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Loss of PTEN expression and AKT activation in HER2-positive breast carcinomas

Perda da expressão do PTEN e ativação da AKT em carcinomas mamários HER2-positivos

Original Article

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Abstract

PURPOSE: To examine the expression of AKT and PTEN in a series of HER2-positive primary invasive breast tumors using immunohistochemistry, and to associate these expression profiles with classic pathologic features such as tumor grade, hormone receptor expression, lymphatic vascular invasion, and proliferation. **METHODS:** A total of 104 HER2-positive breast carcinoma specimens were prepared in tissue microarrays blocks for immunohistochemical detection of PTEN and phosphorylated AKT (pAKT). Original histologic sections were reviewed to assess pathological features, including HER2 status and Ki-67 index values. The associations between categorical and numeric variables were identified using Pearson's chi-square test and the Mann-Whitney, respectively. **RESULTS:** Co-expression of pAKT and PTEN was presented in 59 (56.7%) cases. Reduced levels of PTEN expression were detected in 20 (19.2%) cases, and these 20 tumors had a lower Ki-67 index value. In contrast, tumors positive for pAKT expression [71 (68.3%)] were associated with a higher Ki-67 index value. **CONCLUSION:** A role for AKT in the proliferation of HER2-positive breast cancers was confirmed. However, immunohistochemical detection of PTEN expression did not correlate with an inhibition of cellular proliferation or control of AKT phosphorylation, suggesting other pathways in these mechanisms of control.

Resumo

OBJETIVOS: Avaliar a expressão imuno-histoquímica de AKT e PTEN em uma série de carcinomas mamários invasivos HER2-positivos, e associar seus padrões de expressão com variáveis anatomopatológicas clássicas, como grau histológico, expressão de receptores hormonais, embolização vascular linfática e atividade proliferativa. **MÉTODOS:** Um total de 104 amostras de carcinomas mamários invasivos HER2-positivos foram preparadas em blocos de microarranjos de tecido para detecção imuno-histoquímica de PTEN e AKT fosforilada (pAKT). Cortes histológicos originais foram revistos para avaliação das características anatomopatológicas, incluindo o estado do HER2 e a avaliação da expressão de Ki-67. As associações entre as variáveis categóricas e as numéricas foram feitas com o uso dos testes do chi-quadrado de Pearson e Mann-Whitney, respectivamente. **RESULTADOS:** Co-expressão de pAKT e PTEN foi identificada em 59 (56,7%) casos. Expressão reduzida de PTEN foi detectada em 20 (19,2%) casos, e esses 20 tumores mostraram menores valores de Ki-67. Por outro lado, tumores positivos para pAKT [71 (68,3%)] apresentaram células positivas para valores mais altos de Ki-67. **CONCLUSÕES:** O papel de AKT na proliferação de carcinomas mamários HER-2 positiva foi confirmada. Entretanto, a detecção imuno-histoquímica de PTEN não se correlacionou com inibição da proliferação celular ou controle da fosforilação de AKT, sugerindo outras vias nesses mecanismos de controle.

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Introduction

Breast cancer is a heterogeneous disease including distinct entities according to clinical behavior, pathological features, and molecular subtypes. Prognosis is largely dependent on intrinsic molecular subtype based on gene expression: luminal A, luminal B, HER2 overexpressing, and basal-like[1]. Approximately 20–25% of breast cancer cases involve overexpression of human epidermal growth factor receptor 2 (HER2)[2], a transmembrane receptor with tyrosine kinase activity that regulates several cellular processes such as cell proliferation, differentiation, adhesion, survival, and migration[3]. One of the most potent signaling pathways promoted by HER2 over expression is the phosphatidylinositol 3-kinase (PI3K)/AKT signaling cascade which affects cell cycle progression and can inhibit apoptosis[3]. The PI3K enzyme is a heterodimer composed of a regulatory subunit (p85) and a catalytic subunit (p110). Upon activation by tyrosine kinase receptors, PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2) to produce phosphatidylinositol-3,4,5-triphosphate (PIP3)[4]. PIP3 then recruits the serine/threonine kinase, AKT, to the plasma membrane. Upon phosphorylation of Ser473 of AKT, several kinases are activated, including the mammalian target of rapamycin (mTOR), an important molecule that regulates cell growth, p21 and p27, as well as several other molecules that inhibit apoptosis, such as Bad and caspase proteins[5-7]. It has been reported that activated AKT can be an indicator of poor prognosis, possibly by promoting cell survival[8]. To regulate AKT activity, PI3K is opposed by PTEN (phosphatase and tensin homolog deleted on chromosome 10), which converts PIP3 back to PIP2, thus preventing phosphorylation and activation of AKT. As a result, cellular proliferation is inhibited and tumor formation is suppressed[9].

