HHS Public Access

Author manuscript

Cancer Genet. Author manuscript; available in PMC 2016 January 01.

Published in final edited form as:

Cancer Genet. 2015 ; 208(0): 41–46. doi:10.1016/j.cancergen.2014.11.002.

Esophageal cancer in a family with hamartomatous tumors and germline *PTEN* frameshift and *SMAD7* missense mutations

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Abstract

Germline mutations in the *PTEN* tumor-suppressor gene cause autosomal-dominant conditions such as Cowden and Bannayan-Riley-Ruvalcaba syndromes with variable presentations, including hamartomatous gastrointestinal tumors, dermatologic abnormalities, neurologic symptoms, and elevated cancer risk. We describe a father and son with extensive hamartomatous gastrointestinal polyposis who both developed early-onset esophageal cancer. Exome sequencing identified a novel germline *PTEN* frameshift mutation (c.568_569insC, p.V191S_fs*11). In addition, a missense mutation of *SMAD7* (c.115G>A, p.G39R) with an allele frequency of 0.3% in the Exome Variant Server was detected in both affected individuals. Fluorescence *in-situ* hybridization for *PTEN* in the resected esophageal cancer specimen demonstrated no *PTEN* copy loss in malignant cells, however, immunohistochemistry demonstrated loss of PTEN protein expression. While the risks of many cancers are elevated in the *PTEN* hamartoma tumor syndromes, esophageal adenocarcinoma has not been previously reported. Esophageal adenocarcinoma and extensive polyposis/ganglioneuromatosis could represent less-common features of these syndromes, potentially correlating with this novel *PTEN* frameshift and early protein termination genotype. Alternatively, because simultaneous disruption of both the *PTEN* and *TGF-β/SMAD4* pathways is associated with development of esophageal cancer in a mouse model, and *SMAD4* mutations cause

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Financial Disclosures: None

gastrointestinal hamartomas in Juvenile Polyposis Syndrome, the *SMAD7* mutation may represent an additional modifier of these individuals' *PTEN*-mutant phenotype.

Keywords

PTEN; *SMAD7*; esophageal cancer; Cowden Syndrome; *PTEN* Hamartoma Tumor Syndrome

Introduction

Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) was originally described as a somatically mutated tumor-suppressor gene in brain, breast, and prostate cancers.[1] Germline loss-of-function *PTEN* mutations are responsible for Cowden, Bannayan-Riley-Ruvalcaba, and other syndromes, known collectively as the *PTEN* hamartoma tumor syndrome (PHTS).[2–5] The autosomal-dominant and highly-penetrant PHTS conditions are characterized by a broad range of manifestations including macrocephaly, skin abnormalities, neurologic problems, and hamartomatous or ganglioneuromatous gastrointestinal polyposis.[6,7] Harmartomatous polyps of the stomach and colorectum define the related but distinct autosomal-dominant Juvenile Polyposis Syndrome (JPS), which results from germline mutations of *SMAD4* or *BMPR1A* disrupting signaling through the bone morphogenetic protein (BMP)/SMAD4 pathway.[8,9]

PHTS confers vastly increased lifetime risk of many cancers, including breast (85%), thyroid (35%), colon (9%), kidney (34%), and endometrial (28%) malignancies.[10,11] PTEN terminates growth factor receptor signaling in the phosphatidylinositol-3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway by dephosphorylating phosphatidylinositol-3,4,5-trisphosphate (PIP3).[12] Loss of PTEN function leads to increased cellular growth, proliferation, angiogenesis, and survival signaling.[6,12] In this report we describe a novel *PTEN* frameshift mutation and a *SMAD7* missense mutation occurring in a father and son who had a syndrome of gastrointestinal hamartomatous and ganglioneuromatous polyposis, and who both developed esophageal adenocarcinoma, which has not previously been reported as a feature of PHTS.

