP-2 enhances VEGF gene promoter activity and consequent promotion of angiogenesis by neuroblastoma cells. Endocrinology. 2011;152:3332-3342.

43.   de Groot JF, Fuller G, Kumar AJ, et al. Tumor invasion after treatment of glioblastoma with bevacizumab: radiographic and pathologic correlation in humans and mice. Neuro Oncol. 2010;12:233-242.

44.   Scrideli CA, Carlotti CG,Jr, Mata JF, et al. Prognostic significance of co-overexpression of the EGFR/IGFBP-2/HIF-2A genes in astrocytomas. J Neurooncol. 2007;83:233-239.

45.   Gluz O, Liedtke C, Gottschalk N, et al. Triple-negative breast cancer: current status and future directions. Ann Oncol. 2009;20:1913-1927.

46.   Sarrio D, Rodriguez-Pinilla SM, Hardisson D, et al. Epithelial-mesenchymal transition in breast cancer relates to the basal-like phenotype. Cancer Res. 2008;68:989-997.

47.   Levitt RJ, Georgescu MM, Pollak M. PTEN-induction in U251 glioma cells decreases the expression of insulin-like growth factor binding protein-2. Biochem Biophys Res Commun. 2005;336:1056-1061.

48.   Hers I, Vincent EE, Tavare JM. Akt signalling in health and disease. Cell Signal. 2011;23:1515-1527.

49.   Vazquez F, Ramaswamy S, Nakamura N, et al. Phosphorylation of the PTEN tail regulates protein stability and function. Mol Cell Biol. 2000;20:5010-5018.

50.   Torres J, Navarro S, Roglá I, et al. Heterogeneous lack of expression of the tumour suppressor PTEN protein in human neoplastic tissues. Eur J Cancer. 2001;37:114-121.

▮Table 1 ▮

Antibodies and Antigen Retrieval

Antibody	Supplier	Antigen Retrieval
ERα, clone 6F11	Novocastra, Newcastle-Upon-Tyne, England	MW, Sodium citrate pH6.0
PR (A & B), clone SP2	Lab Vision, Runcorn, England	MW, Sodium citrate pH6.0
HER2, clone SP3	Lab Vision	MW, Sodium citrate pH6.0
Cytokeratin 5/6, clone D5/16B4	Dako, Ely, England	MW, Tris-EDTA pH 9.0
Cytokeratin 14, clone LL02	Novocastra	MW, Sodium citrate pH6.0
PTEN, clone 6H2.1	Dako Ltd	MW, Tris-EDTA pH 9.0
IGFBP2, clone C-18	Insightbio, Middlesex, England	MW, Sodium citrate pH6.0

EDTA, ethylenediaminetetraacetic acid; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; IGFBP2, insulin-like growth factor–binding protein 2; MW, **[Au8: Pls include expansion of MW]** ; PR, progesterone receptor; PTEN, phosphatase and tensin homolog.

▮Table 2 ▮

Factors Associated With PTEN Loss

	Overall	PTEN Loss	No PTEN Loss	*P* for χ^2 Test
No. (%) of patients	89	43	46	
Median age, y	53	47	57	.005[a,b]
Ethnicity, No. (%)				.518[c]
Chinese	53 (59.6)	27 (62.8)	26 (56.5)	
Malay	21 (23.6)	10 (23.3)	11 (23.9)	