PTEN is a tumor-suppressor gene that is located on chromosome 10q23.3[10]. Mutations in the PTEN gene, some of which lead to loss of PTEN protein expression, are related to a variety of human cancers, including prostatic and endometrial carcinomas. Among breast cancers, lack of PTEN protein expression is mainly attributed to loss of heterozygosity or promoter methylation of the PTEN gene[10,11]. In addition, activation of the PI3K pathway, as a result of low levels, or the absence, of PTEN expression, has been associated with resistance to trastuzumab, a recombinant humanized monoclonal antibody that recognizes the extracellular domain of HER2[12-14]. Therefore, reduced PTEN expression in breast carcinomas may reflect a more aggressive biologic behavior[15]. Correspondingly, several studies have demonstrated that deregulation of the PI3K/AKT/PTEN pathway is associated with poor prognosis, an increased incidence of disease recurrence, and a shorter disease-free survival period[15-18]. In this study, immunoexpression of pAKT and PTEN were assayed in a series of HER2-positive primary invasive breast tumor specimens. These expression profiles were then compared with clinicopathologic features of the tumors, including proliferative activity determined by expression of Ki-67.

Methods

Selection of the samples

This project was approved by the Scientific Committee of the Department of Obstetrics and Gynecology, and the Department of Pathology, of the Faculdade de Medicina da Universidade de Sao Paulo (Brazil), and also by the Ethical Committee for Research Projects of the Hospital das Clinicas da Faculdade de Medicina da Universidade de Sao Paulo, Brazil (CAPPesq) (protocol 0756/08).

Formalin-fixed, paraffin-embedded tissue specimens from 104 consecutive patients with HER2-positive primary breast carcinomas diagnosed in 2007 and 2008 were selected from the files of the Consultoria em Patologia (Botucatu, São Paulo), a major reference laboratory in Brazil that receives approximately 6,000 breast specimens per year. Inclusion criteria were: the availability of adequate tissue material, lack of any previous treatment, and HER2 positivity according to the guidelines of the American Society of Clinical Oncology (ASCO) and the college of American Pathologists (CAP)[19]. The mean age of the patients included in this study was 54.0±12.4 years old (range 30–91).

Pathological examination and tissue microarray construction

Two observers (F.M.L.L. and F.M.C) reviewed all slides, and the histologic type was determined based on the World Health Organization classification criteria[20]. Ninety-six (92.3%) tumors were classified as ductal, 3 (2.9%) as mucinous, 2 (1.9%) as micropapillary, 2 (1.9%) as signet ring cell, and 1 (0.9%) as pleomorphic invasive lobular carcinoma. Other histologic features evaluated included grade according to the Nottingham criteria[21], nuclear grade, presence of necrosis (absent or present), and peritumoral vascular invasion. Histologic and nuclear grades 1 and 2 were combined for statistical purposes, since we had only one case histologic and nuclear grade. Immunohistochemical detection of estrogen receptor (ER), progesterone receptor (PR), Ki67, and HER2 were performed using original whole histologic sections. All of the immunohistochemistry assays were performed in the same laboratory, using the same protocol, by the same pathologist.

A representative area of each tumor was selected for the construction of tissue microarray (TMA) blocks and for immunohistochemical studies of PTEN and pAKT. Areas of interest for each tumor were identified

in tissue sections stained with hematoxylin/eosin, and these sections were marked in the corresponding paraffin donor blocks. One cylinder of material (2.0 mm in diameter) was punched from each case and was mounted into recipient paraffin blocks at 2 mm intervals using a precision microarray instrument (Beecher Instruments, Silver Spring, MD). A grid system was established such that each core had an x- and y-coordinate reference for sample identification. Blocks were sealed at 60°C for 10 minutes. Sections (3 µm) from each TMA block were then prepared using standard techniques and mounted on Starfrost® slides. The first histological sections cut were stained with hematoxylin-eosin to ensure that the appropriate sections of the tumor had been obtained.