Materials and Methods

Patients were enrolled under an Institutional Review Board-approved protocol and provided informed consent. Tissues available included blood from both affected patients, a thyroid resection specimen from the proband, and an esophageal resection specimen from the proband's son. DNA was recovered from peripheral leukocytes. *SMAD4* and *BMPR1A* were screened for mutations and deletion/duplications as described.[13,14] Exome sequencing of the proband was performed by Centrillion Biosciences (Palo Alto, CA) using the SureSelect Human All Exon v.4 51Mb kit (Agilent Technologies, Santa Clara, CA) and HiSeq 2000 Sequencer (Illumina, San Diego, CA). Sequence alignment employed the Burroughs-Wheeler Aligner (BWA-MEM),[15] with processing and variant calling by the Genome Analysis Toolkit pipeline.[16] Variant frequencies were from the Exome Sequencing Project Exome Variant Server (EVS).[17] After filtering, candidate mutations included those that were heterozygous (due to presumed autosomal dominant inheritance), were rare in the EVS

population, and were predicted to be damaging (Supplemental Table). Top candidate mutations were confirmed by PCR with Sanger sequencing. Fluorescence *in-situ* hybridization (FISH) was performed using probes for *PTEN* and the chromosome 10 centromere (*CEP10*) according to manufacturer specifications (Abbott Laboratories, Abbott Park, IL). Slides were counterstained with DAPI and 200 interphase nuclei were analyzed. Immunohistochemistry (IHC) for PTEN expression was performed as described with mouse monoclonal antibody 6H2.1 at 1:100 dilution (Dako, Carpinteria, CA),[18] while SMAD7 IHC employed rabbit monoclonal antibody SC-11932 at 1:20 dilution (Santa Cruz Biotechnology, Dallas, TX).

Results

Clinical Features

The proband, a European-American male, presented at age 41 with dysphagia, weight loss, and abdominal pain and was found to have adenocarcinoma of the distal esophagus and multiple gastric, duodenal, and colonic juvenile polyps (Figure 1A, Patient II-2). He underwent esophagectomy, which revealed node-positive disease, followed by adjuvant chemoradiation. Four years later he underwent total thyroidectomy for papillary thyroid cancer. At age 47, colonoscopy revealed persistent colonic polyposis, including a large polyp in the transverse colon, and he underwent subtotal colectomy. Pathology showed generalized juvenile polyposis of the colon. He continued to have regular surveillance and removal of gastric polyps, however, at age 54 he experienced progressive dysphagia and was diagnosed with squamous cell carcinoma at the esophagogastric anastomosis. He underwent palliative chemoradiotherapy and died at age 57.

Due to the proband's presumed JPS diagnosis and development of esophageal cancer at a young age, his son (Patient III-2) had regular upper and lower endoscopic screening, which identified extensive gastroduodenal and colonic polyps and polypoid ganglioneuromas. Of note, Patient III-2 was treated for an intracranial arteriovenous malformation (AVM) at age 21 and had a facial trichilemmoma. With colonic lesions too numerous for endoscopic removal, he underwent subtotal colectomy at age 30. Pathology showed inflammatory polyps, tubular adenoma, and diffuse polypoid ganglioneuromas (Figure 1B). He continued upper endoscopic surveillance and was well until age 33, when a distal esophageal lesion was confirmed as node-positive adenocarcinoma. He likewise underwent esophagectomy and had neoadjuvant chemoradiotherapy. Both patients were lifelong non-smokers who did not abuse alcohol.

Sequencing

The proband's numerous juvenile polyps and lack of PHTS features such as macrocephaly, trichilemmoma, or intellectual disability led to a JPS diagnosis, yet sequencing and multiplex ligation-dependent probe amplification revealed no mutations or deletion/duplications in coding or promoter regions of *SMAD4* or *BMPR1A*. Exome sequencing was therefore performed to search for germline mutations in other potential disease-associated genes. This identified a novel heterozygous single-base insertion in the *PTEN* gene (c.568_569insC, p.V191S_fs*11), predicted to cause a frameshift with premature termination

in the 5th coding exon. Sanger sequencing confirmed the presence of the mutation in both the proband and his son (Figure 1C). This mutation has not previously been reported in large series of PHTS patients or the Online Mendelian Inheritance in Man and Human Gene Mutation databases.[10,19,20]

While this *PTEN* mutation was considered likely to be responsible for the proband's symptoms and multiple cancers, the list of candidate mutations was examined for variants occurring in known GI cancer pathways, which might influence the proband's clinical phenotype. Out of 72 rare variant candidates (Supplementary Table), a germline missense mutation in *SMAD7* (c.115G>A, p.G39R) stood out due to its role in the TGF-β/SMAD4 pathway, mutations in which are known to cause Juvenile Polyposis Syndrome. This mutation was confirmed by Sanger sequencing in Patients II-2 and III-2. The *SMAD7* G39R mutation is reported in dbSNP (rs144204026), but is rare with an allele frequency of 0.3% (36/10904 chromosomes) in EVS.[17] The G39R alteration occurs in a conserved region and *in silico* mutation analysis tools predict it to be damaging.[21]