Indian	12 (13.5)	6 (14.0)	6 (13.0)	
Others	3 (3.4)	0 (0.0)	3 (6.5)	
Median tumor size, cm	3.0	3.5	3.0	.236[b]
Lymph nodes involved, No. (%)				.250
Yes	42 (47.2)	23 (53.5)	19 (41.3)	
No	47 (52.8)	20 (46.5)	27 (58.7)	
Stage, No. (%)				.078
Early (stage 1-2)	55(61.8)	23(53.5)	32(69.6)	
Late (stage 3-4)	34(38.2)	20(46.5)	14(30.4)	
Grade,[d] No. (%)				.145
Grade 2	16 (18.6)	5 (12.2)	11(24.4)	
Grade 3	70 (81.4)	36 (87.8)	34 (75.6)	
Unknown	3	2	1	
Lymphovascular invasion, No. (%)				.780
Present	32 (40.5)	16 (42.1)	16 (39)	
Absent	47 (59.5)	22 (57.9)	25 (61)	
Unknown	10	5	5	
CK14 status, No. (%)				.354
Negative	47 (54)	20 (48.8)	27 (58.1)	
Positive	40 (46)	21 (51.2)	19 (41.3)	
Unknown	87	2	0	

CK 5/6 status, No. (%)				.097
Negative	23 (29.1)	8 (20.5)	15 (37.5)	
Positive	56 (70.9)	31 (79.5)	25 (62.5)	
Unknown	10	4	6	
IGBFP2, No. (%)				.073
Positive[e]	31 (34.8)	19 (44.2)	12 (26.1)	
Negative	58 (65.2)	24 (55.8)	34 (73.9)	

CK, cytokeratin; IGFBP2, insulin-like growth factor–binding protein 2; PTEN, phosphatase and tensin homolog.

[a] Statistically significant (P <.05).

[b] Compared using the Mann-Whitney U test.

[c] Compared using the Fisher exact test.

[d] There were no patients with grade 1 tumor.

[e] Defined as an Allred score of >5.

▮Table 3 ▮

Factors Associated With PTEN Loss in Multivariate Analysis

Factors	OR for PTEN Loss (95% CI)[a]	95% CI for OR		*P* Value
		Lower	Upper	
Age, y	0.95[b]	0.91	1.00	.041
Stage				
Early (stage 1-2)	1.00[c]			
Late (stage 3-4)	3.76[b]	1.17	12.10	.026
Grade				
Grade 2	1.00[c]			
Grade 3	2.95	0.81	10.77	.101

CK 5/6 expression				
No	1.00[c]			
Yes	3.94[b]	1.16	13.34	.028
IGBFP2 expression				
No	1.00[c]			
Yes	3.26[b]	1.04	10.21	.042

CI, confidence interval; CK, cytokeratin; IGFBP2, insulin-like growth factor–binding protein 2; OR, odds ratio; PTEN, phosphatase and tensin homolog.

[a] Derived using a multivariable logistic regression model including all variables with $P < .20$ in univariable analysis; age, stage, grade, CK 5/6, and IGBFP2 status.

[b] Statistically significant ($P < .05$).

[c] Reference category.

■Table 4 ■

Factors Associated With High Levels of IGFBP2 Expression[a]

	Overall	IGFBP2-Positive, No. (%)	IGFBP2-Negative, No. (%)	*P* for χ^2 Test
No. (%) of patients	100	32	68	
Median age, y	53	52	53	.685[b]
Ethnicity				.215
Chinese	63 (63.0)	19 (59.4)	44 (64.7)	
Malay	21 (21.0)	6 (18.8)	15 (22.1)	
Indian	13 (13.0)	7 (21.9)	6 (8.8)	
Others	3 (3.0)	0 (0.0)	3 (4.4)	
Median tumor size, cm	3.0	3.3	3.0	.445[b]
Lymph node involved				.866
Yes	44 (44)	15 (42.9)	29 (44.6)	

