

# Differential Expression of the PTEN Tumor Suppressor Protein in Fetal and Adult Neuroendocrine Tissues and Tumors: Progressive Loss of PTEN Expression in Poorly Differentiated Neuroendocrine Neoplasms

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Genetic alteration and loss of expression of tumor suppressor gene PTEN has been found in carcinomas of the breast, prostate, and endometrium, as well as in gliomas. PTEN expression in neural crest/neuroendocrine (NC/NE) tissues and in neoplasms has not been reported. This study examines PTEN expression in embryonal, fetal, and adult tissues by immunohistochemistry. The authors found high PTEN expression in embryonal, fetal, and adult NC/NE tissues. The authors also study the PTEN expression in NC/NE neoplasms (N = 37), including 5 melanocytic nevi, 2 melanomas, 9 carcinoids, 2 moderately differentiated neuroendocrine carcinomas, 13 poorly differentiated neuroendocrine carcinomas, 2 paragangliomas, 2 pheochromocytomas, 2 medullary thyroid carcinomas, and 1 neuroblastoma. All carcinoid tumors and melanocytic nevi showed moderate or strong immunostaining for PTEN. In contrast, the majority of poorly differentiated neuroendocrine carcinomas (7 of 13) were negative for PTEN (54%); the remainder showed diminished reactivity. The two melanomas studied were also negative for PTEN immunostaining. The paragangliomas, pheochromocytomas, medullary thyroid carcinomas, and neuroblastoma all showed a strong PTEN stain. The authors postulate that PTEN is a differentiation marker for NC/NE tissue and tumors and that loss of PTEN expression may represent an important step in the progression of NE tumors.

**Key Words:** PTEN-tumor suppressor gene—Immunohistochemistry—Neuroendocrine—Carcinoid—Neuroendocrine carcinoma.

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PTEN is a recently identified tumor suppressor gene mapped to chromosome TEN, having both lipid/tyrosine phosphatase activity and molecular homology to tensin (1,2). It is thought to play an important role in the fundamental control of cell growth, death, adhesion, and migration (3–6). Transgenic mice, which harbor dis-

rupted PTEN, manifest early embryonic lethality, whereas heterologous mice that carry the PTEN<sup>+/-</sup> gene spontaneously develop tumors in variety of organs, suggesting a dual role in ontogenesis and tumor suppression (7). Germline mutations of PTEN have been detected in three human autosomal dominant disorders, Cowden disease, L'Hermitte-Duclos disease, and Bannayan-Zonana syndrome, which share some similar features including the formation of multiple benign tumors and an increased susceptibility to malignancy development (8–12). A high incidence of PTEN mutations has also been observed in several tumors, including glioma, endometrial, and breast carcinomas (13–19). Furthermore, loss of PTEN protein expression is frequent in high-grade breast and prostate carcinomas, suggesting that the loss of PTEN is a late event in the progression of these tumors (20–22).

Recently, Gimm et al.(23) found high PTEN expression in fetal and adult central and peripheral nervous systems. These data prompted us to investigate, using immunohistochemical methods, PTEN protein expression in fetal and adult neural crest/neuroendocrine (NC/NE) tissues. We observed that PTEN protein was expressed in the fetal and adult pituitary glands, pancreatic islets, adrenal medulla, peripheral nerve plexuses, and skin melanocytes. We subsequently investigated PTEN expression in a variety of NE neoplasms and found that progressive loss of PTEN protein expression occurred in poorly differentiated tumors.

## MATERIALS AND METHODS

### Tissues and Tumors

The material studied comprised tissues from six embryos and fetuses (gestational age: 8, 11, 13, 17, 22, and 25 weeks), two adults, and 37 NC/NE neoplasms (Table 1) obtained from archival surgical and autopsy specimens in the Department of Pathology, Kings County Hospital Center, Brooklyn, New York. Blocks fixed in 10% buffered formalin and embedded in paraffin were evaluated independently (by C.A.A. and L.W.) for gestational age, tissue type, and diagnosis. Immunostaining

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**TABLE 1.** Grade and semiquantitative scoring of PTEN immunostain in NC tumors