PTEN and *SMAD7* in Tumor Specimens

Immunohistochemistry was performed to determine whether malignant tissues expressed PTEN protein (Figure 2A–C). In Patient II-2's thyroid cancer and Patient III-2's esophageal cancer specimens, PTEN protein was weakly expressed in normal tissue, but completely absent in malignant cells. In contrast to this, IHC revealed expression of SMAD7 protein in tumor that was similar to background expression levels (Figure 2D). To investigate whether *PTEN* allelic loss had occurred, FISH was performed on malignant tissue from Patient III-2's esophagectomy specimen (Figure 2E). Hybridization revealed two *PTEN* copies in all 200 malignant nuclei examined, suggesting that deletion of the wild-type *PTEN* allele is not responsible for disrupting PTEN protein expression in this tumor.

Discussion

The *PTEN* mutation in this family (c.568_569insC) has not been previously observed, but occurs in a frequently-mutated region of *PTEN* exon 5, and is similar to several reported mutations.[10] Of 290 probands with *PTEN* mutations causing PHTS reported in a large registry, 13 had termination or frameshift mutations within 20 residues of this V191S_fs*11 mutation.[6] The same registry reported a higher incidence of colorectal cancer among patients with *PTEN* frameshift mutations compared to missense or promoter mutations, while another registry reported lower rates of thyroid cancer in missense mutation patients.[7,10] The present family demonstrates clinical features consistent with these genotype-phenotype correlations, with thyroid cancer occurring in Patient II-2, and prominent colonic involvement in both affected patients. Although DNA from Parents I-1 and I-2 was not available for analysis, as they are alive and reportedly without symptoms, we speculate that the *PTEN* mutation arose *de novo* in Patient II-2. Notably, the proband survived more than 16 years after diagnosis of Stage III esophageal adenocarcinoma (EAC), which compares favorably with the 2-year median survival expected in Stage III patients.[22] Whether EAC developing in the setting of PHTS is associated with better prognosis is difficult to infer from a single family.

Loss of PTEN protein expression in these patients' malignant tissue matches reports from PHTS-associated tumors of the breast, ovary, cerebellum, and thyroid.[18,23–26] *PTEN* gene dosage affects tumor susceptibility, with the reduced protein expression in patients with germline mutations predisposing them to develop hamartomas, which may retain PTEN expression.[23,27] In cancer cells, however, a second-hit eliminates expression from the wild-type allele. Recognized PHTS second-hit mechanisms include promoter methylation, chromosomal loss of heterozygosity (LOH), and new somatic mutations.[23–25] In Patient III-2, normal *PTEN* copy number by FISH argues against chromosomal loss, but copy-neutral LOH and other genetic or epigenetic changes remain possible.

Whereas PHTS shows high penetrance, expressivity of its diverse features is variable and the proband's phenotype of prominent juvenile polyps led to PHTS initially being less-strongly suspected. In retrospect, Patient II-2's clinical features at presentation for colectomy give a significant 29% risk of having a *PTEN* mutation by the Cleveland Clinic Calculator.[6] Additional PHTS features present in Patient III-2 (AVM, trichilemmoma, ganglioneuromas) raise his risk to >99%. Although whole-exome sequencing, rather than *PTEN* mutational screening, may therefore have been unnecessary to make a PHTS diagnosis, it contributed intriguing additional information in light of both patients' unusual presentation of esophageal cancer at a young age.

Esophageal adenocarcinoma has not been reported in PHTS registries with long-term follow-up.[7,10] In some respects, this is surprising. Although somatic *PTEN* mutations are uncommon in esophageal cancer, alterations in PTEN expression commonly occur in EAC and esophageal squamous cell carcinoma (ESCC).[12,28] In a study of 117 resected EACs, 38% showed absent or markedly reduced PTEN staining by IHC, and PTEN deficiency independently correlated with worse disease-free and overall survival.[29] Similarly, in 97 ESCCs, 50.5% showed loss of nuclear PTEN IHC staining, which also correlated with worse outcome.[30] Patients with germline haploinsufficiency for *PTEN*, who develop other GI cancers at rates many times higher than unaffected individuals, might therefore be expected to show increased susceptibility to esophageal cancer. While EAC could simply be a less-common manifestation of PHTS, its rarity in long-term follow-up of large numbers of PHTS patients makes its presence in these cases suggestive of additional modifying genetic characteristics.