Immunohistochemistry and scoring

The antigens employed were ER (clone SP1, Dako, Carpinteria, USA, dilution 1:1000); PR (clone PgR 636, Dako, Carpinteria, USA, dilution 1:600); HER2 (clone SP3, Neomarkers, Fremont, USA, dilution 1:300); Ki-67 (clone MIB1, Dako, Carpinteria, USA, dilution 1:4800); pAKT (clone LP18, Novocastra, Richmond, USA, dilution 1:200), and PTEN (clone 28H6, Novocastra, Richmond, USA, dilution 1:800). Pressure cooker was the epitope retrieval method for all markers, except for PTEN, for which microwave oven was used. The bound antibodies were detected by goat anti-rabit or anti-mouse horseradish peroxidase-labelled polymerized secondary antibodies (DAKO EnVision TM System). Peroxidase activity was visualized with diamminobenzidine (Dako).

HER2 expression was confirmed on TMA slides and the samples were considered positive if they received a 3+ score according to guidelines from ASCO and CAP[19] (Figure 1A).

Tumors were considered positive for ER and PR expression if at least 10% of the cells were stained, according to the criteria of Park et al.[22]. Ki-67 index values were expressed as the percentage of positive cells present among at least 500 tumor cells in hot spot areas. The highest

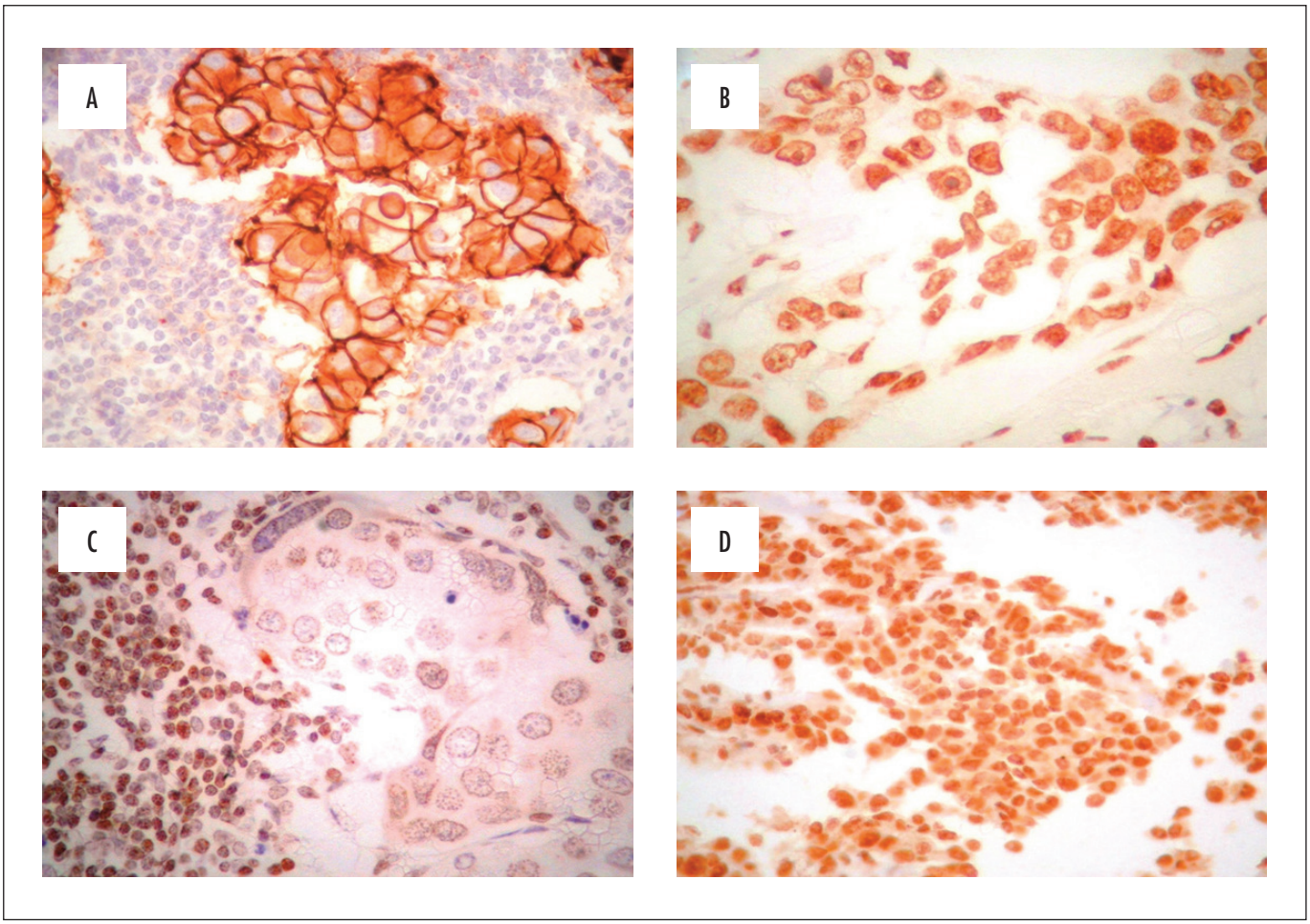

Figure 1. (A) HER2 positive carcinoma with complete and strong membrane expression of the protein in more than 30% of cells; (B) PTEN present characterized by nuclear expression in 100% of neoplastic cells; (C) PTEN reduced characterized by negative reaction contrasting with positive lymphocyte; (D) AKT diffusely positive in the neoplastic cells

value obtained in TMA sections or whole sections was the reported score. Tumors were classified according to the expression of PTEN. "PTEN present" indicated tumors with diffuse and strong positivity in 100% of the nuclei (Figure 1B), while "PTEN reduced" indicated tumors with less than 100% of nuclear positivity (Figure 1C).

The pAKT expression was assessed based on the percentage of nuclear and/or cytoplasmic positive cells in a sample. Tumors with at least 10% of the stained cells positive for pAKT expression were classified as positive according to the criteria of Gori et al.[23] (Figure 1D).