Case	Diagnosis	Staining intensity	Positive cells (%)
1	Carcinoid, thymus	+++	++++
2	Carcinoid, teratoma	++	++++
3	Carcinoid, colon	+++	++++
4	Carcinoid, colon	+++	++++
5	Carcinoid, colon	+++	++++
6	Carcinoid, liver	+++	++++
7	Carcinoid, mediastinum	++	++++
8	Carcinoid, appendix	+++	++++
9	Carcinoid, small intestine	++	++++
10	MDNEC, kidney	++	+++
11	MDNEC, cervix	+	++
12	PDNEC, endometrium	++	+
13	PDNEC, gallbladder	++	++
14	PDNEC, lung	++	+++
15	PDNEC, mediastinum	—	0
16	PDNEC, lung	+	++
17	PDNEC, lung	—	0
18	PDNEC, lung	—	0
19	PDNEC, cervix	++	+
20	PDNEC, lung	—	0
21	PDNEC, cervix	—	0
22	PDNEC, lung	—	0
23	PDNEC, cervix	—	0
24	PDNEC, lung	+	++
25	Pheochromocytoma	+++	+++
26	Pheochromocytoma	+++	++++
27	Paraganglioma	+++	++++
28	Paraganglioma	+++	+++
29	Medullary carcinoma, thyroid	+++	++++
29	Medullary carcinoma, thyroid	+++	+++
30	Neuroblastoma	+++	+++
31	Melanocytic nevus, skin	+++	++++
32	Melanocytic nevus, skin	++	+++
33	Melanocytic nevus, skin	+++	++++
34	Melanocytic nevus, skin	++	++++
35	Melanocytic nevus, skin	+++	+++
36	Melanoma, skin	—	0
37	Melanoma, metastatic	—	0

MDNEC, moderately differentiated neuroendocrine carcinoma; PDNEC, poorly differentiated neuroendocrine carcinoma.

was performed on 5- $\mu$  m sections from one or two representative areas. The diagnosis of neuroendocrine tumors was confirmed by synaptophysin and chromogranin immunostaining. Immunostain for S-100 and HMB-45 was performed on serial sections to confirm the recognition of olfactory cells, nerve plexuses, and melanocytes of skin, respectively.

#### Classification of Neuroendocrine Tumors

The neuroendocrine tumors were classified as carcinoid tumor, well-differentiated neuroendocrine carcinoma, moderately differentiated neuroendocrine carcinoma (MDNEC), and poorly differentiated neuroendocrine carcinoma (PDNEC), according to Axiotis (24). A well-differentiated neuroendocrine carcinoma is equivalent to what the World Health Organization (1999) (25) classifies as an atypical carcinoid or to what Gould et al. (26) classifies as a well-differentiated neuroendocrine

carcinoma grade I and II, and it is characterized by carcinoidlike growth pattern with an apparent loss of architectural organization (2 to 10 mitoses per high-power field and/or small foci of necrosis). Moderately differentiated neuroendocrine carcinoma is equivalent to atypical carcinoid (World Health Organization) or well-differentiated neuroendocrine carcinoma grade III (Gould et al.), and is characterized by a carcinoidlike growth pattern with cellular discohesion and a prominent loss of architectural organization,  $\geq 11$  mitoses per 10 high-power fields, and conspicuous areas of central necrosis. PDNEC is equivalent to small cell/large cell neuroendocrine carcinoma (World Health Organization) or small cell/intermediate neuroendocrine carcinoma (Gould et al.), and it is characterized by the loss of carcinoidlike growth pattern, numerous mitosis, and geographic necrosis. PDNEC can be small cell type, large cell type, or mixed small/large cell type.

#### Reagents

A murine monoclonal antibody (sc-7974; Santa Cruz Biotech, Santa Cruz, CA) that recognizes the last 100 C-terminal amino acids of human PTEN protein was used in all immunohistochemical analyzes. The specificity of this antibody has been shown in previous studies (21,27).