The *SMAD7* G39R mutation could represent such a modifier. SMAD7 negatively regulates the transforming growth factor beta (TGF-β) superfamily pathway in a finely-tuned feedback loop, where it targets TGF-β receptors for ubiquitination and proteasomal degradation and blocks receptor/effector protein association.[31,32] Although the TGF-β and BMP pathways both converge on SMAD4 to exert their effects, they have distinct functions, and whereas mutations in *BMPR1A* and *SMAD4* cause JPS, mutations in TGF-β receptor-associated SMADs (*SMAD2-3* and *SMAD7)*, have not been found in hamartomatous tumor syndromes.[33]

The TGF-β pathway has a complex relationship to cancer development, serving as both a pro- and anti-proliferative and apoptotic signal in different cell types and contexts,[32,34] and recent research suggests an important role for SMAD7 in cancer susceptibility,

progression, and evasion of immune surveillance.[32,34–38] Loss of SMAD7 protein causes decreased colorectal cancer cell growth *in vitro*, but *in vivo* also decreases the ability of tumor infiltrating lymphocytes to induce cancer cell apoptosis, thereby promoting metastasis.[32,34] *Smad7* knockout increases rates of hepatocellular carcinoma (HCC) formation after diethylnitrosamine injection in mice,[38] and negative SMAD7 staining by IHC correlates with worse survival in human esophageal squamous and pancreatic cancers.[39,40] IHC results in Patient III-2 esophageal tumor specimen demonstrate intact levels of SMAD7 protein expression, however, whether the function of this protein is affected by the G39R mutation remains unknown. Strikingly, in a mouse model of conditional dual *Smad4/Pten* keratinocyte-specific knockout, 100% of *Smad4/Pten*-deficient mice developed esophagogastric squamous cell carcinomas by 2 months, while in mice with only *Pten* deficiency, no cancers developed by 8 months.[41] SMAD4 activation increases PTEN protein levels, while Akt directly sequesters SMAD3, down-regulating the TGF-β pathway.[41] Disruption of two closely-regulated and interacting pathways could explain enhanced tumor development in the dual-knockout mouse.[41]

If loss of SMAD4 promotes cancer, dysfunction of a SMAD4 signaling inhibitor might be expected to have the opposite effect, however, data showing tumorigenic effects of SMAD7 loss, and association of single-nucleotide polymorphisms (SNPs) causing decreased SMAD7 function with colorectal cancer in multiple populations,[35,37,42–44] suggest that perturbation of the TGF-β pathway at the level of either SMAD7 or SMAD4 promotes tumorigenesis. Perhaps most germane to this family, the microRNA 216a/217, which is upregulated in recurrent HCC specimens, was found to promote HCC recurrence and sorafenib resistance by direct inhibition of both *PTEN* and *SMAD7*.[45] Finally, sequencing of *SMAD7* in patients with SNP haplotypes deemed at high-risk for colorectal cancer revealed the G39R allele in in 2 of 35 individuals,[37] a frequency significantly higher than in the general population (36 of 10904 chromosomes by EVS, p=0.02 by Fisher exact test). This suggests the *SMAD7* G39R mutation could represent an attenuated allele, which while only weakly tumorigenic by itself, promotes EAC development in this family's *PTEN*-deficient background, similar to the role proposed for succinate dehydrogenase-family (*SDHx*) variants in modifying breast cancer risk in PHTS patients.[11]

In summary, we report novel *PTEN* and rare *SMAD7* mutations in a family with gastrointestinal polyposis and esophageal adenocarcinoma. Emergence of EAC in these patients is possibly influenced by their coexisting *SMAD7* mutation, or may represent a less-common manifestation of PHTS. Exome sequencing of additional affected families could further define the contribution of rare mutations in genes other than *PTEN* to PHTS phenotypic variation. Beyond colonoscopic and other surveillance recommended for PHTS patients,[10] these results support including baseline upper endoscopy, with repeat screening and biopsy of suspicious lesions in patients with upper GI polyps.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We are grateful for our patients' generosity in participating in this research.

Supported by NIH 5T32#CA148062-04 (SKS and JEM).