Statistical analysis

Associations between categorical variables were identified using Pearson's chi-square test, while the Mann-Whitney test was used to compare groups according to numeric variables (e.g., patient age, Ki-67 index values).

A p-value less than 0.05 was considered significant. Statistical analysis were performed using MedCalc for Windows (version 11.5.0.0; MedCalc Software, Mariakerke, Belgium).

Results

Associations between PTEN expression and clinicopathologic features of the cohort studied are listed in Table 1. The only statistically significant difference between PTEN- reduced and PTEN-present tumors was the proliferative activity detected with Ki-67 staining. Furthermore, samples with diffuse expression of PTEN exhibited higher levels of proliferation.

Expression of pAKT was found to be associated with a low histological grade (p=0.03). In addition, an association between expression of pAKT, a lower tumor necrosis, and an ER-positive status were observed. However, these associations were not statistically significant. None of the other clinicopathological features studied were associated with pAKT expression (Table 2). The Ki-67 index values were higher for tumors expressing pAKT compared with tumors that did not express pAKT (45.6±22.1 versus

Table 1. Clinicopathological features of the HER2-positive breast carcinomas examined according to PTEN expression

Features	PTEN reduced n (%)	PTEN present n (%)	p-value
Cases	20 (19.2)	84 (80.8)	
Age (years)	49±10.0	53±13.1	0.26[a]
Histologic grade			
½	9 (45)	37 (44)	0.99[b]
3	10 (50)	47 (55)	
Nuclear grade			
½	4 (20)	20 (23)	0.96[b]
3	15 (75)	64 (76)	
Peritumoral vascular invasion			
Yes	6 (30)	26 (30)	0.84[b]
No	11 (55)	49 (58)	
Tumor necrosis			
Yes	5 (25.0)	30 (35.7)	0.54[b]
No	14 (70.0)	51 (60.7)	
ER			
Positive	8 (40)	42 (50)	0.58[b]
Negative	12 (60)	42 (50)	
PR			
Positive	6 (30)	28 (84)	0.98[b]
Negative	14 (70)	56 (66)	
Ki-67			
Interval	15–90	0–90	0.03[a]
Mean ± SD	35.5±19.7	46±22.7	
Median	37.5	40	

[a]Mann-Whitney test; [b]Pearson´s chi-square test; ER: estrogen receptor; PR: progesterone receptor.