#### Immunostaining

Immunostaining was performed on a Ventana Nexes IHC staining system by using the Nexes Enhanced DAB detection kit Cat#760-003 (Ventana, Tuscon, AZ) according to the manufacturer's recommendations. Deparaffinized, rehydrated 4- $\mu$ m sections briefly underwent microwave antigen retrieval by using Antigen Retrieval Citra solution (BioGenex, San Ramone, CA). The primary antibody against PTEN protein (1:50 dilution) was incubated at 37°C for 32 minutes. The melanin pigments from nevus, skin, or melanoma tissue sections were removed by standard treatment with 10% hydrogen peroxide for 24–48 hours before histochemical immunostaining (28).

#### Antigen retrieval

Antigen retrieval techniques have dramatically improved the staining quality of archival tissue sections (29). An immunohistochemical study of PTEN protein expression in prostate carcinoma that used antigen retrieval techniques by heating tissue sections at 100°C for 10 minutes in a pH 6.0-citrate buffer in archival paraffin-embedded tissue sections has been successful (22). We optimized antigen retrieval conditions with a variety of formalin-fixed, paraffin-embedded archival tissues by heating tissue sections at 100°C for 5 minutes in a commercial citrate solution. A comparable staining intensity was achieved by using a commercial immunostaining kit and an automatic stainer.

### Controls

Parallel sections incubated with PTEN-blocking peptide/PTEN antibody mixture determined the specificity of PTEN protein immunostaining. In detail, the antibody against PTEN protein was preincubated with excess PTEN-blocking peptide (Santa Cruz Biotech) and parallel slides were stained. Sections were also incubated with the immunoglobulin fraction of normal mouse serum (in place of the primary antibody) in the same protein concentration as the primary antibody.

### Quantitation

Cases with staining in more than 10% of cells were considered to have a positive result. PTEN immunostaining was semiquantitatively expressed as percentage of tumor cells stained <10% (0), <25% (1+), <50% (2+), <75% (3+), >75% (4+); and intensity of immunostaining as weak staining (1+), moderate staining (2+), strong staining (3+).

## RESULTS

### Specificity of PTEN Protein Immunostaining

Sections incubated with the anti-PTEN protein antibody all showed diffuse, granular cytoplasmic immunostaining with positive reactivity, except for a case of PDNEC that showed weak staining in both cytoplasm and nuclei. The parallel sections incubated with the anti-PTEN/PTEN-blocking peptide mixture or the normal mouse serum did not show a reaction product (Fig. 1).

### PTEN Immunoreactivity in Fetal and Adult Tissues

The strongest overall PTEN protein immunoreactivity was observed in the central and peripheral nervous systems and in NC/NE tissues. The embryonal, fetal, and adult pituitary glands; pancreatic islets; the adrenal medulla; skin melanocytes; the myenteric and mucosal ganglia of gut; and the paravertebral ganglia stained strongly for PTEN protein (Figs. 2 and 3C). The nerve branches and twigs in peripheral organs and tissues were also strongly positive for PTEN.

PTEN immunostaining in central nervous system (CNS) tissue showed differential expression during human development. In early embryos, the CNS, including the retinal epithelium and primitive nasal olfactory cells, was diffusely and strongly positive for PTEN. Overall, the staining intensity decreases when the fetal CNS matures. Adult brain and spinal cord tissues showed weakly positive staining for PTEN in both astrocytes and neurons (Fig. 3).

In general, PTEN immunostaining results were negative or weak in most nonneural tissues. Blood vessels were the only nonneural/neuroendocrine tissue that showed differential staining for PTEN product. In early fetal development, the aorta and small-vessel wall

muscles tested positive for PTEN protein, whereas the adult aorta, medium-size muscular arteries, and small vessels and capillaries lost PTEN protein expression. Adult thyroid glands, breast tissue, basal layers of skin, the esophagus, and the prostate show focal and weak PTEN protein staining.