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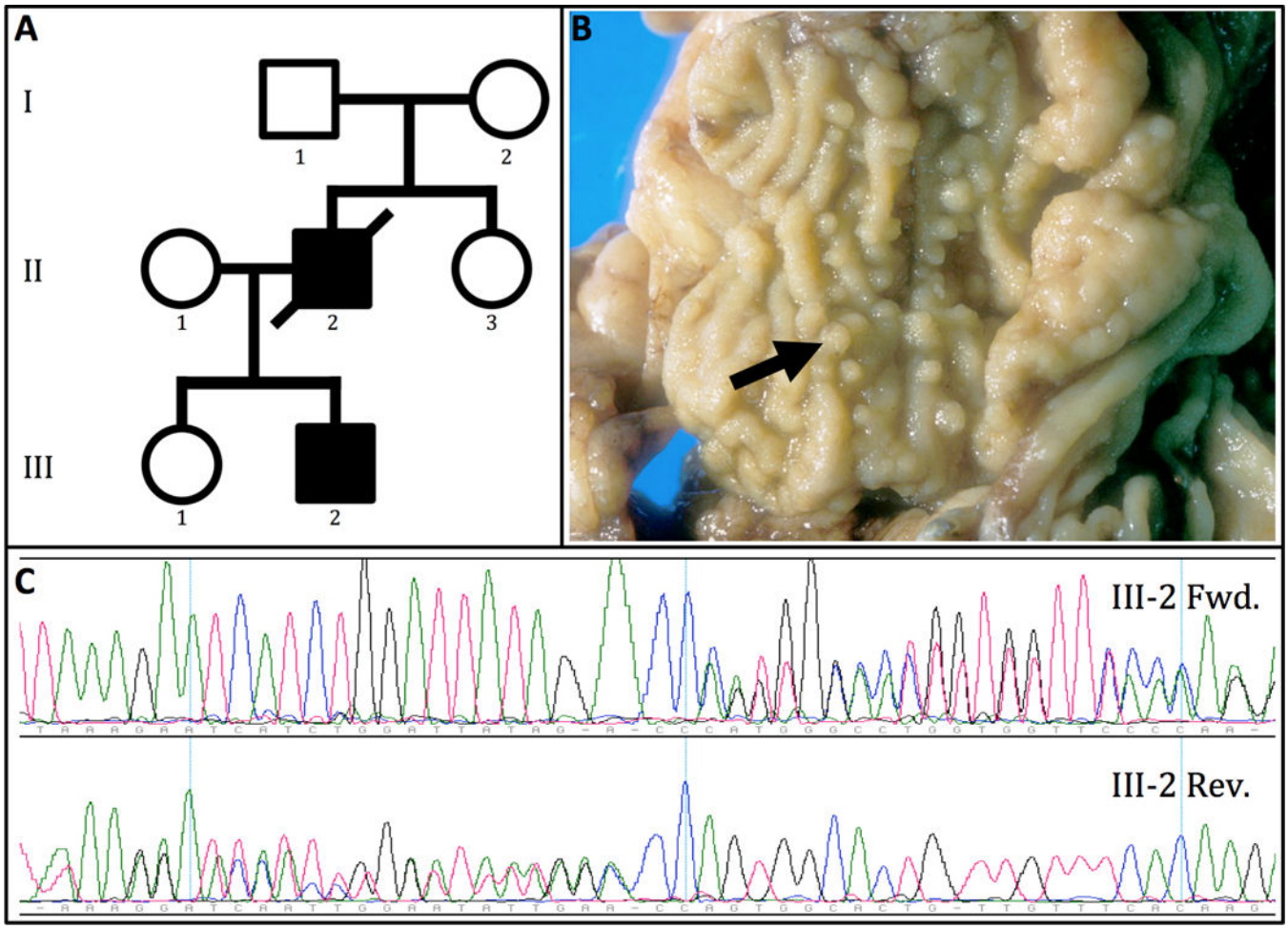

Figure 1.

(A) *PTEN* Hamartoma Tumor Syndrome and esophageal cancer family. Solid shading indicates affected individuals who both had colonic polyposis and esophageal adenocarcinoma. Individuals I-1, I-2, II-3, and III-1 had no apparent symptoms. The proband, (Patient II-2), had esophageal cancer, thyroid cancer, and multiple juvenile polyps of the colon. Patient III-2 also had esophageal cancer, as well as ganglioneuromatous polyps of the stomach, duodenum and colon, and thyroid nodules. (B) Colectomy specimen from Patient III-2, showing diffuse polypoid ganglioneuromas with the arrow marking a representative ganglioneuromatous polyp. (C) Forward (Fwd.) and reverse (Rev.) Sanger sequencing confirmed a germline *PTEN* frameshift mutation in Patients II-2 and III-2.