Table 2. Clinicopathological features of the HER2-positive breast carcinomas examined according to pAKT expression

Features	pAKT positive n (%)	pAKT negative n (%)	p-value
Cases	71 (68.3)	33 (31.7)	
Age (years)	53.2±13.2	54.9±11.9	0.62[a]
Histologic grade			
½	37 (52.8)	9 (27.2)	0.03[b]
3	33 (47.1)	24 (72.7)	
Not assessed	1	0	
Nuclear grade			
½	18 (25.7)	6 (18.1)	0.55[b]
3	52 (74.2)	27 (81.8)	
Not assessed	1	0	
Lymphatic vascular invasion			
Yes	20 (31.7)	12 (41.3)	0.50[b]
No	43 (68.2)	17 (58.6)	
Not assessed	8	4	
Tumor necrosis			
Yes	20 (28.5)	15 (50.0)	0.07[b]
No	50 (71.4)	15 (50.0)	
Not assessed	1	3	
ER			
Positive	39 (54.9)	11 (33.3)	0.06[b]
Negative	32 (45.0)	22 (66.6)	
PR			
Positive	27 (38.0)	7 (21.2)	0.14[b]
Negative	44 (61.9)	26 (78.7)	
Ki-67			
Interval	0–95	15–80	0.22[a]
Mean ± SD	45.6±22.1	40.4±21.5	
Median	40	30	

[a]Mann-Whitney test; [b]Pearson´s chi-square test; ER: estrogen receptor; PR: progesterone receptor.

40.4±21.5), although the difference was not statistically significant. Moreover, diffuse PTEN expression was observed in 59/71 (83.1%) of tumors positive for pAKT, and in 25/33 (75.7%) of tumors negative for pAKT, although the difference was not significant (p=0.06; chi-square=0.4).

Discussion

In this study, expression of PTEN and pAKT was assayed in 104 HER2-positive primary breast tumors. Since these molecules have previously been shown to mediate antagonistic mechanisms, their associations with clinicopathologic features were examined.

Cowden's disease is a hereditary cancer predisposition syndrome that is associated with an elevated risk of breast and thyroid cancer. Germ line mutations in PTEN have been identified in patients with Cowden's disease, and the frequency of sporadic breast cancer with loss of PTEN expression has been reported to range from 30-40%[4,18,24-26]. In the present study, the frequency of breast cancer with loss of PTEN expression was 19.2%. However, loss of PTEN expression was evaluated using immunohistochemistry, and this method may underestimate the proportion of invasive breast tumors that contain an inactivated version of PTEN. For example, in a retrospective study conducted by Bose et al.[18], loss of PTEN expression in a set of 34 sporadic invasive breast tumors was also evaluated using immunohistochemistry. The molecular status of these tumors had previously been established, including the PTEN genotype. For 13 cases with loss of heterozygosity (LOH) at the 10q23 locus, only 6 exhibited lower levels of PTEN expression in immunohistochemistry assays. The remaining seven cases exhibited no loss of PTEN expression. These data suggest that immunohistochemistry assays may not have sufficient sensitivity to detect the decrease in PTEN levels associated with LOH, and this is consistent with the findings of the present study.

Except for proliferative activity, none of the clinicopathological parameters examined was found to be associated with the PTEN results obtained. While similar results have been reported in other studies[11,16,27], significant associations between certain clinicopathological characteristics and PTEN loss have also been reported. For example, Lee et al.[15] found that reduced PTEN expression is correlated with lymph node status, tumor grade, and TNM stage. However, there was no significant difference between PTEN expression and patient age, tumor size, or invasion of the lymphovascular space[15]. Bose et al.[18] also demonstrated that reduced expression of PTEN was associated with a high tumor grade. On the other hand, though, Pérez-Tenorio et al.[4] demonstrated that loss of PTEN was associated with an ER positive status (p=0.001), small tumor size (p=0.02) and low levels of HER2 expression (p=0.01), characteristics that have been associated with a less aggressive phenotype and low proliferative activity. The results of Pérez-Tenorio et al.[4] with the proliferative activity were similar to ours. In addition, it cannot be ruled out that methodological factors and/or the number of cases examined may be responsible for these discrepancies.

