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Protocol for PTEN Expression by Immunohistochemistry in Formalin-fixed Paraffin-embedded Human Breast Carcinoma

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Abstract

The PI3K/PTEN pathway plays a major role in carcinogenesis. Dysregulation of this pathway occurs frequently in breast cancer, and loss of PTEN expression is emerging as a potentially important mechanism of resistance to the widely used anti-HER2 therapy, trastuzumab. However, assays for loss of PTEN expression have suffered from lack of consistency. Here, we describe an automated and reliable protocol for PTEN protein expression by immunohistochemistry in formalin-fixed paraffin-embedded tissue that can be easily incorporated into clinical trials.

Keywords

PTEN protein expression; immunohistochemistry; automated protocol; breast cancer

The PI3K/PTEN pathway is the focus of intense investigation as a potential target for molecular therapy. This pathway controls multiple cellular functions such as cell metabolism, proliferation, apoptosis, migration, and survival. Both proteins are frequently mutated in human cancer, and activation of the pathway by either mutation seems to be sufficient to generate tumors in mice. In breast cancer, there is emerging evidence suggesting that loss of function of PTEN not only plays a role in tumorigenesis, but also that it may be a key event in resistance to targeted therapy.[1–7] Laboratory models have shown that reducing PTEN in breast cancer cells by antisense oligonucleotides can induce resistance to the anti-HER2 antibody, trastuzumab, both in vitro and in vivo; furthermore, patients with ErbB2 overexpressing tumors with concurrent low levels of PTEN expression have a poor response to trastuzumab treatment.[8] The ability to identify the subset of ErbB2 overexpression tumors likely to be resistant to trastuzumab therapy may have a significant impact on treatment planning. Currently, however, reliable and reproducible methods for measuring PTEN expression on formalin-fixed tissue are not standardized.

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Several immunohistochemistry (IHC) protocols have been reported for PTEN. These protocols vary in terms of choice of antibody, methods of tissue fixation, duration of incubation, and scoring method. In addition, all these published protocols use manual staining methods, which leads to greater intersample variability, increases costs, limits use in the high-throughput setting, and potentially introduces bias.[9–12] There is also no standard approach to interpretation and scoring of IHC signal in terms of either intensity of staining or distribution/subcellular localization (Table 1). Here, we report a protocol optimized for automated IHC that allows for accurate and feasible quantitative analysis of PTEN expression suitable for high-throughput screening. Furthermore, we propose a scoring system that classifies PTEN expression both in terms of intensity of expression and localization.

MATERIALS AND METHODS

With the approval of our institutional review board, formalin-fixed paraffin-embedded (FFPE) blocks from breast tumor specimens were retrieved and reviewed to confirm the presence of normal ductal epithelium, ductal carcinoma in situ, and invasive ductal carcinoma. Breast and ovarian cancer cell lines with known PTEN status served as positive and negative controls. The PTEN status in cell lines was confirmed by Western blotting using the anti-PTEN antibody (mouse anti-human, clone 6H2.1, Dako). The breast cancer cell line MDA-MB-468 and ovarian cancer cell line IGROV-1 had no PTEN expression, whereas PTEN wild-type MCF7 and SKOV3 cell lines showed positive PTEN protein expression. Cell pellets were processed and embedded in paraffin using standard techniques. The samples and controls were sectioned (4 mm) and stained on the Dako Autostainer Plus (Dako USA, Carpinteria, CA).