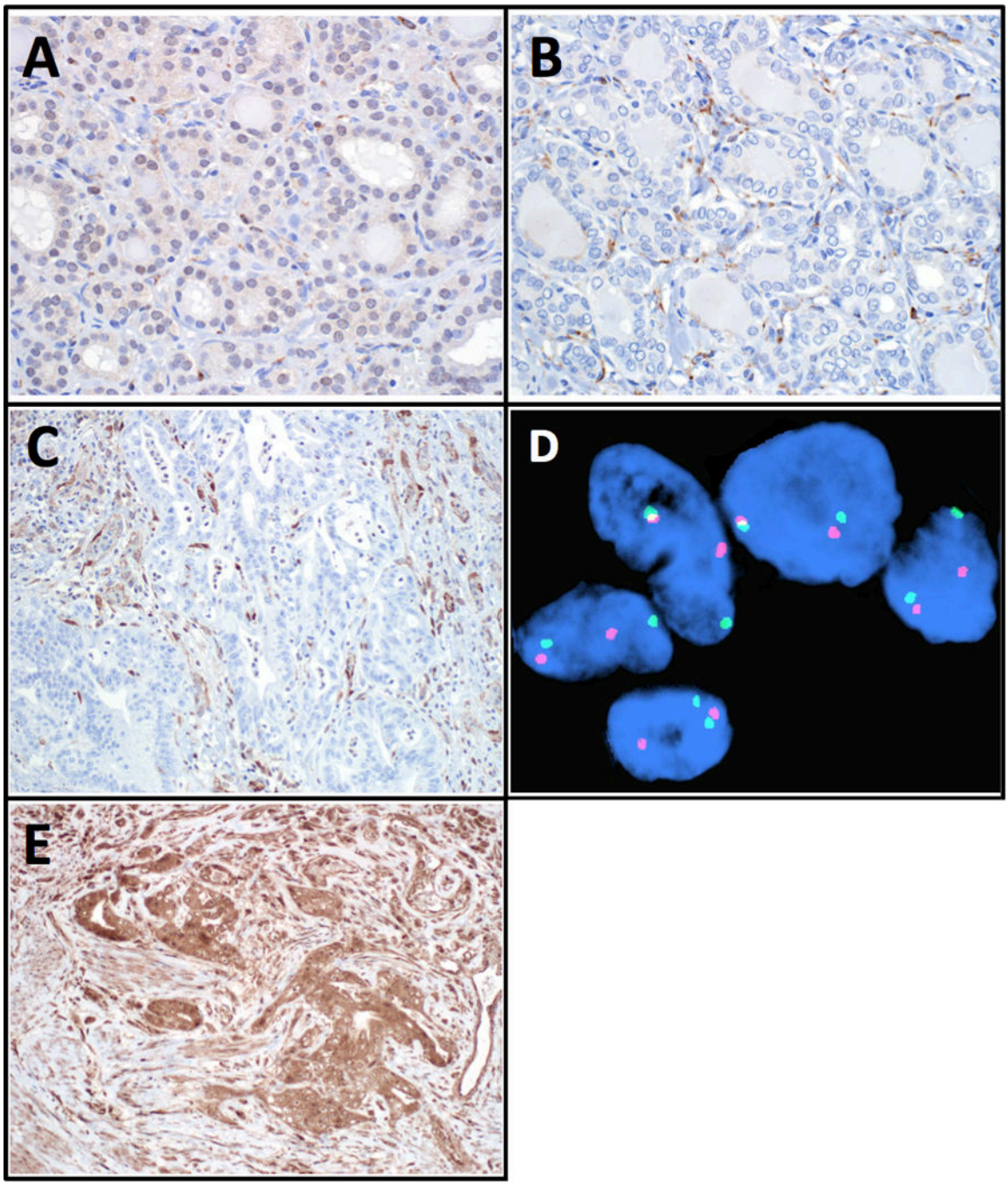

Figure 2.

Immunohistochemical staining reveals weak PTEN protein expression throughout Patient II-2's normal thyroid tissue (A), which is absent in his papillary thyroid cancer (B). Both images magnified 400×. Similar loss of PTEN expression is observed in esophageal adenocarcinoma cells from Patient III-2 (C, 200× magnification). In (B) and (C), non-cancerous endothelial cells retain PTEN expression. Esophageal adenocarcinoma from Patient III-2 demonstrates preserved expression of SMAD7 protein, with staining similar to that seen in surrounding cells (D, 200× magnification). Fluorescent *in-situ* hybridization

demonstrates two *PTEN* copies in malignant esophageal cancer cells from Patient III-2 (E). In these representative cells, red fluorescence marks *PTEN* and green fluorescence marks the chromosome 10 centromere.