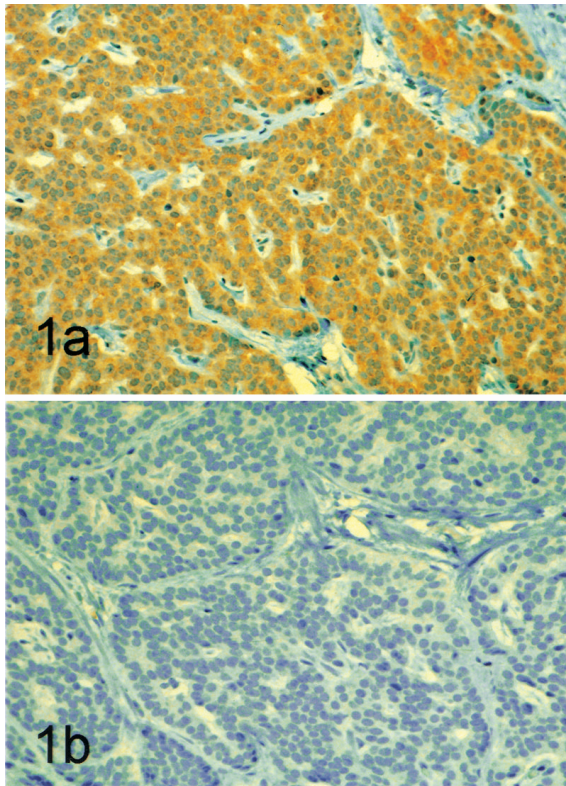
### PTEN Protein Immunoreactivity in NC/NE Tumors

The 37 NC/NE tumors listed in Table 1 were evaluated for PTEN protein immunoreactivity. Figures 4 and 5 are representations of the results obtained. All carcinoid tumors, melanocytic nevi, paragangliomas, pheochromocytomas, medullary thyroid carcinomas, and neuroblastomas showed moderate or strong immunostaining for PTEN protein, and a majority of the tumor cells had positive results (>75%). However, 7 of 13 (54%) PDNECs were negative for PTEN immunostaining; the remaining PDNECs partially lost PTEN expression. Moderately differentiated neuroendocrine carcinomas showed a weak or a moderate PTEN protein stain. The two melanomas examined were also negative for PTEN product.

## DISCUSSION

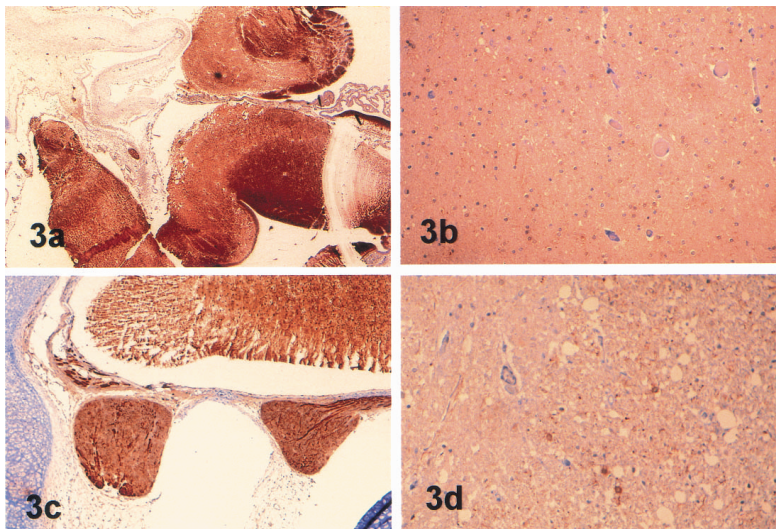
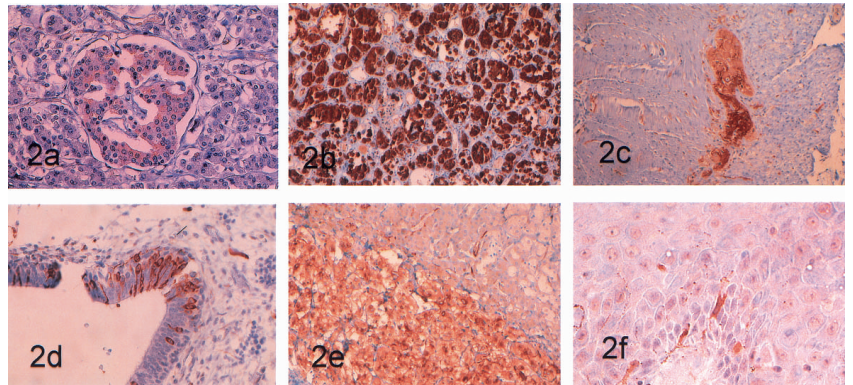
The present immunohistochemical data show 1) differential PTEN expression during CNS development; 2) marked PTEN expression in both fetal and adult NC/NE tissues; and 3) differential PTEN expression in well-differentiated NE tumors and poorly differentiated NE tumors. Because PTEN is not a structural protein, we believe that its expression is important for the differentiation of CNS and NC/NE tissues. PTEN inactivation in a transgenic mouse model results in early embryonic lethality. PTEN<sup>±</sup> and Chimeric mice showed susceptibility to subsequent tumor development, suggesting a dual role in both ontogenic development and tumor suppression (7). However, the formation and development of CNS and NC/NE tissues were not specifically examined in this PTEN knockout animal model. It would be reasonable to speculate that the development of the nervous and vascular systems is mostly likely affected because of the high PTEN expression in these two systems. In addition, Cowden's disease, L'Hermitte-Duclos disease, and Bannayan-Zonana syndrome are characterized by developmental defects such as vascular malformations and features such as dysplastic gangliocytoma of the cerebellum and mental retardation, further suggesting that PTEN plays a role in both neural and vascular development (10–12).