Before immunostaining, the slides were heated (56°C) for 3 hours in a drying oven and then deparaffinized (xylene), washed with alcohol (100% and 95%), and rehydrated in deionized water. Antigen retrieval was performed as follows: the slides were incubated at 98°C for 20 minutes in Target Retrieval Solution pH 9 (Tris/ethylene diamine tetra-acetate buffer, pH 9, Dako Cytomation), then allowed to cool to room temperature before rinsing with Tris-buffered saline wash buffer (Dako). Endogenous peroxidase activity was blocked by incubating the slides for 5 minutes in 0.03% hydrogen peroxide (EnVision/HRP, Dako). After rinsing in wash buffer, the sections were incubated for 30 minutes at room temperature with the monoclonal mouse anti-human PTEN antibody (dilution 1:100, clone 6H2.1, Dako) in Tris-HCl buffer antibody diluent (Dako). Slides were rinsed in wash buffer and incubated for 30 minutes with peroxidase-labeled polymer conjugated to goat anti-mouse immunoglobulins (EnVision/HRP, Dako). The chromogenic reaction was carried out with 3,3′-diaminobenzidine chromogen solution for 10 minutes, resulting in the expected brown-colored signal. Finally, after rinsing with deionized water, the slides were counterstained with hematoxylin, dehydrated, mounted with toluene-based mounting medium (Thermo Scientific Richard-Allan) and coverslipped (Table 2).

RESULTS

The stained slides were reviewed by 2 observers. All cell lines showed concordant results by Western blotting and IHC, confirming the ability of our protocol to accurately demonstrate PTEN expression (Fig. 1). Of note, all positive control cell lines showed a strong cytoplasmic and/or nuclear staining pattern for PTEN.

Normal ductal epithelial cells showed a positive cytoplasmic immunoreaction, whereas the expression in tumor cells (both in situ and invasive) varied. As with the cell line controls, PTEN positive tumor cells showed cytoplasmic and/or nuclear signal localization, whereas

the PTEN negative tumor cells did not show any staining. The surrounding microenvironment served not only as a positive internal control, but also as an internal reference for semi-quantitative scoring of the degree of immuno-reactivity in the tumor cells (Fig. 2). Specifically, the cytoplasmic and/or nuclear immunoreaction was scored based on intensity whereby a score of 2 = positive (equal in intensity to normal epithelial cells), a score of 1 = weak (reduced intensity as compared with normal epithelial cells), and a score of 0 = negative (no immunoreaction).

CONCLUSIONS

In summary, we have optimized an IHC protocol that allows for automated, rapid, and accurate assessment of PTEN status in FFPE samples. Our proposed method of semi-quantitative scoring presents an easily reproducible means of assessing degree of immunoreaction and allows for easy comparison of data across series. As we continue to move toward the identification of patient subgroups that most likely respond to targeted therapies, it is critical that we develop and validate reliable means of assessing protein expression in FFPE tissues. This protocol for PTEN represents a reliable and automated approach that can be easily incorporated into clinical trials.