Surprisingly, Ki-67 index values were found to be higher for tumors with strong, diffuse expression of PTEN compared to PTEN reduced tumors, or tumors negative for PTEN expression. We hypothesize that high PTEN expression represents a positive feedback signal for proliferative activity, which has previously been described for the endometrium. Immunohistochemistry was used to evaluate the expression of PTEN in samples of normal endometrium obtained during different phases of the menstrual cycle. In their study, Mutter et al.[28] demonstrated that endometrial expression of PTEN varies during the proliferative and secretory phases, with levels of PTEN being higher during the first one. This finding was unexpected at the time, and the explanation proposed was that a functional requirement for PTEN-mediated tumor suppressor activity was specific for a highly mitotic, estrogenic environment, while progestin-dominated conditions would inhibit cell division and PTEN activity would be reduced. Although breast tissue and endometrium tissue are quite different, it is possible that this mechanism may be common to both. For example, PTEN positivity in breast tumors that exhibit higher proliferative activity may involve a compensatory mechanism that aims to control cell proliferation to maintain the delicate balance of the intracellular environment.

In the series examined, the proportion of breast cancers positive for pAKT expression (71/104, 68.3%) was similar to that reported in other studies[6,23]. The AKT has been found to mediate protumorigenic effects of hormones and growth factors, and the anchorage-mediated survival of epithelial cells. Accordingly, activation of AKT stimulates glucose metabolism, cell cycle progression, and survival through the phosphorylation of multiple substrates, including GSK3, BAX, and mTOR among others[29]. In the present study, a significant inverse relationship between pAKT positivity and tumor histological grade was observed (p=0.02). Additional inverse relationships between pAKT positivity and lymphatic vascular invasion and tumoral necrosis were also observed, yet these were not statistically significant. These findings are consistent with those of other studies[6,27], yet are in contrast with accumulating evidence that AKT harbors anti-apoptotic properties and promotes tumor progression and tumor growth[29,30]. In addition, other studies have demonstrated a direct relationship between pAKT and tumor histologic grade[30].

It was also observed that some tumors were both pAKT positive and ER positive, although this association did not reach statistical significance. Previous studies have demonstrated

that a complex network of cross-talk occurs between the HER2 activation pathway and ER mediated signaling[31-33]. For example, in an in vitro model, Stoica et al.[34] demonstrated that estradiol can activate the PI3K/AKT pathway in MCF-7 breast cancer cells, and that this effect was ErbB2 dependent. While these results remain to be confirmed in other breast cancer cells *in vitro* and *in vivo*, they are consistent with the positive correlation observed between expression of ER and pAKT in our study, and in other studies[6,27].

Regarding PR expression, Tokunaga et al.[35] previously showed that LOH at the PTEN locus and HER2 overexpression enhance activation of AKT. Since activated AKT has been shown to repress transcription of PR independent of ER expression, loss of PR expression can be induced even in ER-positive breast carcinomas[35]. Accordingly, in the present study, PR expression was lower in tumors positive for pAKT expression, although this correlation was not significant.

An unexpected result was that PTEN expression tended to correlate with pAKT expression. Similarly, in a study by Bose et al.[7] with 145 invasive breast cancers and 140 pure ductal carcinomas *in situ*, AKT and its downstream proteins were found to be activated in approximately 30% of the cancers analyzed independent of PTEN loss. This finding suggests that other mechanisms of AKT activation exist in addition to the activation of AKT by growth factor receptors with tyrosine kinase activity.

The results of the present study confirm that AKT has an important role in cellular proliferation and is associated with low grade carcinomas. However, paradoxically, diffuse expression of PTEN protein was found to be more frequent among tumors positive for pAKT and exhibiting a higher proliferative activity. These data suggest that PTEN is not the primary control mechanism for AKT phosphorylation, and molecules associated with other signaling pathways may be involved. Therefore, while inactivation of PTEN has been described as one of the mechanisms involved in the resistance of HER2 tumors to trastuzumab therapy, it may represent a rare event. Furthermore, we hypothesize that an increase in cellular proliferation may trigger diffuse expression of PTEN in an attempt by the cell to reduce levels of PTEN expression. However, further studies will be needed to investigate this hypothesis.

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