No	56 (56)	20 (57.1)	36 (55.4)	
Stage				.837[c]
Early	65 (65)	24 (68.6)	41 (63.0)	
Late	35 (35)	11(31.4)	24 (37.0)	
Grade[d]				.855
Grade 2	19 (19.6)	7 (20.6)	12 (19)	
Grade 3	78 (80.4)	27(79.4)	51 (81)	
Unknown	3	1	2	
Lymphovascular invasion				.176
Present	34 (34.3)	16 (47.1)	18 (32.7)	
Absent	55 (65.7)	18 (52.9)	37 (67.3)	
Unknown	11	1	10	
CK14 status				.004
Negative	55 (56.7)	26 (76.5)	29 (46)	
Positive	42 (43.3)	8 (23.5)	34 (54)	
Unknown	3	1	2	
CK 5/6 status				.368
Positive	26 (29.5)	11 (35.5)	15 (26.3)	
Negative	62 (70.5)	20 (64.5)	42 (73.7)	
Unknown	12	4	8	
PTEN loss				.073
Yes	43 (48.3)	19 (61.3)	24 (41.4)	
No	46 (51.7)	12 (38.7)	34(58.6)	

Unknown	11	1	10

CK, cytokeratin; IGFBP2, insulin-like growth factor–binding protein 2; PTEN, phosphatase and tensin homolog.

[a] Defined as an Allred Score >5.

[b] Compared using the Mann-Whitney *U* test.

[c] Compared using the Fisher exact test.

[d] No patients with grade 1 tumor.

▮Table 5 ▮

Factors Associated With IGFBP2 Positivity in Multivariate Analysis

		95% CI for OR		
Factor	**OR for IGFBP2 Positivity (95% CI)[a]**	**Lower**	**Upper**	***P* Value**
Lymphovascular invasion				
Absent	1.00 b			
Present	2.56 c	0.95	6.93	.064
CK14 expression				
No	1.00[b]			
Yes	0.23[c]	0.08	0.64	.005
PTEN loss				
No	1.00			
Yes	2.74 c	1.02	7.39	.047

CI, confidence interval; CK, cytokeratin; IGFBP2, insulin-like growth factor–binding protein 2; OR, odds ratio; PTEN, phosphatase and tensin homolog.

[a] Derived using a multivariate logistic regression model including all variables with *P* < .20 in univariate analysis, lymphovascular invasion, CK 14 expression, and PTEN loss.

[b] Reference category.

[c] Statistically significant (*P* < .05).

▮**Image 1** ▮ Patterns of immunohistochemical staining for phosphatase and tensin homolog (PTEN) in three triple-negative invasive ductal carcinomas (IDC). A, Cytoplasmic and nuclear staining of the IDC. B, Weak cytoplasmic and nuclear staining for PTEN in the IDC compared with strong staining in the surrounding stromal tissue. C, Absence of staining for PTEN in the IDC, indicating total loss of PTEN in the tumor compartment, with strong staining for PTEN in the adjacent stromal tissue.

▮**Image 2** ▮ A, Loss of immunohistochemical staining for phosphatase and tensin homolog (PTEN) in a triple-negative invasive ductal carcinoma. B, Strong cytoplamic staining for cytokeratin 5/6 in the same tumor.

▮**Image 3** ▮ A triple-negative invasive ductal carcinoma (IDC) stained with H&E (A), the same IDC showing loss of PTEN staining (B) and strong cytoplasmic staining for insulin-like growth factor–binding protein 2 (IGFBP2) (C).

▮**Image 4** ▮ A, Immunohistocochemical cytoplasmic staining for insulin-like growth factor–binding protein 2 (IGFBP2) in a triple-negative invasive ductal carcinoma. B, The addition of the IGFBP-2 blocking peptide resulted in complete absence of cytoplasmic staining for IGFBP2 in this tumor.

▮**Image 5** ▮ A, A triple-negative infiltrating ductal carcinoma immunohistochemically stained for insulin-like growth factor–binding protein 2 (IGFBP2). B, The same tumor immunohistochemically stained for cytokeratin 14; the normal glands stain positively, the invasive tumor component is negative.

▮**Figure 1** ▮ Relative survival by phosphatase and tensin homolog (PTEN) status.

▮**Figure 2** ▮ Relative survival by insulin-like growth factor–binding protein 2 (IGFBP2) status.