Molecular characterization of PTEN product has shown a high degree of homology to lipid/protein tyrosine phosphatases and tensin (1,30). The lipid phosphatase activity of PTEN product is related to the inhibition of cell cycle progression and G1 arrest through regulation of the PI3K/Akt pathway (31). Akt also is one of the

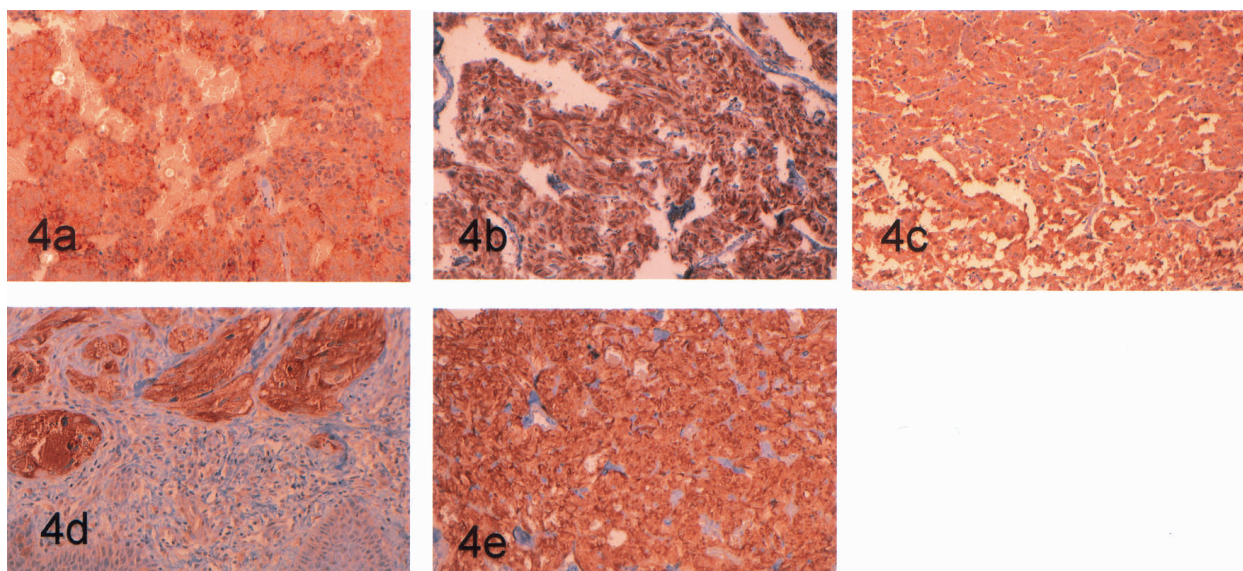


**FIG. 1. A:** Carcinoid incubated with anti-PTEN monoclonal antibody shows strong PTEN reactivity. **B:** The parallel section incubated with anti-PTEN antibody/blocking peptide mixture shows specific blocking of PTEN immunoreactivity.