Acknowledgments

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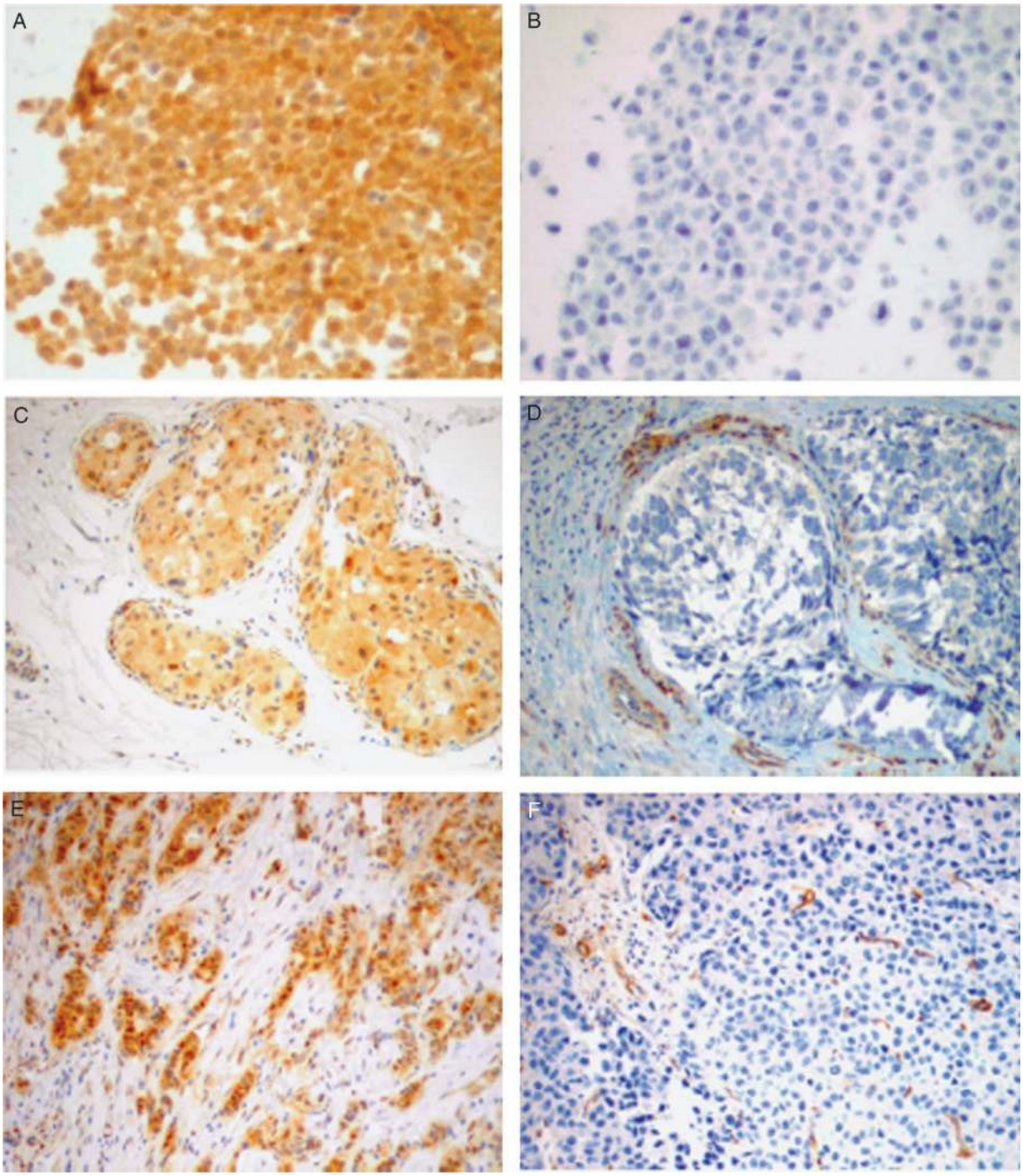

FIGURE 1.

Cell lines: positive PTEN staining in MCF7 breast cell line (A) and negative staining in MDA-MB-468 (B). FFPE breast tissues: positive PTEN staining in ductal carcinoma in situ (C); negative PTEN staining in ductal carcinoma in situ (D); positive PTEN staining in invasive carcinoma (E); negative PTEN staining in invasive carcinoma (F) with normal ductal epithelium and stroma serving as positive internal control. FFPE indicates formalin-fixed paraffin-embedded.

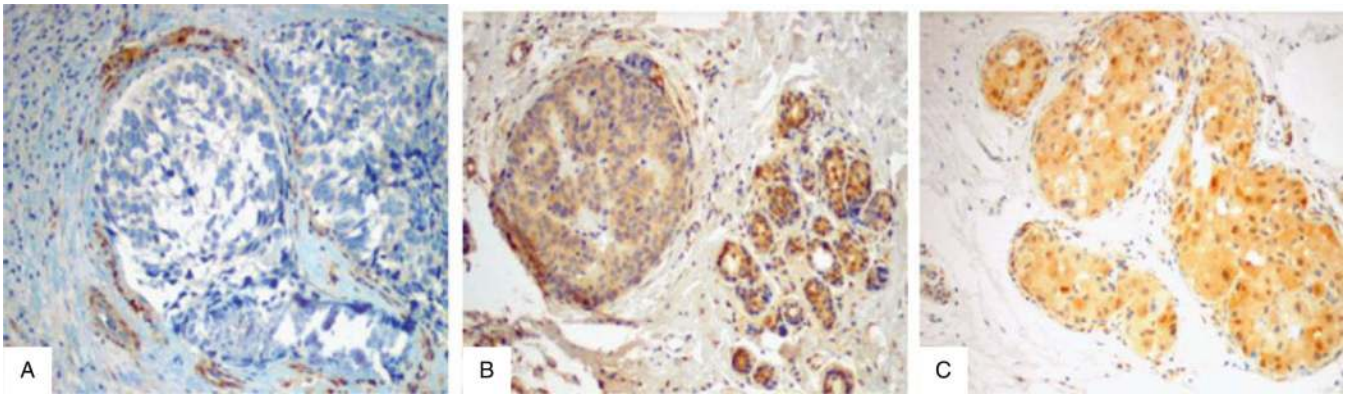

FIGURE 2.
Semi-quantitative scoring: (A) score 0 = staining undetectable in tumor cells but present in surrounding normal ductal epithelial and stromal cells; (B) score 1 = staining weaker than surrounding normal ductal epithelial and stromal cells; (C) score 2 = staining equal to that of surrounding normal ductal epithelial and stromal cells.

TABLE 1

PTEN Protocol Variability in Breast Tissue

Author (Year)	Tissue	PTEN Primary Antibody	Origin	Dilution	Control	Incubation	Procedure	Detection	Staining Location	Scoring
Lee et al (2001)[10]	Tongue	Rabbit polyclonal	Zymed	1:100	Normal gland	Overnight	Manual	Avidin-biotin	Cytoplasmic	Equal to normal, decreased, absent
Tsutsui et al (2005)[9]	Breast	Mouse monoclonal (28H6)	Santa Cruz	1:200	Normal gland	Overnight	Manual	Avidin-biotin	Unspecified	Normal or reduced
Perren et al (1999)[12]	Breast	Monoclonal (6H2.1)	Ziebold and Lees	1:100	Cell lines	1h	Manual	Avidin-biotin		Grade 0, +, + +
Thomas et al (2004)[11]	Prostate	Monoclonal (6H2.1)	Cascade Bioscience	1:300	Normal gland	Overnight	Manual	Avidin-biotin	Unspecified	Scale 0,1,2
Sakr (2010)	Breast	Mouse monoclonal (6H2.1)	Dako	1:100	Cell lines and normal gland	30 min	Automated	Horse radish peroxidase	Cytoplasmic nuclear	Scale 0,1,2

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TABLE 2

PTEN Immunohistochemistry Staining Protocol

1—Heating:
Heating at 55 to 60°C—2 to 3 h
2—Deparaffinization—Hydration at room temperature:
Xylene—5 min (3 times)
Ethanol 100%—5 min (2 times)
Ethanol 95%—5 min (2 times)
Deionized water—5 min (2 times)
Wash buffer—2 min
3—Target retrieval:
Target retrieval solution (pH 9) in water bath at 95 to 99°C—40 min
Cooling at room temperature—20 min
Wash in deionized water
Wash buffer—5 min
4—Autostainer:
Equilibrate reagents to room temperature
Load slides and run the program with 200 μL per slide:
Peroxidase block (Envision Kit)—5 min
Wash buffer—5 min
Primary antibody anti-PTEN (1/100)—30 min
Wash buffer—3 min (3 times)
Polymer-HRP (Envision Kit)—30 min
Wash buffer—3 min (3 times)
DAB chromogen (Envision Kit)—10 min
Wash in deionized water
5—Counterstaining:
Hematoxylin—1 min
Wash in deionized water
Acid alcohol—dip
Wash in deionized water
Ammonia water—dip
Wash in deionized water
6—Dehydration:
Ethanol 95%—3 min (2 times)
Ethanol 100%—3 min (2 times)
Xylene—5 min (2 times)
7—Mounting:
Mounting medium and coverslip

DAB indicates 3,3′-diaminobenzidine.