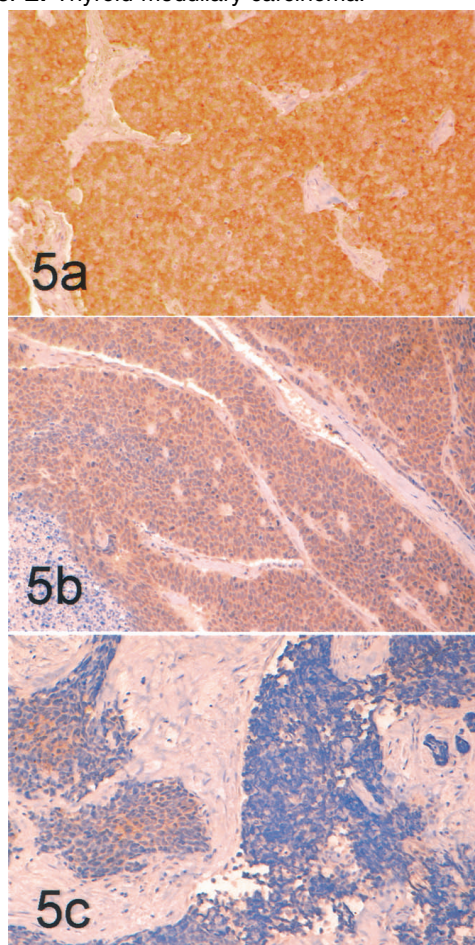
**FIG. 2. A:** Pancreatic islet. **B:** Adenohypophysis. **C:** Nerve plexus of the intestine. **D:** Olfactory cells of nasal mucosa. **E:** Adrenal medulla. **F:** Skin melanocytes. All show PTEN immunoreactivity.



**FIG. 3. A and C:** Embryonic brain and spinal cord cells (11-week gestational age) show strong PTEN immunoreactivity. **B and D:** Adult brain and spinal cord cells show weak PTEN staining.



**FIG. 4.** Positive PTEN immunostaining. **A:** Neuroblastoma. **B:** Pheochromocytoma. **C:** Paraganglioma. **D:** Melanocytic nevus. **E:** Thyroid medullary carcinoma.



**FIG. 5.** **A:** Carcinoid shows strong PTEN immunoreactivity. **B:** Moderately differentiated neuroendocrine carcinoma shows moderate PTEN immunoreactivity. **C:** Poorly differentiated neuroendocrine carcinoma shows weak focal PTEN immunoreactivity.

key regulatory molecules involved in the protection of cells against apoptosis (32–34). Tensin binds actin filaments at focal cellular adhesions, integrin-containing complexes, focal adhesion kinase, and growth factor receptors, all of which have been implicated in cell growth regulation, cell mobility, cell-stromal interaction, and angiogenesis (30,35). Because these events are key elements in both tissue developmental differentiation and tumorigenesis, it is not surprising that the highly homologous PTEN protein molecule may play a dual role in both human development and tumor suppression. Actually, a similar characteristic has been found recently in another important molecule for the development of neural crest derivatives, which is encoded by *c-ret* proto-oncogene (36). It belongs to the membrane receptors of the protein tyrosine kinase family and is expressed during mouse development in a number of tissues, including neural crest derivatives such as dorsal root ganglia, sympathetic and enteric ganglia, and neuroretinal and migrating neural crest cells at earlier stages (37). High levels of *c-ret* transcripts have been found in pheochromocytomas and medullary thyroid carcinomas, which may be associated with a syndrome called multiple endocrine neoplasia (38). The involvement of *c-ret* in these tumors has been supported by the identification of mutations in the *ret* gene. These mutations probably generate dominant tyrosine kinase-activated receptors. Mutations of *c-ret* have also been found in patients with Hirschsprung's disease, which results from a congenital deficiency in the innervation of the posterior gut (39). The nature of the mutations is different in cancer syndromes and in Hirschsprung disease, in which deletions in the *ret* gene suggest a loss of function.

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